EFFECT OF ENVIRONMENTAL SALINITY ON THE AMINO ACIDS OF *BALANUS IMPROVISUS* DARWIN

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ABSTRACT

Free amino acid composition of adductor and depressor muscles of the common euryhaline barnacle, Balanus improvisus, was studied at low (3 to 4 o/oo) and at high (28 to 30 o/oo) salinities by ion exchange chromatography. Of the 16 amino acids identified the mean concentrations of all but one, tyrosine, differed significantly at the two salinities. Total free amino acid concentration more than tripled with an increased environmental salinity of approximately 25 o/oo. Salinity affected not only concentration but also composition of the intracellular amino acid pool. Concentrations of amino acids were unequal as evidenced by a contribution to the total amino acid pool of 82 to 90% by glycine, alanine, proline, arginine, taurine and glutamic acid. Results were compared with relative composition of tissues of other crustaceans. The conclusion was made that in barnacles, as in the decapods, intracellular amino acids may fulfill an osmoregulatory role.

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INTRODUCTION

The euryhaline barnacle *Balanus improvisus* has been reported not only from sea water but also from areas where water is completely fresh at low tide (Pilsbry 1916) or for as long as ten months of the year (Newman 1954). More recently, however, Newman (1962) observed that this species does not occur on the outer coast, at least in the Pacific. Its distribution is worldwide in temperate and tropical waters (Zullo 1963). On the Atlantic Coast it has been recorded from Canada and the Gulf of Maine (Bousfield 1954) and from the Chesapeake Bay (Pilsbry 1916), both intertidally and subtidally (Newman 1962).

In addition to its wide distribution, physiological tests provide further evidence of the strongly euryhaline character of *B. improvisus*. Freezing point depression studies of blood indicate that this barnacle is an osmoconformer, the blood being only slightly hypertonic to salinities down to 1 o/oo in which this species can survive indefinitely (Newman 1962). Survival of *B. improvisus* at low salinities without retaining an appreciable quantity of serum salts leads to the questions: (1) What happens to osmotically active intracellular constituents? (2) Do these constituents fluctuate with changes in salinity?

Literature on the probable osmoregulatory function of free amino acids has recently been reviewed by Awapara (1962) and Potts and Parry (1964). As early as 1904, Fredericq postulated the presence of substantially large quantities of small organic molecules to counterbalance the high inorganic ion concentration of the blood. This postulation was based on his discovery that, although tissues and blood are in osmotic equilibrium, the concentration of inorganic ions is low in the former. In the late nineteenth and twentieth centuries, relatively large quantities of free amino acids, particularly taurine, glycine and arginine, were isolated from invertebrate tissues and were identified primarily by German investigators (Chittenden 1875; Kelly 1904; Kutscher and Ackerman 1926; and others). Indeed, taurine was isolated in 1845 by Karsten but was not reported until 1906 (Mendel and Bradley). Little consideration was given to Fredericq's postulation, however, until the advent of microbiological assay procedures. Microbiological assay involves the use of microorganisms in the qualitative and quantitative estimation of amino acids. Microorganisms require certain amino acids. If fed a synthetic medium lacking an amino acid but supplemented by an unknown solution, the quantity of the missing amino acid can be determined by comparing growth in the presence of the solution to be analyzed to growth in the presence of known amounts of the amino acid in question. Noland (1949) used this technique to quantitatively estimate 18 amino acids in blood and muscles of animals from 5 invertebrate phyla. Camien *et al.* (1951), using the same analytical procedure, determined the concentration of free amino acids in marine and fresh water crustaceans. This comprehensive work stimulated interest in the relationship between free amino acids and salinity.

In 1955 Kermack, Lees and Wood, estimating total α-amino nitrogen (excluding taurine) and not considering salinity, accounted for 84% of non-protein nitrogen (NPN) in lobster (*Homarus vulgaris*) muscle. Their results indicated that approximately 42% of NPN was the α-amino type. The importance of α-amino nitrogen in osmoregulation is evident since it accounted for a large percentage of NPN and exhibited the greatest variation. Without salinity data, however, no correlation with salinity could be made as a feasible explanation for variation in free amino acids.

Shaw (1958) conducted a study of total nitrogen, particularly α-amino nitrogen and taurine, of *Carcinus maenas* muscle. He subjected crabs to normal and 40% sea water and found that the change in concentration was too great to be explained by hydration. Inorganic salts, on the other hand, were simply diluted and tissues and blood were isosmotic.

Individual amino acids were also estimated by chromatography. Belgian biologists attempted to ascertain the osmoregulatory role of amino acids in tissues of euryhaline decapods by altering environmental salinity. Some decapods studied were *Eriocheir sinensis* (Duchâteau and Florkin, 1955 and 1956; Bricteux-Grégoire *et al*. 1962; Duchâteau-Bosson and Florkin 1962), *Carcinus maenas* (Duchâteau and Florkin 1956; Duchâteau, Florkin and Jeuniaux 1959), *Leander serratus* and *Leander squilla* (Jeuniaux, Bricteux-Grégoire and Florkin 1961) and *Astacus astacus* (Duchâteau-Bosson and Florkin 1961). Florkin (1962) stated that all euryhaline invertebrates so far studied exhibited intracellular isosmoregulation whether body fluids regulated or conformed to the external medium.

This investigation is concerned with the differences in free amino acid concentration in muscle of barnacles taken from localities in which the salinity was known to be high or low. Barnacle muscle was chosen both for comparative purposes, since much of the previous work was done on decapod muscles, and because little or nothing had been published on the amino acid composition of muscles of crustaceans other than decapods. A euryhaline species was required and _B_. _improvisus_ was selected rather than the larger _B_. _eburneus_ because previously mentioned work of Newman (1962) indicated that the former can survive indefinitely in 1 o/oo salinity without retaining an appreciable quantity of serum salts.

MATERIALS AND METHODS

Collection and Maintenance

Barnacles of low and medium-salinity waters were collected from oysters dredged in the James River. High salinity specimens were gathered during low tide at Virginia Beach, Virginia, from mussels attached to pier pilings. All collections were made in winter. Water samples were taken at the site of collection for the purpose of maintaining animals and for determining salinity with an RS-7A salinometer (Industrial Instruments Inc.). With the exception of three Virginia Beach samples, animals were kept moist and transported to the laboratory at above freezing temperatures. Both water and animals were placed in the cold room overnight at 5 to 7°C. Virginia Beach barnacles not transported in the above manner were covered with sea water to prevent overheating on that particularly warm day. The following morning the oysters or mussels were sorted and those with the largest barnacles of the desired species were cleaned with a toothbrush and placed in small buckets containing water from the collection site. In most cases, soft tissues of the oysters or mussels were removed prior to placement of the shells in water. Barnacles were maintained at winter water temperatures (6 to 10°C) for 2 to 7 days prior to dissection by surrounding the buckets with running river water. The water was generally changed one or more times daily.

Keys of Zullo (1963) and Pilsbry (1916) were employed in identification.

Dissection

Barnacles displaying cirral motion were marked with a red pencil to designate suitability for dissection. Those measuring 8 to 16 mm in basal diameter were selected from marked individuals and the adductor and depressor muscles of the opercular valves were carefully removed. Contamination with mantle and ovigerous tissues was held to a minimum by exercising extreme caution in dissection. Muscles were transferred immediately to a centrifuge tube containing 1.5 to 2 ml of glass-distilled water precooled in an ice bath. Each barnacle required approximately 10 to 15 minutes dissecting time.

When the quantity of tissue was sufficient for a single analysis (11 to 17 barnacles), the sample was transferred from ice bath or freezer to boiling water bath for 5 minutes to denature the enzymes. Samples that were to be frozen overnight before completion of dissection were similarly boiled. All samples were frozen at -16°C prior to processing for analysis of the free amino acid fraction. If they were to be stored long, they were stored under toluene to prevent bacterial growth.

Processing Samples

Samples were homogenized with a Model LS 75 Sonifier (Branson Instruments Inc.) employing a microtip. The instrument was tuned, with caution, to peak power to achieve maximum disintegration yet to avoid frothing. An aliquot of the resulting homogenate (approximately 0.5 ml) was removed for determination of total Kjeldahl nitrogen (TKN). The remainder of the sample was deproteinized for free amino acid analysis by diluting 1:1 with 10% sulfosalicylic acid (modification of the procedure of Scharff and Wool 1964). The sample was shaken and centrifuged at 1,000 x G for 15 minutes at 0°C. The free amino acid fraction (supernatant) was removed by disposable bulb pipette and stored in the freezer until analysis.

Total Kjeldahl Nitrogen

The nature of dissection was such that the minute quantities of tissue procured were subject to varying degrees of desiccation eliminating the possibility of expressing the quantity of tissue by direct measurement of wet weight. For the purpose of estimating tissue quantity, a modification of the micro-Kjeldahl technique of Hawk, Oser and Summerson (1954) was used to determine TKN.

Procedure: The homogenate or the standard glycine solution was pipetted into a small Kjeldahl tube containing two glass beads. Following the addition of 1 ml of concentrated H_2SO_4 to each tube, including a reagent blank, the samples were digested for one hour on Kjeldahl burners. One to three drops of 30% H_2O_2 were added to each tube one drop at a time until the contents became colorless. Heating was continued for a few minutes to hasten oxidation of color products.

Digested samples were cooled, diluted to the calibrated 20 ml mark with glass-distilled water, and shaken. One milliliter of diluted sample plus 4 ml of glass-distilled water were pipetted into a small test tube. Two milliliters of Koch and McMeekin

Nessler reagent (Hawk, Oser and Summerson 1954, p. 1329) were added and the test tube was shaken briefly. Samples were read immediately at a wavelength of 425 mμ using a two-cell colorimeter (Fisher Scientific Co.). Immediate readings are required since Nessler color reaction is affected by time.

Analysis of Free Amino Acids

Analysis of the amino acid fraction was achieved by means of semiautomatic ion exchange chromatography employing the Amino Acid AutoAnalyzer^R available October 1964 (Technicon Instruments Corp.). The modules and technique have been described elsewhere (Technical manual, Amino Acid AutoAnalyzer, Technicon Instruments Corp.) hence only pertinent specifications will be mentioned here. The AutoAnalyzer^R, originally based on the system developed by Spackman, Stein and Moore (1958), had by this time evolved into a device applying the gradient elution theory of Peterson and Sober (1959) to a single column (Piez and Morris 1960). The 0.6 x 125 cm column was packed with spherical Type B Chromobead^R resin and was operated at 60°C and 150 to 250 psi pressure. The sample and norleucine internal standard, in 12.5% sucrose, were applied to the column by a 1 or 2 ml syringe through a Sample Injection Device^R. Sodium citrate buffers were introduced to the column at the rate of 0.50 ml/min from a nine-chamber Autograd^R producing a continuous pH gradient of 2.875 to 5.00 and a sodium ion concentration of 0.2 to 0.8 M. The sample coming off the column was reacted with ninhydrin and read at 440 and 570 mμ through 15 cm continuous flow cuvettes. The entire analysis required 22 hours.

Amino acids were identified solely on the basis of standard chromatograms. The standard solution contained 17 amino acids plus NH_3 (Technicon Chemical Corp.) and 5 other amino acids were added. Concentrations of amino acids were determined by comparing the area under the peak of a certain amino acid in the barnacle extract to the area under the peak of the same amino acid on the standard chromatogram. The H x W method of Spackman, Stein and Moore (1958) was used to integrate the peaks.

RESULTS

Chromatograms were made of extracts from barnacles maintained for two to seven days in low (3 to 4 o/oo), intermediate (16 to 18 o/oo) and high salinity water (28 to 30 o/oo). Technical difficulties in early experiments prevented the acquisition of accurate data and necessitated the elimination of all data on intracellular amino acids of barnacles kept at intermediate salinities as well as a portion of similar data on barnacles maintained at other salinities. Mean values for each amino acid in µM/mg TKN were calculated from three chromatograms at high and three at low salinities (Fig. 1, Tables I and II). The "t" test was employed to determine if means for a particular amino acid differed significantly at the two salinities (Table II). Significant differences at $P < 0.05$ were found for all amino acids except tyrosine. At the above salinities the most highly significant mean concentration differences ($P < 0.001$) were those of threonine, isoleucine, glycine, leucine, alanine and glutamic acid in that hierarchy. With the exception of tyrosine, lysine and arginine, the means increased, often considerably, at the high salinity. As a result, total mean amino acid concentration more than tripled (2.126 µM/mg TKN at 3 to 4 o/oo to 7.501 µM/mg TKN at 28 to 30 o/oo) with a salinity increment of approximately 25 o/oo. Expressed as percentage TKN (including protein nitrogen), the amino acid nitrogen accounted for 6.02 and 12.63% at the low and high salinities, respectively.

TABLE I

FREE AMINO ACID CONCENTRATION IN μM/mg TKN AT TWO SALINITIES

	3-4 o/oo					28-30 o/oo				
Amino Acid	Replications			$\overline{X}$	S	Replications			$\overline{X}$	S
Taurine	0.278	0.284	0.276	0.279	0	1.473	1.452	0.929	1.285	0.308
Aspartic acid	0.037	0.036	0.041	0.038	0.003	0.071	0.093	0.059	0.074	0.017
Threonine	0.018	0.027	0.028	0.024	0.006	0.115	0.115	0.108	0.113	0.004
Serine	0.064	0.067	0.063	0.065	0.002	0.164	0.159	0.135	0.153	0.015
Glutamic acid	0.166	0.174	0.163	0.168	0.005	0.341	0.310	0.374	0.342	0.032
Proline	0.035	0.026	0.026	0.029	0.005	1.683	1.486	1.847	1.672	0.181
Glycine	0.179	0.126	0.077	0.127	0.051	1.597	1.202	1.325	1.375	0.207
Alanine	0.537	0.479	0.384	0.467	0.077	1.734	1.401	1.717	1.617	0.188
Valine	0.023	0.023	0.024	0.023	0.001	0.102	0.094	0.102	0.099	0.005
Isoleucine	0.009	0.009	0.017	0.012	0.005	0.049	0.046	0.043	0.046	0.003
Leucine	0.014	0.014	0.019	0.016	0.003	0.067	0.054	0.063	0.061	0.007

(Table I continued next page)

TABLE I. (continued)

	3-4 o/oo					28-30 o/oo				
Amino Acid	Replications			$\overline{X}$	S	Replications			$\overline{X}$	S
Tyrosine	0.076	0.050	0.090	0.072	0.021	0.074	0.064	0.064	0.067	0.006
Phenylalanine	0.015	0.010	0.016	0.014	0.003	0.031	0.024	0.026	0.027	0.003
Lysine	0.091	0.086	0.084	0.087	0.003	0.081	0.064	0.070	0.072	0.009
Histidine	0.023	0.023	0.029	0.025	0.004	0.049	0.053	0.039	0.047	0.007
Arginine	0.664	0.689	0.688	0.680	0.014	0.504	0.463	0.386	0.451	0.019
Total	2.229	2.123	2.025	2.126		8.135	7.080	7.287	7.501	

Fig. 1. Concentrations of free amino acids at two collection salinities.

TABLE II

MEAN FREE AMINO ACID CONCENTRATION IN µM/mg TKN AT TWO SALINITIES

Amino Acid	3-4 o/oo	P <	28-30 o/oo
Taurine	0.279	0.050	1.285
Aspartic acid	0.038	0.025	0.074
Threonine	0.024	0.001	0.113
Serine	0.065	0.025	0.153
Glutamic acid	0.168	0.001	0.342
Proline	0.029	0.005	1.672
Glycine	0.127	0.001	1.375
Alanine	0.467	0.001	1.617
Valine	0.023	0.005	0.099
Isoleucine	0.012	0.001	0.046
Leucine	0.016	0.001	0.061
Tyrosine	0.072	N.S.	0.067
Phenylalanine	0.014	0.010	0.027
Lysine	0.087	0.050	0.072
Histidine	0.025	0.010	0.047
Arginine	0.680	0.005	0.451
Total	2.126		7.501

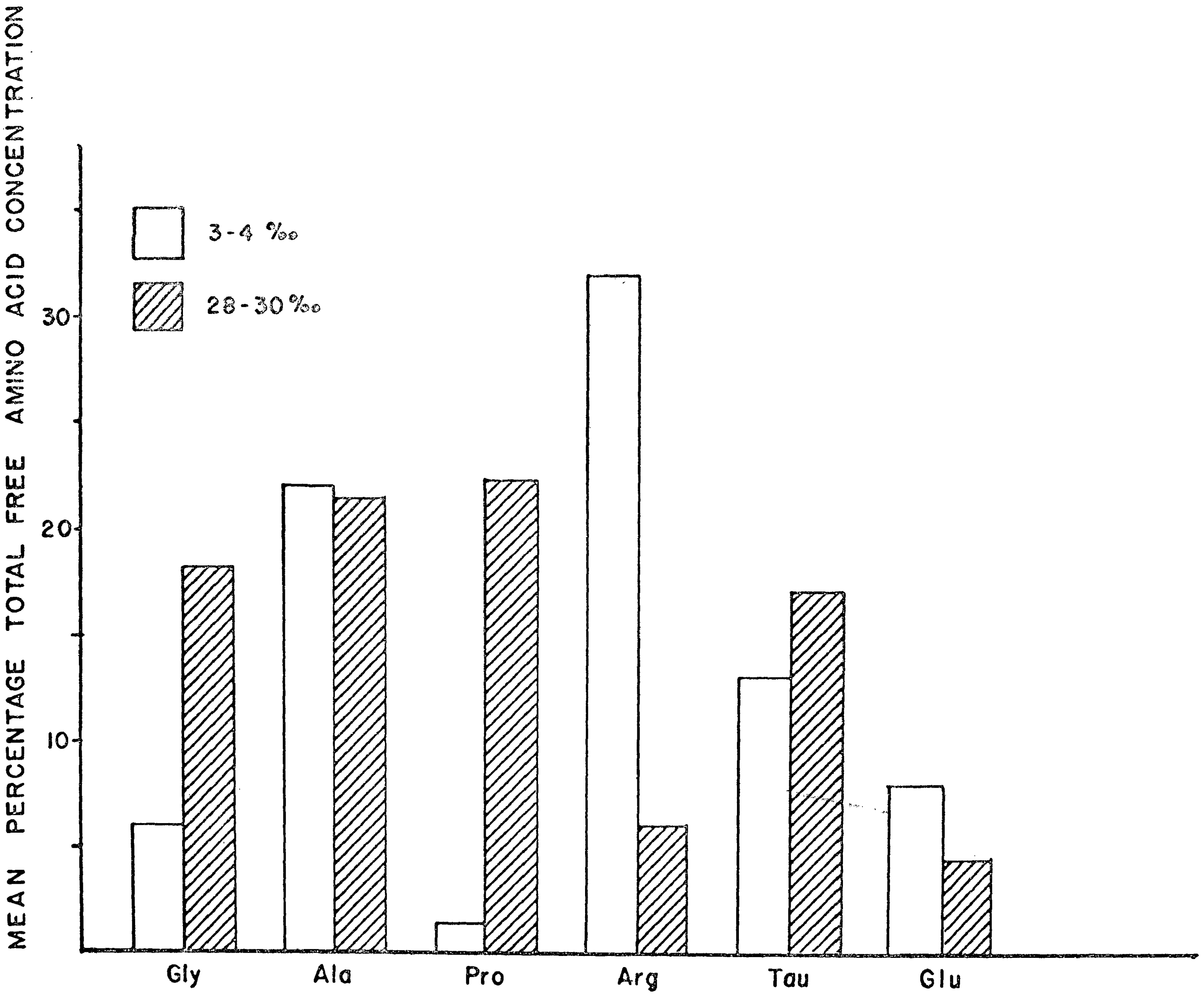

Fig. 2. Relative concentrations of selected free amino acids at two collection salinities.

TABLE III

MEAN PERCENTAGE TOTAL FREE AMINO ACID CONCENTRATION AT TWO SALINITIES

Amino Acid	3-4 o/oo	28-30 o/oo
Glycine	5.97	18.33
Alanine	21.97	21.55
Proline	1.36	22.29
Arginine	31.99	6.01
Taurine	13.12	17.13
Glutamic acid	7.90	4.56
Total	82.31	89.87

Certain amino acids dominated the amino acid pool (Fig. 1). The five most abundant ones at the low salinity were arginine, alanine, taurine, glutamic acid and glycine, in that order. Essentially the same five dominated at the high salinity. Proline, however, which was tenth at the low salinity, became first at the high salinity followed by alanine, glycine, taurine and arginine. Glutamic acid dropped to sixth place at this salinity. Percentage contribution made by the six most abundant amino acids at the high salinity and the same amino acids at the low salinity is summarized in Fig. 2 and Table III. These six amino acids account for 82.31 and 89.87% of total free amino acid concentration at low and high salinities respectively. From these results it is evident that salinity markedly influences concentration and to a lesser degree composition of the intracellular amino acid pool of _B_. _improvisus_ muscle tissue.

Other ninhydrin-positive substances that were detected in small quantities or unmeasurable amounts include the amino acids methionine, cystine and tryptophan as well as γ-aminoisobutyric acid, ammonia and occasionally cysteic acid.

DISCUSSION

Invertebrate tissue, in contrast to that of most vertebrates, has a high intracellular free amino acid content (Awapara 1962). In a comparative study of fish and invertebrates the α-amino nitrogen concentration per 100 g of fresh tissue is 9 to 72 mg for fish and 210 to 370 mg for four species of marine invertebrates (Velankar and Govindan, 1957 and 1958). Mammalian organs contain 10 to 50 mg free amino acid nitrogen per 100 g wet weight (Friedberg and Greenberg 1947), whereas muscles of two species of toads, acclimated to fresh water and 20, 40, and 50% sea water, contain 70 to 148 mg α-amino nitrogen for an equivalent weight (Gordon 1965). Values for mammals and fish are comparable and those for euryhaline toads lie between the foregoing and the concentrations reported for invertebrates. Ranke (1957) conducted a similar comparative study on fish, molluscs and invertebrates and found similar relative concentrations. Prior to recently discovered intermediate values for toads, it appeared that the large free amino acid pool could not be wholly attributed to a marine environment since the pool in fishes, as in other vertebrates, was small relative to that of invertebrates. Indeed the high non-protein amino acid content of tissues was thought to be characteristic of invertebrates (Awapara 1962). Evidence from toads supports the concept of intracellular osmoregulatory function of amino acids in euryhaline animals whether they are vertebrates or invertebrates. Perhaps free amino acids simply have a different function in fishes.

The intracellular amino acid pool is smaller in fresh water species than in marine species of decapod crustaceans (Camien et al. 1951), pelecypod molluscs and worms (Duchâteau et al. 1952). In euryhaline crustaceans such as Eriocheir sinensis (Duchâteau and Florkin 1955), Carcinus maenas (Shaw 1958) and Astacus astacus (Duchâteau-Bosson and Florkin 1961), and in various molluscs (Allen 1961; Lange 1963; Bricteux-Grégoire et al., 1964a and 1964b), a worm (Duchâteau-Bosson, Jeuniaux and Florkin 1961) and toads (Gordon 1965), the amino acid content is lower at low than at high salinities. For example, Carcinus maenas transferred from fresh water to 50% sea water exhibited a mean free amino acid elevation of 82.9 μM/kg water, only 4.2 μM of which could be attributed to dehydration (Shaw 1958). Speculations have been made on the raison d'être for the large intracellular amino acid pool of brackish water and marine invertebrates. The most widely accepted function of this pool and the one supported by the largest body of circumstantial evidence is that of osmoregulation.

Euryhaline invertebrates generally regulate anisosmotically but are sometimes passively isosmotic to the external medium, whereas blood and tissues are, in all known cases, isosmotic (Florkin 1962). Isosmoregulation between extracellular and intracellular fluids prevents excessive hydration and amino acids contribute importantly to this regulation (Florkin 1962). The significance of amino acids in intracellular isosmoregulation is demonstrated by their accounting for approximately one-third of the change in freezing point depression of muscles of Eriocheir sinensis transferred from 33% sea water to 100% sea water (Bricteux-Grégoire et al. 1962) and of Leander serratus and Leander squilla transferred from sea water to 30% sea water (Jeuniaux et al. 1961).

In contrast to muscles, osmotic pressure of invertebrate blood is attributed mainly to inorganic ions and not to organic molecules. Myers (1920) reported this phenomenon in arthropods. Studies of free amino acid composition of marine crustacean blood and blood sera have been performed (Stevens, Howard and Schlesinger 1961) on the horseshoe crab, Limulus polyphemus; rock crab, Cancer irroratus; American lobster, Homarus americanus as well as on Homarus vulgaris, the European lobster (Camien et al. 1951). In the former paper the range was 4.7 to 26.1 mg/100 ml of blood.

To my knowledge no research has been conducted on organic constituents of barnacle blood or mantle fluid. Freezing point depression studies of these extracellular fluids have, however, been carried out on several species of barnacles (Newman 1962). Most crustaceans conform at high salinities and regulate hyperosmotically at low salinities or they remain isosmotic at all salinities within the tolerance range (Lockwood 1962). In contrast to higher crustaceans and to other cirripeds, B. improvisus is essentially a conformer even at 1 o/oo salinity (Newman 1962). Survival by near conformity at such dilutions is unknown in other crustaceans, yet it is quite effective since this species is considered to be one of the most euryhaline of all barnacles (Newman 1962). How can this barnacle tolerate such blood dilutions? In addition to discarding inorganic ions does it also dispose of intracellular amino acids in order to maintain an isosmotic state between blood and tissues?

Although the foregoing questions cannot be answered completely, some information can be offered on the latter. Qualitative and quantitative analysis of intracellular free amino acids of B. improvisus muscle tissue (Tables I and II) demonstrates that there is a very definite and marked decrease in total and individual concentration of amino acids (except tyrosine) with a depression of 25 o/oo external salinity. The magnitude of change (more than threefold) was undoubtedly too great to be ascribed to dilution alone because the relatively impermeable exoskeleton would prevent most water movement. Obviously, then, this species can dispose of its free amino acids in some manner. These data, along with freezing point depression data of mantle fluid from the same species (Newman 1962), support the general conclusion that whenever there is an alteration in blood osmolar concentration there is a corresponding increase or decrease in intracellular amino acids, presumably to prevent hydration or dehydration. This circumstantial evidence suggests that the free amino acid pool plays an important role in osmoregulation. Whether or not the reported change is a direct result of an osmotic function or is simply produced by some other cellular phenomenon has not yet been elucidated.

Unfortunately the foregoing data cannot be compared directly with that of muscle tissue of other crustaceans because most other work is based on wet weight, dry weight, intracellular fluid volume or nitrogen content of the non-protein fraction, whereas these determinations were based on TKN of barnacle muscle homogenate. Amino acid concentration based on TKN is preferable to that based on wet weight because the latter would include the uptake or loss of water and dilution or dehydration of the amino acid pool would have to be considered in comparing concentrations at different salinities. If it were possible to base the concentration on wet weight, and if percentage muscle water were determined, the relative importance of free amino acids to total intracellular osmotic concentration could be estimated in the following manner: (1) establish molar concentration of extracellular fluid from freezing point depression of mantle fluid of *B. improvisus* maintained at the appropriate salinity (Newman 1962); (2) assuming mantle fluid and tissues are isosmotic, calculate percentage contribution of free amino acids to tissue osmotic concentration on the basis of tissue water content.

Early work on the free amino acids of marine and fresh water decapod crustaceans indicated that glycine, proline, arginine, glutamic acid and alanine were the most abundant in muscles of *Homarus vulgaris*, *Eriocheir sinensis*, *Astacus fluviatilis* and *Maia squinado*, the concentrations being higher in marine than in fresh water species (Camien *et al*. 1951). The same amino acids plus taurine were most prominent in the lobster, *Nephrops norvegicus* (Robertson 1961). In the former investigation, taurine content was not studied, yet whenever this amino acid is considered in marine or brackish water forms it is generally one of the most abundant.

Data reported herein for B. improvisus muscle are in agreement with the foregoing investigations with respect to amino acids dominating the intracellular amino acid pool. Although it is impossible to compare actual individual amino acid concentrations of barnacle muscle with those of other crustaceans, concentrations can be compared on the basis of relative contribution of individual amino acids to total amino acid content. A compilation of data obtained by various investigators and recalculated for comparison is made in Table IV. A similar compilation for B. improvisus was made in Table III (Results). In comparing these data some reservations must be made (refer to notes, Table IV).

These data do not support any generalizations on the concentration hierarchy of amino acids in crustaceans. It will be noted, however, that in all species except B. improvisus, glycine was the most abundant amino acid and accounted for one-fourth to one-half of the total amino acid pool. It is interesting that the relative quantity of alanine in barnacle muscle remained constant at the two salinity extremes and this amino acid was more abundant than glycine, especially at the lower salinity. All other crustaceans, with the exception of L. squilla (in which the relative concentrations of all amino acids changed little), exhibited a lower relative concentration of alanine than of glycine and considerable variation of the former was generally noted with salinity changes. The relative quantity of proline in most crustacean tissue was less than 10% except in L. squilla where it was 16 to 17% at both salinities and in B. improvisus at high salinity in which it was the most prominent amino acid, accounting for 22.29% of the total amino acid concentration.

TABLE IV

PERCENTAGE TOTAL FREE AMINO ACID CONCENTRATION

	Astacus pallipes[a]	*Leander serratus*[b]		*Leander squilla*[b]		*Eriocheir sinensis*[c]		*Calanus finmarchicus*[d]
	Approximate salinity, o/oo							
	0	10.5	35	10.5	35	12	35	marine
Glycine	30.49	62.80	49.47	41.55	46.00	31.11	26.47	24.58
Alanine	7.76	1.72	7.50	4.59	4.54	10.63	17.20	8.52
Proline	9.98	4.23	9.63	16.48	16.96	6.91	8.97	8.52
Arginine	26.43	13.68	11.46	18.01	14.84	22.12	16.30	14.12
Taurine	6.10	15.34	11.37	16.33	13.80	10.48	6.08	17.61
Glutamic acid	1.48	0.73	1.42	0	0	2.22	4.10	2.93
Total	82.24	98.50	91.11	96.96	96.14	83.47	79.12	76.28

a Cowey 1961. Composition of unhydrolyzed lobster abdominal muscle of animals maintained in running tap water.

b Jeuniaux *et al*. 1961. Composition of hydrolyzed dialysate of prawn abdominal muscle. Many amino acids were recorded as "trace" or "0" accounting for high percentages of remaining amino acids.

c Bricteux-Grégoire *et al*. 1962. Composition of hydrolyzed dialysate of crab leg muscle.

d Cowey and Corner 1963. Amino acid composition of the unhydrolyzed whole marine copepod, including the exoskeleton. Salinity was not recorded.

In contrast to other amino acids, percentage arginine was depressed at higher salinities although the actual concentration often increased. The reason for this phenomenon is uncertain although the following is a possible explanation. Large quantities of arginine are present in invertebrate muscle primarily as arginine phosphate, a high energy compound comparable to creatine phosphate of vertebrate muscle (Giese 1962). Both phosphagens are very labile and perhaps the elevated salt concentration increases the stability of arginine phosphate. It has been demonstrated, for instance, that proteolysis is completely inhibited by monovalent cations (Na, K and NH_4) at concentrations greater than 0.2 M and to a lesser degree at lesser concentrations (Keil 1962). Possibly these inorganic ions act similarly on phosphagens.

The sulfonic amino acid, taurine, is interesting in that it is generally considered to be more abundant in molluscan tissue than in crustacean tissue and in marine or brackish water forms than in terrestrial or fresh water invertebrates. In a study of 29 species of molluscs, 11 of which were fresh water or terrestrial and 18 of which were marine or brackish water species, taurine was not detected in any of the former by paper chromatography and was found in detectable amounts (greater than 0.1 µM/g) in all of the latter (Simpson, Allen and Awapara 1959). By contrast, taurine occurred at a concentration of 6.6 µM/g (6.1% of total amino acid content) in abdominal muscles of the fresh water crayfish, Astacus pallipes, maintained in tap water (Cowey 1961). In B. improvisus at 3 to 4 o/oo salinity it accounted for 13.14% of the total amino acid concentration.

The origin of intracellular amino acids in invertebrates is unknown. Numerous symposia and reviews have dealt with transport of amino acids and it is generally believed that they are transported through the cell membrane against a concentration gradient (uphill transport, Wilbrandt 1961). There are many theories on the transport mechanism but most evidence supports the carrier transport theory. According to this theory the amino acid combines with a carrier located in the cell membrane and is transported "uphill" across the membrane where it is released, leaving the carrier free again for transport. "Uphill" influx is affected by (1) competition with other amino acids for the same transport system (Ahmed and Scholefield 1962), a phenomenon generally occurring between structurally similar amino acids (Johnstone 1964), and (2) the hormones, insulin, estrogen, and growth hormone, all of which increase intracellular amino acid concentration and protein synthesis in some tissues (Segal 1964). Amino acid transport is indirectly dependent on cation concentrations, particularly sodium, potassium, calcium and magnesium, since they affect ATP content, the latter being required as an energy source (Quastel 1964). Transport reaches a steady state at which point intracellular concentration remains constant and exceeds that of the medium. Whether influx is actually increased, efflux depressed, or both, has evaded discovery. Exchange diffusion requires no energy and may be responsible for selective accumulation of certain amino acids within the cells since it involves exchange of extracellular and intracellular amino acids.

Transport reaction investigations have been performed primarily on mammalian cells or tissues *in vivo* or *in vitro* or on bacteria, although a few studies of this type have employed invertebrates. Stephens and Schinske (1961), on the basis of disappearance of amino acids from solution concluded that arthropods, in contrast to other invertebrates, fail to remove glycine from sea water at a concentration of 150 mg/l. They attributed the failure to impermeability of the exoskeleton and noted that invertebrates capable of removing amino acids from solution are mucous-feeders. The following year through the use of labelled amino acids, it was ascertained that *Fungia scutaria*, a solitary coral, is capable of extracting several amino acids from very dilute solutions. Demonstration of this capability with no decrease in rate of uptake upon plugging the mouth suggested that adsorption of amino acids on mucous is not an effective means of collection (Stephens 1962).

Schoffeniels (1960) investigated the origin of intracellular free amino acids of nerves isolated from *Eriocheir sinensis*. The crabs were adapted to sea water and to 33% sea water after which the nerves were bathed for 24 hours in saline solutions isosmotic with the extracellular fluid. Free amino acid concentration decreased with salinity and Schoffeniels concluded that regulation in isolated nerves could not be hormonally controlled and the amino acids must have originated within the cell. He further postulated intracellular origin by protein hydrolysis and the reversibility of the process by resynthesis. These conclusions seem unwarranted because the difference in amino acid content was probably established upon adapting crabs to the respective salinities prior to isolating the nerves. The new concentration could conceivably be maintained in the electrically isolated nerve. Even with an amino acid leakage, the rate of leakage might have been equal at both salinities sustaining quantitative differences.

If amino acids are not transported into the cell, they must originate intracellularly by synthesis or proteolysis. Cellular metabolism will not, however, be discussed except as it affects amino acid regulation. Intracellular amino acid concentration at the steady state depends upon balance between influx, synthesis and the combined fates of these molecules. Synthesis and degradation of amino acids are probably affected by intracellular cation composition (Gilles and Schoffeniels 1964).

Having dealt with possible sources of intracellular amino acids, we may now discuss their fate. Shaw (1958) suggested that they may combine with other muscle constituents although, in *Carcinus* muscle, they did not yield peptides since the amino acid concentration was not increased by hydrolysis of the trichloroacetic acid extract. Potts (1958) speculated that the free amino acid content is depressed by excretion, polymerization or metabolism.

The foregoing strongly suggests that free amino acids of *B. improvisus* muscle and of tissues of other euryhaline invertebrates are exceedingly important in intracellular isosmoregulation. Whether the species conforms like *B. improvisus* or regulates as do most euryhaline crustaceans to the environmental salinity, intracellular isosmoregulation, primarily by free amino acids, prevents hydration or dehydration of the tissues.

In reviewing these data it must be noted that salinity was the only factor considered in comparing intracellular amino acid concentrations. Other environmental factors affecting amino acid composition are temperature, pollution, diet (organic content of the water), and physiological state. An attempt was made to minimize variation in these factors by collecting and treating specimens as nearly identical as possible.

SUMMARY

1. A study of free amino acid composition of Balanus improvisus adductor and depressor muscles was conducted by semiautomatic ion exchange chromatography.
2. More than 20 ninhydrin-positive substances were detected in the muscle extract. Sixteen of these were quantitatively determined.
3. At low (3 to 4 o/oo) and high (28 to 30 o/oo) salinities the mean intracellular amino acid concentration differed significantly for all amino acids except tyrosine.
4. Total free amino acid composition more than tripled with an increase in salinity of approximately 25 o/oo.
5. Relative composition of the amino acid pool as well as individual and total concentration of intracellular amino acids was affected by salinity.
6. Various publications on free amino acid content of animals representing different phyla and of euryhaline and stenohaline species at different salinities were discussed.
7. Osmoregulatory characteristics of euryhaline invertebrates in general and crustaceans in particular were set forth.
8. Because of the impossibility of basing intracellular amino acid content on wet weight or intracellular fluid volume, the results presented herein could not be compared directly to those obtained by other workers on other crustaceans. Results

were, however, compared indirectly (percentage contribution of individual amino acids to total amino acid concentration) and it was found that in B. improvisus, as in most other crustaceans, six amino acids (glycine, alanine, proline, arginine, taurine and glutamic acid) accounted for approximately 80 to 90% of the total amino acid concentration. With an alteration of salinity, the relative concentration of proline in B. improvisus muscle exhibited the greatest change (1.36 to 22.29%) but generally changed very little in tissues of other crustaceans. Glycine accounted for one-fourth to one-half of the total amino acid pool in most crustaceans but was much less prominent in B. improvisus muscle (6 to 18%). Unlike the majority of amino acids, percentage arginine in most crustaceans including the barnacle, decreased slightly with an increase in salinity. An explanation was offered for this unusual phenomenon. Relative concentration of alanine was much higher in B. improvisus than in other crustaceans and remained relatively constant at both salinities. Relative concentration of taurine changed very little in barnacle muscle or tissues of any of the crustaceans mentioned.

9. The possible intracellular and/or extracellular origin of free amino acids was discussed as was the fate of these molecules.

10. It was concluded that free amino acids of B. improvisus muscle and of tissues of other euryhaline species are important in intracellular isosmoregulation and prevent tissue hydration or dehydration.

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