EVOLUTIONARY TRENDS INFERRED FROM THE ORGANIC TISSUE VARIATION OF MOLLUSC SHELLS

By

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Abstract

I have tried to briefly summarize some highlights of the work we are presently performing at the Department of Chemistry, the Woods Hole Oceanographic Institution. This treatise is by no means a comprehensive account on calcification processes or comparative biochemistry of molluscs. Instead, I have selected a few topics which are of significance to geologists and paleontologists and have avoided to go too far into the actual chemistry and biochemistry. For those who like to learn more about this subject some literature information is added.

INTRODUCTION

Marine invertebrates traditionally have been classified and compared through visual examination of their shell structure. The whole science of Paleontology, involving changes in species by environmental and evolutionary criteria, is dependent upon one technique—*morphological descriptions*. The advent of Molecular Biology, namely the study of molecular changes within the organism, offers a new, powerful tool for understanding how and perhaps why an organism undergoes environmental and evolutionary alterations or convergences.

Studies in Comparative Biochemistry have shown that, for instance, the amino acid sequence of proteins in evolving organisms correlates with phylogeny. That is, the primary structure of such a protein is genetically controlled and will follow a certain pattern. Biochemists are now exploring molecular biological trends in evolving proteins and genes throughout the plant and animal kingdom. Studies on cytochrome *c* and the hemoglobins are already well advanced. For a review and general survey on this subject, one may consult BRYSON and VOGEL (1965) and ZUCKERKANDL (1965).

In the forthcoming discussion, I would like to focus attention on a novel biochemical approach which is of significance to the general area of Paleontology and Comparative Biology. Principally, we will be concern-

[1]) A seminar held by the "Dansk Geologisk Forening" at Copenhagen on February 14, 1966.

ed with calcification processes in biological systems. I will outline a tentative model of how mollusc shells are formed and why these organisms change their shell-morphology in the course of evolution.

SHELL FORMATION IN MOLLUSCS

Prior to carbonate deposition, a highly proteinaceous matrix is secreted from the epithelial tissues in specialized areas of the mantle. Proteins released from the cell vacuoles located along the periphery of the first mantle fold function as a kind of "ion exchange resin," i. e. they concentrate cations and anions at specific sites. Strictly speaking, aspartic acid and glutamic acid functions ($R\text{-}COO^-$) may serve as templates for the fixation of strontium, magnesium, and calcium; in contrast, lysine, histidine, and arginine may act as sites for the concentration of various carbonate ions by virtue of their basic functions ($R\text{–}NH_3^+$). In other words, the spatial conformation of the proteins and the availability of active sites will determine the spacing distance of the initial carbonate nuclei. Thus, the shell morphology is only a macroscopic expression of the molecular biochemical pattern of the calcified tissues.

The mantle also produces the uncalcified periostracum which is secreted from cells positioned roughly in the inner-upper portion of the second mantle fold. Similar to the shell organic matrix, the periostracum is composed of more than 95 percent of protein. Other portions of the mantle are involved in the secretion of byssus fibers, mucus, and the ligament. Again, all of these materials are highly proteinaceous.

In order to examine the chemical nature of the various proteins secreted from the mantle, we have relied on a number of techniques which are briefly outlined below. A more comprehensive account has been prepared by DEGENS and SPENCER (1966), DEGENS et al., (1967), and GHISELIN et al., (1966). These articles also include details on sample selection and computer programs.

ANALYTICAL TECHNIQUES

Principally three methods have been employed: (1) amino acid analysis of total organic matter, (2) protein solubility tests, and (3) factor analysis of amino acid data.

1. Amino acid analysis

Prior to extraction or hydrolysis, the carbonate shells were decalcified by HCl in presence of a 10 percent trichloracetic acid solution. Subsequently, the organic remains were centrifuged, washed, and hydrolyzed with 6 N HCl for 22 hours. The decalcification step was omitted in case of mantle, periostracum, or ligament tissues.

A flow diagram (Fig. 1) illustrates the various steps necessary for the final amino acid analysis. Basically, an aliquot of the hydrolyzate is added to one of the ion exchange columns and citrate buffer solutions

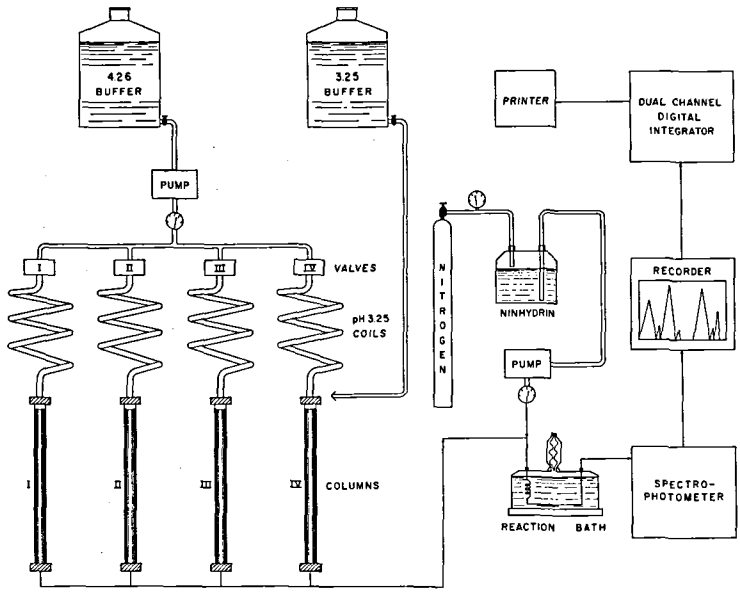


Fig. 1. Outline (Schematic) of Ion-Exchange System (after DEGENS and SPENCER, 1966).

(pH 3.25 and 4.26) are pumped through the system. By carefully controlling water jacket temperature and flow rate, the various amino acids can be effectively separated and they emerge as narrow bands in the column effluent. Ninhydrin is added to the system which in turn reacts with the amino acids, thereby producing characteristic purple, blue, or yellow colors. The color intensities of the reaction products are measured by a spectrophotometer at 440 and 570 mμ. Typical amino acid ion exchange chromatograms of representative samples are presented in Figure 2. A digital integrator and a computer program help in the analytical data handling process. The four column automatic ion-exchange system has a four-sample a day capacity.

2. Protein solubility test

Based on amino acid data and electron microscope studies, a heterogeneous nature for the shell proteins is suggestive. In order to test this idea, we performed a few solubility experiments on the shell organic matrix of *Mercenaria mercenaria*[2]). The protein fiber readily aggregates, a feature

[2]) Recently, we have examined a series of molluscan shell proteins for the physical-chemical properties of the proteins contained in the shells and periostracum and the type of cross-linkages established in these proteins. The results confirm the data obtained from the *Mercenaria* experiments in that all specimens analyzed respond similarly to the dissolution tests.

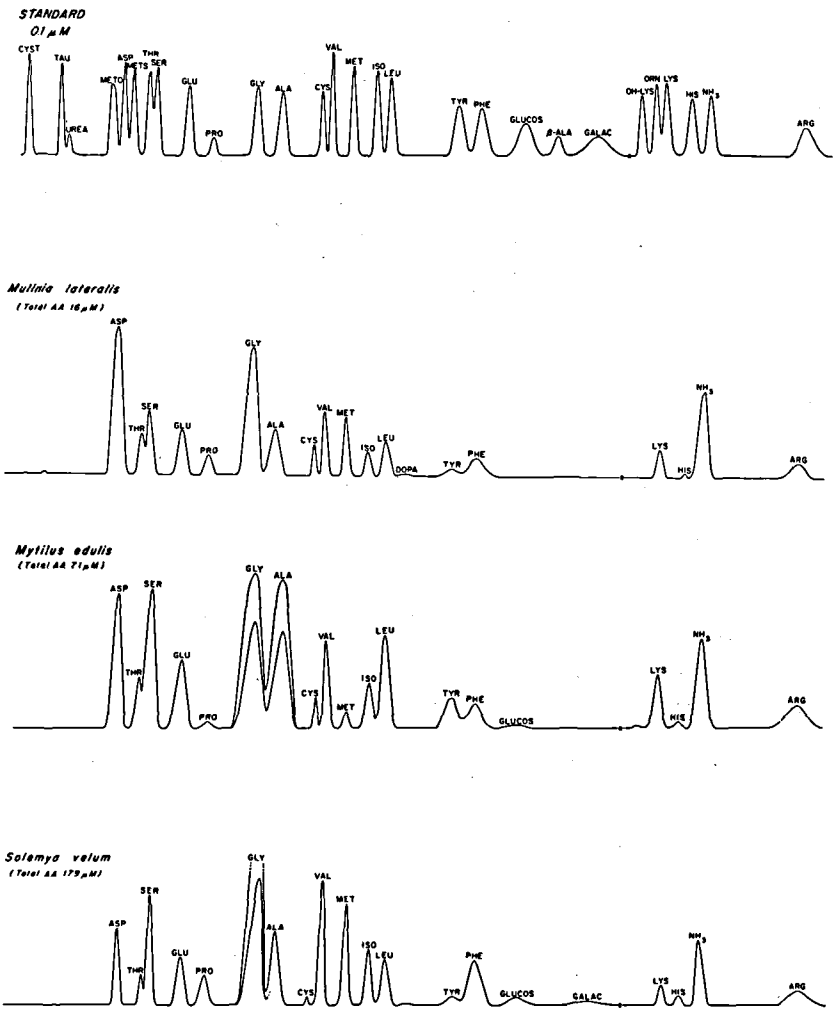


Fig. 2. Representative Ion Exchange Chromatograms.

which is common to collagen and k-m-e-f proteins (keratin-myosin-epidermin-fibrinogen). The results further show that some fractions dissolve in alkaline media, whereas others dissociate in 90 percent formic acid. Subsequent fractionations by means of gel-filtration yield fractions ranging in molecular weight from about 20,000 to 70,000-80,000. The collected fractions were hydrolyzed and analyzed for their amino acid content. Further tests on the protein aggregates with hydroxylamine and hydrazine gave essentially the same results.

Table I. Geometric means of mineralized
(Numbers in paren-

				$CaCO_3$
	Pelecypoda (51)	Gastropoda (40)	Cephalopoda (4)	Brachiopoda (1)
OH-proline	–	.01 (0–0.1)	–	–
Aspartic acid	136 (77–240)	114 (69–185)	98 (75–128)	97
Threonine	28 (18–44)	46 (28–76)	34 (18–64)	39
Serine	66 (43–102)	85 (58–124)	95 (62–146)	70
Glutamic acid	50 (35–72)	87 (52–145)	65 (42–128)	77
Proline	52 (28–98)	61 (36–102)	55 (15–205)	36
Glycine	271 (185–398)	153 (99–238)	153 (89–262)	301
Alanine	66 (43–101)	89 (52–153)	126 (74–216)	76
Cystine (half)	14 (6–33)	9 (4–23)	7 (0–65)	8
Valine	33 (22–49)	48 (34–69)	36 (20–64)	74
Methionine	18 (8–39)	14 (6–31)	8 (5–13)	13
Isoleucine	20 (13–31)	31 (20–46)	21 (17–26)	37
Leucine	34 (26–44)	71 (51–100)	44 (29–67)	47
Tyrosine	11 (2–61)	11 (5–24)	33 (15–75)	4
Phenylalanine	30 (19–48)	24 (14–43)	30 (20–45)	19
OH-lysine	.03 (0–0.6)	.03 (0–0.2)	–	0.1
Lysine	22 (13–38)	26 (17–41)	20 (5–83)	31
Histidine	4 (1–14)	3 (1–16)	5 (0–35)	0.3
Arginine	24 (6–96)	22 (9–52)	23 (10–56)	70
Proteins**	143.97	108.30	1.37	110.97
Hexosamines			(2.88)*	

* Ratio without pen of loligo
** Arithmetic mean

tissues (after DEGENS et al., 1967)
theses are 1σ ranges)

			SiO_2	Chitin	
				Portunid-crabs	
Echinoidea	Bryozoa	Amphineura	Sponges	Calcified	Uncalcified
(4)	(3)	(1)	(3)	(6)	(3)
1	0.1				
(0–36)	(0–7)	–	–		
88	117		123	81	102
(78–98)	(100–136)	128	(109–142)	(73–92)	(97–107)
48	59		47	57	63
(42–55)	(53–66)	60	(30–74)	(53–61)	(57–69)
81	77		102	91	69
(77–86)	(60–98)	68	(58–173)	(80–105)	(65–74)
109	102		83	94	113
(106–112)	(93–112)	98	(67–102)	(85–103)	(106–120)
78	50		64	103	93
(66–92)	(36–67)	93	(49–85)	(89–119)	(85–102)
171	148		134	113	133
(121–241)	(127–172)	131	(99–179)	(101–125)	(99–145)
85	92		94	115	68
(79–91)	(89–95)	88	(79–112)	(102–130)	(63–74)
1	13		5	3	0
(0.5–2)	(4–39)	14	(4–8)	(1–9)	(0–2)
38	48		62	65	44
(31–45)	(38–60)	45	(55–69)	(59–72)	(40–49)
21	16		8	7	6
(18–25)	(14–18)	12	(3–19)	(3–11)	(4–7)
26	31		40	28	37
(21–32)	(25–39)	30	(27–59)	(25–31)	(36–39)
58	59		70	49	47
(47–70)	(46–76)	52	(67–73)	(44–54)	(45–48)
20	23		24	25	28
(16–25)	(21–25)	31	(16–35)	(20–30)	(25–31)
25	28		24	30	46
(19–32)	(24–33)	37	(12–48)	(24–37)	(39–54)
0.6	0.4			3	
(0–11)	(0–8)	–	–	(0–7)	–
45	49		40	52	31
(35–57)	(40–61)	41	(22–72)	(28–97)	(26–37)
16	17		12	26	34
(13–20)	(10–30)	11	(7–23)	(17–39)	(25–44)
69	50		30	35	80
(60–80)	(41–60)	61	(21–42)	(18–68)	(68–96)
33.36	21.23	0.98	10.98	0.40	1.53

Table II. Geometric means of uncalcified
(Numbers in paren-

	Mantle				Perio-	
	Pele-cypoda (14)*	Gastro-poda (2)	Cepha-lopoda (2)	Amphi-neura (1)	Pele-cypoda (12)	Gastro-poda (12)
OH-proline.....	3 (0–75)	31 (20–48)	51 (47–55)	58	–	0.4 (0–26)
Aspartic acid ...	104 (94–116)	108 (99–117)	82 (76–89)	50	31 (13–72)	144 (131–158)
Threonine.....	(50–64)	(33–58)	(48–58)	21	(8–35)	(37–70)
Serine	64 (57–71)	65 (59–71)	66 (64–67)	35	35 (21–59)	101 (81–126)
Glutamic acid ..	(109–133)	(117–137)	(117–121)	69	(10–38)	(119–142)
Proline.....	54 (47–62)	63 (56–71)	74 (65–83)	99	38 (28–54)	37 (32–43)
Glycine.....	126 (101–158)	181 (147–224)	185 (159–215)	264	512 (391–671)	77 (68–87)
Alanine.....	76 (71–81)	80 (76–85)	64 (61–67)	136	37 (23–62)	89 (81–99)
Cystine (half)...	13 (7–23)	4 (2–7)	12 (9–17)	3	5 (1–14)	2 (1–5)
Valine.....	53 (46–60)	50 (45–57)	41 (38–43)	48	30 (16–55)	69 (66–72)
Methionine.....	19 (13–27)	17 (11–26)	16 (15–17)	7	18 (6–56)	6 (4–9)
Isoleucine.....	44 (39–50)	36 (35–37)	41 (38–46)	28	12 (6–26)	57 (49–66)
Leucine.....	72 (65–80)	70 (66–74)	68 (64–72)	65	18 (9–35)	97 (91–103)
Tyrosine	6 (1–37)	5 (1–22)	17 (14–21)	0.6	30 (12–74)	11 (9–14)
Phenylalanine...	28 (22–36)	24 (18–33)	25 (21–30)	19	43 (24–80)	37 (30–46)
OH-lysine.....	0.8 (0–9)	2 (2–3)	4 (3–5)	4	0.1 (0–3)	–
Lysine.....	54 (43–67)	21 (18–26)	23 (18–30)	31	20 (14–29)	28 (20–39)
Histidine.....	12 (5–30)	4 (1–14)	6 (3–10)	0.4	5 (2–11)	1 (0–48)
Arginine	50 (38–66)	55 (44–68)	50 (49–51)	63	27 (13–55)	32 (23–45)
Proteins**	194.70	138.25	18.84	45.96	85.44	18.23
Hexosamines ...						

* Number af amino acid analyses
** Arithmetic mean

tissues (after DEGENS et al., 1967)
theses are 1σ ranges)

stracum		Ligament	Fibrous proteins				
Cephalopoda (1)	Brachiopoda (1)	Pelecypoda (5)	Collagen (5)	Keratin (5)	Fibrinogen (5)	Elastin (3)	Resilin (3)
65	–	–	82 (66–103)	–	–	18 (17–19)	–
224	89	55 (23–130)	49 (47–51)	65 (47–74)	33 (19–55)	17 (8–36)	101 (95–109)
48	31	19 (11–33)	21 (18–25)	58 (51–66)	15 (10–23)	13 (11–16)	31 (30–32)
67	48	41 (21–80)	44 (33–57)	103 (86–123)	131 (105–162)	11 (9–14)	80 (79–81)
101	50	31 (11–86)	74 (71–77)	111 (87–143)	18 (8–41)	46 (29–73)	48 (46–51)
74	49	41 (14–114)	120 (107–135)	75 (59–96)	7 (2–28)	129 (115–145)	79 (78–80)
132	171	376 (211–671)	325 (310–142)	84 (67–107)	292 (218–392)	158 (143–175)	383 (361–413)
46	304	31 (8–114)	113 (105–121)	54 (47–63)	281 (218–364)	151 (128–179)	107 (104–111)
38	1	6 (1–35)	–	114 (88–148)	–	–	–
49	39	22 (11–45)	20 (19–21)	68 (58–79)	24 (12–46)	159 (134–188)	28 (26–31)
3	5	81 (33–199)	6 (4–10)	0.1 (0–2)	–	2 (1–6)	–
28	11	18 (10–33)	12 (11–13)	34 (29–40)	12 (5–31)	36 (30–43)	17 (15–21)
41	26	10 (3–40)	26 (25–28)	74 (64–87)	13 (4–46)	99 (93–105)	23 (20–27)
12	31	11 (1–101)	3 (2–5)	25 (18–35)	46 (14–145)	34 (24–48)	27 (26–28)
19	16	19 (7–48)	14 (12–16)	26 (21–32)	5 (3–10)	77 (76–78)	26 (25–27)
–	–	–	7 (6–8)	–	–	–	–
17	25	11 (4–26)	25 (23–27)	23 (15–34)	7 (3–16)	12 (8–19)	6 (5–7)
–	6	1 (0.5–3)	5 (4–7)	6 (4–10)	3 (1–9)	2 (1–3)	9 (7–12)
35	98	6 (4–10)	48 (46–51)	59 (50–71)	24 (13–44)	20 (12–34)	35 (34–36)
15.83	13.58	171.19	n.d.	n.d.	n.d.	n.d.	n.d.

3. Factor analysis

One of our principal objectives has been to describe and understand the complex interrelationships between the amino acids in the heterogeneous shell organic matrix. To a large extent, we have relied on the multivariate statistical technique known as factor analysis. This program was aimed at determining how the individual amino acids are grouped with respect to species and how these newly derived amino acid groups (factors) systematically vary throughout the molluscan phylum.

COMPARATIVE BIOCHEMISTRY OF MOLLUSCS

In Table I and II the geometric means of calcified and uncalcified organic tissues of various invertebrates are presented. For comparative purposes, amino acid data on a few common fibrous proteins are included. The shell tissues compare with collagen and resilin in that they contain about the same number of non-polar residues, whereas the content of hydrophobic side chains in keratin is about 20 percent less and in fibrinogen and particularly elastin is much higher. The mineralized tissues lack hydroxyproline which is a dominant residue in collagen but contain methionine and cystine. Most noteworthy is the high abundance of aspartic acid and the wide range of amino acid variation in the shell tissues of the molluscs. In contrast, the common fibrous proteins, with exception of resilin, are low in aspartic acid and exhibit a narrow range in amino acid composition.

In comparing, on the other hand, the geometric means of mantle tissues and shell organic matrix of molluscs, one can recognize the following similarities: (1) same number of non-polar residues, (2) high abundance of aspartic acid, and 3) presence of cystine and methionine. They differ principally in that the mantle tissues do contain hydroxyproline and twice the amount in glutamic acid and threonine.

Let us also examine the relationship between the amino sugars and proteins. There is a significant decrease in amino sugars by moving from amphineura to cephalopods, gastropods, and clams. The corresponding mean protein/amino sugar ratios are about 1, 2, 100, and 150 respectively. This relationship is of phylogenetic interest since molluscs, annelids, and arthropods are related to a common annelid-type ancestor. Thus a comparison of the chitin-protein matrices of these groups of animals may be revealing both for the purpose of comparative biochemistry and the elucidation of calcification mechanisms.

Mantle tissues of molluscs are also characterized by the presence of amino sugars. It is interesting to note that cephalopods and amphineura show a higher content in amino sugars relative to the clams and gastropods. These similarities between mantle and associated shell tissues suggests that the organic matrix in shells largely reflects a somewhat modified mantle tissue. This feature would be analogous to the relationships established in the biochemistry of collagens present in vertebrate bones and in the muscle tissues.

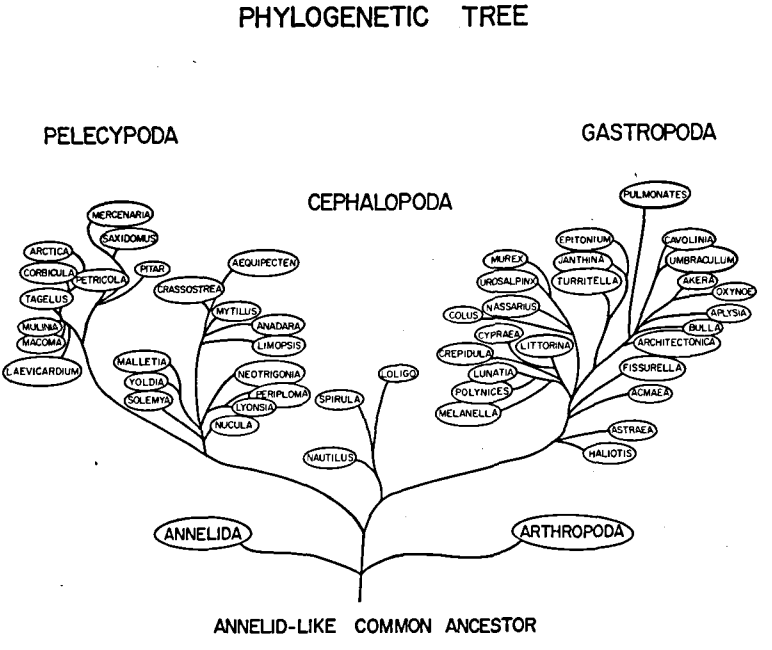


Fig. 3. Phylogenetic Tree of Mollusca based on Factor Scores (after DEGENS et al. 1967).

By taking the amino acid analysis of the individual species and subjecting the data to a factor analysis program, it is possible to reduce the complexities of the data to a few significant variables which have the same information content as the original data. We have discussed at length the significance of such a program in two separate articles (DEGENS et al., 1967; GHISELIN et al., 1966). In principle we could demonstrate that the following co-variant groups of amino acids can be recognized:

threonine, glutamic acid-glycine;
isoleucine, leucine, valine;
aspartic acid;
lysine, histidine, arginine.

The independance of both the aspartic acid and lysine-histidine-arginine factor is remarkable in view of the fact that here we have a means to supply acidic and basic functions for calcification.

The variation in one or the other of the amino acid factors can also be related to molluscan phylogeny and environment. By taking all sources of information into consideration, one can construct a phylogenetic tree for the molluscs which is largely based on the individual factor scores (Figure 3). Furthermore, by investigating a few forms that can live in a wide range of natural habitats, there is a tendency of the organisms to

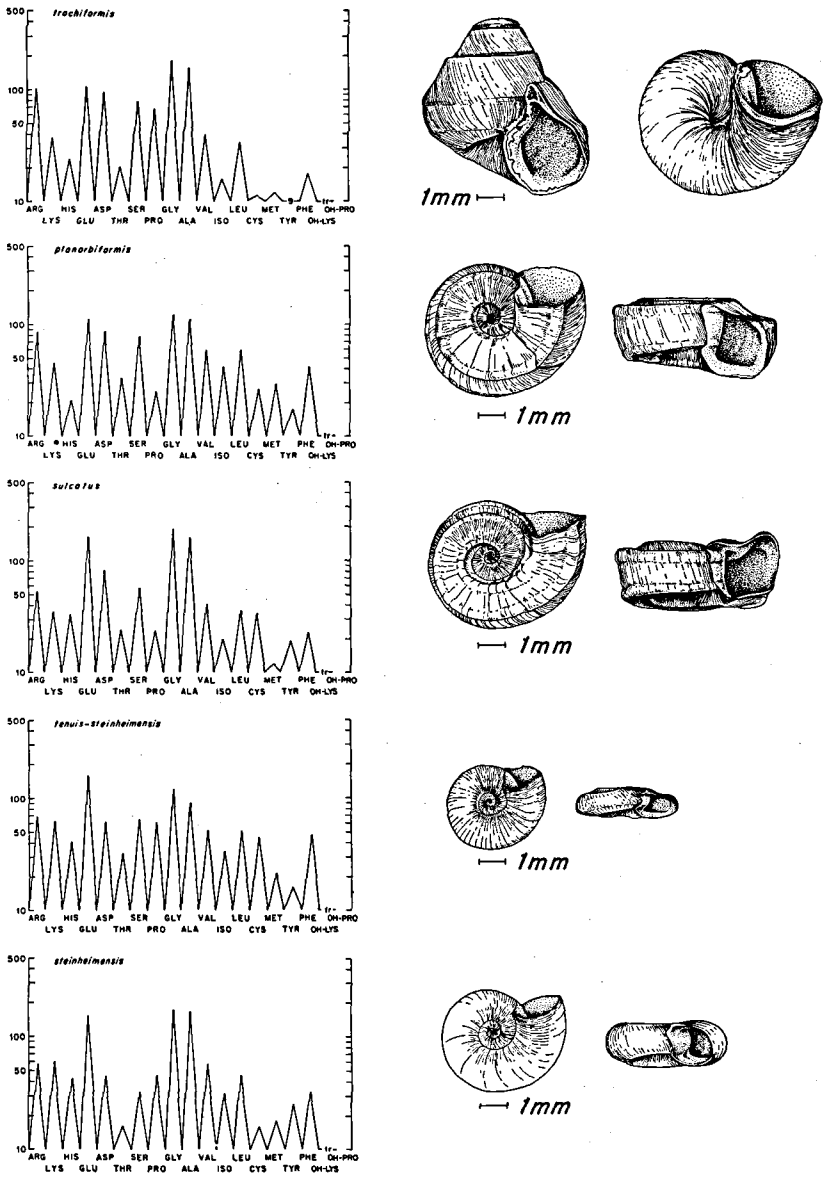
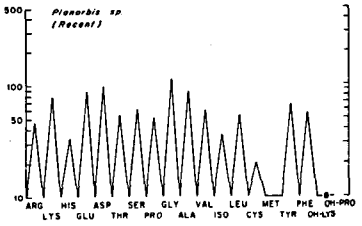


Fig. 4 a-b. Amino Acid Pattern of *Planorbis* sp. from the Miocene of Steinheim, Germany.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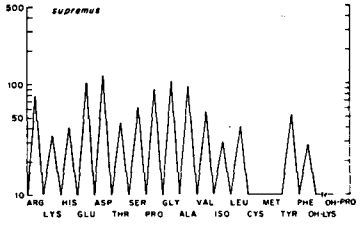
LYS GLU THR PRO ALA ISO CYS TYR OH-LYS


supremus
500
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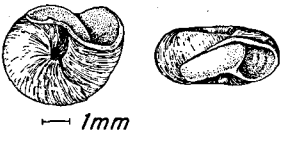
LYS GLU THR PRO ALA ISO CYS TYR OH-LYS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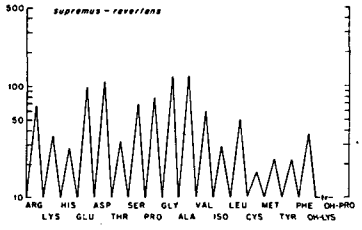
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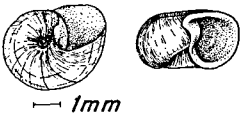
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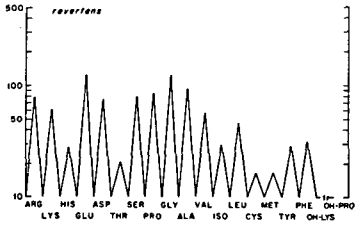
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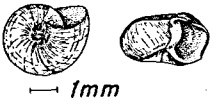
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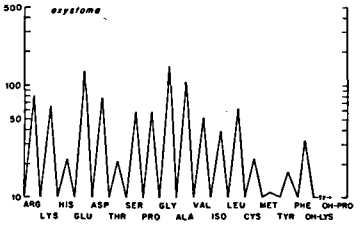
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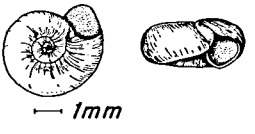
1mm

slightly concentrate onto one or the other of the amino acid factors in response to temperature or salinity fluctuations. The range in temperature or salinity, rather than the absolute temperatures or salt concentrations to which an organism is subjected during its lifetime, appear to exercise control on the secretion pattern of the shell organic matrix. This may mean that these two parameters are a determining factor in the evolution of invertebrates.

In order to show the significance of this type of work to the general area of Paleontology, I have selected a group of snails from the Miocene which, according to the paleontological record have undergone a remarkable degree of modification. The specimens were selected from the Steinheim Basin, Germany. Based on geological and geochemical data, one can infer that the change in morphology of the *Planorbis* sp. coincides with severe fluctuations in salinity or temperature. Two series can be recognized: (1) starting with *steinheimensis* and ending with *throchiformis* (Figure 4 a), and (2) starting with *oxystoma* and ending with *supremus,* (Figure 4 b). In comparing the amino acid composition of the various species, the glutamic acid, aspartic acid, and arginine, lysine, histidine relationships are of special significance. For example, from bottom to top of both series, aspartic acid increases, whereas lysine decreases. Many more differences are recognizable although on first sight the amino acid compositions among the *Planorbis* series appear to be rather similar. For comparison, a recent *Planorbis* specimen has been incorporated.

From these data two things can be learned, i. e. proteins can be preserved in fossil materials, and the acidic and basic functions seem to be a determining factor in controlling the position of the nucleation sites in the organic templates. Consequently the morphology of the shells appears to be only a macroscopic expression of the molecular biology of the shell matrix.

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