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REPAIR OF THE SHELL IN SPECIES OF *ANODONTA*

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(With 2 plates and 3 figures in the text)

The process of shell repair in species of *Anodonta* is re-examined in the light of current views on the mantle/shell relationship in the Lamellibranchia. Experiments indicate that the epithelia of the general outer mantle surface and mantle isthmus normally responsible for the production, respectively, of the inner layer of the valves and of the ligament can regenerate material comparable with that produced by the outer fold of the mantle edge. The process, which is usually preceded by a phase in which amoebocytes are involved, consists initially of the formation of appreciable quantities of organic material (conchiolin). Much of this conchiolin is histochemically comparable with that of the periostracum and the outer layers of the valves and ligament secreted during marginal increment of the shell by the inner and outer surfaces, respectively, of the outer mantle fold. This type of conchiolin is probably rich in quinone-tanned protein (sclerotin). Repair of the valves is completed by the calcification of this organic matrix to form an outer calcareous (prismatic) layer, followed by the desposition of inner (nacreous) layer material.

During the repair process, the form of the general outer mantle epithelium tends to vary according to the type of material being secreted. While regenerating periostracal and outer shell layer components the epithelial cells of the general outer mantle surface and mantle isthmus resemble both histologically and histochemically those of the outer fold of the mantle edge. Completion of valve repair by the deposition of nacre is accompanied by the cells reverting to the form characteristic of the general mantle surface during normal shell growth.

In *Anodonta* and related bivalves, therefore, the relationship which normally exists between the different shell layers and the secretory epithelial zones of the mantle does not appear to be specific and unalterable. The implications of these findings in relation to marginal growth of the mantle and shell are discussed.

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INTRODUCTION

Although much interesting work has been carried out on shell repair in the Mollusca, observations in recent years have been largely confined to gastropods, in particular the land pulmonates (Wagge, 1951 ; Wagge & Mittler, 1953 ; Abolinš-Krogis, 1963). Little work has been done on repair of the shell in the Lamellibranchia since the classic studies on *Margaritana margaritifera* and various species of *Anodonta* by de Villepoix (1892), Rubbel (1911 b) and Rassbach (1912). A re-appraisal of these observations is desirable in the light of the new concept of the relationship of the mantle and shell in the bivalve molluscs elaborated by Owen, Trueman & Yonge (1953) and Yonge (1953, 1957).

MANTLE-SHELL RELATIONSHIP IN THE LAMELLIBRANCHIA

As in the shell of all bivalves, the valves and ligament in *Anodonta* both consist of three layers, i.e. periostracum, outer and inner layers (Fig. 1). Yonge (1962) considers that, as probably in all Unionacea, the ligament has no fusion layer. Each layer of the shell is secreted by a specific region of the underlying mantle (Fig. 1 (a)). The periostracum and outer layers of the valves and ligament are formed, respectively, by the inner and outer surfaces of the outer of the three marginal folds of the mantle (Yonge, 1957). The inner layer of the valves and of the ligament is secreted by the general mantle surface. The inner (in *Anodonta*, nacreous) layer of the valves is formed by the epithelium of the general outer surface of the mantle and the inner ligament layer by the epithelial cells of the narrow neck of tissue, the mantle isthmus, situated beneath the hinge (Fig. 1).

There is, therefore, a specific relationship between the layers of the valves and ligament, which together constitute the shell, and different secretory areas of the mantle (Owen, Trueman & Yonge, 1953 ; Yonge, 1953 ; Owen, 1958). Recent work on *Anodonta cygnea* L. supports this concept on histological and histochemical grounds (Beedham, 1954, 1958 a, b). In this bivalve, the epithelia of the inner and outer surfaces of the outer mantle fold which secrete respectively the periostracum and outer shell layers are alike in many features. Both regions are, however, distinguishable from the epithelia which secrete the inner layer of the valves and of the ligament in that their constituent cells are generally taller, contain denser granular cytoplasm, show greater alkaline phosphatase activity and have a higher ribonucleoprotein content. Similarly, the different shell layers are equally well defined by the chemical and staining reactions of their non-calcareous (conchiolin) components. These reactions are summarized in Table 1. The conchiolin of the outer layers of both valves and ligament is similar in that it is acidophilic and reacts strongly to tests for phenolic groups, including Millon's reaction and Baker's (1956) modification of Folin's method for phenols. This indicates that its protein content is hardened by quinone-tanning (Beedham, 1958 b). The periostracum reacts even more intensely to the argentaffin, xanthoproteic and other phenol tests but is refractory to many stains, probably as a result of further hardening of its protein constituent. On the other hand, the non-calcareous components

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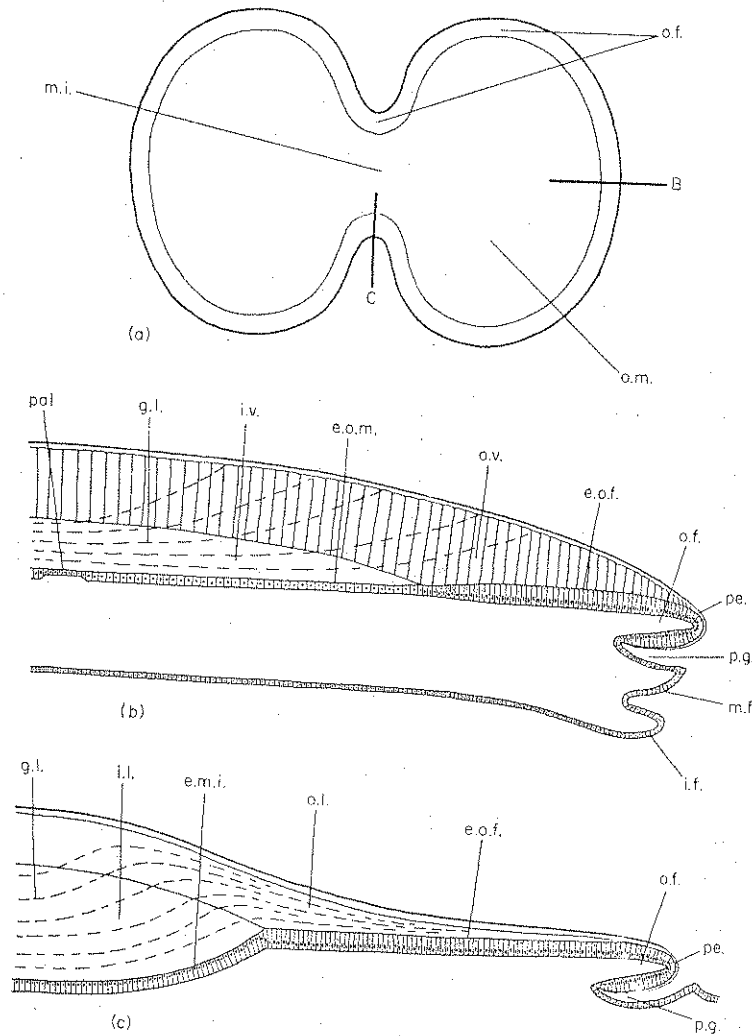mantle isthmu
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Fig. 1. The structure of the valves and ligament of the shell in *Anodonta cygnea* and their relationship to the underlying mantle. (a) The mantle of a hypothetical primitive bivalve spread out flat and viewed from its outer surface showing the two thin pallial lobes of the mantle responsible for the secretion of the valves connected by the mantle isthmus (m.i.) underlying the ligament. (b) & (c) Sections through the valve and ligament in *Anodonta* in the respective planes illustrated in (a) to show the relationship between the layers of the shell and the secretory areas of the mantle. Not to scale

e.o.f., epithelium on outer surface of outer mantle fold ; e.o.m., epithelium of general outer mantle surface ; e.m.i., epithelium of mantle isthmus ; g.l., growth lines ; i.f., inner fold ; i.l., inner layer of ligament ; i.v., inner (nacreous) layer of valve ; m.f., middle fold ; m.i., mantle isthmus ; o.f., outer fold of mantle edge ; o.l., outer layer of ligament ; o.m., general outer surface of mantle ; o.v., outer (prismatic) layer of valve ; pal., pallial line ; pe., periostracum ; p.g., periostracal groove.

of the inner layers of the shell appear to contain less highly-tanned protein and stain moderately strongly with basic dyes, Sudan black B and Alcian blue (Steedman, 1950) (Table I).

TABLE 1—A summary of the staining and histochemical reactions of the non-calcareous components of the shell of *Anodonta cygnea*

| Test | | Valves | | | Ligament | |
	Perio-stracum	Outer layer	Inner layer	Perio-stracum	Outer layer	Inner layer
Mallory's stain	Colourless	Red	Blue	Colourless	Red	Blue
Masson's stain	Colourless	Red	Green	Colourless	Red	Green
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
Arginine (Baker, 1947)	XX	XXX	XXX	X	X	XXX
Sudan Black B	tr	XXX	XXX	tr	XX	XX
Periodic acid/ Schiff (Hotchkiss, 1948)	0	?tr	X	0	X	X
Alcian blue (Steedman, 1950)	0	0	XXX	0	0	XX

No. of X's indicates intensity of reaction ; tr, trace ; 0, no recognizable response.

These properties, as does the mantle/shell concept in general, apply to bivalves during normal growth conditions. They pose the interesting problem, however, of the specificity of shell secretion, i.e. whether the relationship which has been shown to exist between the various secretory zones of the mantle and the different layers of the shell is permanent and specific, or whether it can be altered experimentally. This has been investigated by studying the process of repair of the valves and ligament in *Anodonta*, with particular reference to (i) the nature of the non-calcareous material produced in damaged regions of the shell and (ii) the appearance of the mantle epithelia concerned with shell repair.

MATERIAL AND METHODS

The experiments were carried out on medium-sized specimens of *Anodonta cygnea* L. and *A. anatina* L. The structure and properties of the mantle and shell in these species are basically the same and they accomplish repair in a similar manner.

The experimental animals were divided into three groups each concerned with a particular aspect of shell repair (Fig. 2). In the first group, a study was made of repair of the valves within the pallial line, i.e. in the region bordered by the line of attachment of the pallial muscles, the adductor muscles and the ligament. Small holes 2–3 mm in diameter were carefully drilled in the valves, usually one per valve in each specimen. This exposed the general outer surface of the mantle which normally is concerned with the secretion of the inner calcareous layer of the valves. The position of drilling was
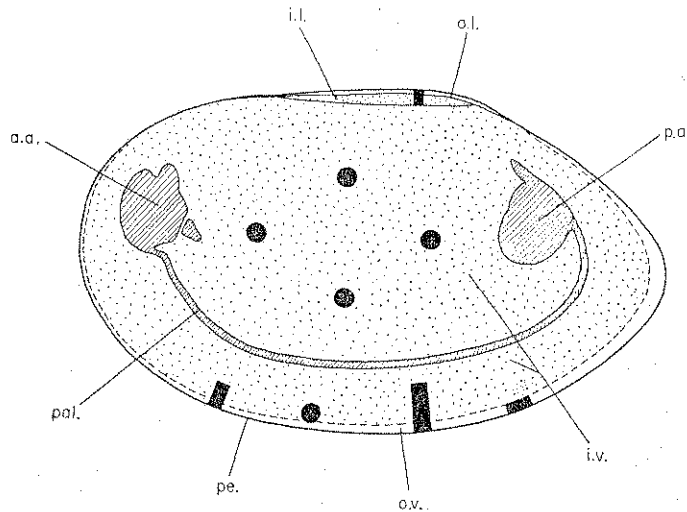

Fig. 2. The right half of the shell in *Anodonta cygnea* viewed from its inner surface to show the regions in which repair was investigated. Portions of shell (shown in black) were removed from (i) the area of the valve inside the pallial line, (ii) the margin of the valve outside the pallial line, or (iii) the mid-region of the ligament. Areas of shell removal indicated are not drawn to scale. a.a., anterior adductor muscle; p.a., posterior adductor muscle; other lettering as in Fig. 1.

varied (Fig. 2), but there appeared to be no significant difference in the results obtained. The damaged region on one valve of each specimen was covered with a small piece of cover-glass or celluloid attached to the adjoining shell, a procedure previously adopted by Rassbach (1912) and other investigators. However, in order to ascertain repair under more natural conditions the damaged zone in the other valve was left unprotected.

The second group of animals was used to investigate repair of the marginal region of the valves outside the pallial line (Fig. 2). Pieces of shell varying in size were cut or drilled out of the margin, the damaged regions invariably

being left uncovered. In the third group, a study was made of repair of the central part of the ligament (Fig. 2). Small pieces 2–3 mm long were cut out of the hinge thus revealing the epithelium of the mantle isthmus which in normal growth secretes only the inner ligament layer. The damaged regions in approximately half of the specimens were covered with celluloid, the remainder being left without protection.

The animals survived well and were maintained in their natural habitat. Specimens were removed from each group for examination every 3–4 weeks over a total period of almost 12 months. In addition, the immediate effects of exposure of the mantle by damage to the overlying shell were observed in animals maintained in the laboratory.

After preliminary observations followed by fixation in Bouin's fluid, neutral formalin, or other routine fixatives, the regions of repaired shell and the underlying mantle were for practical reasons processed separately. The latter were treated in a standard manner and embedded in paraffin wax but the former required in many cases to be decalcified and then embedded in ester wax (Steedman, 1947) or, in the specimens of ligament repair, celloidin. Mallory's and Masson's triple stains were preferred for routine examination of sections because of the ease with which they differentiate between the various non-calcareous components of the shell (Table 1). The other specific tests recorded in Table 1 were used to identify the types of conchiolin produced during repair.

REPAIR OF THE VALVES INSIDE THE PALLIAL LINE

The exposed general outer surface of the mantle, which appears smooth and relatively transparent immediately after removal of the overlying shell, gradually becomes opaque and irregular in appearance. This is due to the aggregation of large numbers of amoebocytes in the sub-epithelial tissues (Pl. 1 (1)), a phenomenon which is known to occur widely in molluscs subjected to experimental shell damage (Takatsuki, 1934 ; Wagge, 1951, 1955 ; Dunachie, 1963). The exposed epithelium may begin to break down in places and groups of amoebocytes extrude on to the outer mantle surface. Although these effects tend to be minimized at the margins of the damaged zone where the mantle is closely apposed to the cut edges of the shell, the exposed epithelium may in some cases almost cease to exist as an organized layer and is largely replaced by closely-packed amoebocytes (Pl. 1 (1)). It seems likely that these characteristic reactions of the exposed mantle are a direct result of contact with a changed environment.

The amoebocytes found at the exposed region are of different types. Some are small, active, irregularly-shaped cells, 8–12 μ across, with deeply-staining basiphilic granular cytoplasm, whereas others are larger, more spherical cells, 20 μ in diameter, with weakly-staining vacuolated cytoplasm. The latter tend to aggregate in large flocculent masses at the exposed surface. Since intermediate forms exist between both these varieties no attempt is made to differentiate critically between them. The term amoebocyte is used here in a non-specific manner. It is known that many different kinds of

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amoebocytes (leucocytes) are found in lamellibranchs (de Bruyne, 1896 ; Kollman, 1908) but it seems likely as pointed out by Yonge (1926) that some of these are functional variants of the same type.

The massing of amoebocytes at the affected surface of the mantle in *Anodonta* is probably of protective value. Not only do the amoebocytes form a barrier between the environment and the exposed mantle tissues but they also remove waste matter from the unprotected surface. As shown by de Villepoix (1892), they are able to ingest foreign particles falling on the mantle surface and as a result keep it free from detritus.

Repair of the damaged regions by the secretion of new shell material takes place relatively slowly. This is known to be the case also in shell regeneration in other aquatic molluscs (Heaysman, 1959 ; Wagge & Mittler, 1953). It would seem likely that the effect of damage is not so immediately critical in freshwater and marine species as in *Helix aspersa* and other terrestrial molluscs where the dangers of desiccation are such that rapid repair of exposed areas of the mantle is vital. In general it was found also in *Anodonta* that artificial protection of experimentally induced damage to the shell does not necessarily hasten the process of repair. Indeed, in many cases, the secretion of new shell material appears to be actively stimulated by the exposed mantle coming into direct contact with the environment.

Repair commences under the protection of the shell at the margin of the damaged region and then spreads gradually across the exposed mantle (Fig. 3). The regenerated material ultimately becomes attached to the inner surface of the intact shell. The material formed initially consists of thin, uncalcified, loosely connected laminae with basiphilic staining properties. These membranes are probably secreted by the amoebocytes which can be seen closely associated with them in sections of the regenerating region (Pl. 1 (2)). The amoebocytes principally involved are the small, active variety which have cytoplasmic contents with similar basiphilic staining properties. It is interesting to note that these amoebocytes show strong alkaline phosphatase activity, a property also exhibited by certain of the amoebocytes concerned with shell repair in *Helix* (Wagge, 1951).

If it has been affected by exposure, the outer mantle epithelium now reforms under the protection of the newly secreted membranes. This process appears to be accomplished by the spread of epithelial cells, possibly by amoeboid movement, from the margins of the intact epithelium adjacent to the exposed area. It is known that in the molluscs epithelial cells from the outer mantle surface are capable of independent movement whilst shell repair is taking place (Wagge, 1955).

A complete outer mantle epithelium is responsible for all subsequent repair, the various stages of which are illustrated in Fig. 3. Initially the epithelium may secrete small amounts of an uncalcified material basically similar in composition to that already produced by the amoebocytes (Pl. 1 (2)). All this conchiolin exhibits basiphilic staining properties, reacts faintly to tests for phenols and in general shows histochemical reactions similar to those given by the non-calcareous matrix of the inner (nacreous) layer of the valves (Table 1). These properties, coupled with its natural
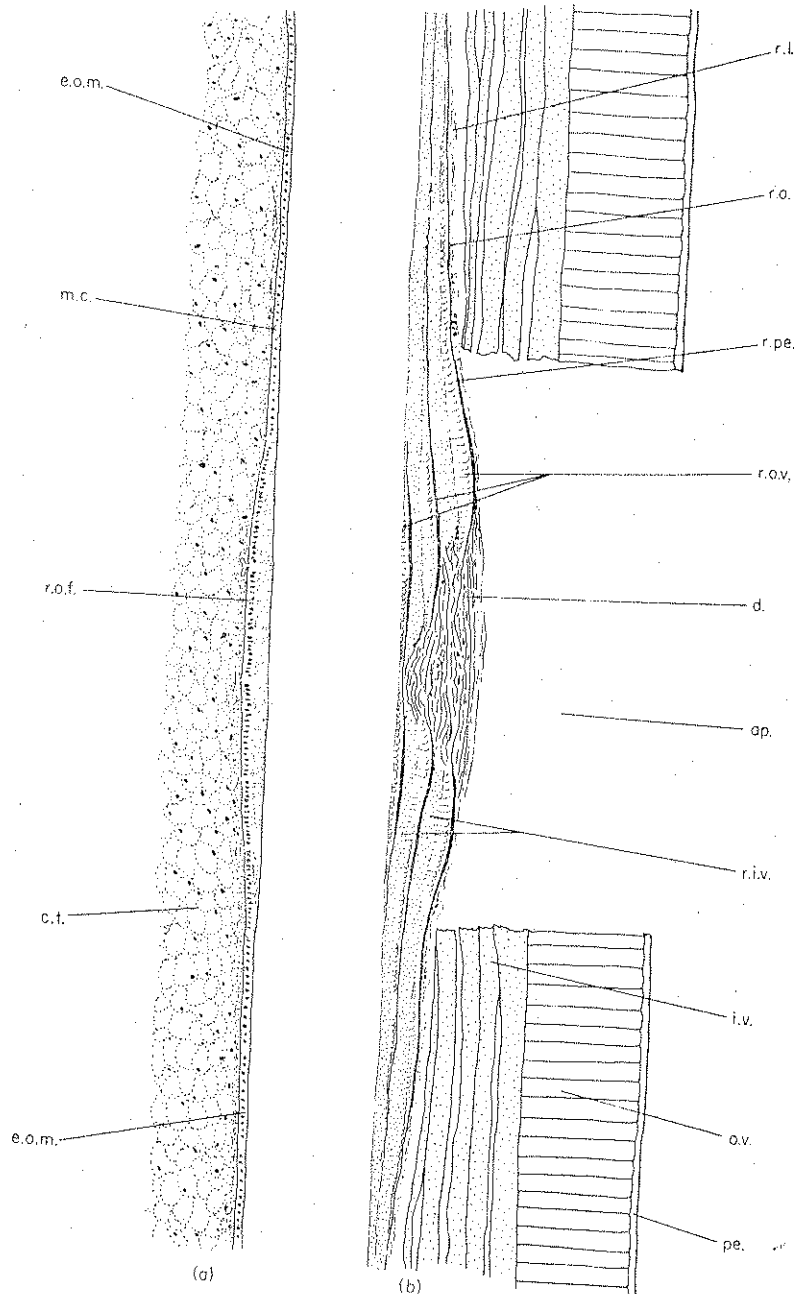
Fig. 3. Semi-diagrammatic representation of sections through (a) the mantle and (b) the shell (decalcified) in the region of an uncovered aperture in the valves inside the pallial line of a specimen of *Anodonta* which has been regenerating for 5 months. Not to scale.

ap., aperture in valve ; d., detritus and irregular layers of conchiolin ; c.t., connective tissue of mantle ; m.c. mucous cell ; r.i.v., regenerated inner (nacreous) layer ; r.l., regenerated initially-formed laminae ; r.o., regenerated outer layer type conchiolin ; r.o.f., epithelial cells resembling those of the outer mantle fold which are regenerating outer layer material ; r.o.v., regenerated outer (prismatic) layer ; r. pe., regenerated periostracal-like conchiolin ; other lettering as in Fig. 1.

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pale yellowish colour, suggest that it consists mainly of protein only slightly hardened by quinone-tanning. The epithelial cells which secrete this material resemble those of the adjacent general outer mantle surface, but they are slightly taller and contain more deeply-staining cytoplasm (Pl. 1 (2)).

Although the secretion of this type of conchiolin may form a fairly consistent feature in the early stages of repair, it is always followed by the production of non-calcareous material with quite different properties (Pl. 1 (3–5)). The latter constitutes firm, membranous conchiolin which in section colours red with Mallory's and Masson's stains. It reacts moderately strongly with histochemical tests for phenolic substances concerned in tanning and shows similar properties to the organic matrix of the outer calcareous (prismatic) layer of the valves (Table 1). The innermost laminae of the material gradually lose their reactivity to stains and appear clear and slightly amber-coloured in section (Pl. 1 (3)). These laminae respond much more vigorously to tests for phenolic substances, a change consistent with further extensive hardening of their protein content by quinone-tanning. The properties of this conchiolin are almost indistinguishable from those of the periostracum (Table 1) normally produced only at the mantle edge.

Periostracal and outer layer types of conchiolin, which in *A. cygnea* differ in composition from each other in degree rather than in kind (Beedham, 1958 b), now form the most prominent components of the still uncalcified material being used for repair. They are particularly prominent in those specimens in which the damaged regions of shell were not artificially protected. However, although non-calcareous material is an important and effective component of the initial repair process, it is usually further strengthened by calcification, as at the mantle margin, to form a true prismatic layer (Fig. 3 and Pl. 1 (4)). Calcified layers in the mollusc shell are typically formed in this way, i.e. by the elaboration of an organic membrane on which calcium carbonate crystals are deposited (Bevelander & Benzer, 1948; Bevelander, 1952). The prisms appear initially as rudiments on the inner surface of the periostracal type of conchiolin (Pl. 1 (4)) and their subsequent enlargement accompanied by further secretion of outer layer conchiolin produces a shallow but prominent prismatic layer (Fig. 3).

The secretion during repair of periostracal and outer layer components by regions of the mantle usually concerned solely with the formation of nacre is accompanied by significant changes in the outer mantle epithelium. The epithelial cells regenerating these components resemble closely those on the inner and outer surfaces of the outer fold of the mantle edge. They are tall and columnar with basally-situated nuclei (Fig. 3) and granular, deeply-staining basiphilic cytoplasm (Pl. 1 (6)). In these properties, which are all characteristic of the normal epithelial cells of the outer mantle fold in *A. cygnea* (Beedham, 1958 a), they are clearly distinguishable from the adjoining cells of the general outer mantle surface which secrete the inner layer of the valves (Fig. 3 a, Pl. 1 (6)). Tsujii (1960) has also noted changes in appearance of the epithelial cells of the pallial mantle during shell repair in *Anodonta* species.

Repair is completed, as in normal shell growth, by the deposition of inner (nacreous) layer material (Fig. 3). It consists of thin alternating layers of

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crystalline calcium carbonate and non-calcareous material, the latter reacting
to histochemical tests in the manner shown in Table 1. This conchiolin
appears similar in composition to that produced in the earliest stages of repair
although differing from it in that it becomes associated almost immediately
with mineral deposits. Nacre production in the repaired shell completes the
necessary strengthening of the damaged zone although it may be many months
before a completely rigid structure is produced. The secretion of nacre is
accompanied by the underlying epithelial cells reverting to the form
characteristic of the remainder of the general outer surface of the mantle.
The epithelium carrying out repair is now indistinguishable from that of
adjoining areas.

There is, therefore, a clear cycle of events in *Anodonta* during repair of
the valves inside the pallial line in which a restricted portion of the general
outer mantle epithelium can secrete in succession the non-calcareous as well
as the calcified components of all the layers of the valves. Moreover, this
cyclical process may be repeated several times in the one region resulting in
the complex, multiple type of repair structure illustrated in Fig. 3. The
pronounced layered effect is not uncommonly found in specimens regenerating
shell without protective cover and is probably caused by further natural
damage to the exposed portion of the repaired shell before it is complete and
fully strengthened. The mantle epithelium would be subjected more than
once to exposure to the surrounding water and would therefore tend to repeat
partly or wholly the secretory cycle originally initiated by removal of the
overlying shell. A similar effect is shown in Pl. 1 (5). In this specimen,
which also illustrates the actual bulk of non-calcareous material which may be
produced during repair, regeneration has been accomplished by the alternate
production of several layers of the outer and inner layer types of conchiolin
by the same area of the outer mantle epithelium.

REPAIR OF THE VALVES OUTSIDE THE PALLIAL LINE

Repair of damage to the valve margin takes a relatively predictable course
since it is carried out by those regions of the mantle edge which are concerned
with normal shell growth. The mantle margin withdraws as far as possible
behind the damaged region thus minimizing the effects of exposure on the
mantle tissues. In general, these effects are conditioned by the extent of the
damage inflicted. Where comparatively small pieces of shell are removed, the
mantle is only slightly affected. If the damage extends back almost as far
as the pallial line (Fig. 2), the outer mantle epithelium shows signs of
degeneration. In all cases, amoebocytes are active and present in large numbers
at the exposed surface.

Actual repair commences under the shelter of the intact valve adjacent
to the damaged zone (Pl. 1 (8)). Thin, non-calcareous laminae similar in
composition to the organic matrix of the nacre are produced initially in the
proximal parts of the affected area. They are probably secreted by the
amoebocytes active at the exposed surface. The remainder of repair, however,
is carried out by the outer mantle epithelium. The epithelial cells of the

outer mantle fold secrete initially non-calcareous material consisting principally of outer layer conchiolin. This material may be irregularly arranged at first but ultimately a firm, complete layer is produced (Pl. 1 (7)). As in repair inside the pallial line, the innermost laminae of this material subsequently lose their affinity for acid dyes, show increased reactivity to the argentaffin and other tests for phenolic substances and become comparable in appearance and properties with normal periostracum. It is likely that this process resembles periostracum formation during marginal increment of the shell although usually the whole sequence would take place within the confines of the periostracal groove (Fig. 1 (b)). The fact that periostracal conchiolin can be secreted by the outer surface of the outer fold of the mantle edge as well as by its inner surface tends to support the view that all the epithelial cells of the outer mantle fold in *A. cygnea* have basically the same structure and secretory properties (Beedham, 1958 a, b).

The regenerated membrane is now calcified to form a prismatic layer (Pl. 1 (8)) and the outer mantle fold extends the repair process gradually towards the original border of the shell. As this extension occurs, nacre is deposited on the inner surface of the proximal region of the regenerated shell by the epithelium of the general outer surface of the mantle and the whole structure becomes firmly attached to the undamaged shell.

REPAIR OF THE LIGAMENT

Regions of the mantle isthmus exposed by the removal of small portions of the ligament undergo similar modifications to those exhibited by the exposed pallial mantle. The epithelium may degenerate in parts and large groups of amoebocytes aggregate at the exposed surface (Pl. 2 (3 & 4)). Although these effects may be caused at least partly by direct exposure, it is not unlikely that the epithelium is physically damaged and parts of it torn away when the overlying ligament is removed. There is in *Anodonta*, as in certain other bivalves, a close natural continuity between the epithelial cells of the mantle isthmus and the matrix of the inner layer of the ligament (Beedham, 1958 a). The epithelium adjacent to the damaged region, however, remains closely apposed to the ligament and is relatively unaffected by the experiments.

Repair commences by the secretion of thin, non-calcareous laminae at the margin of the aperture (Pl. 2 (1)). These are probably secreted initially by the amoebocytes which can be seen closely associated with them (Pl. 2 (1)), but the material is thickened and extended (Pl. 2 (2)) by the underlying epithelium which reforms beneath it (Pl. 2 (4)). The rather delicate laminae produced initially exhibit staining and histochemical properties similar to those shown by the organic matrix of the inner layer of the normal ligament (Table 1). Much of the conchiolin used in thickening, however, is quite different. It is firmer in consistency and reacts to stains and histochemical tests in a manner comparable to the outer ligament layer (Table 1).

The outer layer type of conchiolin effectively completes the coverage of the damaged zone and may ultimately form the bulk of the material used in

ligament repair (Pl. 2 (5 & 6)). As in repair of the valves, this type of material with its high content of quinone-tanned protein appears to be secreted more rapidly and extensively in damaged areas which are not artificially protected. These firm, laminated membranes project into the aperture to form an arc (Pl. 2 (5 & 6)) which follows the natural contours of the underlying mantle isthmus (Pl. 2 (8)). The regenerated structure often extends a considerable distance under the ligament anterior and posterior to the damaged region and is firmly attached laterally to the remnants of the intact ligament or to the adjoining ligament ridge (Pl. 2 (5 & 6)). As this attachment becomes established, the repaired structure is increasingly subjected to the same lateral tensile stresses as those imposed on the outer layer of the intact ligament by the closing movement of the valves. This is marked by the constituent laminae of the repair membrane exhibiting birefringence to polarized light. The birefringence occurs parallel to the outer surface of the regenerated ligament, as seen in transverse section, and it may ultimately become as intense as in the outer layer of the normal ligament. It is interesting to observe that in certain specimens there are signs of what may be incipient prism formation on the inner surface of the margins of the regenerated membranes. However, unlike repair of the valves, no complete calcification of the outer layer conchiolin ever takes place.

The production of outer layer material is directly associated, as in valve repair, with a change in appearance of the underlying mantle epithelium (Pl. 2 (7 & 8)). The cells are taller and contain more deeply-staining, granular cytoplasm than the adjacent cells of the mantle isthmus concerned with the formation of the inner layer of the ligament. They appear essentially similar in structure and properties to the epithelial cells of the outer mantle fold which normally secrete the outer ligament layer only. The sharp line of demarcation which exists between the epithelium secreting the repaired structure and that adjacent to it (Pl. 2 (7)) is as well-defined as that which occurs between the epithelia of the outer mantle fold and mantle isthmus during normal ligament growth (Fig. 1 (c)).

Although in certain specimens there is some secretion of laminae on the inner surface of the repair membranes with properties resembling the conchiolin of the normal inner ligament layer, no complete calcified inner layer was regenerated during the course of the present experiments. It is possible that the relatively slight damage inflicted on the ligament has had no appreciable effect on the elastic functional properties of the intact inner ligament layer and there is, therefore, no immediate urgency for its replacement once a firm protective membrane has been regenerated.

DISCUSSION

Although amoebocytes probably play a limited secretory role in the early stages of shell repair in *Anodonta*, the greater part of the repair process is accomplished by the outer mantle epithelium. Dunachie (1963) has shown that amoebocytes take part in the repair of damage to the periostracum in the *Mytilus edulis* by supplying material to the secretory epithelium although

they are not themselves directly concerned in regeneration. In land snails, amoebocytes would appear to be more extensively involved in the necessarily very rapid repair of damaged areas of the shell (Wagge, 1951 ; Wagge & Mittler, 1953). One important function of the amoebocytes which aggregate during repair in *Anodonta* is that of protection of the exposed mantle surface. This role is particularly valuable in view of the comparatively long period which may elapse before repair is completed.

The results of the present study, which show that the epithelium of the general outer surface of the mantle in *Anodonta* can secrete, as well as nacre, material which closely resembles that of the periostracum and outer (prismatic) layer, agrees with observations by Rubbel (1911 b), Rassbach (1912), van Deinse (1912), de Waele (1930), Tsujii (1960) and others on shell regeneration in related freshwater bivalves. The regenerated layers can be identified however not only on their morphological appearance, as shown by previous investigators, but also by the characteristic histochemical and staining properties of their non-calcareous (conchiolin) components. These properties are as clearly differentiated in the structures produced during repair as in the normal shell (Beedham, 1958 b). Non-calcareous material constitutes the bulk of the new shell secreted in the early stages of repair with the yellowish-brown periostracal and outer layer types of conchiolin forming the major components. The observations on ligament regeneration show similar results. The epithelium of the mantle isthmus, normally concerned with the production of the inner layer of the ligament only, secretes during repair large quantities of conchiolin much of it comparable with that of the outer layer. This material resembles its counterpart in the normal ligament not only chemically but also in its birefringent properties.

The support and protection imparted to the regenerated shell by the periostracal and outer layer types of conchiolin is probably due largely to their high content of quinone-tanned protein (sclerotin). Only later, in the case of repair of the valves, is the material further strengthened by calcification. Scleroproteins concerned with structural support are widespread in the invertebrates, particularly in the cuticles of insects and other arthropods (Pryor, 1940 ; Dennell, 1947 ; Wigglesworth, 1948 ; Dennell & Malek, 1955) and in the shell and byssus of many bivalves (Trueman, 1950 ; Brown, 1950, 1952 ; Beedham, 1958 b). The extensive production during repair in *Anodonta* of conchiolin with a high sclerotin content appears to be actively stimulated by direct contact with the environment. It is in general found to be better developed in those specimens which are allowed to regenerate shell naturally and without artificial protection. The brownish patches of a similar type of conchiolin quite frequently seen in normal adult specimens of *Anodonta* on the inner surface of the nacre near the umbones and in some cases underneath the anterior end of the ligament may result from the same kind of external stimulus. Water could infiltrate through cracks in the anterior portion of the hinge of older specimens or permeate directly through the valves in the region of the umbones where the protective periostracum tends to be worn away. Similar conditions may operate during the initial stages of pearl formation in *Anodonta* and related bivalves. Rubbel (1911 a, 1912) records the presence

of brownish periostracal-like conchiolin in the " nucleus " of many natural pearls found in freshwater bivalves and the initial stimulus for the formation of this material has been attributed to irritation of the mantle tissues by grains of sand or other foreign bodies from the external environment (for summary see Tsujii, 1960).

The ability of the general mantle surface and mantle isthmus to secrete during repair in *Anodonta* types of material found normally only in the periostracum and outer shell layers indicates that they can carry out when necessary the secretory functions of the outer fold of the mantle edge. Moreover, in doing so, their epithelial cells usually become histologically and histochemically comparable with those lining the inner and outer surfaces of the outer mantle fold. It should be stressed that during repair as in normal shell growth, the mantle tissues within the pallial line are relatively static in relation to the shell. This differs from regeneration at the mantle margin where, unless the damage is extensive, the process is usually initiated by the outer fold of the mantle edge which withdraws behind the damaged area. Subsequently, repair of the edge of the valves takes place basically in the same way as marginal increment of the shell. The process, nevertheless, is conditioned by the extent to which the mantle edge can be withdrawn. This is limited in *Anodonta* by the position of the pallial line (Fig. 2), although in some bivalves, e.g. *Pinna carnea* (Yonge, 1953), the possible extent of withdrawal may be very much greater. However, proximal to the pallial line in *Anodonta* no such movement of the mantle surface relative to the shell is possible by reason of the insertion of the adductor and pallial muscles. This is enhanced in the region of the mantle isthmus by the almost physical continuity which exists between the epithelial cells there and the inner surface of the inner ligament layer, a feature found in several bivalves (Trueman, 1949 ; Beedham, 1958 a). It follows that in any one region of the general outer mantle surface or mantle isthmus in *Anodonta* the same area of epithelial cells must basically be responsible for all stages of repair.

Regeneration experiments indicate, therefore, that there is no absolute difference in secretory properties between the various epithelial zones of the mantle in *Anodonta* and their relationship with the different layers of the shell is apparently not specific and unalterable. These results have some bearing on the relationship which is maintained between the mantle and shell during marginal growth. Growth of the mantle could be attributed either to a general increase in numbers of the epithelial cells by division over the whole outer mantle surface, or to an active proliferation of cells at the mantle edge, possibly at the proximal end of the inner surface of the outer fold where secretion of the periostracum commences. In the former case, the epithelial cells of the different areas of the mantle would continue to secrete the same shell material during marginal increment of the mantle and shell, i.e. they would tend to retain their secretory identity. However, if the latter condition prevails, the cells formed in the meristematic zone at the mantle edge would pass round the outer mantle fold and along the outer mantle surface and in doing so would secrete in succession the periostracum, outer and inner shell layers. Dunachie (1963) considers that the nature of the growth of the periostracum in *Mytilus edulis* is consistent with the second

hypothesis, although he points out that there is at present no conclusive evidence for the existence of a specific region of cell generation at the mantle margin.

With the same reservations, the present study may lend some further support to this view. The epithelial cells of the mantle in *Anodonta* appear to be capable of the type of cyclical change in secretory activity which must operate at the mantle edge should this be the active growing point of the mantle. Such a cycle commences, as appears also to be the case during repair of the shell and pearl formation, with the production of periostracal and outer layer material by cells of the characteristic outer fold type. It is not known how the cycle, which would culminate in the deposition of the inner shell layers accompanied by a change in form of the secretory cells, is controlled although it seems possible that external factors, for instance, degree of permeability of the shell, may be involved.

The characteristic form of shell repair in *Anodonta* and related freshwater bivalves is not necessarily found universally in the Mollusca. Indeed, although Boutan (1923) records that *Haliotis* can regenerate the whole of its shell, in many gastropods periostracum is not regenerated when pieces of shell are removed from regions other than the extreme margin (Levetzow, 1932 ; Andrews, 1934, 1935 ; Wagge, 1951 ; Abolinš-Krogis, 1963). Andrews (1935) considers that there is extreme specialisation of the mantle edge in gastropods and this accompanies a compensating inability of other regions of the outer mantle epithelium to secrete all the layers of the shell.

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KEY TO LETTERING

am.	amoebocytes
e.m.i.	epithelium of the mantle isthmus
e.o.f.	epithelium of outer surface of outer mantle fold
e.o.m.	epithelium of the general outer surface of the mantle
e.r.	exposed region of mantle
i.l.	inner layer of ligament
i.v.	inner (nacreous) layer of valve
l.r.	ligament ridge
o.v.	outer (prismatic) layer of valve
p.	prism rudiments
r.e.	re-forming epithelium
r.i.	regenerated inner layer type conchiolin
r.i.v.	regenerated inner (nacreous) layer
r.l.	regenerated laminae
r.o.	regenerated outer layer type conchiolin
r.o.f.	regenerating epithelium resembling that of the outer mantle fold
r.o.v.	regenerated outer (prismatic) layer
r. pe.	regenerated periostracal-like conchiolin

EXPLANATION OF THE PLATES

PLATE 1

Sections illustrating repair of the valves in *Anodonta*. 1–6 : repair inside the pallial line. 7 & 8 : repair outside the pallial line. In all photomicrographs the outer surface of the shell is orientated towards the top of page.

1. Portion of the pallial mantle exposed to the environment for 8 days showing amoebocytes (am.) massed in the exposed region (e.r.) whereas the adjacent epithelium of the general outer mantle surface (e.o.m.) is relatively unaffected.

2. Specimen repairing for $3\frac{1}{2}$ weeks (uncovered) showing formation of non-calcareous laminae (r.l.) secreted initially by amoebocytes (am.) and thickened by the epithelium of the general mantle surface (e. o. m.).

3. Specimen repairing for $7\frac{1}{2}$ weeks (uncovered) showing regeneration of periostracal-like conchiolin (r. pe.) on the inner surface of conchiolin of the outer layer type (r.o.). The initially-formed laminae (r.l.) are visible on the outer surface of the regenerated structure.

4. Portion of the repaired structure produced in the region of an uncovered aperture after 4 months in which prism rudiments (p.) appear on the inner surface of a thick membrane of periostracal (r. pe.) and outer layer type (r.o.) conchiolin.

5. Part of a thick, non-calcareous membrane produced in a specimen after $6\frac{1}{2}$ months regeneration (uncovered) showing the alternate production of several layers of outer layer (r.o.) and inner layer (r.i.) type conchiolin.

6. Region of mantle underlying a specimen regenerating for $7\frac{1}{2}$ weeks showing the marked difference in appearance between epithelial cells resembling those of the outer fold at the mantle edge (r.o.f.) secreting outer layer type conchiolin and the adjoining epithelium of the general outer mantle surface (e.o.m.).

7. A portion of the repair structure completed by the mantle margin of a specimen after three months showing periostracal-like conchiolin (r. pe.) being secreted on the inner surface of material resembling the non-calcareous matrix of the outer (prismatic) layer (r.o.) by the epithelium on the outer surface of the outer mantle fold (e.o.f.).

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8. Specimen regenerating for ten months showing repair of a damaged valve margin (at the right-hand side of the photo-micrograph) by the secretion of new outer (prismatic) layer material (r.o.v.) followed by inner (nacreous) layer (r.i.v.). The repaired structure is firmly attached to the intact valve (o.v., i.v.).

PLATE 2

Sections illustrating repair of the ligament in *Anodonta*. In all the specimens the dorsal surface of the ligament is uppermost.

1. Transverse section of specimen regenerating for three weeks (covered aperture) showing formation of thin, incomplete laminae of inner layer type conchiolin (r.l.) with which large numbers of amoebocytes (am.) are associated. Laterally is situated a remnant of the inner layer (i.l.) of the intact ligament.

2. Transverse section of 7-week-old specimen (covered) showing aperture almost protected by the initially-formed laminae (r.l.) which are, however, being thickened laterally by the firmer, outer layer type conchiolin (r.o.).

3. Transverse section of mantle isthmus underlying specimen illustrated in (1). The epithelium of the dorsal surface of the isthmus has been largely replaced by amoebocytes (am.) although it is being reformed laterally (r.e.).

4. Transverse section of mantle isthmus underlying repair structure illustrated in (2). Amoebocytes (am.) are still present but much of the epithelium has been re-formed (r.e.).

5. Transverse section of specimen after seven months repair (uncovered) showing the formation of a complete protective membrane of outer layer type conchiolin (r.o.) attached laterally to the ligament ridge (l.r.).

6. Transverse section of a thick repaired structure consisting mainly of outer layer conchiolin (r.o.) produced after ten months repair in the region of an uncovered aperture.

7. Longitudinal section of mantle isthmus underlying specimen illustrated in (6) showing the sharp demarcation line between the epithelial cells conducting repair which resemble those of the outer mantle fold (r.o.f.) and the adjacent cells of the mantle isthmus which secrete the inner layer of the normal ligament (e.m.i.).

8. Transverse section of mantle isthmus underlying repair structure illustrated in (6) showing epithelium resembling that of the outer mantle fold (r.o.f.) which is concerned with the regeneration of outer layer type conchiolin.