The cuticle and associated structures of *Podura aquatica* at the moult

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With 4 plates (figs. 3 to 6)

Summary

At the beginning of a moult a foam secretion appears between the epidermal cell surface and the old cuticle. This secretion is involved only with the lifting away of the old cuticle and formation of the exuvial space. A granular secretion then appears in the exuvial space. The granules contain inactive moulting enzymes, which become active only after the completion of the new cuticulin layer, and then digest the soft parts of the old cuticle. The rest of the new cuticle is laid down extracellularly and in lamellae below the cuticulin layer. The sculpturing of the new cuticle occurs under the influence of the epidermal cells. Subsequent hardening of certain areas of the new cuticle involves disruption of the lamellae and complete reorganization. Setae are formed early and rapidly, but nervous connexions with the old setae are maintained until just before ecdysis. Muscle insertions into the new cuticle are formed early, but attachment to the old cuticle is retained until ecdysis. The cuticulin layer is involved in the formation of the new attachments.

Introduction

COLLEMBOLA, in common with other apterygotes, continue to moult throughout their lives, even after they become sexually mature. Few studies have been made on the life-history of Collembola, but Maclagen (1932) in his study of the life-history of *Smynthurus viridis* L. quoted an average instar length of 3 to 5 days for the early instars, though the instars varied in length from 2 to 10 days and became longer in the latter part of the life-history. He also showed that a definite number of instars (8) occurred in the life-history, though this is not to be expected for all Collembola. In *Podura aquatica* I have found that the instar length varies, usually within the limits of 2 to 6 days, the most frequently occurring instar lengths being 3 to 5 days.

Earlier studies on the cuticle in Collembola have been restricted mainly to a consideration of the fully formed intermoult cuticle, and no attempts have been made to describe the deposition of the new cuticle at the moult along the lines of studies made on pterygotes (compare Wigglesworth, 1933, 1947, 1948*b*; Way, 1950; Wolfe, 1954*a, b*). Philiptschenko (1907) noted the occurrence of unicellular exuvial glands in the epidermis, which change their appearance at the onset of ecdysis, becoming foam-like. Willers and Durken (1916) suggested that these large cells may be oenocytes. Ögel (1958) regarded them as probable dermal glands which poured out the material forming the cement layer. She further noted that just prior to ecdysis the epidermal...
surface along with the new cuticle was folded, thus achieving a greater surface area and allowing for growth in the subsequent instar. The exuvial space between old and new cuticles was narrow. The muscle attachments were restricted to the epidermis, and did not change at the moult.

![Diagram of culture setup]

**FIG. 1.** The arrangement of the culturing tubes in the small glass aquarium used to rear *P. aquatica*. The *Lemna* which was used to provide food is shown on the surface of the water in the tubes.

In the absence of prior detailed considerations of the events occurring at the cuticle during the moulting cycle, this aspect has received greater attention in the present study on *P. aquatica*. Specimens of all stages throughout the instar have been examined histologically.

**Materials and methods**

*P. aquatica* L. (Collembola, Isotomidae) was collected from the surfaces of ponds and ditches in the Cambridgeshire area and kept in small aquaria in the laboratory. These aquaria formed the stock cultures. The duckweed *Lemna* was added to the aquaria to provide food and the aquaria were filled with tap-water, or ditch-water, to a depth of 2 to 3 inches. Individual specimens were isolated under small, inverted tubes in shallow aquaria over which glass plates were placed to prevent evaporation of the water. To each inverted tube was added one or two pieces of duckweed to provide food. Ten tubes were kept in each aquarium. This culture method allowed the moulting cycle to be followed over many instars in individual insects. The arrangement of the tubes in the aquarium is shown in fig. 1.

Specimens were prepared for the light and electron microscopes as outlined in an earlier paper (Noble-Nesbitt, 1963).

**Results from light microscopy**

The basic structure of the intermoult cuticle has been given in an earlier paper (Noble-Nesbitt, 1963). This structure is attained very shortly after ecdysis within an hour or so of the shedding of the old cuticle. The first changes at the cuticle involved in the next moult are apparent only after 80 to 100 h following ecdysis, though this varies with the length of the instar.
At this time the old cuticle is lifted away from the epidermis, and evidence of a new cuticle overlying the epidermis is seen as a refractile membrane which is slightly amber-coloured, very thin, and tuberculate. There is no marked folding of the new cuticle and epidermis apart from the tubercles (compare Ögel, 1958). The folding inherent in the tubercles will obviously allow for much subsequent expansion, especially if the ‘pitch’ of the tubercles is greater in the newly forming cuticle. It may be noted that Ögel described only minor tubercles in *Folsomia candida* (Willem), so that distinct epidermal folding when the new cuticle is being formed may be important in this insect to allow for subsequent expansion.

At this earliest recognizable stage in light-microscope preparations, the lifting old cuticle differs in no major respect from the intermoult cuticle. No evidence of dissolution of the endocuticle is seen. The new cuticle is extremely thin, less than 1 μ thick, and in general there are no secondary layers present. The cuticle at this stage apparently consists of epicuticle only, and therefore represents a very early stage in the moulting cycle and in the laying down of the new cuticle. However, a hint of a bluish coloration is seen over the clypeus and over the ventral tube vesicles in Mallory-stained preparations. It also becomes evident over the ventral groove at a slightly later stage. Over the clypeus, it is possible that already the thicker cuticle of this area is becoming evident, so that the blue coloration may be due to the first lamellae of the endocuticle. As the cuticle over the ventral tube vesicles even in the fully formed stage is very thin, its staining reactions are not readily determined, and little weight can be attached to them.

A consideration of the newly forming setae shows up an interesting sequence, which may be seen in a single specimen. Most of the newly formed setae, which are made to lie along the new cuticle in the limited exuvial space, stain blue in Mallory’s triple stain, probably indicating that the cuticle is soft at this time (Dennell, 1946; Dennell and Malek, 1955 a, b; Wolfe, 1954b). Some, however, are blotchy red-blue, or completely red, while 1 or 2 even at this stage are amber-coloured. These differences in staining properties follow the scheme suggested by Dennell (1946) and Dennell and Malek (1955 a, b) for the sequence of the hardening process in the tanning of the cuticle in the blowfly and cockroach. At a later stage, all the setae are amber-coloured, the staining property they show after ecdysis. This has been described for Diptera by Weismann (1864), Wolfe (1954b), and Cottrell (1960), though, as Cottrell shows, the sequence continues through to a final colour much darker than the light amber attained in *P. aquatica*. Cottrell further indicates that this sequence occurs at different times over different parts of the body, as though a wave of initiation passes along the body. It is not possible to say if such a wave occurs in *P. aquatica*, but the setae do appear in different stages in the sequence at the same time in different areas. From these observations it would appear that the sensory hairs are prepared well in advance of ecdysis, and they presumably will be functional immediately after ecdysis, resulting in a minimal period when sensory inflow may
be disrupted (see also Wigglesworth, 1953b; Wolfe, 1954b). Further evidence from electron microscopy will be presented later in this paper to show that this early preparation does, indeed, occur. Furthermore, strands may be seen during the earlier stages of the cycle passing out to the old setae, suggesting that nervous contact is maintained during this period.

On emergence from the old cuticle (i.e. just post-moult), or actually before ecdysis is completed, the new cuticle shows the whole range of cuticular structure seen in fully formed intermoult cuticle. It thus appears that the new cuticle is more or less complete at ecdysis. This would account for the fact that Collembola emerge from their cast skins and jump and walk immediately. This was observed in *Orchesella villosa* (as well as in *P. aquatica*) and a specimen of *O. villosa* fixed immediately after emergence showed the same completely formed new cuticle.

Cast cuticles prepared in the normal way, and stained in Mallory, show all the layers seen in the fully formed intermoult cuticle, but everywhere the inner blue layer (soft endocuticle) appears reduced, further indicating that resorption occurs.

**Results from electron microscopy**

The earlier work done with the light microscope, described in the above section, had indicated the nature of the problem. The moulting cycle of cuticular deposition and resorption characteristic of arthropods in general and pterygote insects in particular occurs also in Collembola. But though conforming in general principle to the sequence of events already known for these, the moulting cycle of *P. aquatica* also seemed to diverge a little, especially in the timing of any hardening that occurs. Normally, in pterygotes, a period of expansion and hardening occurs after ecdysis; in *P. aquatica*, where only localized hardening occurs, there is not the same need for expansion before hardening, which therefore need not be delayed until after ecdysis. Cottrell (1960) has shown that in Diptera certain areas are pre-hardened and darkened before ecdysis. These areas are associated with movement and protection. While fairly extensive, they do not interfere with the expansion of the cuticle in general after ecdysis and prior to final hardening and darkening.

Further, the very thinness of the old and new cuticles makes resolution difficult in light microscopy. Hence detailed consideration of the sequence of events during the lifting away and partial dissolution of the old cuticle, and the formation of the new cuticle, was carried out using the electron microscope, the stages already seen with the light microscope being used to correlate the two studies.

**The lifting of the old cuticle**

The plasma membrane of the epidermal cells is in intimate contact with the base of the old cuticle before the onset of moulting. Electron micrographs
show that it is thrown into numerous small folds, the tips of these being very close to, but distinct from, the lowermost lamella of the cuticle (see fig. 3, A).

Before the first layers of the new cuticle can be laid down, therefore, it is first necessary that the old cuticle be lifted away from the cell surface. Though this necessity has long been recognized, and the lifting away of the old cuticle mentioned as a prerequisite to the laying down of the new cuticle in many papers dealing with moulting (Wigglesworth, 1933, 1953a; Way, 1950; Richards, 1951, 1953; Passonneau and Williams, 1953; Wolfe, 1954 a, b), as far as is known no full description of the phenomena associated with this process has been given. Such accounts as there are refer only to the retraction of the epidermis from the old cuticle, though dissolution of the endocuticle is also suggested as the loosening mechanism (Richards, 1951, 1953).

When this lifting away occurs, a space is made between the plasma membrane and the old cuticle, and this space must be filled by something, if only extracellular fluid. Certainly, the exuvial space between new and old cuticles has been shown to contain a 'moulting fluid' with enzymatic properties (Tower, 1906; Wigglesworth, 1933; Dennell, 1946; Way, 1950; Passonneau and Williams, 1953; Wolfe, 1954 a, b). It will be shown here that before a fluid-filled space of this sort is produced, the plasma membrane becomes remote from the old cuticle due to a secretion produced by the cell, and only then does the plasma membrane become far enough away from the old cuticle to start producing a new cuticle, which is separate from the old cuticle. Though this initial lifting away is only for a short distance (approximately 800 \( \mu \)), it may well be sufficient to overcome physical forces that otherwise might bind the new cuticle to the old. Its brief occurrence alone, followed quickly by the appearance of tubercles on the new epicuticle, may account for Way (1950) concluding that no exuvial space is present until after the new epicuticle is laid down in Diataraxia.

The 'foam' secretion. The earliest stage in the moulting cycle discernible at the cuticular surface of the epidermis is the production by the epidermal cells at approximately 72 h post-moult of a foam secretion between the plasma membrane and the old cuticle, which is thereby lifted away from the plasma membrane (see figs. 2, A; 4, A). This foam secretion is composed of small vesicles surrounded by smooth membranes. The vesicles vary from about 20 to 50 \( \mu \) in diameter, with some scattered larger vesicles of varying size, but up to approximately 200 \( \mu \) in diameter. As far as can be interpreted from the electron micrographs, these vesicles are formed as out-pushings of the plasma membrane which is then pinched off, leaving vesicles bounded by smooth membranes free of the cell surface (see fig. 4, A). The epidermal cells producing this secretion are filled with endoplasmic reticulum bearing ribose nucleic acid (RNA) granules (zymogen granules) characteristic of protein-synthesizing cells (Palade, 1955; Sjöstrand and Hanzon, 1954). It is to be emphasized that this foam secretion occurs only at the surface of the epidermal cell. Therefore it is distinct from the foam-like cells of Philiptschenko (1907) in which the whole cell becomes foam-like. This foam secretion produces an
Noble-Nesbitt—Moulting cuticle of Podura

Fig. 2
extracellular space which separates the plasma membrane from the base of the old cuticle. As will be seen, its subsequent fate seems to play little part in the moultng cycle. It is a phenomenon involved only in the lifting away of the old cuticle.

Soon after it has been fully formed, the foam secretion also lifts away from the plasma membrane and disperses, presumably into solution in the exuvial, or moulting, fluid, which comes to fill the space produced by the foam secretion. The distance between old and new cuticles may then be increased by a buckling away from the epidermis of the old cuticle where no attachment of it to the epidermis remains (compare Way, 1950).

*The 'granular' secretion.* Shortly after the first appearance of the foam secretion, a further secretion at the epidermal surface becomes apparent. This is characterized by very dense granules, and is termed the 'granular' secretion (see figs. 2, B, C; 4, B).

The granular secretion is continually produced over a short period, probably for a few hours. At first, it probably appears along with the last of the foam secretion, and certainly it can be seen to occur mixed with the foam secretion (see figs. 2, B; 4, C). Later, at the cessation of production of foam secretion, the granular secretion occurs alone and the foam secretion lifts away (see figs. 2, C; 3, B). The granular secretion continues to appear as the first layer of the new cuticle is laid down and the foam secretion disappears (see figs. 2, D; 4, D). Only when this cuticular layer is completed does the secretion of the granules end, though the granules persist in the exuvial space for a short time after this. As this granular secretion appears to take an important part in the events of the moulting cycle, its production, nature, and function will now be considered.

The granules of the secretion are seen only outside the usual limits of the plasma membrane (see figs. 4, D, E). Nowhere have similar granules been seen lying deeply in the epidermal cells. The epidermal cells contain the usual cellular components of nucleus, mitochondria, and endoplasmic reticulum, with pigment granules (which give the characteristic colouring to the insect) and a few scattered vesicles (see figs. 2; 3, B; 4, A–E). Perhaps most noteworthy of these components is the endoplasmic reticulum, the granular

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**Fig. 2.** Diagram showing the sequence of events at the epidermal surface during the early part of the moulting cycle. The plasma membrane is shown by a thin double line, and the endoplasmic reticulum, mitochondria, pigment granules, cytoplasmic vesicles, and part of a nucleus are diagrammatically represented. The outer parts of the old cuticle are omitted from the diagrams of stages C to F. A, the foam secretion between the plasma membrane and the old cuticle. B, the mixed granular and foam secretions in the exuvial space. C, the dispersal of the foam secretion, and the appearance of granule-bodies and the cuticulin layer of the new cuticle. D, the secretion of a granule body through a pore in the new cuticulin layer and the commencement of the digestion of the old cuticle. E, the completion of the cuticulin layer and the dispersal of the moulting granules, with the formation of a faintly granular moulting fluid and the digestion of the old cuticle. F, the digestion of the old cuticle, leaving the ecdysial membrane; and the production of the sculpturing pattern of the new cuticle, with epidermal projections passing to the tubercles. A vesicle enclosed by epidermal projections underlies the major tubercle.
membranes of which fill most of the cell (see fig. 3, B). As many workers have shown (Karrer, 1960; Palade, 1955; Sjöstrand and Hanzon, 1954), the endoplasmic reticulum is associated with the synthesis of proteins, secretory cells having a large amount of endoplasmic reticulum. The granules on the membranes of the endoplasmic reticulum are thought to be the site of RNA (Palade, 1955), probably the sites of protein elaboration. The large amount of endoplasmic reticulum in the epidermal cells therefore correlates well with the high secretory activity shown by these cells during the moulting cycle.

One of the first of these secretory activities is the production of the granular secretion. At those points on the surface of the cell where the granules appear, the endoplasmic reticulum can be seen penetrating the plasma membrane and passing up into the mass of granules, or granule body. This is a feature of the secretion: the granules are produced in clumps, so forming a granule-body (see figs. 2, D; 4, E). Moreover, the granules appear to be elaborated at this point, i.e. actually on the surface of the epidermal cell, apparently under the influence of the endoplasmic reticulum which presumably utilizes precursors from the bulk of the cytoplasm.

It is thus possible to build up a picture of the mechanism of secretion of these granules. The endoplasmic reticulum, with its synthesizing sites, penetrates the plasma membrane, which appears to break down at these points. Presumably carrying through with it the necessary precursors, probably within the vesicles enclosed by the endoplasmic reticulum, the endoplasmic reticulum then elaborates and sets free the granules, which come to lie in the exuvial space. Once this is accomplished, the plasma membrane re-unites over the tiny pore through which the endoplasmic reticulum passed. Further evidence for this method of secretion has been put forward in a recent paper by Karrer (1960), who distinguishes two types of secretory mechanisms in embryonic chick aorta tissues, one of which resembles the condition described here.

Information as to the nature of the granular secretion has been obtained indirectly from a consideration of the substructure of the granules and of its effects during the moulting cycle. Its action during the moulting cycle, and hence its function, will be described in detail later in this paper, but the conclusions drawn then will be freely used in this present section.

The granular secretion appears as granule-bodies of varying size, with the numbers of granules in each granule-body varying accordingly. Each granule-body consists of a number of dense granules with a much fainter granular matrix between the large granules (see fig. 4, B). It is considered that the matrix probably represents merely a spilling-over of materials which are expelled from the cell along with the granules, though a physiological role for the matrix should not entirely be ruled out (see below). The granules themselves are spherical with a diameter of approximately 100 m\(\mu\). When closely examined, they show evidence of being composed of still smaller spheres, of diameter approximately 10 to 20 m\(\mu\), though it is not always possible to see these subunits. The first impression is of a virus particle. This
impression is not without its uses. The pattern exhibited by the subunits forming the shell of a virus is important in packing these subunits tightly, using a minimum number of subunits, and giving an external covering of maximum area with a minimum of subunits. These subunits are protein molecules, or molecular-aggregations (see Horne, Brenner, Waterson, and Wilby, 1959; Horne and Nagington, 1959; Crick and Watson, 1956; Picken, 1960). Similarly, it is at least possible that the granules under consideration here are such tightly packed arrays of protein-bearing subunits. Such packing may well so modify the properties of the contained proteins as to render them physiologically inactive while they are so packed. Evidence will be presented to show that the secretion becomes physiologically active only after the granules disperse.

The structure of the granules thus fits in with the idea that they are well-packed arrays of subunits which are within the range of size of large protein molecules. These subunits, therefore, could well be enzymes in an inactive state: it is as if the cell produces and secretes prepacked quantities of enzymes which only later are liberated and able to perform their enzymatic functions. That the products of the granule-bodies do, in fact, perform enzymatic functions in the digestion of the old cuticle will be discussed further below, and it is evident that the granular secretion contains the digestive enzymes of the moulting fluid, but as yet in an inactive state.

The granular secretion first becomes evident towards the end of the production of the foam secretion (see fig. 4, c) but is most abundant when the foam secretion is disappearing, having performed its function. Its production continues while the first layer of the new cuticle is laid down, through small pores left in that layer. As discrete granules, however, it disappears soon after the completion of the cuticulin layer of the new cuticle and the exuvial space then exhibits a faintly granular appearance (see figs. 2, D; 3, c). At the same time, evidence of the commencement of digestion of the old cuticle becomes apparent (see fig. 3, d). It seems probable, therefore, that the granules, as soon as the new cuticulin layer is completed, liberate the enzymes, which begin to attack the old soft cuticle. This finding is borne out by observations made on granule-bodies at this time. In fig. 4, f we see a granule-body adjacent to the old cuticle when the new cuticulin layer was almost completed. Already here we can see evidence of the old cuticle being eaten away where it is in contact with the granule-body. The old cuticle at this point appears to be breaking down into a disorganized, faintly granular material. This is probably the first stage in the digestion of the old cuticle. A granule or two in the granule-body may already have liberated their enzymes in an active state. It is only to be presumed that the remaining discrete granules would similarly release their enzymes within a short period of time after this.

The function of the granular secretion therefore seems to be to carry out into the exuvial space, in an inactive form, the enzymes necessary for the digestion of the old cuticle, and to liberate them in an active form only when
most of the new cuticulin layer has been laid down. This raises the interesting question of why the secretion of the enzymes is not delayed until the cuticulin layer is complete, and then undergone with the enzymes in an active state, so that they function immediately. A partial answer to this may lie in the size of the enzymatic molecules. If these are large protein molecules, then they presumably will not pass through the cuticulin layer, and this layer certainly seems to protect the new endocuticle, being therefore impermeable to the enzymes. This, of course, could be overcome by leaving small pores in the cuticulin through which the enzymes could pass, as indeed we have seen is the case in the later stages of the secretion of the granules. The answer would appear to lie in the function of the cuticulin layer and the activity of the enzymes.

As earlier workers on insect moulting have shown (compare Wigglesworth, 1933, 1948a, 1957), the cuticulin layer of the new cuticle is resistant to the action of the enzymes of the moulting fluid and serves to protect the underlying new soft cuticle as it is being laid down, as well as the epidermis. Now all the layers of the cuticle in all regions are unhardened at this time of deposition: they are all, therefore, presumably sensitive to the enzymes of the moulting fluid. To prevent attack, it is important that the cuticulin layer overlying and protecting them should be complete before they are laid down and before the moulting fluid enzymes are active. But the large amounts of material to be digested from the old cuticle requires that a great deal of enzymatically active fluid be present and that it should act for some considerable time. Some idea of the amount that must be secreted can be obtained by looking at the amount of granular secretion (see figs. 3, E; 4, B–D). Almost the whole of the surface is at some time concerned, and possibly for a fair period of time (approximately 4 h). Passing the active enzymes through the cuticulin

Fig. 3. (plate). A, section through epidermis and soft cuticle showing the narrow space between the surface of the epidermal cell and the innermost lamella of the endocuticle.
B, section showing the foam secretion lifting away and breaking down, with the granular secretion occurring in discrete clumps. The cuticulin layer is beginning to appear, and there is extensive endoplasmic reticulum in the epidermal cells. Part of a nucleus is included.
C, section showing the faintly granular moulting fluid in the exuvial space. The new cuticulin layer is almost complete and the granule-bodies are no longer present.
D, section showing the digestion of the old cuticle, leaving the faint ecdysial membrane.
E, survey electron micrograph showing large amounts of the granular secretion in the exuvial space, with the cuticulin layer just beginning to appear. Large mitochondria are present in the epidermal cell, and a nucleus with nucleolus is included.
F, section showing the almost complete cuticulin layer following the contours of the epidermal surface.
G, section showing a very early stage in the deposition of the cuticulin layer. Both the granular and foam secretions are still present in the exuvial space.
H, section showing the vesicles under the major tubercles of the new cuticle, which is still being secreted. The ecdysial membrane has been formed at the base of the old cuticle.
cutic. l., cuticulin layer; ec dys. mem., ecdysial membrane; endocut., endocuticle; epicut., epicuticle; epid., epidermis; epid. pr., epidermal projections; exuv. sp., exuvial space; foam, foam secretion; gr., granular secretion; maj. tub., major tubercle; m. fl., moulting fluid; mit., mitochondrion; new cut., new cuticle; nl., nucleolus; nucl., nucleus; old cut., old cuticle; pig. gr., pigment granule; p.m., plasma membrane; ves., vesicle.
FIG. 3

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layer would cause this to be no more than a network: the active enzymes would not be confined to the exuvial space but would be able to pass back into these pores and attack the new soft cuticle and the epidermal cells before the breaches in the cuticulin layer could be repaired. This is distinct from effects while the enzymes are passing outwards, which presumably could be prevented by the cytoplasmic filaments in the pore canals elaborating the enzymes only at the cuticulin surface of the new cuticle, thereby avoiding the necessity of passing active enzymes through the newly forming endocuticle (compare Richards, 1951). It is, therefore, obviously much better to secrete the enzymes in an inactive form; and, further, to complete the majority of the secretion before commencing to lay down the cuticulin layer, so that finally few pores have to be sealed off. This is the situation found in \textit{P. aquatica}.

An obvious corollary of this latent period between secretion of the granules and the liberation of their enzymes is the question of control of the timing of activation. Though it is not possible to give a complete answer to this, there are one or two methods by which this timing mechanism could operate. It is possible that the matrix of the granule bodies may contain a principle which takes time to act and that this, then, begins the liberation of the enzymes. Perhaps a much more feasible timing mechanism would operate from the epidermal cells. While these are still exposed to the exuvial space it is possible that a principle inhibiting the release of the enzymes is passed into the space. On completion of the cuticulin layer, this supply would cease. Alternatively, a small molecular ‘key’ substance may be passed through the completed cuticulin layer to commence the liberation of the enzymes.

It is to be noted that in this present study, as in previous studies (Wigglesworth, 1933; Dennell, 1946; Way, 1950; Wolfe, 1954 \textit{a}, \textit{b}; Passonneau and Williams, 1953), the first evidence of digestion of the old soft cuticle (endocuticle) appears after the completion of the new cuticulin layer, and continues while the remainder of the new cuticle is being laid down. This has led to the view that the moulting fluid (containing the digestive enzymes) is secreted by the epidermis at this stage of the moulting cycle (Wigglesworth, 1948\textit{a},

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{(plate). \textit{A}, section showing the foam secretion between the plasma membrane of the epidermal cell and the old cuticle. Vesicles just being formed are shown by the arrows. \textit{B}, section showing the granular secretion in the exuvial space. The incomplete cuticulin layer of the new cuticle overlies the plasma membrane of the epidermal cell. \textit{C}, section showing the granular secretion appearing amongst the foam secretion (bottom left) and also occurring alone over a neighbouring cell (upper right). The cuticulin layer is being formed in isolated patches. \textit{D}, section showing the granular secretion alone in the exuvial space, beyond the limits of the plasma membrane. The cuticulin layer is discontinuous. \textit{E}, section showing the formation of a granule body outside the plasma membrane. The new cuticulin layer overlies the rest of the epidermal surface, and the epidermal cell is filled with endoplasmic reticulum. \textit{F}, section showing the digestion of the old cuticle commencing where it is in contact with a granule body.}
\end{figure}

cutic. I., cuticulin layer; epid., epidermis; exuv. sp., exuvial space; foam, foam secretion; gr., granular secretion; old cut., old cuticle; p. c., pore canal; pig. gr., pigment granule; p. m., plasma membrane; ves., vesicle of foam secretion.
The objections to secretion occurring at this time have been outlined above and, indeed, they have been realized by earlier workers (Richards, 1951). It may now be possible to reinterpret those findings, using the situation found in *P. aquatica* as the basis for the reinterpretation. Since the digestion of the old cuticle commences only at this stage and since the fluid in the exuvial space only then becomes enzymatically active (Passonneau and Williams, 1953), it is necessary to postulate that the moulting fluid at first contains no active enzymes for digesting the old cuticle. However, this does not preclude the occurrence of these enzymes in an inactive 'bound' state. This is the form in which it is suggested they occur in the granule-bodies. It therefore becomes unnecessary to postulate the actual secretion of these enzymes through the newly forming cuticle, thereby circumventing the objections already mentioned. Instead, activation of the enzymes already present in the moulting fluid in a 'bound' inactive state in the granular secretion is all that need be postulated. The activating substance need have no profound effect on the newly forming cuticle through which it may have to be passed. Supporting this hypothesis is the work of Passonneau and Williams (1953), who found that in *Cecropia* pupae a proteinaceous gel already present in the exuvial space becomes converted into a sol containing digestive enzymes after 14 days.

*The secretion of the new cuticle*

The production of the foam and granular secretions essentially takes place during the lifting away of the old cuticle, though we have seen that the production of the granular secretion overlaps the phase that will now be considered in greater detail: the laying down of the new cuticle. Further, the effects of the granular secretion on the digestion of the old cuticle first become apparent during this phase.

Towards the end of the production of the granular moulting secretion the first signs of the secretion of the cuticulin layer are seen (see figs. 2, D; E). No detailed investigation of the mechanism of the actual secretion of this (or any other) cuticular layer has been made. It seems probable, however, that the secretion occurs in much the same way as Karrer (1960) has shown for the secretion of extracellular materials in the embryonic chick; that is to say, in much the same way as for the granular moulting secretion. The precursors are presumably liberated from the cisternae of the endoplasmic reticulum at the cell surface and form the cellular layers.

The first dense layer of the new cuticle (corresponding to the cuticulin layer of the fully formed cuticle) is not produced simultaneously over the whole of the surface, but first appears as numerous small areas which enlarge and only finally coalesce (see figs. 3, F, G; 4, E). Even before final coalescence, this layer is thrown into gentle folds (see fig. 3, F) which sometimes follow the contours of the cell surface, but not always. These are evidently the beginnings of the minor tubercles. Despite many attempts, the short phase between completion of the cuticulin layer and the laying down of the first
endocuticular lamellae has not yet been successfully fixed for the electron microscope, the specimens proving to be just too early or just too late in the moulting cycle. It is therefore not possible to trace the development of these minor tubercles, since they are complete by the time the first endocuticular lamella appears. However, we must not rule out the possibility that they are formed while the cuticulin layer is still being laid down. Previous considerations of the production of ridges (compare Wigglesworth, 1933; Locke, 1957, 1958) have indicated a swelling of the layer subsequent to its completion, giving a greater surface area, which is accommodated by ‘wrinkling’. It would equally be possible to produce a greater surface area by adding to the edges of the layer before these coalesce, hence invoking no mechanism of ‘swelling’. The gentle folds (see above) noticed in *P. aquatica* before the completion of the layer lend weight to this argument. And as the layer is produced in scattered islands which enlarge by addition round their margins, there is at least the basis for an excessive production of cuticulin by this method. Without intermediate stages, however, it is not possible to decide what actually occurs. It may be added that whichever way the cuticulin area is increased, by swelling or addition, the result will be a puckered surface. It is possible that this puckering may be used and moulded by the epidermis to produce the precise surface sculpturing pattern. There seems to be some evidence to support this view (see below).

Subsequently, further cuticular material is added below the new cuticulin layer, as lamellae which appear successively. These lamellae may be produced because of the molecular forces within the cuticular materials (compare Picken, Pryor, and Swann, 1947), or they may be the expression of successive bursts of cuticular secretion from the epidermal cells, each secretion forming a lamella before the next one appears. There seems to be reason to suppose that each lamella is, indeed, laid down separately and at the same time over the whole body surface, since electron micrographs show that each lamella is completed before the next one arises (see fig. 5, A). If molecular forces were acting alone, we would expect to find the deposition of homogeneous thick regions of cuticle, which only subsequently would become organized into lamellae (compare Picken, Pryor, and Swann, 1947; Wolfe, 1954 a, b). This is not the case. Rather, it would seem that any molecular forces act on the secretion immediately it arises, forming the lamellae and binding them together. In this way, the thickness of the cuticle is built up. As we shall see, some areas attain a greater thickness because of more rapid secretion occurring over a slightly longer period.

The formation of the surface sculpturing pattern. During the early stages of the secretion of the new cuticle, the surface sculpturing pattern is produced. We have seen that the minor tubercles appear before the first lamellae underlying the cuticulin layer appear. At first, they seem to be puckerings due to the cuticulin layer having a greater area than the underlying epidermis. Later, however, they appear to be fixed in a definite pattern with a definite structure, under the influence of the secretions of the epidermis. The next ‘layer’ of
the cuticle to appear after the cuticulin layer follows closely the pattern of the puckered cuticulin layer, and there is some slight evidence that the cell surface also follows the course of the cuticulin, microvilli seeming to pass up the folds (see figs. 2, F; 5, A). Thus an even secretion over the whole cell surface will give a cuticular layer which is sculptured in the same way as the cuticulin, without any need for expansion subsequent to its secretion and elaboration. However, the final form of these minor tubercles requires that this layer is altered at least under the tubercles; it is also altered between the tubercles in some areas (e.g. over exocuticle; see Noble-Nesbitt, 1963). There is reason to suppose that this alteration (which may be a hardening process) occurs under the influence of the epidermal projections: as will be seen, these are usually maintained throughout cuticular deposition and even during the intermoult period (see below; see also Noble-Nesbitt, 1963). Accordingly, this change conceivably could occur at any time; that it occurs during the formation of the exocuticle is most likely (compare Locke, 1957, 1958).

The formation of the major tubercles can be followed with greater certainty. The first indication of their formation is the appearance of extracellular vesicles at the surface of the epidermis at regular intervals (see fig. 3, H). These vesicles on closer examination are seen to be enclosed by numerous projections of the cell surface (microvilli) (see figs. 2, F; 5, B), and the cell surface is, therefore, effectively sculptured. The fact that the lamellae of the cuticle already laid down are everywhere penetrated by cytoplasmic strands means that the extra length of the lamellae could be accommodated by addition at the edges of these pores. The effective extra cell surface due to the microvilli enclosing the sculpturing vesicles takes care of subsequent lamellae, which follow the outline produced by the microvilli. In this way sinuous lamellae result, and the sculpturing seen in the general body surface is produced. The pitch of the tubercles is greater than in the intermoult cuticle (see fig. 5, A). This allows for subsequent growth and expansion.

It may be noted that we have here no evidence as to the contents of the sculpturing vesicles, and it can only be assumed that they are filled with extracellular fluids. An observation of interest, however, is that in certain plants a similar mechanism is used to produce the surface pattern, and in this case the vesicles contain an oil (van Herck, personal communication). It can only be inferred that this oil is secreted by localized areas of the epidermal surface, even by part of the surface of a single epidermal cell, and that therefore the basic pattern for the production of the surface sculpturing resides in the epidermal cells. An aqueous fluid rather than an oil would be expected to seep into the vesicles if they were formed passively owing to expansion of the cuticle of the plant. Similarly, the enclosing of the vesicles by the microvilli in P. aquatica would not be expected if the vesicles were formed by cuticular expansion. Though these results are at best tentative and indirect, nevertheless, the role of the epidermal cells in the formation of the surface sculpturing pattern cannot be easily dismissed (compare Wigglesworth, 1933, 1954; Locke, 1957, 1958, 1959, 1960).
In thicker regions of the cuticle, the sculpturing pattern is reflected by sinuous lamellae only in the outer parts of the fully formed cuticle (Noble-Nesbitt, 1963). Obviously, a further change must occur at the cell surface. In the thin cuticle the vesicles do not persist: eventually the epidermal cell surface becomes less deeply folded and takes on the more gentle folding of the tubercles, the microvilli of the vesicles being reduced in size to the more normal small folds seen over the whole surface (see fig. 6, A). In the thicker cuticles, these large microvilli become more bunched together proximally, and splay out in the region of the original vesicle, producing lamellae between (see fig. 6, B). This produces proximally a cord of cellular projections which spread out distally through pores left in the lamellae (see fig. 5, c). This effectively straightens the cell surface, leaving only very localized projections of bunched cytoplasmic filaments. If the cytoplasmic filaments now stop secreting cuticle, then the later lamellae will be essentially straight, with pores left where the cytoplasmic filaments pass out to the earlier formed regions of the cuticle. The result is a cuticle with the sculpturing pattern obvious only in the outer parts.

This sequence of events also demonstrates that pore canals (which in the cuticle of *P. aquatica* pass mainly out to the centre of the major tubercles; see Noble-Nesbitt, 1963) are the result of incomplete cuticle deposition leaving passages round the persistent cellular filaments. In this way, cellular connexions are maintained with outer regions of the cuticle, and these may be of the utmost importance in the subsequent changes occurring in these regions.

The formation of thicker regions of the cuticle. As we have seen, each lamella of the newly formed cuticle is laid down more or less simultaneously over the whole surface of the body. Some thicker regions have a greater number of lamellae and it is of interest to look at the boundaries of such regions. Fig. 6, c shows such a boundary in the clypeal region. The outer lamellae of the thick region are continuous with the lamellae of the thin region. A few inner lamellae have no counterpart in the thin region. Thus the increase in thickness occurs by deposition of thicker and extra lamellae in the specialized region.

A further point of interest here is that the lamellae in continuity with the lamellae of the thin cuticle are at least part of those which will be transformed later into hardened exocuticle (see below). That is to say, these seemingly continuous lamellae in one region will become exocuticle and in another will remain as endocuticle. The endocuticle of the thick cuticle consists of the inner lamellae, some of which are not continuous with the lamellae over the general body surface. This argues well for the subsequent control of the hardening process by the epidermal cells (through the cytoplasmic strands in the pore canals) and against the proposition that there is a ‘built-in’ system for subsequent hardening when the cuticular material is secreted. If this were the case, it would be expected that a burst of secretion by the specialized area would occur much earlier and interpolate the lamellae with this ‘built-in’
system between the epicuticle and endocuticular lamellae in continuity with the endocuticular lamellae of the thin cuticle.

The nature of the hardening process. Though no attempt has been made to study exhaustively the process of hardening, which occurs only in very localized regions, some features of interest have been noted during this process in *P. aquatica*; these are included here principally in the hope that they may aid the interpretation of this process in insects in general. The remarks made here refer only to the cuticle of the clypeus region. Further specialized areas, notably at the sensilla, will be discussed later in this paper.

The fully formed cuticle of the clypeus has been described earlier (Noble-Nesbitt, 1963). It will be noted, in its structure as seen in the electron microscope, the exocuticle differs from the endocuticle in the absence of lamellae, and in its homogeneous appearance. In the forming cuticle, however, no such distinction can be seen either in *P. aquatica* (see above) or in other insects (Wigglesworth, 1933; Dennell, 1946; Way, 1950; Wolfe, 1954 a, b) up to the moment of ecdysis. Shortly after ecdysis, however, hardening occurs (compare Wigglesworth (1933), Dennell (1946), and Wigglesworth (1948a, 1953a, 1957) for reviews). The structures seen at this time are therefore of considerable interest and importance.

In specimens fixed within half to one hour of ecdysis the outer regions of the thick cuticle are undergoing structural changes (see fig. 5, D). The lamellae present before this stage appear to be replaced by a structureless material—one that is much more homogeneous. This appears to be a true replacement, not just a more rigid binding of the lamellae, nor a mere interpolation of new materials between the lamellae (compare Pryor, 1940). The material already present in the lamellae, of course, is presumably incorporated into the new structure, but in a very intimate association such that it loses its

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**Fig. 5 (plate).**

A, section through parts of two new major tubercles, showing the first endocuticular lamella to be formed. Cellular projections pass through the lamella to the minor tubercles (lower right). The 'pitch' of the major tubercles is greater than in the old cuticle, which is beyond the limits of the electron micrograph.

B, section showing epidermal projections enclosing the vesicles under the major tubercles of the new cuticle.

c, section through a major tubercle showing the branching pore canals in the outer sinuous lamellae of the cuticle.

D, section showing the structural changes which occur in the cuticular lamellae during the sclerotisation of the exocuticle. To the right of the two vertical fold-marks, the lamellae are distorted and are being disrupted.

E, section through a newly forming seta, showing the underlying tormogen and trichogen cells which demarcate the differentiation of the new cuticle, and the persistent nerve-strand passing out through the exuvial space at the tip of the new seta. The old cuticle is beyond the limits of the electron micrograph. The new cuticle is at a very early stage of its deposition, and the exuvial space is filled with faintly granular moulting fluid.

F, section showing the persistent nerve-tracts to old setae, and a new seta forming.

cutic. l., cuticulin layer; endocut., endocuticle; epid., epidermis; epid. pr., epidermal projections; exocut., exocuticle; exuv. sp., exuvial space; gr., granular secretion; lam., endocuticular lamella; min. tub., minor tubercle; new cut., new cuticle; old cut., old cuticle; old sens., old sensory processes; p. c., pore canal; sens., sensory processes; sin. lam., sinuous lamellae; torm., tormogen cell; trich., trichogen cell; ves., vesicle.
FIG. 5

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previous lamellar structure and forms a homogeneous matrix probably in
association with new materials added at this stage. The extensive ramifi-
cations of the cytoplasmic filaments obviously could provide the channels for
transport and secretion of such new materials to this site.

Fig. 5, D shows an intermediate stage in this process. The lamellae in the
exocuticle region are becoming disorganized; they seem to be disintegrating
as orderly lamellae and to be forming a ground substance. This then is
moulded into the final homogeneous form of the exocuticle.

From this picture of the formation of the exocuticle we can draw up a
scheme for the events by which endocuticular lamellae are transformed into
typical exocuticle. The cytoplasmic processes secrete substances which react
with the cuticular lamellae, bringing about their disorganization: this is
equivalent to reversing the stage passed through in the elaboration of the
lamellae from the cellular secretion, though, of course, it may be a different
chemical process. The residuum of cuticular materials (presumably protein
and chitin; compare Richards, 1951; Wigglesworth, 1957) resulting from
this process forms the ground substances for the exocuticle. This groundwork
then becomes impregnated with bonding materials secreted by the cyto-
plasmic filaments, and the result is a homogeneous, hard structure. It is,
of course, possible that these bonding, or tanning, agents, may also have the
effect of disrupting the lamellae when they come into contact with them;
it is then necessary to postulate only one secretion from the cytoplasmic
filaments.

We must now see how this fits in with other studies made on hardening
and darkening. A system with the cuticular tanning agents 'built-in' at the

**Fig. 6 (plate)**. 
A, section showing the shortening epidermal projections in the diminishing
vesicle underlying a major tubercle of the new cuticle, which is nearing completion.
B, section through two major tubercles showing pore canals splaying out in the outer part
of the cuticle.
C, section through the boundary between the thick clypeal cuticle and the surrounding
thin cuticle. The lamellae are continuous between the two regions, but they are broader in
the clypeal cuticle, and they disappear in the exocuticular layer of the clypeal cuticle.
D, section showing a gap in the cuticulin layer of the new cuticle over the sensory cells,
with the faintly granular moulting fluid present in the exuvial space.
E, section showing the origin of a new seta at the base of the old sensillum, which is heavily
sclerotized. Note the intucking of the plasma membrane at the tip of the new seta, and down
its centre. The cuticulin layer of the new cuticle is just being formed.
F, section showing the tonofibrillae maintaining their connexions to the old cuticle, which
is only slightly lifted away at the muscle insertion.
G, section showing tonofibrillae passing through the new cuticle and out to the old cuticle,
across the exuvial space. To the left is a depression in the new cuticulin layer where a tono-
 fibrilla pierces it.
H, section showing persistent tonofibrillae in the old cuticle which has undergone extensive
digestion. The ecdysial membrane runs alongside the black line of a fold in the section.

**cutic. l.,** cuticulin layer; **ecdys. mem.,** ecdysial membrane; **endocut.,** endocuticle; **epicut.,**
epicuticle; **epid.,** epidermis; **epid. pr.,** epidermal projections; **exocut.,** exocuticle; **exuv. sp.,**
exuvial space; **gr.,** granular secretion; **m. fl.,** moulting fluid; **new cut.,** new cuticle; **old cut.,**
old cuticle; **old endocut.,** old endocuticle; **p. c.,** pore canal; **p. m.,** plasma membrane; **sens.,**
sensory processes; **ton.,** tonofibrilla; **torm.,** tormogen cell; **trich.,** trichogen cell.
time of deposition of the cuticle, and which therefore requires only a ‘trigger-
ing’ to bring about hardening and darkening, has been discussed on p. 383 and it seems unlikely. The extension of the epidermis as cytoplasmic filaments through the endocuticle obviates the necessity of pouring the tanning agents through the substance of the cuticle (compare Way, 1950). The spreading out of these pore canals and cytoplasmic filaments within the presumptive exocuticle also obviates the need to postulate a great deal of lateral diffusion of the tanning agents. The course taken by the tanning agents thus calls for no special resistant properties of the inner endocuticular lamellae. The deep penetration of the cytoplasmic filaments into the presumptive exocuticle allows that the tanning agents can be produced locally, in small quantities, and therefore that their effects can be localized within fine limits. The changes in structure noted above indicate that tanning does not involve merely the impregnation of bonding materials between the molecules of these lamellae, but the actual disruption of the lamellae and the incorporation of their substance into a more rigidly bound matrix; resulting in a homogeneous layer in this region, penetrated by the pore canals surrounding the persistent cytoplasmic filaments. Where hardening is not complete a structural scheme different from that outlined here may be found.

At the time of the formation of the exocuticle the ‘inner layer’ of the epicuticle also takes on its final appearance. It seems to be formed by a change in the outermost lamella of the endocuticle, immediately below the cuticulin layer. Over the exocuticle the layer seems to be continuous (Noble-Nesbitt, 1963) and sometimes separated from the underlying exocuticle by a persistent endocuticular lamella. This indicates the fine control exercised by the cytoplasmic filaments over the process of hardening. The formation of the ‘inner layer’ over other parts of the cuticle further illustrates this. As a discrete layer it often can be distinguished only within the immediate vicinity of the minor tubercles (Noble-Nesbitt, 1963). If this layer is hardened in the same manner as the exocuticle, then its occurrence in the tubercle regions would obviously be of great importance in the support of these tubercles, protecting them from mechanical deformation (Noble-Nesbitt, 1963). An interesting parallel is seen with the taenidia of insect tracheal systems (compare Locke, 1957, 1958), where the cuticular ridges are underlain by a hardened region: in this case though, it is the hardened region itself and not the ridges which are of especial importance.

The formation of the seta and its insertion. During the earliest stages of the moulting cycle, when the cuticulin layer is being formed and the digestive moulting fluid is being liberated from its granules, there appears to be a certain delay in the deposition of new cuticle over the sensory cell areas (see fig. 6,D). Presumably these cells are more resistant to moulting fluid (compare Wigglesworth, 1953b). Certainly these areas are the last to be invested with new cuticulin, and a nerve-strand even then retains its connexion with the old sensillum (compare Wolfe, 1954b; Slifer, Prestage, and Beams, 1959).
Despite this initial delay, however, the subsequent events are very rapid. By the time the first one or two endocuticular lamellae are produced below the cuticulin layer, not only is the sensillum apparent, but also many of its structural details are complete, and already it has the form of the sensilla seen in the fully formed cuticle (see Noble-Nesbitt, 1963; and fig. 5, f). The influence of the underlying cells is also evident. The socket boundaries are delimited by the boundaries of the tormogen cell; those of the setal base by the trichogen cell. The structureless (and therefore presumably tanned) regions of the rim of the socket and the setal walls are present, with soft 'elastic' cuticle between them. In some cases the persistent nerve-tract to the old sensillum can be seen (see fig. 5, f). Sections passing through its junction with the nerve-tract to the new sensillum have been obtained and this (as shown also by Slifer, Prestage, and Beams, 1959) occurs near the base of the new sensillum (see fig. 6, e), though the possibility that in some instances it continues from the tip of the new hair cannot be ruled out (see fig. 5, e). Slifer, Prestage, and Beams (1959) have shown a number of different connexions between old and new sensilla of different types, and this may also be true of *P. aquatica*.

The early production of the complete sensillum, with early tanning, is surprising. It illustrates how very local control of this process is possible. It will be remembered that the full range of the tanning series in sensilla at this pre-ecdysis stage was observable in the light microscope. Since sensilla are doubtless of great importance to the insect, those which function as soon as possible after ecdisis will be important assets. It is perhaps then not surprising to find that *P. aquatica* has completed its new sensilla before ecdisis.

The maintenance of the nerve-connexion with the old sensillum, as Wolfe (1954b) pointed out for the blowfly, limits the period during which sensory inflow is disrupted before ecdisis. This, too, is expected to be an important asset to the insect.

**The muscle insertions during the moulting cycle**

It has been shown earlier (Noble-Nesbitt, 1963) that the muscles inserting on to the cuticle do so through what are evidently inward projections of epicuticular material. These tonofibrillae pass through the main body of the cuticle and pierce the epidermis, eventually becoming Y-shaped at the end proximal to the muscle. A group of myofilaments attach to the cup so formed.

During the formation of the new cuticle these connexions are maintained. It is obvious that there must be some extension in length of the tonofibrillae to accommodate the extra width of the new cuticle, though, of course, no measurements of this increase in length are possible in one of these connexions. There is evidence, however, that this length increase is kept to a minimum. Thus, in the areas of muscle attachment the old cuticle is only very slightly lifted away, while in neighbouring areas it is lifted well away (see fig. 6, f). This also goes to show that the connexions are still good and therefore doubtless still functional; they remain so until at least very close to ecdisis.
animal, therefore, can use its double cuticle as one (in muscular efforts) right up to ecdysis (compare Wolfe, 1954b, on Calliphora.) We have seen also that the new cuticle is more or less complete by the time of ecdysis and that mobility is present as soon as ecdysis is complete. This necessitates the firm attachment of the muscles to the cuticle.

Since the tonofibrillae retain their connexions between the old cuticle and the muscle, they must pass through the epidermal surface, where the new cuticle is being laid down (see fig. 6, c). The first layer of the new cuticle is the epicuticular layer of cuticulin, which, like the tonofibrillae, shows up densely in the electron micrographs. Further, it is this layer which seems to be projected inwards to form the tonofibrillae of the fully formed cuticle (Noble-Nesbitt, 1963). It is therefore suggested that the new cuticulin layer becomes fastened to the old tonofibrillae and that muscle attachment to the new cuticle is accomplished at this early stage (see fig. 6, c). Wigglesworth (1959) has shown that cementing occurs in the tracheal system, and it is possible that a similar process occurs when the tonofibrillae are fastened to the new epicuticle. Also, since the epidermal cells are secreting cuticulin (tonofibrillar material), there is the possibility of addition of material to the tonofibrillae and, therefore, of their increase in length.

We have thus seen the establishment of the new muscle insertion. It will be noted that no mechanism for the production of new insertions is postulated; the new insertions are essentially the same as the old.

These connexions to the epicuticle through the old cuticle remain unaffected by the moulting fluid where they cross the exuvial space (see fig. 6, F, G) and even where they pass through layers of the old cuticle that are undergoing extensive digestion (see fig. 6, H). This resistance is shared with the epicuticle, a further reason for regarding the tonofibrillae as epicuticular projections. The old connexions, then, remain up to ecdysis. It is obvious that they must break before ecdysis is completed. It is probable that they break due to the muscular effort of the insect as it crawls out of its old skin. We have already seen above that the new epicuticle is probably cemented on to the tonofibrillae. It is possible that this process involves an intimate union with the tonofibrilla at that point, the region proximal to the muscle becoming, in effect, in continuity with the new epicuticle. This would allow the growth of the tonofibrilla to accommodate for the increasing thickness of the new cuticle, by continued synthesis of cuticulin. Similarly, the connexion with the distal region could be maintained, though perhaps with a weak point at the new cuticulin surface, because of the setting up of the new connexion to the new cuticle. At ecdysis, breakage at this point would be expected.

Wolfe (1954b) has shown that in the blowfly, Calliphora erythrocephala, the tonofibrillae, which he suggested contained sulphur, and were prolongations of the myofilaments, likewise maintained their connexions with the old cuticle, through pores in the new epicuticle. At ecdysis, they broke off at the surface of the new epicuticle. He also stated that they became invested in endocuticular materials, and were resistant to the moulting fluid. In view
of the fact that the tonofibrillae of *P. aquatica* are continuous with the cuticulin layer (Noble-Nesbitt, 1963), it is probable that they are invested with cuticulin, which will afford resistance to the moulting fluid. The apparent tubular nature of the tonofibrillae (Noble-Nesbitt, 1963) may mean that the core is a muscular strand which is invested with cuticulin. However, the Y-shaped connexion with the muscle suggests that the muscle elements end there, and do not penetrate the integument further.

**Discussion**

Some of the phenomena reported here may well profit from further investigation in other insects. The suggested method of secreting the moulting enzymes into the ecdysial space in the form of moulting granules may be more widespread. This method of secreting the enzymes certainly resolves many of the problems of the protection of the new cuticle.

Some of the observations of Passonneau and Williams (1953) on the moulting fluid in *Cecropia* may be explained in the light of the results obtained in the present study. Passonneau and Williams were unable to decide, on the basis of their observations, whether the moulting enzymes were secreted through the new cuticle into the late moulting fluid, or were already present in an inactive state in the early moulting fluid. The presence of moulting granules in *P. aquatica* suggests that their second alternative holds. The timing of the activation of the enzymes is linked to the sclerotization process in *Cecropia* (Passonneau and Williams, 1953), but this cannot be generally applicable since not all insects sclerotize most of their cuticle, and it is evident that a timing mechanism may be linked to different processes in different insects. It may be added that the breakdown of the old cuticle may be delayed until the new one can perform its functions of support and protection. The layer of the new cuticle resistant to the moulting fluid is the cuticulin layer, and this layer alone is sufficient to provide for the protection of the underlying layers of the new cuticle from attack by the moulting enzymes. Therefore, the delay in the activation of the moulting fluid until the new cuticle is sclerotized in *Cecropia* may be only a reflection of the timing mechanism, and not of a newly acquired resistant property of the new cuticle as Passonneau and Williams suggested. Passonneau and Williams also suggested that the epidermis synthesized and secreted moulting fluid and cuticle simultaneously. While this is true for a short period in *P. aquatica*, the bulk of the synthesis and secretion of the moulting granules is carried out before most of the new cuticle is laid down. Further, their suggestion that the early moulting fluid acts as a protection for the delicate underlying insect may be questioned because of the protective role of the old and new cuticles. The gel-like moulting fluid at this stage subserves the important function of holding the moulting enzymes in abeyance, in the space at least partially formed by the foam secretion during the earlier stage when the old cuticle is lifted away from the plasma membrane.

The lifting away of the old cuticle has been a much-neglected aspect of the
moulting cycle, and it is possible that specialized secretions such as the foam secretion found in *P. aquatica* may occur in other insects as well. Passonneau and Williams (1953) suggested that a surface tension generated in the epidermis as a whole, due to the elongation of the cells, may account for the retraction of the epidermis. On the other hand, the evidence from the apterygote *P. aquatica* suggests that the epidermal cells secrete a foam between the plasma membrane and the cuticle, so forming a space into which the moulting granules are secreted. These secretions result in the plasma membrane becoming remote from the old cuticle, and able to secrete a new cuticle distinct from the old. The possibility that a similar mechanism may operate in pterygotes deserves consideration.

The activity of the epidermal cells in moulding the cuticle into its characteristic sculpturing pattern may also be more widespread than previous investigations (Wigglesworth, 1933, 1954; Locke, 1957, 1958, 1959, 1960) suggest, and may help to resolve some of the problems posed by the determination and formation of cuticular patterns in insects (compare Locke, 1959, 1960). Richards (1951) considered the question of whether the cuticle was an extra or intracellular secretion, and concluded that a full answer could only be given if the plasma membrane at the cuticular surface were located positively at all stages of development or if its absence between the cytoplasm and the cuticle were demonstrated. In the present study the plasma membrane has been located positively between the cuticle and the cytoplasm at all stages of the moulting cycle. The cuticular materials are elaborated only beyond its limits, and it is suggested that the precursors are liberated through the plasma membrane from the cisternae of the endoplasmic reticulum. The cuticle, therefore, is produced extracellularly.

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