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SOME ASPECTS OF AMINO ACID METABOLISM IN MYTILUS EDULIS L.,
1758, AND RANGIA CUNEATA GRAY, 1831

by

Kenneth W. Allen

A THESIS
SUBMITTED TO THE FACULTY
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Houston, Texas
November, 1959
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>1</td>
</tr>
<tr>
<td><strong>CHAPTER I: GENERAL INTRODUCTION</strong></td>
<td>2</td>
</tr>
<tr>
<td>1. Historical</td>
<td>2</td>
</tr>
<tr>
<td>2. Development of the Problem</td>
<td>11</td>
</tr>
<tr>
<td><strong>CHAPTER II: GENERAL MATERIALS AND METHODS</strong></td>
<td>17</td>
</tr>
<tr>
<td>1. Materials</td>
<td>17</td>
</tr>
<tr>
<td>2. Methods</td>
<td>19</td>
</tr>
<tr>
<td>a. Paper Chromatography</td>
<td>19</td>
</tr>
<tr>
<td>b. Dry Weight Determinations</td>
<td>24</td>
</tr>
<tr>
<td>c. Nitrogen Determinations</td>
<td>25</td>
</tr>
<tr>
<td>d. Glycogen Determinations</td>
<td>25</td>
</tr>
<tr>
<td>e. Formula for Standard Deviation</td>
<td>25</td>
</tr>
<tr>
<td><strong>CHAPTER III: THE EFFECT OF SALINITY ON THE PRODUCTION</strong></td>
<td>26</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>26</td>
</tr>
<tr>
<td>2. Historical</td>
<td>26</td>
</tr>
<tr>
<td>3. Methods</td>
<td>30</td>
</tr>
<tr>
<td>4. Results</td>
<td>31</td>
</tr>
<tr>
<td>5. Discussion</td>
<td>35</td>
</tr>
</tbody>
</table>
### TABLE OF CONTENTS (Cont'd)

**CHAPTER IV: TAURINE FORMATION IN *RANGIA CUNEATA* AND *MYTILUS EDULIS*** .......................... 42

1. Introduction ........................................ 42
2. Historical .......................................... 42
3. Materials and Methods .............................. 50
4. Results ............................................. 52
   a. Formation of Cystathionine ..................... 52
   b. Formation of Cystine ............................ 53
   c. Oxidation Products of Cysteine ............... 56
   d. Formation of Sulphate and Taurine ......... 61
5. Discussion ......................................... 68

**SUMMARY AND CONCLUSIONS** ............................................. 71

**LITERATURE CITED** .................................................. 73

**APPENDIX: THE ISOLATION OF β-AMINOISOBYTIC ACID FROM *MYTILUS EDULIS*** .............................. 85

1. Introduction ........................................ 85
2. Methods ............................................ 88
3. Results ............................................. 90
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table Number</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Results of Taurine Studies</td>
<td>14</td>
</tr>
<tr>
<td>2.</td>
<td>Amino Acids in Feces of Snails Before and After Feeding Taurine</td>
<td>15</td>
</tr>
<tr>
<td>3.</td>
<td>Per Cent Dry Weights of <em>R. cuneata</em> from Different Salinities</td>
<td>32</td>
</tr>
<tr>
<td>4.</td>
<td>Per Cent Ash Weights of <em>R. cuneata</em> from Different Salinities</td>
<td>32</td>
</tr>
<tr>
<td>5.</td>
<td>mg N/gm Tissue of <em>R. cuneata</em> from Different Salinities</td>
<td>33</td>
</tr>
<tr>
<td>6.</td>
<td>Amino Acid Concentration in <em>R. cuneata</em> from Different Salinities</td>
<td>34</td>
</tr>
<tr>
<td>7.</td>
<td>Per Cent Nitrogen of <em>R. cuneata</em> in Different Salinities</td>
<td>37</td>
</tr>
<tr>
<td>8.</td>
<td>Per Cent Glycogen in <em>R. cuneata</em> from Different Salinities</td>
<td>37</td>
</tr>
<tr>
<td>9.</td>
<td>Rf Values of Standard BAIB and Unknown from <em>M. edulis</em></td>
<td>90</td>
</tr>
<tr>
<td>10.</td>
<td>Rf Values for DNP Derivatives of Standard BAIB and Unknown from <em>M. edulis</em></td>
<td>91</td>
</tr>
<tr>
<td>11.</td>
<td>Concentration of BAIB in Organs of <em>M. edulis</em></td>
<td>94</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure Number                                      Follows Page

1. Fractionation Technique of Amino Acids .......... 22

2. Amino Acid Concentrations from Various
   Salinities of *Rangia cuneata* ..................... 36

3. Per Cent Glycogen and Nitrogen from Various
   Salinities of *R. cuneata* .......................... 37a

4. Methionine Metabolism Diagram ...................... 49

5. Formation of Cystathionine in *M. edulis* ....... 55

6. One Dimensional Chromatogram Showing Radio-
   active Taurine in *R. cuneata* ..................... 57

7. One Dimensional Chromatogram Showing Radio-
   activity in Taurine, Cysteinesulfinic acid
   and Cysteic Acid in *Rangia cuneata* .............. 58

8. Periodic Extracts of *R. cuneata* Following
   Injection of S-35 Methionine ....................... 60

9. Decrease of Activity Following Injection of
   S-35 Methionine in *R. cuneata* .................... 62

10. Infrared Analysis of Isolated S-35 Taurine
    from *M. edulis* and Cold Taurine ................. 66

11. Chromatogram of *Mytilus edulis* Showing
    \(\beta\)-aminoisobutyric Acid ......................... 92

12. Amino Acid of *Volsella dimissus* ................. 93
ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Jorge Awapara for his guidance, invaluable aid and timely criticisms during the course of this investigation. Thanks are also extended to Mr. Paul McGee for his aid in collecting and caring for the experimental animals used in this study.
CHAPTER I

GENERAL INTRODUCTION

1. Historical

The information available on nitrogen metabolism in microorganisms and vertebrates is considerable compared to the information available for invertebrates. The paucity of knowledge on this subject is even more apparent when one seeks information concerning nitrogen metabolism in one particular invertebrate phylum such as the Mollusca.

Nitrogen catabolism in molluscs has been studied especially in the gastropods. Clementi (1918) demonstrated for the first time in an invertebrate the enzyme arginase. In this work he surveyed many animal species including some vertebrates; only one invertebrate - Helix pomatia - had arginase. It was on the basis of this work that Clementi used the words ureotelic and uricotelic for the excretion of urea and uric acid respectively. The presence of arginase according to Clementi was an indication of ureotelism. The absence of arginase would conversely indicate uric acid excretion. Strohl (1924) reviewed the literature and reported that a high concentration of uric acid existed in molluscs including H. pomatia. Later Hunter (1925) exam-
ined a starfish, *Pisaster ochraceus*, a crab, *Cancer productus*, and a clam, *Saxidomus gigantus*, for arginase, but found none. He concluded that *H. pomatia* was an exception to Clementi's rule for, although arginase was present, uric acid was excreted in large amounts by this species. Albrecht (1923) and Delauney (1931) examined numerous species of invertebrates and reported traces of urea in their tissues and urine. *H. pomatia* was included in this study, and urea was found in small amounts in the tissues of this organism. Delauney also reported values for the amino nitrogen in molluscan urine indicating the excretion of these compounds by many invertebrate species.

In view of the values reported for urea and the presence of arginase in *H. pomatia* Baldwin and Needham (1934) examined this snail hoping to demonstrate the ornithine cycle of Krebs (1932). If this cycle should be present, Clementi's hypothesis that arginase and ureotelism were related would be supported. Their findings however were confusing for the only source of urea appeared to be the arginine in the food. Ornithine incubated with tissue slices did not increase the amount of urea above the normal level for the tissue involved, nor did ornithine and arginine produce more urea than arginine
alone. Since there were no numerical values given for arginase in *H. pomatia*, Baldwin (1935a) reopened the question of urea synthesis in *H. pomatia* and results of this study showed the arginase activity in this organism were as high as that reported for mammalian liver. Baldwin then postulated that urea was synthesized in the snail, but that its low levels indicated that it was converted to something else almost as fast as it was formed.

a) Urea Formation.

Needham (1935) showed a remarkable correlation between environment and the production of uric acid in snails. Baldwin (1935a) compared the concentration of uric acid in numerous species with the activity of arginase, and observed that there was a direct correlation between the level of arginase and uric acid concentration. This suggested that arginase in snails may well play a role in uric acid formation. It seemed possible that urea may still be formed by the ornithine cycle, but that it was converted to uric acid by the condensation reaction described by Wierner (1902) for higher animals.
Baldwin (1935b) investigated this possibility in vitro and found that slices of hepatopancreas of *H. pomatia* did on the addition of hydroxy malonic acid and urea produce uric acid. In view of the work of Howes and Wells (1934) in which snails were shown to undergo alternate periods of dessication and hydration, Baldwin (1936) suggested that *H. pomatia* may alternate between ureotelism and uricotelism depending on the degree of hydration. It is well known that during stages of estivation the water content of *H. pomatia* decreases con-
siderably and that during this period large quantities of uric acid are retained in the animal. However, neither the alternation of excretion nor the method of uric acid synthesis has been corroborated in these animals. In more recent studies the interest has been only in reporting values for uric acid in molluscs rather than investigating the metabolic pathways; for example, Lal et al. (1952) and Saxena (1956) reported the concentration of uric acid in Pila globosa, and Meenakshi (1955) for Pila virens. Saxena (1953) studied arginase activity in Achitina fulica.

b) Amino Acid Oxidase.

The first record of an amino acid oxidase in molluscs was reported by Blaschko and Hawkins (1951). The study was made with organ preparations and racemic mixtures of amino acids were used as substrates. Activity was demonstrated in the hepatopancreas of Octopus vulgaris and Sepia officinalis. Extending the number of molluscs containing this enzyme Blaschko and Hawkins (1952a) examined H. pomatia, M. edulis and Anodonta sp. The results were positive for all three species, but a considerable difference in activity as well as substrate selectivity was observed between the three
species. In all cases racemic mixtures of amino acids were used. In 1952 Roche et al. (a and b) described an enzyme in the hepatopancreas of M. edulis that oxidized L-arginine. The oxidation products obtained in vitro and in vivo were guanidobutyric and keto guanidovaleric acid. These results prompted Blaschko and Hope (1956) to re-examine M. edulis again in order to determine whether a D or L amino acid oxidase was present. The results showed that the enzyme was an L-amino acid oxidase acting on basic amino acids, particularly arginine. However, since the gut in molluscs is intimately associated with the hepatopancreas the possibility of the enzyme being bacterial in origin has not been excluded.

Robin et al. (1957) reported what is apparently the same enzyme from Lymnaea stagnalis. By means of paper chromatography they isolated the oxidation products reported earlier by Roche. Blaschko and Himms (1955) reported D-glutamic and D-aspartic acid oxidase in the hepatopancreas of O. vulgaris and Sepia officinalis. The role of these enzymes in molluscan metabolism has not yet been determined.

c) Amine Oxidases.

The presence of amine oxidases in molluscs and other invertebrates has been reported by Blaschko et al. in
a series of papers (1937, 1941, 1952b, 1953, 1954, 1957). Earlier Henze (1913) isolated tyramine from the salivary gland of *O. vulgaris*. Sereni (1930) concluded from the work of Henze plus observations of his own that the salivary glands in *O. vulgaris* were endocrine in nature for they secreted tyramine into the circulation. Blaschko in the above mentioned series of articles showed tyraminase activity in *S. officinalis*, *Patella vulgata* and *Asterias rubens*. Amine oxidases are considered to be important in molluscs in connection with neurophysiology (e.g., Welsch, 1954; 1957). Welsch considers serotonin as a neurotransmitter in molluscs and thus postulates that the role of amine oxidases in these animals is one of control on the sympathomimetic amines. However, Blaschko and Himms (1954) think that the wide distribution of monoamine oxidases suggest some function for amines in metabolism not yet recognized. Reviews on amine oxidases in general, which include invertebrate studies, have been given by Page (1958) and Davison (1958).

d) Isolation of Nitrogenous Compounds.

Besides these studies on catabolism and certain enzyme systems pertinent to nitrogen metabolism, the inform-
ation on nitrogen metabolism in molluscs is mainly concerned with the isolation and demonstration of nitrogenous compounds. Interest in isolation of various compounds from animal tissues developed from the idea that biochemical dissimilarities would throw light on the phylogenetic position of organisms in the animal kingdom. For example, Kutscher and Ackerman (1926) demonstrated that creatine phosphate was present only in vertebrates and arginine phosphate was limited to invertebrates. Later work by these same authors as well as Florkin (1949) has shown that this is not such an exclusive characteristic as first indicated. A review on the subject of evolution of phosphagens has been given by Ennor and Morrison (1958). Since the present paper is mainly concerned with amino acids, further discussion of other nitrogenous compounds is not presented.

e) Free Amino Acids in Invertebrates.

Duchâteau et al. (1952) made the first comprehensive study of the free amino acid concentrations in a molluscan species. By microbiological assay, free amino acids were determined in the muscles of *M. edulis* and *Ostrea edulis*. They reported high values for alanine, arginine and glycine
for both species. Proline was also present in high concentration in O. edulis. Later using the same technique Duchateau and Florkin (1954) reported values for the concentration of free amino acids in the foot of the marine gastropod Buccinum undatum. Alanine, arginine, glycine and proline were present in highest concentrations. Kirk et al. (1954) attempted to use paper chromatography to classify snails. They based their classification of species on the number of fluorescent compounds obtained from an alcoholic extract of snail muscle. The species involved were Theba pisana, Helix aspersa and Austrosuccinia contenta. The use of paper chromatography in separating and determining relationships between these three species seems redundant since morphology is more readily adaptable for the species in question. Lewis (1952) reported that free amino acids accounted for over 20% of the dry weight of nerve tissue in cephalopods. He reported values for aspartic acid, glutamic acid, alanine, taurine and glycine for Sepia officinalis and Octopus vulgaris. Mead and Kemmerer (1953) determined the amino acid content of Achatina fulica to establish the value of snail meal as live stock food. Unfortunately, it is difficult to tell from the paper whether the compounds reported were free amino acids or the components of protein. Melnick (1958) studied collagen form-
ation in *H. aspersa* and reported the presence of hydroxyproline in the mantle of this snail. This is the first report of the existence of this compound in snails, and it is unfortunate that no quantitative values were given. Noland (1949) determined some of the free amino acids in *Mactra solidissima*, *Loligo peali* and *Busycyon canaliculatum*. High values for tryptophane, tyrosine and histidine were recorded for *L. peali* and only traces of these compounds were found in the other two species.

On the basis of the available information on amino acids in molluscs, a comparative study of amino acids in several species of snails was undertaken in order to provide ground work for future studies in amino acid metabolism and the physiological function of these compounds in molluscs.

2. Development of Problem.

The work reported in this thesis was the outcome of several observations made while surveying amino acids in the freshwater snails, *Lymnaea palustris*, *Marisa cornuarietis* and *Pomacea bridgesi*, and the terrestrial snail, *Otala lactea*. It was noted that these species, without exception, had no taurine. It had been reported earlier that this compound is present in high concentration in marine molluscs.
(Kelley, 1904; Jansen, 1917; Mendal and Bradley, 1906). To determine whether this apparent difference was real, a number of molluscan species from freshwater, marine and terrestrial habitats were examined for taurine. The results of this survey have already been reported by Simpson, Allen and Awapara (1959). From this work the molluscan species could be separated into two groups, regardless of class: (1) marine forms which contained taurine in high concentrations and (2) freshwater and land forms which had none detectable by paper chromatography. The listing of the species examined is in Table #1.

This definite separation of molluscs into two groups based on the presence of or absence of taurine relative to a marine vis à vis freshwater environment may have important taxonomic implications. To follow this fact to wider conclusions would necessitate the examination of a large number of species under all possible natural conditions. However, for the present study the problem to be answered was why taurine was either absent or in such low concentration that it was undetectable by chromatography in freshwater and terrestrial molluscs. Two approaches to this problem were made. The first was to test the effect of increasing salinity on a
species which could withstand a wide range of salinity in its environment. The species selected for this phase of the investigation was Rangia cuneata, a brackish water pelecypod. This study was done in order to determine if a saline environment would enable the animal to establish detectable concentrations of taurine in its tissues. The second phase of the study was a comparison of the metabolism of sulfur amino acids in R. cuneata and M. edulis. The latter species was noted for its high concentration of taurine while the former was apparently devoid of this compound.

Following this survey for taurine, feeding experiments were performed on L. palustris, M. cornuarietis, P. bridgesi and O. lactea in order to establish that the absence of taurine in the tissues of these snails was not a dietary factor. These animals had previously been fed lettuce which does not contain taurine. The animals were taken from the stock tanks and starved for five days. After this time they were placed in individual 50 ml. beakers with 5 ml. of water. Fish food rich in taurine was supplied, and as the first strings of feces appeared they were collected for chromatographic analysis. Five hours after the first feces appeared the animals were extracted for amino acid analysis. O lactea, a ter-
### Table 1

**Results of Taurine Studies**

<table>
<thead>
<tr>
<th>Mollusca</th>
<th>Environment</th>
<th>Taurine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gastropoda</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lymnaea palustris</em></td>
<td>Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Marisa cornuarietis</em></td>
<td>Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Pomacea bridgesi</em></td>
<td>Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Rumina decollata</em></td>
<td>Terrestrial</td>
<td>-</td>
</tr>
<tr>
<td><em>Otala lactea</em></td>
<td>Terrestrial</td>
<td>-</td>
</tr>
<tr>
<td><em>Mesodon thyroideus</em></td>
<td>Terrestrial</td>
<td>-</td>
</tr>
<tr>
<td><em>Bulimulus alternatus</em></td>
<td>Terrestrial</td>
<td>-</td>
</tr>
<tr>
<td><em>Murex fulvescens</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Oliva sayana</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Littorina irrorata</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Thais haemastoma</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><strong>Pelecypoda</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anadonta grandis</em></td>
<td>Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Quadrula quadrula</em></td>
<td>Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Lampsilis</em> sp.</td>
<td>Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Elliptio</em> sp.</td>
<td>Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Rangia cuneata</em></td>
<td>Brackish Freshwater</td>
<td>-</td>
</tr>
<tr>
<td><em>Donax variabilis</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Volsella dimissus</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Venus mercenaria</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Brachiodontes recurves</em></td>
<td>Brackish-marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Dosinia discus</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Crassostrea virginica</em></td>
<td>Brackish-marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Arca incongrua</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Arca campechiensis</em></td>
<td>Marine</td>
<td>+</td>
</tr>
<tr>
<td><em>Noetia ponderosa</em></td>
<td>Marine</td>
<td>+</td>
</tr>
</tbody>
</table>
restrial snail, was treated in the same manner with the exception that it was placed in a five gallon aquarium which contained moist paper toweling for a floor. A cover was placed over the tank in order to maintain a sufficient humidity so that the snails would remain active and feed. The results are shown in Table #2.

Table #2

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acids in Feces Before Feeding Taurine</th>
<th>Amino Acids in Feces After Feeding Taurine</th>
<th>Taurine in Tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. palustris</td>
<td>Glycine</td>
<td>Glycine</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>Alanine</td>
<td>Taurine</td>
</tr>
<tr>
<td>M. cornuarietis</td>
<td>Glycine</td>
<td>Glycine</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>Alanine</td>
<td>Taurine</td>
</tr>
<tr>
<td>O. lactea</td>
<td>Glutamic acid</td>
<td>Glutamic acid</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>Alanine</td>
<td>Taurine</td>
</tr>
<tr>
<td>P. bridgesi</td>
<td>Glycine</td>
<td>Glycine</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>Alanine</td>
<td>Taurine</td>
</tr>
</tbody>
</table>

These results show that the absence of taurine in tissues of freshwater and terrestrial snails is not necessarily due
to the lack of taurine in the diet. The observation that other amino acids were also present in the feces is interesting for without exception the amino acids in the feces were the ones in highest concentration in the individual species. From these results it appears that the problem of the origin of taurine in molluscan tissue must be examined from an environmental as well as a metabolic standpoint.

While investigating the amino acid composition of M. edulis by paper chromatography, a compound appeared on chromatograms with Rf values identical to the Rf values of \( \beta \)-aminoisobutyric acid. Since this compound was not known to exist in invertebrates, and in view of its role in pyrimidine metabolism, it was isolated and identified from this species. Although this compound bore no relationship to the original problem, its role in amino acid metabolism prompted the inclusion of its identification from M. edulis in this study.
CHAPTER II
GENERAL MATERIALS AND METHODS

1. Materials

*Mytilus edulis* was obtained from the Marine Biological Laboratory in Woods Hole, Massachusetts. On arrival these organisms were immediately placed in a fifty gallon aquarium containing sea water from Offatts Bayou, Galveston, Texas. The sea water before use had been placed in a cold room at 40° F. The mussels shipped via air freight were not out of sea water longer than thirty six hours. Although the salinity of the previous habitat was unknown they adapted favorably to the laboratory environment.

*R. cuneata* in the adult stage was collected in the San Jacinto River, Harris County, Texas, near highway #90. Smaller forms were obtained from upper Trinity Bay near Baytown, Texas. They were always found in abundance at these locations in about three feet of water just below the surface of the sandy bottom. This species was transported to the laboratory and kept at approximately 70° F. (room temperature in the air conditioned animal room) in large aerated fifty gallon aquaria. Both *M. edulis* and *R. cuneata* were allowed at least three days to adapt to the aquarium before being
used in experimental work. In all cases the viability of the animal used was determined by several criteria: (a) the retractability of the shell when pried open, (b) the irritability of the foot on probing and (c) when the entire animal was removed the heart was closely watched for movement.

To test the effect of salinity on the amino acid concentration in *R. cuneata*, six five-gallon aquaria were set up, each containing water of a higher salinity. Filtered sea water was diluted with filtered pond water on the basis of per cent sea water. Later a more exacting figure of salinity was obtained, using the Mohr technique for the determination of chloride concentration. Thus, in each tank salinity was expressed as sodium chloride concentration in parts per thousand (0/00). Specimens of *R. cuneata* were transferred from stock tanks to the first experimental tank containing fresh water (3 0/00). After two days the animals were removed to the next tank leaving six to twelve specimens in the original tank as controls. This process was repeated until all of the tanks contained a dozen specimens. Amino acid extracts were made from individual animals from each tank and chromatograms were prepared. This procedure permitted the clams to remain several days in each tank in
order to acclimatize to the new environment and insured a
current starvation time for all animals used in a particular
experiment. To correct for evaporation from the experimental
tanks water levels were marked on each tank, and the volume
maintained by adding small amounts of pond water as needed.

2. Methods

Several techniques were used during this study; therefore, to avoid repetition, the general methods will be stated here. Any modifications or special techniques will be discussed in context of the experiments.

a) Paper Chromatography.

(1) Extraction procedure.

The free amino acids and related nitrogenous compounds were extracted from snails and clams according to the method of Awapara (1948). Specimens involved in any particular experiment were homogenized in a Serv-All Omnimixer containing in ratio 1 gm. of tissue to 20 ml. of 80% ethanol. The broken cell preparation was heated in a boiling water bath to complete protein precipitation, and then centrifuged at 2000 X g. for twenty minutes. The supernatent was transferred to a centrifuge tube containing 15 ml. of chloroform and thoroughly mixed. This mixture was then
centrifuged at 2000 X g. for twenty minutes and the chloroform-alcohol layer removed and discarded. The remaining water layer was placed in 10 ml. beakers and evaporated on a steam bath or under vacuum at 48° C. The latter was employed during the investigation of intermediate compounds after the injection of radioactive methionine, since many sulfur amino acids are readily oxidized. The dried material was dissolved in 1 ml. of glass distilled water.

(2) Fractionation of amino acids.

The amino acid extracts were fractionated by means of two ion exchange resins depending on the degree of separation desired in any particular experiment. This fractionation eliminated streaking in paper chromatograms caused by inorganic salts. Also this treatment permitted a separation of the total amino acid extract into acidic and neutral amino acids. This aided in identification of sulfur containing amino acids.

The general procedure for fractionation followed the scheme shown in Figure #1. The extract was usually run first through Dowex 50 H+, a cation exchange resin. By the method of Smith (1958), two fractions were obtained from this resin. The effluent contained sulfonic and sul-
finic acids along with other anions. Ammonium hydroxide eluted the neutral and basic amino acids from the resin bed. In some of the isotope studies it was found more convenient to fractionate the acid fraction further by running this extract through Dowex 2 (OH⁻ form) and eluting successively with acetic acid and hydrochloric acid. This separated the stronger acids from the weaker acids, and aided in the identification of any sulfur-containing compound which was acidic, other than taurine.

(3) Chromatography.

The amino acids were analyzed by two dimensional ascending paper partition chromatography using the apparatus of Williams and Kirby (1948). For qualitative studies Whatman filter paper #3 Mm was used, whereas for quantitative work Whatman #4 was employed; the latter was found to produce less cellulose fiber in the extraction solution for the quantitative procedure than the 3 Mm paper.

The solvent mixtures used were 72% phenol and 65% lutidine. Both solvents were redistilled before use. The amino acids were visualized on the papers by dipping in a solution of 0.5% ninhydrin made up in acetone (w/v). To preserve the papers for future study the chromatograms were
Figure #1

**Amino Acid Extract**

- Amino Acids
- Carbohydrates
- Other Nitrogenous Compounds
- Water Soluble Compounds

---

**Dowex 50 H⁺**

---

**Effluent**

- Acidic Amino Acids
- Non-polar Substances
- Anions

**Dowex 2 OH⁻**

---

**1 N Acetic Acid**

- Taurine
- Taurocyamine

**1 N HCL**

- Cysteic Acid
- Cysteine Sulfinic Acid

**4N NH₄OH Eluate**

- Neutral and Basic-Amino Acids
treated as suggested by Block, Burrum and Zweig (1958, pp. 126). After this treatment the spots remained visible for several months.

(4) Quantitative analysis of amino acids.

Alanine, aspartic acid, glycine and glutamic acid were quantitatively determined by the method of Awapara (1950) with one modification. After localization of the amino acids on the chromatogram the area containing the compound to be measured was outlined with pencil, and the paper was then sprayed with 1% KOH in methanol rather than adding NaOH directly to the tube. The spraying with KOH followed the technique described by Moore and Stein (1948); therefore, since this step was followed in the beginning of the study it was carried on throughout. After drying the sprayed paper in air, the penciled squares were cut out and placed in test tubes. The tubes were put in a vacuum desiccator over concentrated sulfuric acid for three hours. Color was developed with the reagent of Moore and Stein (1948). The tubes were read at 570 μm on a Coleman Junior Spectrophotometer. The machine was zeroed with tubes containing paper blanks. Calculation of the quantity of amino acids present was based on values obtained from individual
standards of the compounds which had previously been used to establish a calibration curve.

b) Dry Weight Determinations.

To determine the gain or loss of water resulting from a change in salinity the dry weight of the animal from different environments was determined. The procedure consisted of drying stainless steel cups or planchets, normally used as containers for radioactive material, in an oven at 110° C. for three hours. Following the drying process the planchets were placed in a dessicator, then weighed. This process was repeated until a constant dry weight was obtained. The total animal or individual tissues were then removed from the specimens which were under investigation, and the excess water removed by blotting with filter paper. The planchets and tissue were then weighed followed by drying in an oven at 110° C. until a constant dry weight was reached.

Ashing: Ash weights were determined on individual specimens by placing the dried tissues in crucibles of constant dry weight. The weights of the crucibles and tissues were recorded. The crucibles and tissues were placed in a muffle furnace and gradually heated to 1000° C. The difference in dry and ash weight was expressed as per cent ash weight.
c) Nitrogen Determinations.

Total nitrogen was determined by the Micro-Kjeldahl technique as described in Hawk, Oser and Summerson (1954; pp. 380-381). The steam distillation method with titration of the distillate with sulfuric acid was followed exactly as described.

d) Glycogen Determinations.

Glycogen was determined by the method of Carroll et al. (1956). This technique consisted of a trichloroacetic acid precipitation of protein followed by an alcoholic precipitation of glycogen from the filtrate. The anthrone reagent of Roe (1955) was used for the colorimetric determination of glycogen. The measurement of the anthrone reaction was done on a Coleman Junior Spectrophotometer at 520 μm.

e) Determination of Standard Deviation of the Mean. (StDM).

The Standard Deviations shown in the result sections were determined by the following formula:

\[ \text{St.DM} = \pm \sqrt{\frac{\sum_{i=1}^{n} (x_i - M)^2}{n-1}} \]

- \( n \) = number of determinations
- \( x \) = individual determinations
- \( M \) = mean
CHAPTER III

THE EFFECT OF SALINITY ON THE PRODUCTION OF TAURINE
AND AMINO ACID COMPOSITION

1. Introduction

The results of the survey on the occurrence of taurine in molluscs showed that this compound was exclusive to animals from a marine environment. The first approach to the possible reasons for this phenomena was to move an animal from a lesser to a greater saline environment. Closely related to this problem, if not the cause, was an observation made in this laboratory between the concentration of amino acids in marine and freshwater molluscs. The results of Simpson's (1959) study showed that the amino acids were in higher concentration in marine forms than in the freshwater snails L. palustris, M. cornuarietis and P. bridgesi. For this reason the literature discussed below is concerned with the effect of increased salinity on amino acid concentration in invertebrates. Literature pertaining to the taurine problem does not exist.

2. Historical

The contrast in concentration of amino acids between freshwater and marine molluscs appeared to be related to
osmoregulation. This had been suggested as early as 1904 by Frederique, when he observed that the muscle and other tissues of marine molluscs were very dilute with respect to inorganic ions. The tissues are, however, in osmotic equilibrium with the blood which in the case of most marine molluscs is nearly identical in inorganic composition with sea water. Frederique therefore postulated that small organic molecules may well make up the difference in osmotic activity between blood and tissues.

The first significant study on the amino acids in invertebrates from habitats of different salinities was made by Camien et al. (1951). In comparing the amino acid constituents of Homarus vulgaris, the lobster, with that of the freshwater crayfish, Astacus fluviatilis, it was noted that the major difference was one of concentration. The amino acids were found to be much higher in the sea-dwelling lobster than the freshwater crayfish. In 1955a Duchâteau and Florkin examined this phenomenon experimentally by moving the euryhaline crab, Eriocheir sinensis, from salt to brackish water and measuring the amino acids from each environment. There was a definite decrease in the concentration of amino acids as the animal passed into the more dilute environment.
Potts (personal communication) observed a similar change in the amphipod *Gammarus zoddachi*, but carried the experiment further by gradually moving animals from sea water into 1% sea water and then back again. The increase and decrease of amino acid nitrogen was observed to be reversible. The concentration changed from 250 millimoles of amino nitrogen/kilogram of animal tissue in salt water to 80 millimoles of amino nitrogen/kilogram of animal tissue in 1% sea water. Potts (1958) published his observations on the effect of salinity on *M. edulis* and *Anodonta cygnaea*. Since neither of these animals could withstand a wide range of salinity the observations were limited, but in *A. cygnaea* the amino nitrogen increased constantly up to a concentration of 18% sea water while it decreased in *M. edulis* as this animal passed from sea water to 50% sea water. Shaw (1958a) reported studies of *Carcinus maenas* under varying salinities. Although there appeared to be a reduction in the tissue water as the animal approached higher salinities, the amount of amino acid gained did not balance with the theoretical quantities determined on the basis of a simple concentration gradient. This led Shaw to postulate that Crustacea lose amino acids as they go into environments of lower concentration so that
the loss of salts and gain of water is kept to a minimum. Shaw (1958b, 1959a,b) published a series of papers on osmo-
regulation in crabs and demonstrated that the amount of salt
present could not account for the osmotic equilibrium that
existed in these animals. He demonstrated that the amino
acids based on amino nitrogen determinations accounted for
approximately 40% of the osmotic activity in the crabs, C.
maenas but to a lesser extent in Potaman niloticas. He thus
hypothesized that the ability of an animal to utilize amino
acids as osmoregulators may be a legacy from the early marine
ancestry of the species. Duchâteau et al. (1959) studied the
effect of ecdysis on amino acid concentration in C. maenas.
The animals were kept at constant salinity, and the results
followed the general pattern already demonstrated, e.g. hy-
dration decreases the amino acid concentration while dehy-
dration increases the amino acid concentration when expressed
on a wet weight basis. Since C. maenas goes through a cycle
of hydration and dehydration during the process of ecdysis
the amino acids fluctuated accordingly. Duchâteau and Flor-
kin (1955b) also studied the effect of temperature on the
amino acid concentration in E. sinensis and noted that as the
temperature decreased the amount of proline also decreased.
There is no explanation for this phenomenon and further studies need to be done on the effect of temperature on amino acids. In another study Meenakshi (1956) suggested that the fluctuation of amino acids is not restricted to aquatic molluscs, for he noted an increase in glutamic acid in the nervous tissue of the amphibious snail, *Pila virens*, during hibernation. Although this was the only amino acid studied, this may well coincide with the effect of hydration-dehydration of tissue on amino acid concentration on the basis of Howes and Wells (1934) investigation. They found that in hibernating snails there is a considerable loss of body water, and that activation is followed by hydration.

In view of the findings reported above, a series of experiments were performed on *R. cuneata* in order to determine the effect of salinity on the production of taurine as well as the concentration of amino acids.

2. Methods

A series of five gallon aquaria were set up in consecutive order of increasing salinities which ranged from 30/00 to 250/00. Filtered sea water was diluted to the appropriate volume with distilled water. Salinities were determined by the Mohr technique as described in Standard Methods (1955).
Several tests were made on *R. cuneata* to determine how rapidly they could be moved from one salinity to another. It was found that if the animals were allowed to remain in a particular tank for two days, they could be transferred without fatalities.

In order to insure correct controls for starvation a group of clams were started together in the freshwater tank. After two days all but a dozen of the original group were moved to the next tank of higher salinity. This was repeated until all the tanks contained a dozen clams. After the final tank was reached, two days were allowed to elapse prior to making amino acid extracts from representatives of each tank. The effects of leaving clams in one tank over a long period were checked and found that up to twenty days no measurable change in the amino acid concentration could be observed.

3. Results

In view of the possible relationship between amino acid concentration and the hydration of tissues the dry weight, total nitrogen and ash weights were determined on specimens of *R. cuneata* taken from environments of different salinities. The results are shown in Tables #3, #4, and #5.
Table #3
Per Cent Dry Weights of *R. cuneata*
from Different Salinities

<table>
<thead>
<tr>
<th>Salinity</th>
<th>3.0/00</th>
<th>6.0/00</th>
<th>10.0/00</th>
<th>17.0/00</th>
<th>20.0/00</th>
<th>25.0/00</th>
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<td></td>
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<td>25.96</td>
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<td>25.03</td>
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</tr>
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<td>25.71</td>
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<td>23.70</td>
<td>24.04</td>
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<tr>
<td>Mean</td>
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<td>20.36</td>
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<td>25.75</td>
<td>25.70</td>
</tr>
<tr>
<td>Std DM</td>
<td>±0.36</td>
<td>±0.41</td>
<td>±0.51</td>
<td>±0.95</td>
<td>±0.33</td>
<td>±0.32</td>
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</table>

Table #4
Per Cent Ash Weights* of *R. cuneata*
from Different Salinities
(Dry Weight)

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<thead>
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<th>Salinity</th>
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<th>6.0/00</th>
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<td>4.61</td>
<td>6.31</td>
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<td>3.32</td>
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<td>4.97</td>
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<td>4.79</td>
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<td>Mean</td>
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<td>5.13</td>
<td>5.84</td>
<td>5.27</td>
</tr>
<tr>
<td>Std DM</td>
<td>±0.36</td>
<td>±0.13</td>
<td>±0.24</td>
<td>±0.35</td>
<td>±0.22</td>
<td>±0.23</td>
</tr>
</tbody>
</table>

* Per cent ash weight expressed in terms of dry weight of animal
Table #5

mgN/gm Tissue of *R. cuneata*
from Different Salinities
(Wet Weight)

<table>
<thead>
<tr>
<th>Salinity 3 0/00</th>
<th>5 0/00</th>
<th>10 0/00</th>
<th>17 0/00</th>
<th>20 0/00</th>
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<td>25.70</td>
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<td>35.32</td>
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<td>41.73</td>
</tr>
<tr>
<td>27.85</td>
<td>29.32</td>
<td>40.52</td>
<td>42.64</td>
<td>44.66</td>
<td>44.49</td>
</tr>
<tr>
<td>28.98</td>
<td>27.65</td>
<td>30.13</td>
<td>42.57</td>
<td>42.79</td>
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</tr>
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<td>27.65</td>
<td>28.10</td>
<td>35.18</td>
<td>41.72</td>
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<td>26.65</td>
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<td>38.25</td>
<td>41.20</td>
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<td>43.22</td>
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<tr>
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<td>28.60</td>
<td>35.88</td>
<td>41.79</td>
<td>43.30</td>
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<tr>
<td>Std DM</td>
<td>±0.50</td>
<td>±0.63</td>
<td>±1.74</td>
<td>±0.30</td>
<td>±0.34</td>
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</table>

From the dry weight studies it is obvious that as the clams progress from a dilute to a more saline environment there is a loss of water. The ash weights on the other hand increase slightly in the higher salinities reflecting the increase of inorganic constituents. These results show that determinations of tissue constituents should be based on dry weight in order to avoid errors caused by dehydration. The amino acids measured from *R. cuneata* were based on dry weight and the results are shown in Table #6. Graphic demonstration of these results is shown in Figure #2. Although the amino acids measured showed a definite increase in concentration from fresh water to water of 25 0/00 taurine was never observed on the chromatograms.
Table #6

Amino Acid Concentration in *R. cuneata*
from Different Salinities
(Expressed as µMoles/gm Tissue Dry Weight)

<table>
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<tr>
<th></th>
<th>3 0/00</th>
<th>5 0/00</th>
<th>10 0/00</th>
<th>17 0/00</th>
<th>20 0/00</th>
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<tbody>
<tr>
<td>Aspartic Acid</td>
<td>1.91</td>
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<td>7.21</td>
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<tr>
<td>Acid</td>
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<td>9.54</td>
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<td>11.28</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
<td>2.12</td>
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<td>7.68</td>
<td>16.22</td>
<td>10.30</td>
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<tr>
<td>Std DM</td>
<td>±0.38</td>
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<td>±0.47</td>
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<td>Alanine</td>
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<td>Glutamic Acid</td>
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<td>37.81</td>
<td>54.45</td>
<td>26.61</td>
<td>20.86</td>
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<tr>
<td></td>
<td>6.48</td>
<td>18.42</td>
<td>29.00</td>
<td>47.07</td>
<td>36.00</td>
<td>25.60</td>
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<tr>
<td></td>
<td>6.73</td>
<td>15.32</td>
<td>38.40</td>
<td>45.93</td>
<td>33.67</td>
<td>24.40</td>
</tr>
<tr>
<td>Mean</td>
<td>5.14</td>
<td>18.94</td>
<td>34.84</td>
<td>51.57</td>
<td>32.10</td>
<td>23.66</td>
</tr>
<tr>
<td>Std DM</td>
<td>±0.68</td>
<td>±2.11</td>
<td>±1.65</td>
<td>±2.43</td>
<td>±1.54</td>
<td>±0.75</td>
</tr>
</tbody>
</table>
However the increase of alanine was such that it prompted a digression from the main problem for a time. In view of the known interconversions of carbohydrate and amino acids, glycogen was measured in animals from the different salinities to see if the increase in alanine in any way was related to carbohydrate metabolism. The results of these determinations are shown in Table #8 and Figure #3. The nitrogen values based on dry weight are shown in Table #7 and Figure #3.

4. Discussion

The results of the dry weight determinations show an increase in the per cent dry weights of the clams as they go from a dilute to a more concentrated salt environment. This was expected since the early work of Frederique (1901), Bottazzi (1908), Bethe (1929), Krogh (1939) and Fox (1941) has shown that euryhaline molluscs establish an equilibrium with their environment which is easily observed by a volume and weight change. In fact this phenomena assumes a practical significance in that commercial oyster fishermen for years have enticed shell fish gourmets with opulent specimens of oysters which have resided for sometime in dilute sea water. This change in weight and volume is a reversible process
Figure #2

Amino Acid Concentrations from Various Salinities of R. cuneata

▲ - Alanine
□ - Glycine
○ - Glutamic Acid
● - Aspartic Acid
Table #7

Per Cent Nitrogen of *R. cuneata*
in Different Salinities
(Expressed on basis of dry weight)

<table>
<thead>
<tr>
<th>Salinity</th>
<th>3 0/00</th>
<th>5 0/00</th>
<th>10 0/00</th>
<th>17 0/00</th>
<th>20 0/00</th>
<th>25 0/00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.53</td>
<td>15.13</td>
<td>15.24</td>
<td>17.34</td>
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<td>14.40</td>
<td>17.48</td>
<td>18.11</td>
<td>17.34</td>
<td>17.31</td>
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<td></td>
<td>15.25</td>
<td>13.58</td>
<td>13.00</td>
<td>18.08</td>
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<td>15.60</td>
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<td>17.50</td>
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<td>16.82</td>
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<tr>
<td>Mean</td>
<td>14.41</td>
<td>14.05</td>
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<td>17.75</td>
<td>17.00</td>
<td>15.59</td>
</tr>
<tr>
<td>Std DM</td>
<td>±0.34</td>
<td>±0.28</td>
<td>±0.75</td>
<td>±0.15</td>
<td>±0.30</td>
<td>±0.33</td>
</tr>
</tbody>
</table>

Table #8

Per Cent Glycogen in *R. cuneata*
from Different Salinities
(Expressed on basis of dry weight)

<table>
<thead>
<tr>
<th>Salinity</th>
<th>3 0/00</th>
<th>5 0/00</th>
<th>10 0/00</th>
<th>17 0/00</th>
<th>20 0/00</th>
<th>25 0/00</th>
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<tr>
<td></td>
<td>11.62</td>
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<td>13.81</td>
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<td></td>
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<td>12.04</td>
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<td>11.78</td>
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<tr>
<td>Mean</td>
<td>13.60</td>
<td>12.95</td>
<td>15.28</td>
<td>12.15</td>
<td>10.64</td>
<td>14.32</td>
</tr>
<tr>
<td>Std DM</td>
<td>±0.70</td>
<td>±1.38</td>
<td>±0.59</td>
<td>±0.45</td>
<td>±0.43</td>
<td>±0.26</td>
</tr>
</tbody>
</table>
Figure #3

Relations of Glycogen and Nitrogen in *R. cuneata*

from Different Salinities

0 - Glycogen
● - Nitrogen

Values are expressed as % of dry weight.
directly correlated with the osmotic pressure of the environment so that the increase in dry weight of *R. cuneata* from near fresh to near salt water is not amazing. Likewise the increase in nitrogen values based on the wet weight of tissues indicates that water is lost with a concurrent retention of the nitrogenous compounds in the tissues, and the changes observed become those of dehydration and concentration. In view of this observation it is imperative that measurements of amino acids be based on dry weight of tissue in order to determine actual increases of the compounds rather than an increase actually produced by the loss of water.

Boyle and Conway (1941), Hodgkin (1951) and Shaw (1955) have shown that a modified Donnan equilibrium is established by many vertebrates and invertebrates in the maintenance of ion concentrations in striated muscle. The increase in ash weight in *R. cuneata* may result from an increase of inorganic ions in the more saline environments. This increase of inorganic ions was shown to occur in *Anodonta cygnea* by Potts (1958), when this species was placed in higher salinities.

The elevation of nitrogen determined on a dry weight basis is actually an increase due to redistribution, for as has been shown the carbohydrate decreases in the higher
salinities with the apparent increase of nitrogen. In the highest salinity (25 0/00) the values for these two substances tend to readjust to previous concentrations. This suggests that there is a shifting of the carbohydrate concentration which reflects a false increase in nitrogen when both of these substances are reported as per cent of dry weight.

The shift in carbohydrate is interesting. One can give two possible explanations for this. First, carbohydrate may furnish the carbon chains of amino acids, and therefore in the higher salinities where the increase of amino acid concentration is greatest there is a drop in the amount of carbohydrate present in the total animal. Second, the increase in osmotic pressure demands expenditure of energy by the animal in order to maintain an internal concentration equal to the gradient in the environment. This energy necessary to keep the concentration within the tissue equal to that of the environment or more correctly the blood may be supplied by the carbohydrate. However, this problem can only be answered with experimental evidence, and although the results in this study are suggestive of several possibilities no single explanation can be established on the strength of the work reported here.
The effect of increased salinity on the amino acid concentration appears to have a general effect on all the amino acids, and is not selective in any sense except with alanine. Alanine was found to be in highest concentration in each change of environment. When comparing ratios of one amino acid to the other there appears to be no established ratio which holds in each salinity. A general ratio does exist, however, in that the following concentrations were maintained by *R. cuneata* throughout the various salinities; alanine > glycine > glutamic > aspartic. This relationship of one amino acid to the other in regard to concentration may be a very important characteristic of the free amino acid pool of invertebrate species. Yet this also shows that when reporting values for amino acids, in molluscs at least, the environment plays an important role as to their significance, for the values of alanine for *R. cuneata* would be considerably different if collections of specimens were made from two different salinities.

The results of this study indicates, however, that a more basic and important relationship exists between amino acids within one particular species than necessarily occurs between two different species. If the ratios of amino acids
remain anywhere near constant within a species the most im-
portant comparisons will be quantitative rather than a char-
acterization based on the presence of a particular compound.
The only reason that taurine is of importance by its absence
from some molluscs and its presence in others is that this
is associated with one common factor, the salinity of the
environment. From the present study it is now apparent that
the absence of this compound in freshwater forms is due to
some inherent difference in the metabolism in freshwater and
marine species. For this reason a study of the metabolism
of methionine in *R. cuneata* and *M. edulis* was begun and re-
ported in the next section.
CHAPTER IV

TAURINE FORMATION IN R. CUNEATA AND M. EDULIS

1. Introduction

A marine environment has no apparent effect on the ability of *R. cuneata* to maintain or establish sufficient concentrations of taurine in its tissues to be detected by paper chromatography. In view of the results obtained on the amino acids of this animal under varying salinities it is apparent that taurine is either absent or is present in extremely low concentrations. Since taurine is one of the end products of sulfur amino acid metabolism in vertebrates, it was decided to compare the metabolism of sulfur amino acids in two molluscan species in which the concentration of taurine was found to be high in one (*M. edulis*) and absent in the other (*R. cuneata*).

2. Historical

The metabolism of sulfur amino acids with the intermediate formation of taurine has been extensively studied in higher animals. In Figure #4 are shown the reactions for taurine formation from methionine. Reviews on the subject of sulfur amino acid metabolism have been given fairly recently by Greenberg (1954), Singer and Kearney (1955) and
The demethylation of methionine to homocysteine was first demonstrated chemically by Butz and du Vigneaud (1932). At that time they suggested that the demethylation of methionine might be a preliminary step in the utilization of methionine by animals. Later feeding experiments on rats by du Vigneaud and associates (1939a,b,c; 1941) showed that homocysteine could replace the cystine requirement in the diet, but not methionine. Cantoni (1955) remarked that, "One peculiar feature of homocysteine (homocysteine) is the fact that despite all the evidence pointing to its important metabolic function... there is no conclusive evidence that this compound occurs naturally as a free amino acid." However, later work by de la Haba and Cantoni (1959) and Cantoni and Scaran (1954) has shown that the enzymatic transmethyllations of methionine to homocysteine occurs and requires ATP. This is not a phosphrylating reaction. Rather, the ATP forms a S-adenosyl methionine which is chemically active. This latter form of methionine is the one which permits the transfer of the methyl group.

Nutritional studies and isotope experiments by Tarver and Schmidt (1939), Stetten (1942), Binkley et al. (1942) and
du Vigneaud et al. (1944) have shown that only the sulfur of methionine is utilized in the formation of cystine whereas the carbon chain of the latter arises from serine. Further studies on the metabolic reactions involved in the conversion of methionine to cystine (Horowitz, 1947; Binkley, 1951) have shown that the probable pathway of cystine formation from methionine begins with the demethylation of methionine to homocysteine, the condensation of homocysteine with serine to form cystathionine and cleavage of cystathionine to cysteine. The above reactions are collectively termed transulfuration.

The interconversion of cystine and cysteine via hydrolytic dismutation has been suggested by Pirie (1933). It has also been shown by Keilin (1930) and Medes (1939) that this interconversion is catalyzed by cytochrome oxidase in the presence of cytochrome c. It is still doubtful, however, that this enzyme system is essential for the reaction; cysteine is readily oxidized by oxygen and heavy metals to cystine, and likewise cystine is reduced by numerous compounds to cysteine. Thus, the interconversion of these amino acids may depend on the oxidation-reduction state of the cell. In view of this interconversion a common pathway for their oxidation is accepted.
Pirie (1934) demonstrated the formation of inorganic sulfate from cysteine, cystine and methionine in rat liver slices. He postulated that cysteine and cystine are oxidized to cysteinesulfenic acid with an unstable precursor, cysteinesulfenic acid. The postulation of the formation of cysteinesulfenic acid as a major compound in cysteine-cystine oxidation has received considerable support over the years.

Fromageot (1947, 1955) reviews the work on the oxidation of organic sulfur in animals up to that time, and the investigations establishing the central position of cysteinesulfenic acid in cysteine-cystine metabolism are reported in that work.

The oxidation of cysteinesulfenic may take one of three possible routes depending on the animal.

a) Oxidation to Sulfate.

Singer and Kearney (1953) demonstrated from extracts of Proteus vulgaris a cysteinesulfenic acid transaminase which, in the presence of alpha-ketoglutaric acid, formed B-sulfinylpyruvic acid. The latter compound in turn is desulfinated to pyruvate and sulfur dioxide which is further oxidized to sulfate.
b) Oxidation to Cysteic Acid.

Greenberg (1954) describes the oxidation of cysteinesulfinic acid to cysteic acid as an alternate and competing pathway in the metabolism of cysteinesulfinic acid. Awapara and Doctor (1955) demonstrated the formation of cysteic acid and cysteinesulfinic acid in rat liver mitochondria from S-35 cysteine. Singer and Kearney (1953) demonstrated a similar reaction in *Proteus vulgaris*.

c) Decarboxylation to Hypotaurine.

Awapara (1953) and Awapara and Wingo (1953) established the decarboxylation of cysteinesulfinic acid to hypotaurine following injections of cysteine and cysteine S-35 in the rat. No cysteic acid was found in the livers of rats injected with S-35 cysteine, but it appeared in kidneys and spleens of animals injected with cysteine. Chatagner and Bergeret (1951) indicated that a similar if not the same compound as hypotaurine is formed from cysteinesulfinic acid by the action of acetone-dried liver preparation. They also reported the same amino acid compound was formed in the livers of rats receiving cysteinesulfinic acid. The relationship of this compound in the formation of taurine is discussed in the following paragraph.
Taurine formation may follow two different pathways. The first route reported for taurine formation was a cysteic acid decarboxylase. Virtue and Doster-Virtue (1939) demonstrated an increase in taurocholic acid in dogs after feeding cysteic acid and cholic acid. Later Blaschko (1942) showed by in vitro methods a cysteic acid decarboxylase in dog liver. Taurine is also formed from the oxidation of hypotaurine. This second pathway of taurine formation appears actually to be the one of most importance, as indicated from the work of Awapara and Wingo (1953), Bergeret et al. (1952a,b) and Chatagner et al. (1951).

The work reported in the above section was based entirely on experiments performed on vertebrates. The investigation of sulfur metabolism in lower forms does not present such a complete picture. Robin and Roche (1954) reported hypotaurine from a number of coelenterates and sponges. The occurrence of this compound in the different species coincided with the presence or absence of taurine. Hilchey et al. (1955) studied the sulfate metabolism in the German cockroach, Blatella germanica, and reported the formation of radioactive methionine and cystine following injections of radioactive sulfate. No other intermediates were reported
and methionine synthesis was speculated to follow the reversal of the pathway reported for sulfate formation in vertebrates. Shibuya and Shunji (1957) reported the presence of hypotaurine in *Septifer virgatus*, and Ouchi (1959) extended this study by investigating numerous species of molluscs. His results were similar to Robin and Roche in that wherever taurine occurred in high concentrations hypotaurine was detected. This is rather interesting in view of the fact that hypotaurine has never been observed in vertebrate tissue without addition of a precursor. Cotty (1958) studied sulfur metabolism in the house fly, *Musca domestica*, and his observations indicated that taurine was formed from methionine and cysteic acid. When S-35 taurine was fed to flies the radioactivity remained in the taurine molecule. Likewise when S-35 sulfate was placed in the diet only radioactive sulfate was isolated from fly tissues. The formation of taurine was greater when methionine was added to the diet of flies than when cysteic acid was used. These results suggest strongly that the decarboxylation of cysteinesulfinic acid to hypotaurine and the further oxidation of the latter compound to taurine may well be the most important pathway of taurine formation in flies as well as vertebrates.
The diagram in this figure shows the metabolism of methionine to sulfate and taurine. The numbers shown in the Figures are described below under the appropriate number.

Reaction No.

1 - Methionine demethylates to homocysteine.

2 - Homocysteine condenses with serine to form cystathionine. This reaction is referred to as a transulfuration reaction.

3 - Cystathionine is split either by a reductive or hydrolytic reaction to cysteine (cystine).

4 - Cysteine is oxidized to cysteine sulfinic acid.

5 - Cysteine sulfinic acid is decarboxylated to hypotaurine.

5 - Cysteine sulfinic acid is oxidized to cysteic acid.

7 - Cysteine sulfinic undergoes transamination forming B-sulfinyl pyruvate and R-amino acid.

8 - Hypotaurine is oxidized to taurine.

9 - Cysteic acid is decarboxylated to taurine.

10 - B-sulfinyl pyruvate is desulfinated to pyruvic acid and sulfur dioxide.

11 - Sulfur dioxide is further oxidized to sulfate.
In view of these studies on taurine formation in invertebrates methionine S-35 was injected into *M. edulis* and *R. cuneata* to determine if taurine was formed in both animals and if not, to what degree they differed in their metabolic pattern.

3. Materials and Methods

*R. cuneata* and *M. edulis* were injected with S-35 methionine obtained from Abbott Laboratories, Oak Ridge, Tennessee. The methionine was made up to a volume so that 0.1 cc contained 500,000 counts \((2.5 \times 10^4 \text{ counts}/0.1 \text{ mg/min.})\). Each animal throughout these experiments were injected with 500,000 counts of S-35 methionine. The injections were performed on *M. edulis* by first spreading the shell slightly with a wooden wedge. Wound spreaders were then inserted between the open edges of the shell and traction was applied so that the opening was kept at a convenient width for injecting, approximately one quarter of an inch. A 1.0 cc syringe with a 22 gauge needle was used for injections. The foot of the mussel was cautiously moved so that it was in a perpendicular position to the operator and the needle was inserted approximately one quarter of an inch into the foot. Amazingly enough the animal did not react violently to the injection,
but considerable difficulty was encountered in opening the shell.

*R. cuneata* could not be handled in this manner, so that a different approach was used. Large specimens of this species (averaging 5 grams) were used throughout this work. In order to inject these clams their shells were ground on an emery wheel. The clams were pressed against the wheel for short periods of time and then dipped in cold water in order to avoid overheating. This process was repeated until a "pier hole" appeared in the center of the ground area of the shell. The remainder of the area was then easily chipped off with forceps until an area approximately one half inch was uncovered. By this method the lateral edges of the mantle were exposed without injury. From previous dissections the approximate position of the viscera could be determined. The needle was then inserted through the mantle and gill into the fleshy part of the viscera. The injection was made with extreme care so that unnecessary tearing of the tissue was avoided.

Extracts of the injected animals were prepared as described in the section on general methods. The extracts were fractionated on columns of Dowex 50 H⁺ as previously described,
and two dimensional chromatograms were made of the neutral fractions. The acid fractions were run on 1 1/2 inch strips of paper one dimensionally in phenol and the small strips were analyzed on a chromatogram strip counter.\textsuperscript{1} This technique decreased considerably the time involved in obtaining results in contrast to the radioautograms prepared from the two dimensional chromatograms of the neutral fraction. Auto-grams of the neutral fraction were made on Ansco Non-Screen X-ray Film and were exposed for two to three weeks depending on activity.

In some cases the acid fractions were further fraction-ate on Dowex 2 OH\textsuperscript{-} form in order to separate cysteic acid and taurine. The experiments in which this fractionation was used will be so noted in the text.

4. Results.

a) Formation of Cystathionine.

Both M. edulis and R. cuneata were injected with S-35 methionine (approximately 500,000 counts). After twenty-four hours the organisms were extracted and the extracts were fractionated. The neutral fraction was chromatographed from

\textsuperscript{1}Appreciation is expressed to Dr. Robert Hulbert of M. D. Anderson Hospital for the use of the strip counter and analyzer during these experiments.
each species and radioautograms were prepared. The chromatograms were two dimensional with the first solvent being 72% phenol and the second 65% lutidine. The radioautograms of R. cuneata had no cystathionine. However, the exclusion of this compound cannot be accepted as absolute for it may be a transient intermediate in this particular species. The radioautograms of M. edulis showed activity to be located in the following compounds: hypotaurine, cystathionine, methionine and methionine sulfone. Figure #5 shows the radioautogram of M. edulis. The identification of these compounds was established by their Rf values.

b) Formation of Cystine.

Specimens of M. edulis and R. cuneata were injected with S-35 methionine and after five hours were extracted. The extracts were fractionated, and the neutral fractions were used for the identification of cystine. Cystine is very insoluble and lends itself to isolation. The neutral fractions of each species were treated in the following manner: The extract was made up to 1 cc volume to which 3 drops of 30% hydrogen peroxide were added to oxidize all cysteine present. The solution was then taken to near dryness under vacuum. A solution of cold cystine (100 mg.) was made up in 1 cc of
1 N HCl. This acid solution was added to the amino acid extract. A solution of saturated sodium bicarbonate was made and added to the acidified extract drop wise until the solution was neutral. When neutrality was reached a precipitate of white crystals appeared in the solution. Five cc's of ethanol were added and the entire mixture was placed in the refrigerator for several hours. The crystals were removed by filtration, washed in ice cold ethanol and finally in water. They were dried in a desiccator and several milligrams were placed in a planchet and the radioactivity determined in a gas flow counter. The results are shown below.

<table>
<thead>
<tr>
<th>Species</th>
<th>Wgt. of Crystals</th>
<th>Counts/min.</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. edulis</td>
<td>6</td>
<td>406</td>
<td>67.6 c/m/mg</td>
</tr>
<tr>
<td>R. cuneata</td>
<td>5</td>
<td>596</td>
<td>119.2 c/m/mg</td>
</tr>
</tbody>
</table>

Cystine or cysteine is formed in both species. If one assumes that transulfuration takes place in these animals, then cystathionine and cysteine are the intermediates. However, cystathionine was not detected in R. cuneata. If this compound is not formed then another mechanism for the formation of cysteine must be postulated, and no other mechanism
Figure #5

Formation of Cystathionine in *M. edulis*

Following injections of S-35 methionine in *M. edulis* the following radioautogram was obtained. The spot in the lower left hand corner is where the extract was applied for chromatography. P and L represent the direction of the solvent flow for phenol and lutidine respectively.

#1 - methionine

#2 - methionine sulfone

#3 - hypotaurine

#4 - cystathionine
is presently known.

c) Oxidation Products of Cysteine.

Extracts of *R. cuneata* and *M. edulis* were fractionated on Dowex 50 H⁺. The acid fraction from this column was then run through Dowex 2 OH⁻ and two fractions were obtained. The first fraction was made by elution with acetic acid and the second with HCl. The fractions were then chromatographed on narrow strips of paper with standard cysteic acid and cysteinesulfinic acid using 72% phenol. The strips were analyzed with a continuous strip counter and then developed with ninhydrin. Figures #6 and #7 show the results obtained from *R. cuneata*. *M. edulis* gave only one radioactive compound other than methionine and that was taurine. The results show that cysteic acid is formed in rather large quantities in *R. cuneata*. Cysteinesulfinic acid was also formed and a compound with the same Rf as standard taurine appeared in both the acetic acid and HCl acid fractions. If this was taurine the fractionation on Dowex 2 OH⁻ was not complete for there was carryover of this compound in the HCl fraction. A two dimensional chromatogram was run with both eluates and the compound which was suspected of being taurine was confirmed. Since taurine could not be demonstrated in
Figure #6

The figure on the left is the strip of paper used for the chromatogram. The letter "O" represents the origin or area where extract was applied to the paper. The area marked "T" represents the standard taurine and "CA" the standard cysteic acid. The lined block on the right shows the record of the analyzer showing radioactivity in the area of taurine.
Figure #7

This is the same type of diagram as shown in Figure #6.

0 - represents origin of chromatogram

CA - standard cysteic acid

CSA - standard cysteinesulfinic acid

T - taurine
the tissues of this species under normal conditions these results were rather surprising. An experiment was then performed in order to determine the time needed for the formation of these intermediates. S-35 methionine was injected into several specimens of R. cuneata and extracts were made at the end of 1, 5, 10, 15 and 24 hours. The extracts were fractionated and the acid fraction of each chromatographed on narrow strips. Radioautograms were made and the results are shown in Figure #8. Taurine was formed only after cysteinesulfinic and cysteic acid were formed, and it took five hours to form sufficient taurine to be detected. At the end of 10 hours two S-35 labelled compounds appeared. Compound D has an Rf value identical with that of taurocyamine, but gave a negative Sakaguchi test on paper. Another Sakaguchi test was performed on the whole extract and this gave a positive Sakaguchi reaction, showing that a guanidine compound is present. Taurocyamine has a guanidine group present in its structure. At the end of 24 hours most of the end products have disappeared from the animal which shows that taurine is formed but not held.

To determine the length of time R. cuneata will retain taurine, a number of specimens were injected with 5000 counts
Figure #8

Periodic Extracts of *R. cuneata* Following Injection of S-35 Methionine

The letters on the left side of the page represent the following compounds:

A - cysteic acid
B - cysteinesulfinic acid
C - taurine
D - unknown (possibly taurocyamine).

The numbers at the bottom of the picture represent hours of extraction as follows:

1 - Extraction 1 hour following injection
2 - Extraction 5 hours following injection
3 - Extraction 10 hours following injection
4 - Extraction 15 hours following injection
5 - Extraction 24 hours following injection.
of S-35 taurine and extracts were made every 24 hours. The extract without fractionation was counted and the results are shown in Figure #9. Although one may argue that the animal is simply ejecting an exogenous substance which was injected in abnormal amounts, the previous work showing the formation of taurine indicates that this is not the case. What does appear to be true is that taurine is endogenously formed and is rapidly excreted as taurine or some other compound, possibly taurocyamine.

d) Formation of Sulfate and Taurine.

To determine the presence or rather formation of radioactive sulfate in the two species of molluscs under investigation the following procedure was used. Extracts of S-35 methionine-injected *R. cuneata* and *M. edulis* were evaporated to dryness under vacuum and brought up to 50 cc volume. To this solution 1.5 cc of 10% Na₂SO₄ were added. The mixture was heated near boiling on a hot plate. Hydrochloric acid (0.1 N) was added until the solution was acidic. Saturated BaCl₂ was added slowly while the solution was still hot. A white precipitate formed, and the BaCl₂ was added drop-wise until 5 cc's had been delivered. The solution was then placed on a steam bath for one hour after which time it
Figure #9

Decrease of Activity Following Injection of
S-35 Taurine

(Total Counts Injected 5000)

The counts represented in this graph are
total counts taken from unfractionated amino acid
extracts. The points on the graph are averages
of counts taken from aliquots of individual amino
acid extracts.
was tested with more BaCl₂ to determine the completeness of the precipitation. The precipitate was then removed from the solution by filtration, was washed with hot water followed by a dilute solution of BaCl₂. A final wash with hot water in order to remove excess BaCl₂ was also performed. The filter paper containing the precipitate was dried in an oven at 100°C, and then ashed in a muffle furnace at 1000°C. The activity of the isolated barium sulfate was determined on a gas flow counter. The results are shown below.

<table>
<thead>
<tr>
<th>Species</th>
<th>Wgt. of Tissue in Extract</th>
<th>Counts Injected</th>
<th>c/m minus Bkg of Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. edulis</td>
<td>3.3 g</td>
<td>500,000</td>
<td>35</td>
</tr>
<tr>
<td>R. cuneata</td>
<td>2.5 g</td>
<td>500,000</td>
<td>35</td>
</tr>
</tbody>
</table>

The formation of taurine from methionine S-35 has already been shown for R. cuneata. M. edulis on the other hand contains very large amounts of taurine and demonstration of its formation from administered precursors would be difficult. The approach which appeared to be the most feasible was to isolate it from the animal. Specimens of M. edulis were injected with 500,000 counts of S-35 methionine and extracts were made 48 hours following injections. The extracts were fractionated on Dowex 50 H⁺. The acid fraction was used for
isolation of taurine. The acid fraction was first reduced in volume under vacuum to approximately 1 cc. Ice cold ethanol was added slowly with the simultaneous formation of small crystals. After adding approximately 5 cc of ethanol the solution containing the crystals was placed in the refrigerator for several hours. The solution was then filtered, the crystals were dried in a desiccator under vacuum, and then recrystallized from glass distilled water. Chromatograms of the isolated material showed the crystals to be taurine, and an infra red analysis was run\(^1\) to determine the purity of the isolated taurine. The results of the infra red analysis are shown in Figure #10. The isolated taurine was pure. The activity of this compound is shown in the chart below. In view of the low activity of the isolated taurine more supporting evidence was needed in order to establish that the activity observed was actually from taurine and not a contaminant. Further evidence was obtained by isotope dilution. The isolated taurine was mixed with equal quantities of unlabelled taurine, dissolved in water and then recrystallized with alcohol. Following recrystallization the activity

\(^1\)Thanks to Dr. T. Patton, M. D. Anderson, for the infra red analysis.
of the unlabelled-labelled taurine mixture was determined as shown below.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount Counted</th>
<th>Counts/min. minus Bkg.</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-35 taurine</td>
<td>6 mg</td>
<td>40</td>
<td>6.6 c/min/mg</td>
</tr>
<tr>
<td>S-35 taurine plus unlabelled taurine</td>
<td>12 mg</td>
<td>45</td>
<td>3.8 c/min/mg</td>
</tr>
</tbody>
</table>

The specific activity of taurine was so low in this experiment that the results appear to be insignificant. To see if this is just a function of metabolic rate two specimens of this mussel were injected as above, but left for five days prior to extraction. The results are shown below.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount Counted</th>
<th>Counts/min. minus Bkg.</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-35 taurine</td>
<td>6 mg</td>
<td>160</td>
<td>26.6</td>
</tr>
<tr>
<td>S-35 taurine plus unlabelled taurine</td>
<td>12 mg</td>
<td>145</td>
<td>12.2</td>
</tr>
</tbody>
</table>

The crystals isolated from the second group of mussels were chromatographically pure, and the specific activity was increased to the point where there is little doubt that taurine had been formed in the injected animal. The counts for taurine formed from S-35 methionine are low, but the large
Figure #10

Infra red Analysis of Isolated S-35 Taurine
from *M. edulis* and Cold Taurine

A - Standard cold taurine

B - Isolated S-35 taurine from *M. edulis*
quantities of endogenous taurine in this species (approximately 4% of wet tissue weight) adds considerable difficulty in determining either rate or amount of this compound which is formed. There is the added difficulty that the isotope used for the number of steps involved in the formation of taurine increases the dilution of the final product. It is felt that the results obtained in the above experiment are not doubtful and that taurine was formed from the S-35 methionine which was injected.

An earlier experiment performed on *M. edulis* involved the injection of S-35 taurine to determine the efficiency of the injection technique. At the time this experiment was performed no particular metabolic significance was attached to it. When it was recognized that *R. cuneata* forms taurine but excretes it rather rapidly, the injection of taurine in *M. edulis* assumed added significance. Several specimens of *M. edulis* were injected with S-35 taurine and after 10, 24, 48 and 72 hours extracts were prepared. Two dimensional chromatograms were made from the whole extract without fractionation, and radioautograms were made. The results showed that taurine was not metabolized to any other compound, and visual inspection indicated that the amount of activity ap-
pearing on films from the various hourly extractions did not decrease. These results show that taurine in *M. edulis* unlike taurine in *R. cuneata* is apparently an end product and that it is stored rather than excreted as in the latter species.

5. Discussion

The results of this study show that taurine is formed from methionine in both *M. edulis* and *R. cuneata*. The main difference in metabolism appears to be in the formation of hypotaurine. *M. edulis* had been reported by Ouchi (1958) as one of the molluscs in which he demonstrated the presence of hypotaurine chromatographically. In the present study the chromatographic evidence for this compound was weak but radioautographs following S-35 methionine injections gave faint spots of activity in the area of hypotaurine. In this same species cysteic acid could not be demonstrated. However, in *R. cuneata* large amounts of cysteic acid were detected both chromatographically and on radioautographs. In this latter species hypotaurine never appeared even after administration of S-35 methionine. On the basis of these results it appears that taurine formation in *M. edulis* follows the
hypotaurine pathway while taurine in R. cuneata is formed via cysteic acid.

The major point investigated in this study was whether or not R. cuneata forms taurine. The positive answer to this question poses another involving the fate of metabolically formed taurine. From these studies it appears that taurine formation in the brackish-water species is rather rapid and that it is further metabolized to a Sakaguchi positive compound, possibly taurocyamine, and then either is lost through the excretory system or is simply excreted through the body surface. In contrast the formation of taurine is slow in M. edulis, but once it is formed it is no longer metabolically active, and apparently plays a very strong role in osmoregulation. The predominance of alanine as the amino acid in highest concentration in R. cuneata resembles the role of taurine in M. edulis.

On the other hand the comparison of the two species indicates a difference of extreme interest. For if the absence of taurine in all freshwater species studied to date is in reality one of rapid turnover and then loss, a relationship on the basis of activity of cysteic acid decarboxylase may well be of considerably more importance in estab-
lishing relationships between freshwater and marine molluscs than any method we have to date. The reasons why there should be this difference between such forms demand a great deal more study. It does appear now that the small carbon chain compounds postulated so long ago by Fredericq as important factors in osmoregulation have taken on identity as amino acids. Why certain species from different environments will have one main amino acid in extremely high concentration compared to the others in the pool has yet to be determined.
SUMMARY AND CONCLUSIONS

It has been shown in this study that taurine is absent in a great number of molluscs all of which are freshwater or terrestrial inhabitants. Such a phenomenon has been shown to be due to the retention of taurine in the marine species M. edulis while taurine is formed and eliminated from the brackish-water species R. cuneata.

The effect of increased salinity on brackish-water molluscs is a loss of water from the tissues, and an increase in the amino acid concentration. The marine environment did not effectuate the production of taurine to measurable levels in R. cuneata. However, the marine environment produced a higher concentration of amino acids. The increased concentration of amino acids followed a definite pattern which was already established prior to the change into higher salinities. The pattern for R. cuneata is alanine > glycine > glutamic > aspartic regardless of environment.

The major difference between sulfur metabolism in freshwater molluscs and salt-water forms is that taurine is apparently formed from hypotaurine in marine species while in the freshwater form it is produced from the decarboxylation of cysteic acid. In R. cuneata taurine is metabolized to
another compound, taurocyamine, while in *M. edulis* it is an end product of sulfur metabolism. Thus the amino acid which is maintained in high concentration is alanine in *R. cuneata* in contrast to taurine in *M. edulis*. The role of amino acids in osmoregulation has been discussed in the text.
LITERATURE CITED


Clementi, A. 1913 See Baldwin, E. 1935a


Krebs, H. A., and Henseleit, K. 1932 Untersuchungen über
die Harnstoffbildung im Tierkörper. Zeit. f. physiol.

Krogh, A. 1939 Osmotic Regulation in Aquatic Animals. Cam-
bridge Univ. Press.

Kutscher, F., and Ackerman, D. 1926 Vergleichend-physiologis-
che Untersuchungen von extrakten verschiedener Tierklas-
sen auf tierische alkaloiide eine Zusammenfassung.

Lal, M. B., and Saxena, B. B. 1952 Uricotelism in Pila

Lewis, P. R. 1952 The free amino acids of invertebrate nerve.

Mead, A. R., and Kemmerer, R. A. 1953 Amino acid content of
117: 138-139.

Meenakshi, V. R. 1955 Protein catabolism in developing eggs

Meenakshi, V. R. 1956 Physiology of the hibernation of the

Medes, G. 1939 Metabolism of sulfur. VIII. Oxidation of the
sulfur-containing amino acids by enzymes from the liv-

Meister, A. 1957 The Biochemistry of the Amino Acids. Aca-

Melnick, S. C. 1958 Occurrence of collagen in the phylum

Mendal, L. B., and Bradley, H. C. 1906 Experimental studies
on the physiology of the molluscs. Amer. Journ. Physi-
ol. 17: 167-176.


Page, I. H. 1958 Serotonin (5-hydroxytryptamine); the last four years. Physiol. Rev. 38: 277-335.


Roche, J., Thoai, N., and Glahn, P. E. 1952 Sur la L-amino
cide oxydase de nombreux invertébrés marins. Ex-
perientia 8: 428-429.

Roe, J. H. 1955 The determination of sugar in blood and
Chem. 212: 335-343.

Saxena, B. B. 1953 Arginase and uric acid in Achatina fulica.

Saxena, B. B. 1956 Excretory constituents of the blood of
Pila globosa. Arch. Intern. Physiol. et Biochim. 61:
578-582.

Sereni, E. 1930 The chromatophores of cephalopods. Biol.

Shaw, J. 1955 Ionic regulation in the muscle fibres of Car-

Shaw, J. 1958a Osmoregulation in the muscle fibers of Car-

Shaw, J. 1958b Further studies on ionic regulation in the
35: 902-919.

Shaw, J. 1959a Solute and water balance in the muscle fibers
of the east African fresh water crab, Potamon niloticus.

Shibuya, S., and Shunji, O. 1957 Isolation of 2-aminoethane-

Simpson, W. J. 1959 A comparative study of free amino acids
in marine invertebrates. Master's Thesis, Rice Insti-
tute, Houston, Texas.

Simpson, J., and Allen, K. W., and Awapara, J. 1959 A quanti-
tative study of the amino acid concentration in inverte-


APPENDIX

The Isolation of $\beta$-aminoisobutyric Acid from *Mytilus edulis*

1. Introduction

While running two dimensional paper chromatograms of the free amino acids of *M. edulis* preparatory for studies on sulfur metabolism, a compound with the same Rf values as $\beta$-aminoisobutyric acid appeared on the chromatograms. In view of its significance in pyrimidine metabolism in higher animals and because it had not previously been reported in invertebrates, it was felt that it would be of importance to establish the identity of this compound.

Fink (1951a) observed an unidentified ninhydrin positive compound in human urine which appeared from their studies to be related to cancer. Later the same year (1951b) Fink *et al.* studied the excretion of this compound in normal and cancerous patients. Preliminary studies on the metabolism of $\beta$-aminoisobutyric acid (BAIB) by these workers indicated that a 5-methyl pyrimidine was its precursor. Independently Crump-ler *et al.* (1951) reported the occurrence of BAIB in human urine, and based on the number of individuals in their study it appeared that the excretion of this amino acid may be genetically controlled.
The early evidence concerning the formation of BAIB in animals was based on diet studies in rats by Fink et al. (1951b). Rats normally do not have BAIB in their urine in detectable quantities, but when animals were fed diets containing high quantities of DNA and thymine, BAIB was excreted in the urine. Fink et al. (1952) continued their studies on the formation of BAIB in rats and found that dihydrothymine increased the production of BAIB above that which was observed when thymine alone was fed to the animals. The next year, 1953, Fink et al. studied BAIB formation in vitro and results with dihydrothymine concurred with the in vivo studies of a year earlier. From this evidence they postulated that thymine was reduced to dihydrothymine with the subsequent hydrolysis of the latter to ureido BAIB, followed by the production of BAIB. Canellakis (1956) examined this pathway in vitro in rats and found the first reduction step to be TPNH dependent.

Reinvestigating the possibility of other intermediates in the formation of BAIB, Fink et al. (1956) found supernatent fluids of rat liver homogenates capable of enzymatically converting dihydrothymine to carbamyl β-aminoisobutyric acid. Wallach et al. (1957) purified the enzyme which converted
dihydrothymine to carbamyl $\beta$-aminoisobutyric acid from calf livers and found that this enzyme was also capable of converting dihydrouracil to carbamyl $\beta$-alanine. The enzyme was called hydropyrimidine hydrase. The postulated reductive pathway of pyrimidine degradation to BAIB is shown below.

Studies by Awapara and Shullenberger (1957) showed that leukemic patients excreted large quantities of this amino acid only when given nitrogen mustard (methyl-bis[$\beta$-chloroethyl]amine hydrochloride) or thymine. Determination of the presence of this compound in other animal tissues has been done by Campbell (personal communication) in which he demonstrated BAIB to be present in the tapeworms Moniezia expansa and Cittotaenia perplexa. Other reports from animal and
plant tissue are reviewed by Meister (1957). Asen et al. (1959) isolated this \( \beta \)-amino acid from the bulbs of Iris tingitana. Campbell (personal communication) found BAIB in the free living turbellarians Bdelloura candida, the parasitic trematodes Macraspis cristata and Entobdella bumpusi as well as numerous cestodes. In this work the author studied the formation of \( \beta \)-alanine and BAIB via the pyrimidine reductive pathway described earlier in this section. The production of \( \beta \)-amino acids followed the pathway described from higher animals.

2. Methods

Following the tentative identification of the ninhydrin positive compound on chromatograms of M. edulis as BAIB, 100 grams of pooled M. edulis were extracted with 80% ethanol. The isolation of the amino acid fraction followed the procedure described in the section on general methods. Following the extraction procedure the water extract was treated first with Amberlite CG -50 H\(^+\) to remove basic compounds. The eluate collected from this resin column was placed on a column containing Dowex 50 H\(^+\) in order to remove the large quantity of taurine and other strongly acidic substances. The amino acids which remained on the resin were displaced from
the column with 4 N NH₄OH. The effluent was evaporated to
dryness under vacuo. The residue was dissolved in 4 ml of
glass distilled water, and decolorized with activated char-
coal.

Before isolating the compound suspected of being BAIB
another test was performed. Aliquots of the neutral fraction
of amino acids from M. edulis were spotted on two separate
papers. On one paper 0.25 μMoles of standard BAIB were added
to the spot containing the extract. The same amount of stand-
ard was added to the same paper parallel with the origin, but
to the far right side of the paper. To the second paper the
standard solution was added only on the extreme right side
of the paper. The chromatograms were developed two dimen-
sionally, but the lutidine front was not allowed to reach the
area where the standard solution was paralleling the extract
in phenol. The results showed an increase in concentration
in the ninhydrin reacting substance suspected of being BAIB
in the paper which had the standard solution added at the ori-
gin. The Rf of the standard on the right side of the paper
was the same as the suspected compound in phenol.

To isolate the unknown from all other ninhydrin posi-
tive compounds the entire extract was streaked along the lower
edge of Whatman filter paper #4 with one edge of the paper containing standard BAIB. This paper was chromatographed in phenol. Approximately three inches of the paper containing the standard BAIB and a small edge with the extract were cut off and developed in 0.5% ninhydrin in acetone. The area corresponding to the standard was then cut out from the entire chromatogram and eluted with 50% ethanol. Several chromatograms were prepared in this manner in order to accommodate the entire extract. The ethanol eluates were pooled and the entire process was repeated until one ninhydrin spot of the same Rf as the standard amino acid was obtained.

3. Results

The Rf values of the isolated compound and standard \( \beta \)-amino isobutyric acid are shown in Table #9.

Table #9

Rf Values of Standard BAIB and Unknown from *M. edulis*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Std. BAIB</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>65% Lutidine</td>
<td>.34</td>
<td>.34</td>
</tr>
<tr>
<td>75% Phenol</td>
<td>.58</td>
<td>.58</td>
</tr>
<tr>
<td>Butanol: Acetic Acid: Water 120:30:50</td>
<td>.43</td>
<td>.43</td>
</tr>
<tr>
<td>Butanol: Formic Acid: Water 75:15:10</td>
<td>.48</td>
<td>.48</td>
</tr>
</tbody>
</table>
Table #10 shows the Rf values for the two compounds after DNP derivatives were made and run in four different solvents.

Table #10

Rf Values for DNP Derivatives of Standard BAIB and Unknown from *M. edulis*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Std. BAIB</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanol: Ethanol: Water 40:10:50</td>
<td>.71</td>
<td>.72</td>
</tr>
<tr>
<td>Water saturated Butanol</td>
<td>.53</td>
<td>.53</td>
</tr>
<tr>
<td>Butanol saturated with 1% ammonia</td>
<td>.40</td>
<td>.41</td>
</tr>
<tr>
<td>Cresol saturated with 0.3% ammonia</td>
<td>.74</td>
<td>.74</td>
</tr>
</tbody>
</table>

From these results it was concluded that the unknown ninhydrin positive compound in *M. edulis* was $\beta$-aminoisobutyric acid. *Volsella dimissus*, another mussel which for a long time was considered as belonging to the same genus, was examined chromatographically and a ninhydrin spot located in the area of BAIB was found to be present. Chromatograms showing the location of BAIB are shown in Figures #11 and #12.
Figure #11

Chromatogram of *Mytilus edulis* Showing $\beta$-aminoisobutyric Acid.

1. Aspartic acid
2. Cysteic acid
3. Ethanolamine phosphate (?)
4. Glutamic acid
5. Glycine
6. Serine
7. Taurine
8. Unknown (Basic compound)
9. Glutamine/Citrulline
10. $\beta$-alanine
11. Threonine
12. Arginine (Basic compound)
13. Proline
14. $\beta$-aminoisobutyric acid
15. Valine
16. Methionine
17. Tyrosine
Figure #12

Amino Acid of *Volsella dimissus*

1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Glycine
5. Serine
6. Taurine
7. Glutamine/Citrulline
3. $\beta$-alanine
9. $\alpha$-alanine
10. Threonine
11. Arginine (Basic compound)
12. Proline
13. $\beta$-aminoisobutyric acid
14. Valine
15. Unknown
16. Tyrosine
17. Methionine
The quantity of BAIB in various organs of *M. edulis* are tabulated below in Table #11.

Table #11

<table>
<thead>
<tr>
<th>Organ</th>
<th>µMoles/100 gms Fresh Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mantle</td>
<td>44</td>
</tr>
<tr>
<td>Gill</td>
<td>103</td>
</tr>
<tr>
<td>Viscera</td>
<td>52</td>
</tr>
<tr>
<td>Foot</td>
<td>93</td>
</tr>
</tbody>
</table>

**Literature Cited**

References used in this section are included in the bibliography.