Observations on the Non-calcareous Component of the Shell of the Lamellibranchia

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SUMMARY

A comparison has been made between the staining reactions and histochemical properties of the non-calcareous material (conchiolin) from the different layers of the shells of *Anodonta cygnea*, *Mytilus edulis*, and *Ostrea edulis*. Acid hydrolysates of the conchiolin protein have been analysed qualitatively by paper chromatography.

The composition of the conchiolin in *Anodonta* confirms the view that corresponding layers of the valves and ligament represent modifications of the same layers of the shell. In this bivalve, the properties of the outer layers of the valves and ligament are closely comparable with each other, and also with those of the periostracum and the fusion layer of the ligament. All these regions consist of a quinone-tanned protein, hydrolysates of which are rich in phenolic amino-acids, especially tyrosine, and in glycine.

Much of the periostracal conchiolin in *Mytilus* shows basically the same properties as the periostracum in *Anodonta*. However, the outer layers of the valves and ligament in *Ostrea* and *Mytilus* each exhibit progressively greater specialization compared with the situation in *Anodonta*. This is most marked in *Mytilus* where these components differ completely in character.

The conchiolin in the inner shell layers differs markedly in composition from that in the outer layers in *Anodonta* and *Ostrea* and from the periostracum in *Mytilus*. Hydrolysates of its protein constituent contain appreciably more aspartic acid and glutamic acid but much smaller amounts of phenolic amino-acids. The protein is only lightly tanned. Although in these properties the corresponding inner layers of the valves and ligament appear fundamentally alike, each component has certain specialized features. It is suggested that the modifications shown by the protein of the inner ligament layer, which is characterized by a high content of proline and methionine, are correlated with the specialized function of this region of the shell.

INTRODUCTION

IT has been maintained on morphological grounds that, in the Lamellibranchia, the outer and inner layers of the valves and of the ligament are to be regarded as representing local modifications of the same two layers of the shell (Owen, Trueman, and Yonge, 1953). However, little attempt has yet been made to determine to what extent the non-calcareous components of these corresponding layers of the valves and ligament are comparable chemically. This non-calcareous material, originally termed conchiolin by Frémy (1855), makes up the bulk of the ligament and is also a variable but important constituent of the valves. Trueman (1949) has shown that in *Tellina tenuis* the conchiolin in the outer layer of the ligament differs in composition from that in the inner layer, and that these two types of conchiolin appear to correspond respectively with those of the periostracum and the inner complex layers of the valves. He considered, however, that the chemical

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properties of the various forms of conchiolin could be more effectively studied in bivalves which have larger ligaments and less highly calcified valves than is the case in *Tellina*.

In this paper, the properties of the non-calcareous material in three such bivalves are described. Particular reference is made to the shell conchiolin of *Anodonta cygnea*, the properties of which have already been outlined (Beedham, 1954), and additional observations are made on *Mytilus edulis* and *Ostrea edulis*. The staining reactions and histochemical properties of the non-calcareous components of the different layers of the shell are compared in detail and their protein contents analysed qualitatively by means of paper chromatography. Sections of the conchiolin were prepared from shells fixed in Bouin's fluid, 4% aqueous neutral formalin, or other routine fixatives, and decalcified in dilute hydrochloric acid. Ester wax (Steedman, 1947) was found to be the most suitable embedding medium, although in the case of the ligament, which tends to become extremely brittle during wax embedding, sections were often cut directly on a freezing microtome.

Staining Reactions

The non-calcareous components of the outer and inner calcareous layers of the valves in *Anodonta* and *Ostrea* can readily be differentiated in section both by their appearance, owing principally to the well-marked prismatic structure of the former, and by their reactions to triple stains. With Mallory's or Masson's stains, the outer layer always colours red and the inner layer blue or green respectively. The thin, whitish laminae of the inner layer are also distinguished by the fact that they colour relatively more strongly with Delafield's or Ehrlich's haematoxylin and show slight metachromasia with aqueous toluidine blue, although these differences between the layers are less pronounced in *Ostrea* than in *Anodonta*. The outer layer in *Anodonta* is continuous with the overlying periostracum, up to 15 μ thick, which has a natural amber colour and is refractory to stains. In contrast, the extremely thin periostracum in *Ostrea* is hardly distinguishable overlying the shell, and its properties are not recorded here.

Although the outer and inner calcareous layers of the valves in *Mytilus edulis* differ in crystalline structure (Field, 1922; White, 1937), their conchiolin components have a similar appearance in section. Unlike the corresponding regions in *Anodonta* and *Ostrea*, they have the same staining reactions and both colour blue with Mallory. The whole of this ground substance of the valves is sharply distinguished in structure and properties from the superficial thick periostracum.

The periostracum in *Mytilus*, which is secreted by the inner epithelium of the outer fold at the mantle edge, consists basically of three layers (fig. 1). To avoid confusion with the main layers of the shell, these will be referred to as the external, middle, and internal layers. The thin external layer is formed at the extreme base of the periostracal groove. At its origin this layer shows affinity for the acid fuchsin in Mallory's stain, but the reaction fades as the

external surface of the periostracum comes into contact with sea-water (fig. 1). The middle layer is secreted next; it constitutes the bulk of the periostracum and is up to 80 μ thick. It consists of clear, yellowish conchiolin, mostly uncoloured by routine stains, in which lies a central vacuolated region (fig. 1).

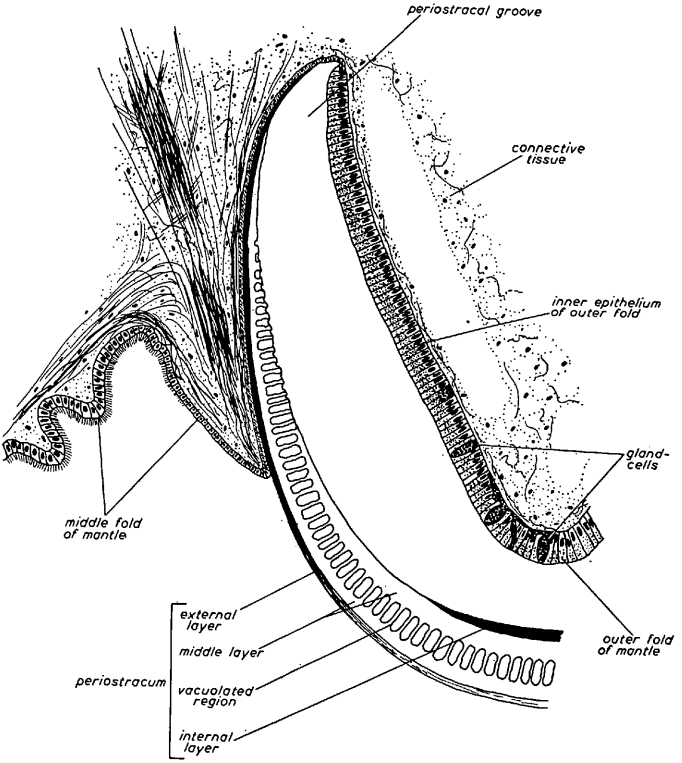

FIG. 1. Diagram of a transverse section through the mantle edge of *Mytilus edulis* to show the structure of the periostracum. Not to scale.

Finally, the internal layer is deposited by the epithelium towards the tip of the outer fold. It has a natural brownish colour and in both Mallory and Masson preparations it stains deep red. The products of the underlying gland-cells in this region of the outer mantle fold (fig. 1) are considered to have a lubricatory function (Beedham, 1958), and do not appear to be directly concerned in periostracum formation.

As in other lamellibranchs described by Trueman (1950*b*), the outer and inner layers of the ligaments in the bivalves investigated are rapidly differentiated by Mallory, the former colouring with the acid fuchsin and the latter with aniline blue. A similar distinction is obtained with Masson's trichrome stain. The inner layer is also comparable with the corresponding region of the valves in that it shows affinity for Ehrlich's haematoxylin and exhibits metachromasia with toluidine blue. In contrast, the periostracum which extends over the ligament in *Mytilus* (Yonge, 1955) and the amber-coloured fusion layer of the ligament in *Anodonta* (Beedham, 1958) are refractory to most staining techniques.

The characteristic reaction of the conchiolin to triple stains suggests that the outer and inner layers differ in composition and that, in certain lamellibranchs, corresponding layers of the valves and ligament have basically the same properties. Trueman (1951) observed, moreover, that these reactions emphasize the homologies of corresponding layers of the shell in different bivalves. Consequently, it seemed worth while to investigate the staining properties of the shell layers further by comparing their reactions to specific acid and basic dyes in a series of buffer solutions (fig. 2). Although Levine (1940) has shown conclusively that this technique of staining at controlled pH does not, as had been previously claimed, determine the isoelectric points of components of tissue sections, it remains, nevertheless, a useful method for comparing the staining properties of different elements within the same section.

Since differentiation of the shell layers with trichrome stains is invariably obtained whatever the method of fixation, the observations were made under the same conditions on material preserved in routine fixatives. Sections were stained for 24 to 36 h in 0·0001 M solutions of the acid dye (ponceau 2R) and the basic dye (methylene blue) in standard buffers ranging from pH 1·6 to pH 11·0. The intensity of staining of the conchiolin was estimated arbitrarily on the scale 0 to 8 by visual comparison with colour charts prepared with solutions of ponceau 2R and methylene blue of different concentrations (Levine, 1940). The values obtained were plotted on graph paper with staining intensity on the ordinate and pH of the dye-buffer solutions on the abscissa (fig. 2).

The results show that, in general, the outer layers of both valves and ligament always stain more strongly with the acid dye than do the inner layers (fig. 2), a feature which can be correlated with the greater affinity which they show for the acid fuchsin in Mallory's stain. However, this difference varies considerably in extent. It is most pronounced in the ligaments of *Mytilus* and *Ostrea*, in which the outer layers stain intensely with ponceau 2R over a very wide pH range (fig. 2). In the valves of *Anodonta*, on the other hand, the difference in acidophily is small, although it is interesting to note that the distinction between the layers can be enhanced by treatment with tap-water. This procedure, which is normally adopted during Mallory's technique, causes rapid decolorization of the inner layer, whereas the acid dye tends to be retained by the outer layer.

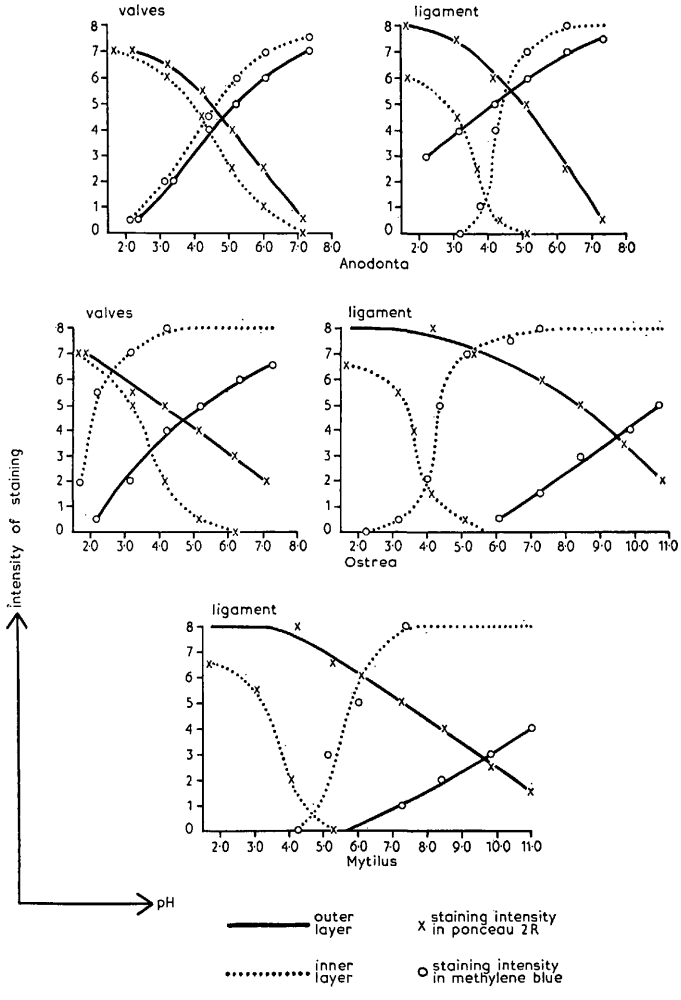

FIG. 2. Diagram showing the staining reactions at controlled pH of the outer and inner layers of the shell in *Anodonta*, *Ostrea*, and *Mytilus*. Intensity of staining in the acid dye, ponceau 2R, is marked with a cross and that in the basic dye, methylene blue, with a circle. The buffers used, namely, Walpole's sodium acetate / HCl (below pH 2·2), Clark and Lubs's standard buffers (pH 2·2 to pH 10·0), and Sörenson-Walbum's glycine and NaCl/NaOH (above pH 10·0), were all employed at one-tenth their standard concentration. Staining curves drawn with unbroken lines represent the outer layers of the valves and ligament; those with broken lines the inner layers.

A similar variation is observed in the degree of differentiation obtained between the shell layers with methylene blue (fig. 2). The difference in basiphilia between the layers in *Anodonta* is not unusually large, but in the valves of *Ostrea*, the inner layer is sharply distinguished from the outer, and also from the rest of the conchiolin investigated, in that it shows marked affinity for the basic dye. In *Mytilus* and *Ostrea*, the outer and inner layers of the ligament are similarly well defined, but this is due to the exceptionally weak reaction of the former to methylene blue (fig. 2).

The difference in staining properties which undoubtedly exists between the outer and inner layer conchiolin is, therefore, subject to some variation. As a result, the reactions of corresponding layers of the valves and ligament may appear somewhat similar, as is the case in *Anodonta*, or they may show appreciable differences, as in *Ostrea* (fig. 2). It is evident that the properties of individual layers can differ from those of their homologues both in the same shell and in the shells of other bivalves, these modifications not necessarily being revealed by Mallory's and other routine staining techniques.

Histochemical Properties

Anodonta cygnea

The chemical and histochemical reactions of the different layers of the shell are compared in detail in table 1. As previously observed (Beedham, 1954), the conchiolin consists mainly of protein, although the colouring of the outer and inner layers with Sudan black and their weak reaction to the periodic acid / Schiff test (Hotchkiss, 1948) indicates respectively that lipid material and possibly some polysaccharide may also occur. The mucin stain, alcian blue (Steedman, 1950), reacts with the inner layers but not with the remainder of the shell conchiolin. It is interesting to observe that fragments of conchiolin from certain parts of the shell resist treatment with hot, saturated potassium hydroxide and react to the chitosan / iodine test for chitin (Campbell, 1929) (table 1). Chitin has already been recorded in the shell of *Anodonta* and other bivalves by Wester (1910). Nevertheless, even if chitin occurs it is present only in very small quantities. Analyses by Schlossberger (1856), Wetzel (1900), and many others have shown that conchiolin has a nitrogen content of approximately 16% in contrast to 6·5% for chitin, which indicates that its principal constituent is protein.

When treated with a series of reagents devised by Brown (1950*b*) for determining the types of linkages present in structural proteins, the whole of the non-calcareous material in the shell dissolves almost completely in the sodium hypochlorite reagent only (table 1). This suggests that much of the conchiolin protein is hardened by quinone-tanning. As is well known, the presence of quinone-tanned proteins has been established in many invertebrate skeletal structures, including the shell and byssus of certain lamellibranchs (Brown, 1950*a*, 1952; Trueman, 1950*a*), and also in the egg capsules of selachians (Brown, 1955; Threadgold, 1957).

The outer layers of the valves and ligament, which have remarkably similar

TABLE 1

A summary of the results of chemical and histochemical tests on the non-calcareous material in the different layers of the shell of Anodonta cygnea

Intensity of the reaction is represented arbitrarily by number of Xs; *tr* indicates trace amount; O indicates no recognizable response. Details of the tests used are given in the text

	Valves			*Ligament*		
Test	*Perio-stracum*	*Outer layer*	*Inner layer*	*Fusion layer*	*Outer layer*	*Inner layer*
HCl, conc., room temp., 8 h	All persist					
HCl, conc., 55° C, 8 h	Persists		Slowly dissolves	Persists		Quickly dissolves
KOH, hot, sat. .	Dissolves		Fragments persist	Fragments persist		Dissolves
10% sodium hypochlorite	All mostly dissolve					
Sodium sulphide	No apparent effect					
Millon . .	XXXXX	XXXX	X	XXXXX	XXXX	X
Xanthoproteic .	XXXXX	XXX	X	XXXXX	XXXX	X
Folin (Baker) .	XXXXX	XXXX	tr	XXXXX	XXXX	X
Argentaffin .	XXXXX	XX	? tr	XXXXX	XXX	X
Sakaguchi (Baker)	XX	XXX	XXX	X	X	XXX
Sulphur . .	tr		O	tr		XXXX
Sudan black B .	tr	XXX	XXX	tr	XX	XX
Periodic acid / Schiff	O	? tr	X	O	X	X
Alcian blue .	O	O	XXX	O	O	XX
Chitosan (Campbell)	O	O	+ve	+ve		O

histochemical properties, both differ markedly from the inner shell layers in their reactions to tests for phenolic groups (table 1). The outer layers and, to an even greater extent, the periostracum and fusion layer always react strongly to the Millon and xanthoproteic tests and to Baker's (1956) modification of Folin's method for phenols, which indicates that all these regions of the shell contain a large proportion of phenolic groupings and are highly tanned. As will be shown later, hydrolysates of the protein contents of these regions contain considerable amounts of phenolic amino-acids, especially tyrosine.

As observed by Trueman (1950*a*), the presence of an orthoquinone which may be responsible for hardening the conchiolin is suggested by the fact that, even after boiling, sections of the outer layer of the ligament are still able to oxidize the Nadi reagent (dimethyl-*p*-phenylenediamine and α-naphthol). However, attempts to detect, either in the conchiolin or in the mantle tissues, a specific polyphenol which might be the precursor of the tanning agent were rather unsuccessful. The argentaffin test (Lison, 1953), which has been widely used to indicate the localization of polyphenols in quinone-tanning systems, reacts moderately with the outer layers and intensely with the periostracum and fusion layer (table 1), but this reaction, which is also given by the

epithelial cells of the outer mantle fold (Beedham, 1958), is not specific. Moreover, the response of the conchiolin to the argentaffin and other phenolic tests is apparently unaffected by the prolonged treatment with acid, alcohols, &c., involved in the preparation of decalcified sections, which suggests that it is due to firmly bound aromatic groups rather than to free polyphenols. The chromaffin test for polyphenols (Lison, 1953) reacts with the fusion and outer layers of fresh undecalcified sections of the ligament, but the more specific potassium iodate reaction, and the ferric chloride and ammonium molybdate tests for orthodiphenols (Lison, 1953), were found to give mainly negative results.

A free orthodiphenol may, of course, occur in *Anodonta* but not in sufficient quantities to be detected histochemically. Possibly this orthodiphenol is initially present in the tissues in a masked condition, as is known to occur in the case of protocatechuic acid, the precursor of the tanning agent in the oothecae of *Blatta* and *Periplaneta* (Brunet and Kent, 1955), although this would not appear to explain the failure to locate free polyphenols in the immediate vicinity of the shell. However, the presence of an enzyme capable of oxidizing polyphenols is indicated by a number of techniques. If fresh sections of the ligament are incubated with *l*-tyrosine in buffer at pH 8·0, the incubation medium slowly darkens and laminae of the fusion layer and parts of the outer layer turn brownish black. This reaction, which is inhibited by potassium cyanide or by boiling, indicates the occurrence of a thermolabile polyphenol oxidase (Brown, 1952). Smyth's (1954) catechol technique for the detection of polyphenol oxidase also reacts positively with these and certain other regions of the ligament.

Although it can be assumed from these results that the conchiolin in the periostracum and in the fusion and outer layers consists largely of a quinone-tanned protein, the nature of the tanning process is obscure. The rich tyrosine content of the protein indicates, as already suggested by Roche, Ranson, and Eysseric-Lafon (1951) in observations on the valve conchiolin of certain lamellibranchs, that hardening may be partly effected by the tanning action of an orthoquinone produced by the oxidation of the side chains of this tyrosine component. It has been suggested that this form of aromatic bonding, in which a phenolic protein acts both as substrate and as tanning agent, i.e. undergoes 'self-tanning', occurs in the byssus of *Mytilus* (Brown, 1950*a*, 1952; Smyth, 1954) and the cuticles of myriapods and insects (Blower, 1951; Dennell and Malek, 1956). However, theargentaffin reaction of the conchiolin indicates the presence, especially in the periostracum and fusion layer, of a more powerful reducing agent than a phenolic protein. Dennell and Malek (1955*b*) suggest that such a reaction could well be caused by oxidation products of polyphenols. They demonstrated that fully hardened cuticles of *Periplaneta americana* still give an intense argentaffin reaction even after all free dihydroxyphenols present have been extracted. It is provisionally suggested, therefore, that the phenolic protein in the periostracum, fusion layer, and outer layers is initially 'self-tanning', but that at least part of it sub-

sequently undergoes phenolic tanning in the manner visualized by Pryor (1940, *a*, *b*). The situation in *Anodonta* may be analogous to that in the cuticle of *Periplaneta*, in which tanning is considered to occur in two such stages (Dennell and Malek, 1955, *a*, *b*, 1956). Whatever the tanning method, there is little doubt that final hardening is most pronounced in the amber-coloured conchiolin of the periostracum and fusion layer, rendering it refractory to stains.

In contrast to the conchiolin described above, that in the inner layers of the valves and ligament reacts weakly to all tests for aromatic groupings (table 1). These properties, coupled with the fact that hydrolysates of the inner layers contain only small amounts of phenolic amino-acids (see below), suggests that the inner layer protein is relatively lightly tanned. The difference in stability between this protein and the highly tanned component of the remaining shell layers is illustrated by the reaction of the conchiolin to concentrated mineral acids. Although all the shell conchiolin shows some resistance to concentrated hydrochloric acid at room temperature, that in the inner layers dissolves more quickly when the acid is heated (table 1).

In these and other properties, the conchiolin in the inner layer of the valves corresponds closely with that in the inner ligament layer. Both these regions react moderately to Baker's (1947) modification of the Sakaguchi test for arginine, and are distinguishable in this respect from the fusion and outer layers of the ligament (table 1). However, the inner layer of the valves differs from its homologue in that it responds to the chitosan reaction, whilst the inner ligament layer is characterized by the fact that it reacts positively to the test for sulphur described by Hawk, Oser, and Summerson (1954) (table 1). Portions of conchiolin from each shell layer were heated with potassium nitrate and sodium carbonate and after dissolving in warm water and filtering, the filtrate was acidified with hydrochloric acid and boiled. On adding barium chloride, a faint but distinct white precipitate formed with the product from the inner ligament, whereas the reaction was found to be much weaker or negative with the other layers (table 1). As will be shown later, there is evidence that an amino-acid containing sulphur occurs in appreciable amounts in hydrolysates of the inner ligament. However, since all regions of the shell appear to be unaffected by an alkaline solution of sodium sulphide (table 1) or by thioglycollate solution (Goddard and Michaelis, 1934), it is likely that even if disulphide bonds occur in the inner layer of the ligament, they are not concerned in the stabilizing of its protein structure (Brown, 1950*b*, 1952).

Mytilus edulis *and* Ostrea edulis

Similar tests were applied to the conchiolin in *Mytilus* and *Ostrea* and the results are summarized in tables 2 and 3 respectively. In many cases, the reactions of the various layers of the shell are comparable with those described for their homologues in *Anodonta*. As shown by Brown (1952), the whole of the periostracum in *Mytilus* is intensely argentaffin and consists of a quinone-tanned protein. The properties of the external and middle layers, especially

their intense reactions to all tests for phenolic substances (table 2), suggest that they are both similar in composition to the periostracal conchiolin of *Anodonta*. The ability of the external layer to stain with Mallory is probably due to it being less completely hardened at its origin than is the middle layer. In contrast, the internal layer of the periostracum reacts only moderately to the Millon, xanthoproteic, and Folin (Baker) tests, and appears to differ more fundamentally in composition from the conchiolin of the middle layer.

TABLE 2

A summary of the results of chemical and histochemical tests on the non-calcareous material in the shell of Mytilus edulis

Nomenclature as in table 1

	Valves				*Ligament*		
	Periostracum						
Test	*Ext. and middle layer*	*Int. layer*	*Outer layer*	*Inner layer*	*Perio-stracum*	*Outer layer*	*Inner layer*
HCl, conc., room temp., 8 h	All persist						
HCl, conc., 55° C, 8 h	Persists		Slowly dissolves		Persists	Quickly dissolves	
KOH, hot, sat.	Dissolves			Fragments persist	Dissolves		
10% sodium hypochlorite	All mostly dissolve						
Sodium sulphide	No apparent effect						
Millon	XXXXX	XX	X	X	XXXXX	X	? tr
Xanthoproteic	XXXXX	XXX	X	X	XXXXX	XX	? tr
Folin (Baker)	XXXXX	XX	tr	tr	XXXXX	X	O
Argentaffin	XXXXX	XXXXX	? tr	? tr	XXXXX	X	XX
Sakaguchi (Baker)	XXX	XX	XXX	XXX	XXX	XXX	tr
Sulphur	O		O	? tr	O	O	XXXX
Sudan black B	X	tr	tr	tr	O	XX	tr
Periodic acid / Schiff	? tr	X	tr	tr	O	O	O
Alcian blue	O	O	XXX	XXX	O	O	XX
Chitosan	O	O	O	+ve	O	O	O

The outer layers of the valves and ligament in *Ostrea* correspond closely to each other and to their counterparts in *Anodonta* in that they react moderately to the argentaffin test and strongly to other tests for aromatic groups (tables 1 and 3). This suggests that these layers are composed of a quinone-tanned protein probably similar to that in the outer shell conchiolin in *Anodonta*. However, as in their staining reactions at controlled pH, each of these components in *Ostrea* is specialized in particular ways. The conchiolin in the outer layer of the valves, for example, is distinguished by the fact that it shows affinity for alcian blue and reacts positively to the chitosan test (table 3).

TABLE 3

A summary of the results of chemical and histochemical tests on the non-calcareous material in the shell of Ostrea edulis

Nomenclature as in table 1

Test	*Valves*		*Ligament*	
	Outer layer	*Inner layer*	*Outer layer*	*Inner layer*
HCl, conc., room temp., 8 h	All persist			
HCl, conc., 55° C, 8 h.	Persists	Quickly dissolves	Persists	Quickly dissolves
KOH, hot, sat.	Fragments persist		Dissolves	
10% sodium hypochlorite	All mostly dissolve			
Sodium sulphide	No apparent effect			
Millon	XXXX	XX	XXXXX	X
Xanthoproteic	XXXX	X	XXXXX	XX
Folin (Baker)	XXXX	XX	XXXX	X
Argentaffin	XXX	tr	XXX	tr
Sakaguchi (Baker)	XXX	X	XXXX	XXX
Sulphur	tr	O	X	XXXX
Sudan black B	XX	XX	XX	XX
Periodic acid / Schiff	O	XX	O	? tr
Alcian blue	XX	XX	O	XX
Chitosan (Campbell)	+ve	+ve	O	O

In *Mytilus*, as was to be expected, the non-calcareous component of the outer layer of the valves was found to differ totally in character from that of the corresponding layer of the ligament (table 2). This is not, however, due solely to the former being almost identical in composition with the matrix of the inner calcareous layer of the valves. The outer ligament layer is also unusual in that it dissolves rather easily in warm, concentrated hydrochloric acid, and gives a weak reaction to phenolic tests (table 2). Its protein content is obviously considerably less hardened by tanning than that in the homologous layers of *Anodonta* and *Ostrea*.

In common with the corresponding regions in *Anodonta*, the inner shell layers in both *Mytilus* and *Ostrea* show only slight signs of tanning. Their histochemical properties indicate that they contain a relatively small proportion of phenolic groupings (tables 2 and 3). In these features, the inner layers of the valves and ligament are readily differentiated from the outer shell layers in *Ostrea* and from the periostracum in *Mytilus*. Other notable points of comparison between homologous layers of the shells in different bivalves are that, as in *Anodonta*, the conchiolin of the inner ligament layer in *Mytilus* and *Ostrea* is specialized in that it gives a positive reaction for sulphur, while the inner layers of the valves all respond to the chitosan test for chitin (tables 1–3).

THE COMPOSITION OF THE SHELL PROTEIN

In order to examine the protein complements of the different shell layers in greater detail, each was analysed qualitatively by paper chromatography.

It was found that the outer and inner layers of the valves in *Mytilus* have almost the same amino-acid composition, a feature which accounts for the similarity in their staining and histochemical reactions. Consequently, the amino-acid content of the inner layer only is described here and is contrasted with that of the periostracum. In all other cases, the inner layer conchiolin is compared with that occurring in the remaining shell layers. The latter are referred to as the 'outer regions' of the shell, this term replacing 'outer layers' previously employed (Beedham, 1954). In addition to the true outer layers, these regions incorporate periostracal material, and in the case of the ligament of *Anodonta*, the fusion layer. It was not found possible to separate these layers sufficiently well to treat them as separate units.

After being purified by extraction with boiling ether, samples of conchiolin from the different regions of the shell were hydrolysed in sealed tubes with 6 N hydrochloric acid for 24 h at 100° C. Excess hydrochloric acid was removed by evaporation and the hydrolysates were then taken up in 10% *iso*-propanol to produce in each case a final concentration of 10 mg of the original purified conchiolin per 1 ml solvent. The amino-acids were separated on Whatman no. 1 filter paper; the solvents used were aqueous phenol and butanol / acetic acid / water (4:1:5) (Block, Durrum, and Zweig, 1955). Estimates of the amounts of individual amino-acids in the different hydrolysates were made, usually on one-dimensional chromatograms, by visual assessment of the size and intensity of the spots, these being assigned an arbitrary value on the scale 0 to 11. Most of the estimates were carried out on chromatograms developed with ninhydrin, but in certain cases more specific reagents were employed. Arginine was determined by the α-naphthol / bromine test (Acher and Crocker, 1952) and proline by spraying with isatin in acetone (Block, Durrum, and Zweig, 1955). Jepson and Smith's (1953) technique for the detection of hydroxyproline was also applied to the chromatograms, but with negative results.

The estimates of 11 of the amino-acids detected in the hydrolysates are summarized in fig. 3. Other amino-acids identified were histidine, lysine, threonine, and valine. This analysis demonstrates conclusively that the protein in the inner layers of both valves and ligament differs markedly in composition from that in the remaining shell layers. Glycine, for example, is abundant in most of the hydrolysates but it occurs in relatively higher concentration in those of the 'outer regions' of the shell and of the periostracum in *Mytilus* (fig. 3). This difference is particularly evident in the ligament, in which the glycine content of the inner layer is comparatively low. In contrast, the hydrolysates of the inner layers of the valves and ligament contain relatively larger amounts of aspartic acid, glutamic acid (fig. 3), and lysine.

As observed earlier, an important feature for distinguishing between hydrolysates of different regions of the shell is their relative content of phenolic amino-acids. Tyrosine and phenylalanine are much more abundant in the 'outer regions' of the valves and ligament and in the periostracum of *Mytilus* than in the inner layers (fig. 3). In the case of tyrosine, this agrees with previous

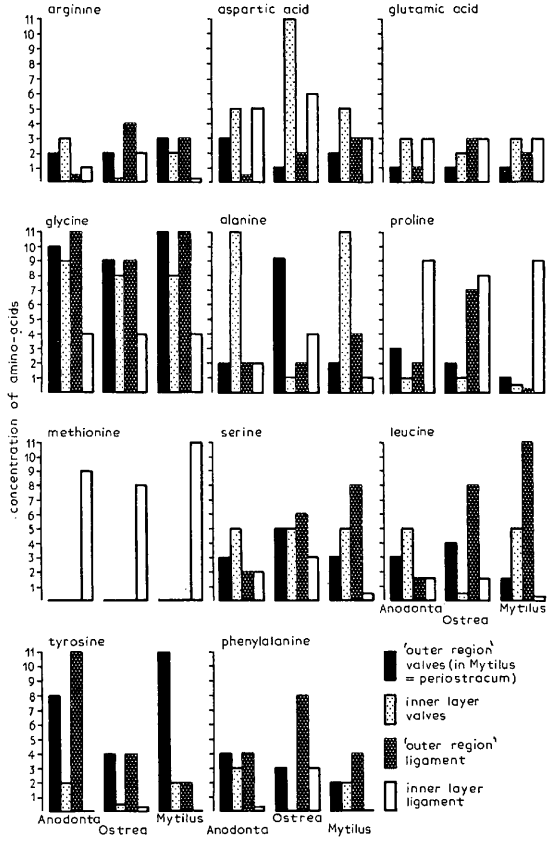

FIG. 3. Histograms showing the relative amounts of 11 amino-acids occurring in hydrolysates of the protein contents of the different shell layers in *Anodonta*, *Ostrea*, and *Mytilus*. Black represents the 'outer region' of the valves (except in *Mytilus* where it represents the periostracum); dense stippling the 'outer region' of the ligament. The inner layers of the valves and ligament are indicated by light stippling and white respectively.

analyses of the valve conchiolin in *Anodonta cygnea*, *Meleagrina margaritifera*, and species of *Pinna* (Friza, 1932; Roche, Ranson, and Eysseric-Lafon, 1951). These results can also readily be correlated with the histochemical reactions of the different shell layers to Millon's and other tests for aromatic groups. Unlike the corresponding regions in other shells, the 'outer region'

of the ligament in *Mytilus* contains relatively little tyrosine. In contrast, the exceptionally rich tyrosine content of the 'outer regions' of the shell in *Anodonta* and of the periostracum in *Mytilus* suggests that this amino-acid plays an important role in the tanning of the conchiolin of these regions. If this is so, the method of hardening of the periostracum in *Mytilus* may differ from that of the byssus, where, as shown by Brown (1952), the precursor of the tanning agent is a phenolic protein or amino-acid, but not tyrosine. Hydrolysates of byssal material were, in fact, found to contain only small quantities of tyrosine compared with those of the periostracum. It may well be that the periostracal conchiolin in *Mytilus*, or more specifically that of the external and middle layers, is hardened in a manner similar to that suggested for the periostracum of *Anodonta*, with which it is closely comparable histochemically.

In addition to showing that the 'outer regions' and inner layers of the shell contain two different types of protein, the present analysis confirms impressions gained from the observations on staining and histochemical reactions concerning the relative composition of homologous layers of the shell. Whilst in *Anodonta* the hydrolysates of the 'outer regions' of the valves and ligament have fundamentally the same composition, these components in *Ostrea*, although similar, show individual modifications (fig. 3). The former is distinguished by a high alanine content, whereas the latter is rich in proline. Also, the 'outer region' of the ligament in *Ostrea* exhibits certain characteristics in common with its homologue in *Mytilus*, a feature which recalls the similar staining reactions of the outer ligament conchiolin in these species. The hydrolysates of both these regions contain appreciably larger amounts of leucine and serine than those of the other components analysed (fig. 3).

The composition of the protein contents of the inner layers of the valves and ligament appear basically alike although each shows a certain amount of specialization. That in the inner layer of the valves in *Ostrea*, for example, contains a particularly large concentration of aspartic acid (fig. 3), a feature which seems to be consistent with the strong basiphil properties exhibited by this layer. The inner valve layers in *Anodonta* and *Mytilus*, which are almost identical in composition, are both characterized by a high content of alanine. On the other hand, the hydrolysates of the inner ligament, which show striking similarity in amino-acid content in all the bivalves investigated, are distinguished from those of the corresponding region of the valves in that they are rich in proline and in the sulphur-containing amino-acid, methionine (fig. 3). The presence of an appreciable concentration of methionine is unusual, since although this amino-acid is known to occur widely in proteins, it usually forms only a small percentage of the total amino-acids formed on hydrolysis (Fruton and Simmonds, 1953). The occurrence of methionine, which can be correlated with the positive sulphur reaction given by the inner layer of the ligament (tables 1–3), was determined by its ability to reduce Feigl's sodium azide-iodine reagent, and by the identification of its derivatives, methionine sulphone and methionine sulphoxide after oxidation with hydrogen

peroxide (Block, Durrum, and Zweig, 1955). These reactions gave mainly negative results with all the other hydrolysates.

Discussion

In comparing the composition of homologous layers of the shell, the nature of the non-calcareous material in *Anodonta* is of particular interest. There is no doubt that, in this bivalve, the conchiolin of the outer layer of the ligament is essentially similar in its staining and histochemical reactions to that of the outer calcareous layer of the valves. In addition, the properties of the periostracum and fusion layer differ in degree rather than in kind from those exhibited by the outer layers. All these regions consist mainly of a quinone-tanned protein which contains a high proportion of phenolic residues, especially tyrosine, and which differs markedly in composition from the protein contents of the inner layers of both valves and ligament.

These properties may well be correlated with the characteristic zonation of the secretory epithelium of the mantle in *Anodonta cygnea* (Beedham, 1958). The epithelia on the outer surfaces of the outer mantle fold forming the outer layers of the valves and ligament are comparable histologically and histochemically both with each other and with those on the inner periostracal-secreting surface of the outer fold and the outer surface of the fused outer folds which secretes fusion layer. All these epithelial zones are readily distinguishable from the epithelia concerned with the deposition of the inner shell layers. In *Anodonta* the outer mantle fold (i.e. both inner and outer surfaces) is to be regarded, therefore, as a complete secretory unit whose products differ in composition from those formed by the remainder of the outer surface of the mantle.

The situation in *Anodonta* provides confirmation of the view that corresponding layers of the valves and ligament are basically identical and are, in fact, locally modified regions of the same layers of the shell (Owen, Trueman, and Yonge, 1953). In the other bivalves investigated, the non-calcareous components of the outer layers of the valves and ligament undergo more extensive modifications, the degree of specialization being relatively slight in *Ostrea* but extremely pronounced in *Mytilus*. It should be pointed out, however, that the conchiolin of the major part of the periostracum in *Mytilus* appears to have a similar constitution to that of its homologue in *Anodonta*.

Whereas the conchiolin of the periostracum or of the outer layers of the valves and ligament is hardened and stabilized by quinone-tanning to form an efficient protective cover over most of the external surface of the shell, the non-calcareous matrix of the inner shell layers contains a low proportion of aromatic groupings and is relatively lightly tanned. Grégoire, Duchâteau, and Florkin (1955) have demonstrated that only a part of the protein in the inner calcareous layer of the valves in *Pinctada* (*Meleagrina*) *margaritifera* consists of a scleroprotein, and that there is in addition a protein soluble in water and a polypeptide.

In these and other features the inner layers of the valves and ligament are

fundamentally the same, which fully supports similar conclusions based on morphological evidence (Owen, Trueman, and Yonge, 1953). However, not unexpectedly, the protein content of each layer is found to undergo some modification. The high proline content of the protein of the inner ligament layer, coupled with the fact that it contains a very low percentage of phenolic amino-acids, suggests that it is somewhat similar in composition to collagen (see also Trueman, 1949). However, it differs from collagenous proteins in that it contains appreciable quantities of methionine, a relatively small proportion of glycine, and little or no hydroxyproline. Since all these characteristics invariably appear in each of the species investigated, it is probable that the modifications exhibited by the protein in the inner ligament are correlated with the highly specialized function of this region of the shell. Unlike the remainder of the shell, the inner layer of the ligament is constantly subjected to compressional stresses due to the closing action of the valves.

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