AMINO ACIDS AND AMINO SUGARS IN CALCIFIED TISSUES OF PORTUNID CRABS

WOODS HOLD OCEANOGRAPHIC INSTITUION
NSR-22-014-001

by

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The various regions in the exoskeleton of portunid crabs exhibit a wide range of hardness and rigidity. The most rigid structure is the dactylus of the chela; most flexible and soft are the unmineralized joints between the limbs. The peropodus, the carapace and the pleopods represent intermediate stages of rigidity. Since the extent of mineralization largely determines the flexibility and hardness of the exoskeleton, we were interested in learning the important factors in this calcification process.

Previous work on mineralization in biological systems[1] has shown that the proteinaceous matrix in calcified tissues provides a set of highly specific templates. Most essential in nucleating a mineral phase appears to be the availability of free carboxyl and amino groups provided by certain acidic and basic amino acids. In the light of these results we decided to determine the amino acid and amino sugar composition of representative regions in the exoskeleton and to relate these data to the calcification phenomena.

The animals selected for this study were four specimens of *Callinectes sapidus*, one of *Ovalipes ocellatus*, and one of *Carcinides maenas*, all intermolt males. The regions sampled were the soft uncalcified joint membrane between the carpus and merus of the cheliped, the flexible paddle of the pleopod, the cardiac and gastric regions of the carapace, the propodus and dactylus of the

cheliped. All samples were freed of extraneous tissues and subjected to decalcification, hydrolysis, and ion-exchange chromatography[2]. Data on calcium, magnesium and strontium obtained by atomic absorption spectroscopy[3] and on phosphate by colorimetry[4] were used as a measure of the degree of calcification of the individual organic matrix. The analytical results are summarized in Tables 1 and 2. In order to explore the interrelationships both within the amino acids and between the amino compounds and $CaCO_3$, the technique of factor analysis has been employed[5]. However, as our calcium data are restricted to the sample of Callinectes, we confined our factor analysis only to the calcified tissues of these specimens. In all, we analyzed the relationships between 18 amino acids, the amino sugar to protein ratio, and the calcium content in all samples. The analysis was performed by a GE 225 computer using a program written by Spencer[6]. A principal components solution followed by a varimax rotation showed that three factors account for 83% of the variance of the data. The varimax factor matrix and the varimax factor score matrix are reproduced in Tables 3 and 4. Figure 1 is a plot of the factor scores of factor 1 and factor 2.

About 80% by weight of the samples when expressed as carbonates, amino acids, and acetylglucosamine polymer could be accounted for after the chemical analysis. The missing portion partly represents humin and partly water; chitin is known to retain up to 10% water even when dried to constant weight at 105°C[7].

Based on the factor analysis, the following interpretations are offered. Factor one is related to the actual calcification process. In this factor, proline, lysine, and the amino sugar to protein ratio, form a covariant group strongly correlated with the calcium content and negatively correlated with aspartic acid, threonine, serine, glycine, tyrosine and phenylalanine. Factor two forms a covariant group involving isoleucine, leucine, valine, glutamic acid and ala-

nine. Factor two forms a covariant group involving isoleucine, leucine, valine, glutamic acid and alanine. Noteworthy is the fact that individual number 12 (Table 4) generally scores higher on factor two than either individual number 11 or 13, as illustrated in Figure 2. Although the pleopod, propodus and carapace of these individuals contain larger quantities of the amino acids involved in this factor, factor two appears to have no connection to the actual calcification process. Because of the differences in individuals it seems most likely to be an environmental factor (e.g. water temperature, pH, Eh, salinity, or diet). Factor 3 has a loading on basic amino acids. In comparison with factors 1 and 2, it contributes little to the factor score.

The increase in lysine and OH-lysine with progressing calcification agrees with the concept[1] that both amino acids may provide nucleation sites for crystal growth. Inasmuch as chitin may also contain free amino groups[7-8], the higher yields of glucosamine in the most mineralized regions of the exoskeleton may also be linked to calcification. Should dicarboxylic acids be essential in providing negative sites for the fixation of calcium, their effect is masked by other factors.

Crustacean cuticles are hardened by both tanning and mineralization processes. As a general rule on increase in tanning is accompanied by a reduction in mineral deposition and vice versa. Thus at least two distinct protein matrices are contained in the rigid structures of the exoskeleton. This phenomenon is analogous to the occurrence of mineralized proteins and the periostracum in mollusk shells. A study of the amino acid composition in tanned proteins of gastropods and cephalopods is informative for the interpretation of the covariant group in factor one, i.e. aspartic acid, threonine, serine, glycine, tryosine and phenylalanine. Essentially the same amino acids characterize the periostracum of these two classes of molluscs[9]. This similarity is further underlined by the high abund-

ance of amino sugars in the tanned shell proteins. In the light of these data, factor one (Table 3) can be regarded as a reflection of the different mixing ratios of mineralized and tanned proteins in crustacean cuticles. The association of aspartic acid with the tanned proteins and of proline with the mineralized tissues does not necessarily imply that the former is not involved in the actual calcification process whereas the latter is. It may be that because of processes unrelated to calcification the mineralized tissues contain less aspartic acid and more proline than their tanned counterparts. The proportions of tanning and mineralization vary from region to region as shown by representative values for the thickness of tanned and mineralized layers in *Calinectes* presented in Table 5. The strong negative correlation between the two groups of amino acids in factor one is probably related to the changing ratio of tanned to mineralized cuticle in the various regions. The other two specimens included in this report, namely, *Ovalipes ocellatus* and *Carcinus maenas* show essentially the same factor structure and biochemical relationships we discussed before. The enrichment in lysine and amino sugars with progressive calcification is even more pronounced than in *Callinectes* and may be a species characteristic.

Based on the amino acid composition, the joint membrane differs in some aspects from the mineralized structures so far considered. For this reason, we excluded the data in our factor program. Particularly noteworthy is the high abundance of proline, acidic, aromatic and basic amino acids.

In relating the present data on mineralized tissues in portunid crabs with previous results largely inferred from electronmicrographs[10] we offer a tentative model on the calcification in this biological system: The cuticle of crustacea is a layered structure composed of minerals deposited in and between matted layers of chitin and protein fibrils. The fibrils mainly lie in the plane of the

layers, but some branch up and down to connect adjacent layers. The vertical fibrils line the walls of the numerous pore canals which run through the cuticle in a direction perpendicular to its surface. Organic materials form a diffuse matrix between the fibrils and in the pores. Mineralization proceeds along the fibrils but the resulting crystals are always small in size. Larger crystals are restricted to the interstices and lumen of the pore canals. It is inferred that the formation of multitudes of small mineral seeds in the fibers represent the first crystallization stage. The deposition of large minerals comes later as nucleation sites in the matrix become gradually available. The larger size of the crystals in the second mineralization phase is simply a consequence of the fewer nucleation sites available along the organic templates. The individual layers are spaced further apart in the thick, highly calcified regions compared to the less mineralized regions. The proteinaceous matter is principally contained in the layers of matted fibers, and consequently, the most calcified regions have a greater proportion of mineral to organic matter. This implies that with advancement of calcification the organic matrix becomes a more effective template. In a sense this is a duplication of what we observe in mollusk shells by going from primitive to highly advanced forms[9]. With evolution, progressively less organic matrix is required for the nucleation of calcium carbonate. Whereas a *Nautilus*, *Haliotis*, or *Mytilus* may require a few per cent organic matter for the deposition of their shell structure, some highly evolved gastropods such as *Architectonica* or *Bulla* get along with just 0.01%.

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11. This work was sponsored by grants No. 22-001-014 from the National Aeronautics & Space Administration and No. 816 from the National Science Foundation.

Table 1

Distribution of Amino Acids, Glucosamine and Mineral Matter in Cuticles of Portunid Crabs

Residues/1000 A.A.	Callinectes sapidus					Ovalipes ocellatus				Carcinides	
	Joint Membrane	Pleopod	Carapace	Propodus	Dactylus	Joint Membrane	Pleopod	Carapace	Propodus	Joint Membrane	Carapace	Propodus
Aspartic Acid	116	91	87	77	72	99	87	91	75	100	95	87
Threonine	71	59	55	54	48	57	46	59	54	66	53	56
Serine	59	104	81	73	64	73	111	109	74	70	99	90
Glutamic Acid	118	84	101	88	95	111	90	92	92	106	112	88
Proline	101	115	109	144	165	104	82	98	115	90	87	92
Glycine	122	127	108	101	103	124	155	130	103	130	120	114
Alanine	67	108	125	124	115	67	73	130	T07	74	98	107
Cystine	1	3	2	2	4	0.3	6	1	7	3	6	10
Valine	47	64	81	77	77	39	60	77	60	47	62	63
Methionine	8	5	12	12	10	5	6	11	4	5	6	4
Isoleucine	39	28	30	27	28	35	24	33	27	39	29	28
Leucine	49	39	52	49	50	46	42	54	45	45	48	55
Tyrosine	31	41	27	25	16	26	40	24	18	32	33	21
Phenylalanine	59	44	39	39	32	39	40	27	21	46	36	27
OH-Lysine	2	3	3	5	4	0.4	0.4	2	3	0	0.5	9
Lysine	37	27	34	46	55	38	35	31	87	28	42	107
Histidine	21	21	17	20	23	42	36	18	49	37	22	25
Arginine	71	34	28	36	34	95	67	13	60	81	50	18
Glucosamine	561	749	1297	1227	1527	1113	1150	1738	3870	775	2290	5430
Weight % *												
Protein	54	22	13	13	7	36	22	1.9	0.6	44	2.2	0.7
Chitin	46	42	24	20	19	64	43	5.7	4.1	56	8.3	6.0
Mineral	0	36	62	67	74	0	35	92	95	0	89	93

* Adjusted to 100%

Table 2

Element Composition in Various Regions of the Exoskeleton of *Callinectes*

Weight %	Joint Membrane	Pleopod	Carapace	Propodus	Dactylus
Calcium	0.05	13.5	23.4	25.2	27.7
Magnesium	0.03	0.87	1.01	1.19	1.26
Strontium	-	0.27	0.23	0.22	0.26
Phosphorus		1.7	0.9	1.3	1.2

Table 3

Varimax Factor Matrix

	Factor 1	2	3
Aspartic Acid	0.923		
Threonine	0.751		
Serine	0.927		
Glutamic Acid		0.819	
Proline	-0.736	-0.560	
Glycine	0.825		
Alanine		0.729	
Cystine			
Valine		0.919	
Methionine	-0.668	0.401	
Isoleucine		0.877	
Leucine	-0.599	0.730	
Tyrosine	0.926		
Phenylalanine	0.897		
Hydroxylysine			0.911
Lysine	-0.847		
Histidine	-0.468		0.842
Arginine		-0.344	0.896
Amino Sugar/Protein	-0.831		
Calcium	-0.926		

Table 4

Varimax Factor Score Matrix

Sample No.	Region of Exoskeleton	1	2	3
13	Pleopod	1.320	-0.956	1.456
12	Pleopod	1.299	-0.080	-1.243
11	Pleopod	0.999	-1.408	-0.777
12	Propodus	0.022	0.703	1.657
11	Propodus	-0.948	-0.534	-0.488
13	Propodus	-0.766	-0.793	-0.180
12	Carapace	-0.125	1.778	-0.695
11	Carapace	0.593	1.416	-0.493
13	Carapace	0.171	0.371	0.121
12	Dactylus	-1.730	-0.610	-0.619
11	Dactylus	-0.835	0.113	1.261

Table 5

Thickness of Tanned and Mineralized Regions in *Callinectes*

Region	Epicuticle and Pigmented Layer (mm)	Endocuticle (mm)
Pleopod	0.06	0.25
Carapace	0.06	0.45
Propodus (chela)	0.06	0.75
Dactylus (chela)	0.07	1.30

The epicuticle and pigmented layers are tanned and calcified. The Endocuticle is calcified.

THE LOADING OF FACTOR I VERSUS FACTOR II

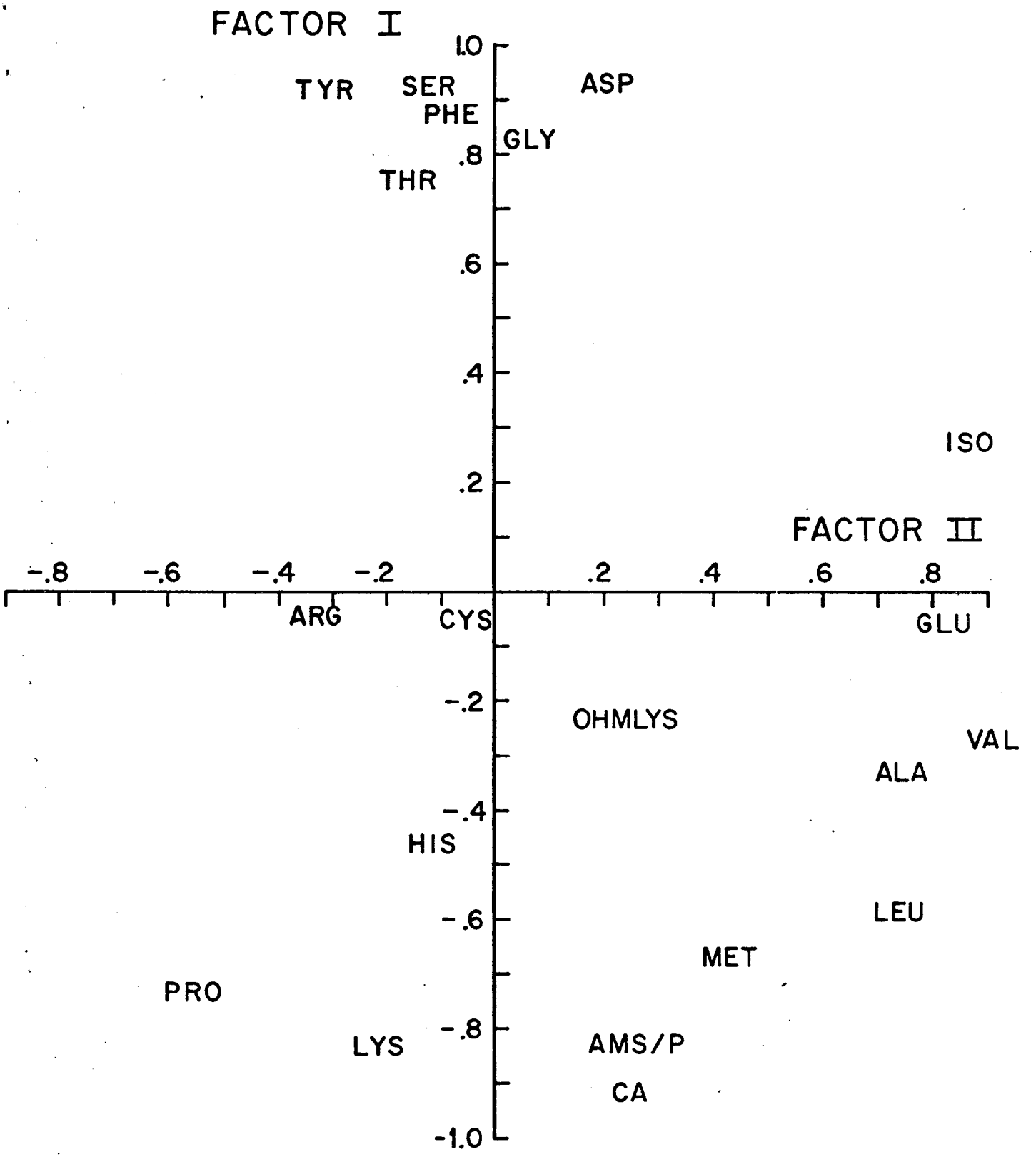
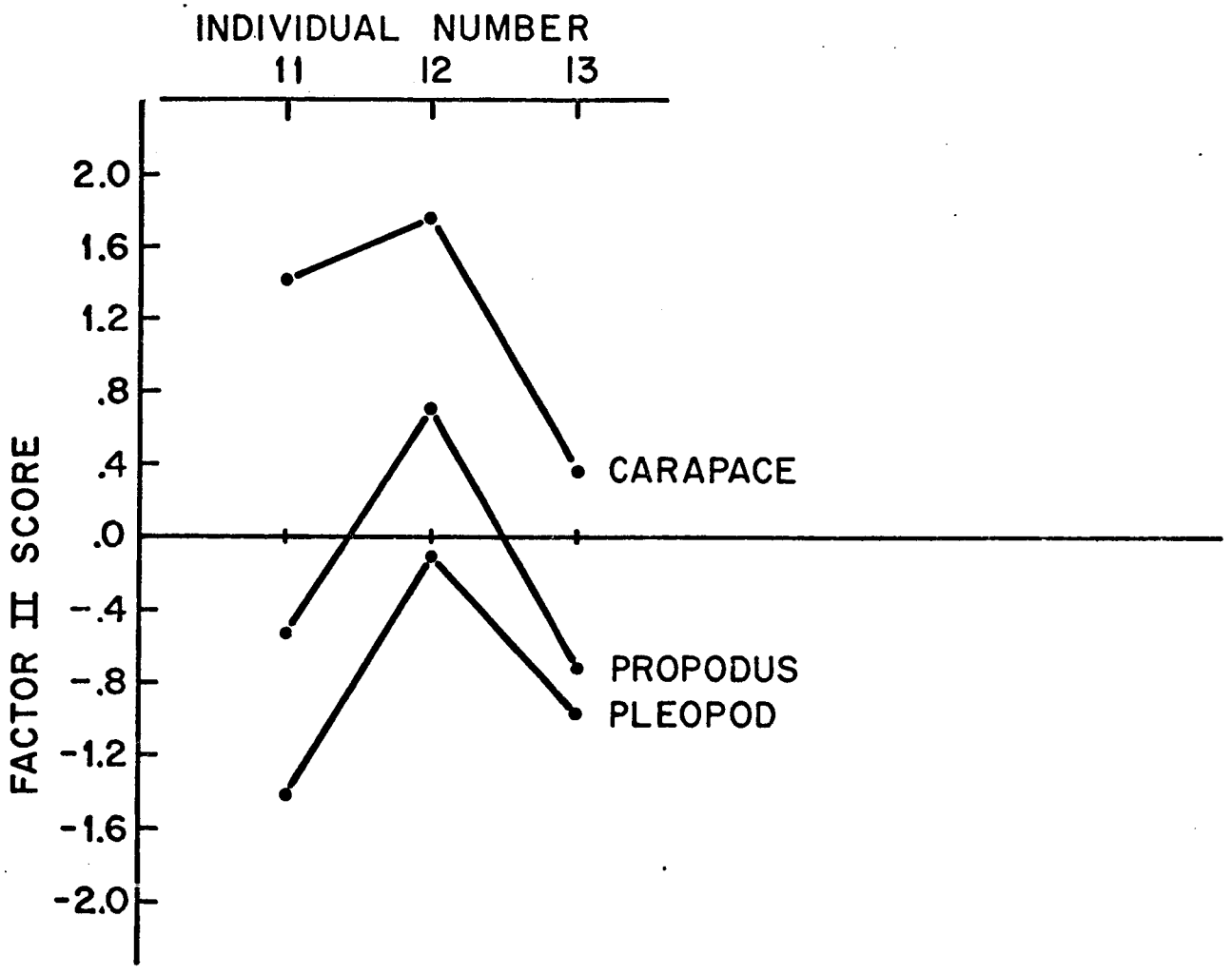


FIG.1



FACTOR II SCORES IN 3 INDIVIDUALS (11, 12 AND 13) OF CALLINECTES SAPIDUS.

FIG. 2