PORE CANALS AND RELATED STRUCTURES IN INSECT CUTICLE

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ABSTRACT

The fine structure and the distribution of an esterase have been studied in the cuticle of Galleria larvae, Tenebrio larvae and pupae, and in the wax-secreting cuticle of the honey bee, and compared with those in the cuticle of the caterpillar of Calpodes. In Galleria and Tenebrio the pore canals are spaces passing through the lamellate endocuticle from the epithelium to the epicuticle. They contain a filament from the cells which may be concerned in their formation. The shape of the pore canal is probably determined by the orientation of the fibres making up the lamellae in the endocuticle and is not a regular helix. The pore canals also contain numerous filaments of another sort which pass on through the epicuticle and are believed to be the origin of the surface wax. They are particularly abundant in the pore canals of the honey bee wax-secreting cuticle and extend into the cell in long pockets surrounded by an envelope of the plasma membrane. The esterase is probably concerned with the final stage of wax synthesis, for its distribution is similar to that of the lipid filaments.

INTRODUCTION

This paper is one of several upon the structure of the insect integument. It will be limited to a description of structures which may be concerned in the synthesis and transport of cuticular waxes. Only the structure of the cuticle has been studied, not its genesis.

The integument in insects consists of a single layer of cells and the cuticle (Fig. 1 A) (Wigglesworth, 22, 29, 31, 33, Richards, 18, 20). In most integuments the cuticle is perforated by pore canals running from the epithelium up to but not through the epicuticle (Denell, 5, 6, Richards and Anderson, 21, Locke, 11). The wax layer in the epicuticle is of great physiological interest, for it determines many of the surface properties and forms the main barrier to water loss (Wigglesworth, 30, Beament, 2).

In the formation of the cuticle the epithelium secretes the cuticulin first and then the lamellate endocuticle. The exocuticle is derived later by quinone tanning of the outer lamellae of the endocuticle. The wax layer is one of the last layers to appear, and the wax or its precursors must traverse the endocuticle. Later still, the wax is covered by the cement secreted by the dermal glands.

The problem is how to account for the appearance of wax on the outside of the cuticulin when it is separated from the epithelium by a solid hydrophilic endocuticle. It has been supposed that the pore canals might be the pathway for the movement of wax (Wigglesworth and Kramer, 36), but this has been doubted as a general explanation, for in many insects, notably in the caterpillars of Calpodes (Locke, 14, 16) and Diataraxia (Way 27), wax appears outside the cuticle not penetrated by pore canals.
Experiments on the caterpillar of *Calpodes ethlius* (Lepidoptera, Hesperidae) suggested that wax was not transported across the endocuticle but that at least the final stage of wax synthesis was very close to the surface, probably in or immediately below the epicuticle, where an esterase was detected (Locke, 14). It was inferred that the wax traversed two layers of the epicuticle (the dense layer and the cuticulin, Fig 1B) by way of filamentous structures, 60 A in diameter, termed wax canals (Locke 16). Filaments similar to the wax canals of *Calpodes* also occur in other insects in spaces in the cuticle. The term wax canal will be retained for the space through the epicuticle, and wax canal filament for the dense contents, which may appear filamentous or tubular on emergence from the epicuticle. This is a terminology of convenience which one hopes to amend when the molecular nature of the structures concerned is better understood.

In *Calpodes*, pore canals are not necessary for wax secretion, but this may not be true generally; the mechanism in *Calpodes* could be a specialisation resulting from a more permeable endocuticle. The aim of this paper has been to relate the data upon *Calpodes* to those in other insects before attempting any general hypothesis correlating esterase distribution and fine structure with wax synthesis.
FIGURE 2

Diagram of the structure of the abdominal cuticle in *Galleria* larvae. The inset shows the regions which can be distinguished in the epicuticle. In addition to the regions shown a thick lipid layer is known to be present at the surface.

MATERIALS AND METHODS

(a) Histochemistry

There are two problems. The hardness of the cuticle makes it difficult to cut the thin sections necessary for the resolution of the pore canals, and its denseness and impermeability reduce the penetration of reagents. The following method gave good resolution and localization of an esterase.

Small pieces of integument were fixed in neutral ice cold formalin for 2 to 24 hours, washed in ice cold water, and then incubated at 20°C in 5-bromo-indoxyl acetate at pH 7.6 for 1/2 to 12 hours (Pearse, 17). They were then embedded in agar, dehydrated, and re-embedded in esterwax. One-micron sections were cut from a small block face with a Cambridge Rocking microtome or a Jung microtome. (Wigglesworth, 35).

In the dense cuticle the blue indigoid dye rarely appeared granular, and localization of the esterase was much better than in the cells. Control fragments of cuticle were completely inhibited by 1 to 4 hours pre-incubation with $10^{-4}$ M AgNO₃. The effect of other inhibitors and activators is forming part of a separate study, but it is of interest to note here that the oxidation catalysts ($10^{-3}$ M potassium ferri-cyanide + $10^{-3}$ M potassium ferrocyanide and $10^{-3}$ M CuSO₄) usually used with this reagent may cause complete inhibition of cuticular esterases.

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(b) Electron Microscopy

Small fragments of integument were fixed at 0–4°C in 1 per cent osmium tetroxide made isotonic with sucrose at pH 7.4 for 1 to 4 hours in the usual manner. Isotonic 1.2 per cent potassium permanganate at pH 7.6 was also used as an alternative to osmic fixation. Fixed cuticle was rinsed in 70 per cent alcohol and dehydrated rapidly before embedding in Araldite (Glauert and Glauert, 9) or, via propylene oxide, in Epon 812. Hard methacrylate was also used but some structures were then frequently absent. The main difficulty is in section cutting. Arthropod cuticle can be exceedingly hard, up to 3 on Mohs' scale (Bailey, 1). It may scratch silver and calcite. Glass knives rapidly break up after the first few sections. In addition to being hard, the epicuticle has little affinity for the embedding medium from which it almost always breaks away. I have found no remedy for these difficulties but perseverance. Surface replicas of chloroform-extracted cuticle were prepared either by shadowing with carbon directly and dissolving the cuticle in strong hydrochloric acid, or by carbon shadowing a collodion impression. The first collodion impression frequently stripped off the cement layer so that the second presumably showed the pattern of the hard epicuticle beneath.

Many structures were barely visible without lead staining (Watson, 26) which was used routinely to increase contrast.

As an aid to the interpretation of electron microscope sections, osmium tetroxide-fixed material was treated for 24 hours with ethyl gallate and sectioned at 1 μ as above for observation by phase contrast (Wigglesworth 34, 35).

Photographs were taken at 50Kv on an RCA EMU 2B or at 80Kv on a Siemens Elmiskop I at magnifications of 10 to 20,000.

(c) Material

Cuticle from several insects has been examined, but most time has been spent on the cuticle of the following: (1) the larva of Galleria mellonella (Lepidoptera, Galleriidae), the bee moth caterpillar. Mid-final instar larvae were chosen as examples of caterpillar cuticle not specialised for extra wax secretion. (2) Larvae and pupae of Tenebrio molitor (Coleoptera,
Tenebrionidae), the meal worm. *Tenebrio* was selected to represent a commonly occurring hard type of cuticle. (3) The wax-secreting cuticle from the abdominal sternites of the honey bee, *Apis mellifica* (Hymenoptera, Apidae). This last cuticle was chosen because any structures within it concerned with wax secretion would be expected to be hypertrophied.

RESULTS

The Cuticle in Galleria Larvae (Fig. 2)

The main features are seen in Fig. 5. The cuticle is rugose over most of the larva. Each rugosity has a deep root of dense material extending into the endocuticle, and is capped by a diffuse mass of melanin above which the epicuticle is much thickened. In insects in which the integument does not stretch between instars the exocuticle is a continuous layer, but in *Galleria* the cuticle stretches during growth and the endocuticle is only reinforced to become exocuticle in the bases of the rugosities.

The endocuticle is made up of 10 to 30 lamellae about 0.5 to 1 μ apart, the larger number being found later in the instar. The lamellate appearance is due to the arrangement of fibrils. These have not been satisfactorily resolved but they give the impression of an arrangement as in Fig. 3. The endocuticle next to the epithelium does not have this ordered fibrillar structure. The fibrillar structure has already been briefly reported for *Calpodes* larvae (Locke, 16).

Pore canals extend through the endocuticle. Some also pass through the dense roots into the thickened epicuticle above. The Epithelium and the Inner Surface of the Endocuticle: Schmidt (22) has described a layer staining intensely with aldehyde fuchsin between the epithelium and the endocuticle in a number of insects. This corresponds to the layer of cuticle: (1) containing fibrils (about 80 A thick) (Fig. 2, H) is, therefore, tentatively fixable by OsO₄, would show as a thin dark line, continuous with the pore canal filaments. They resemble the structures termed wax canals and their contents, the wax canal filaments, which pass through the epicuticle in *Calpodes*. In *Calpodes* the wax canals were inferred to be the route by which wax reaches the surface. Their nature is discussed below.

The Pore Canals and Their Contents: The pore canals are nearly always cut obliquely, appearing as clear crescentic areas. They are found throughout the cuticle between the epithelial cell layer and the epicuticle, and their typical arrangement is shown in Fig. 4. They terminate below the thin epicuticle between the rugosities, but where the epicuticle is much thicker over the top it is penetrated by a number of finger-like spaces more or less circular in cross-section and continuous with the pore canals in the endocuticle.

Within each pore canal is a filament about 150 to 200 A in diameter. It originates beneath the plasma membrane of an epithelial cell and may extend to the peripheral end of the pore canal.

Near the surface the pore canals contain numerous filamentous structures of a different sort about 80 A in diameter. These filaments emerge from the pore canals and pass through nearly all of the epicuticle (Fig. 8). They do not appear to be continuous with the pore canal filaments. They resemble the structures termed wax canals and their contents, the wax canal filaments, which pass through the epicuticle in *Calpodes*. In *Calpodes* the wax canals were inferred to be the route by which wax reaches the surface. Their nature is discussed below.

The Epicuticle: Six regions can be distinguished. On the very outside is an irregular “fluffy” layer. This might be contamination from the food of the larva but it will be referred to as the “cement” (Fig. 2, I) although only future work can tell whether or not it is the secretion of the dermal (Verson’s) glands. It resembles the cement in *Rhodnius* (Locke, 11) and *Calpodes* (Locke, 16) both in position and texture. In carbon replicas prepared from the first collodion stripping, similar “dirt” is always present. Below this is a dense, well defined layer of very uniform thickness (about 60 A) (Fig. 2, II). There is good physiological evidence that one lipid layer at the surface of the epicuticle is a well ordered monolayer or bilayer (Beament, 3). Such a layer, if fixable by OsO₄, would show as a thin dark line, and this layer (Fig. 2, II) is, therefore, tentatively homologised with the oriented lipid layer inferred from physiology. Favouring this hypothesis, layer II and the cement are the only layers of the epicuticle not penetrated by the wax canals. Also supporting this interpretation is the uniform clear layer (about 80 A thick) (Fig. 2, III) below it.
Galleria Larval Cuticle

FIGURE 4
Electron micrograph of a transverse section of the abdominal cuticle from a mid-last instar larva of Galleria showing the fibrous structure of the lamellae in the endocuticle and pore canals each containing an axial filament.
*pcf*, pore canal filament; *pc*, pore canal.
(Osmium tetroxide, Araldite). × 42,000.

FIGURE 5
Electron micrograph of a thick transverse section to show the main features.
*Ep*, epicuticle; *End*, endocuticle; *Epith*, epithelium.

FIGURE 6
Photomicrograph of 2 μ section after incubation in 5-bromoindoxyl acetate to demonstrate esterase. Nearly all the black part in this picture is due to the indigo produced.
(cf. Fig. 36.)
Phase contrast. × 1,500.

FIGURE 7
Surface view of whole mount of the epicuticle treated as in Fig. 6 and showing the esterase localised most densely in the pore canals.
Phase contrast. × 1,500.
FIGURE 8

Electron micrograph of a transverse section through the epicuticle between two rugosities.

m, melanin; d, dense layer; c, cuticulin; cm, cement; pc, pore canal containing wax canal filamer
of oriented lipid layer.
(Osmium tetroxide, Araldite). X 50,000.
FIGURE 9
Diagram of the structure of the tergal cuticle in Tenebrio larvae. In some pore canals the pore canal filament extends almost to the epicuticle.

These layers (II and III) may well be part of a lipid protein complex of the sort studied by Stoeckenius (24), but for the moment they will be referred to as the oriented lipid layer. Below layer III is a thicker, less regular, dense layer (Fig. 2, IV) which may have been secondarily derived from the thick (0.2 μ) homogeneous dense layer below (V) which makes up the bulk of the epicuticle. Layer IV is probably the homologue of the cuticulin layer in Calpodes. Layer V is greatly thickened to form the caps to the rugosities. The non-committal term dense layer was coined for it in Calpodes. Within it there is a region (VI) with a looser texture containing islands of layer V where the pore canals end and the wax canal filaments ramify.

The Distribution of Esterase: Figs. 6 and 7 show the distribution of esterase. The pore canals show up clearly close to the epicuticle and around and within the roots. The epicuticle is also strongly coloured. The failure of the inner ends of the pore canals to colour in this sort of cuticle is probably not an artefact. In the hard brown sclerite at the base of the leg the cuticle is different and the pore canals contain an esterase throughout their length. These results are summarised in Fig. 36.

Tenebrio Pupal Cuticle (Thoracic and Abdominal Tergites 48 Hours after Moulting)

Many of the features described in Galleria can be seen in the survey picture (Fig. 13): epithelium with finger-like processes, Schmidt's layer, a wide lamellate endocuticle with pore canals cut obliquely, but with a smoother and more uniform epicuticle.

The texture of Schmidt's layer distinguishes it very sharply from the lamellate endocuticle (Fig. 12). The granularity has an orientation similar to that in the lamellae and usually the pore canals already have the crescentic cross section characteristic of the endocuticle. Sometimes the pore canal filament traverses this layer without any space accompanying it. In Calpodes the pupal cuticle is very like that in Tenebrio, and Fig. 15 of Calpodes shows two well defined pore canal filaments which are not surrounded by a discrete pore canal until they reach the endocuticle.

The pore canals contain wax canal filaments over a much greater part of their length than in Galleria. The epicuticle is exceedingly hard and all the detail seen in Galleria could not be resolved in the sections obtained, but the general pattern is similar. The pore canals terminate below and
Electron Micrographs of Galleria Larval Cuticle

**Figure 10**
Transverse section in the region between the epithelium and the endocuticle showing the pore canal filaments emerging from the cell and traversing the layer of unoriented material before passing into the endocuticle.
(Osmium tetroxide, Araldite). × 50,000.

**Figure 11**
A thicker section from the region between the cells and the endocuticle showing how the oriented fibres of the endocuticle merge into the poorly oriented granular layer above the finger-like projections from the cells. The endocuticle has very little contrast, and fibre orientations are difficult to make out on thin sections.
(Osmium tetroxide, Araldite). × 50,000.
Electron Micrographs of Tenebrio Larval and Pupal Cuticle

**FIGURE 12**
Slightly oblique transverse section of *Tenebrio* pupal cuticle in the region between the cell and the endocuticle. Note the crescent shape of the pore canal space even in the poorly oriented granular layer above the finger-like projections from the cells, and the contrast in textures between endocuticle and the granular layer.

*pcf*, pore canal filament; *pc*, pore canal; *end*, endocuticle; *g*, granular layer—endocuticle in the process of formation; *d*, desmosome.

(Osmium tetroxide, Araldite). X 50,000.

**FIGURE 13**
Transverse section of pupal cuticle to show the main features, cf larval cuticle in Fig. 14.

*ep*, epicuticle; *end*, endocuticle; *pc*, pore canals; *g*, granular layer—endocuticle in the process of formation.

(Osmium tetroxide, Epon). X 2,500.

**FIGURE 14**
Transverse section of larval cuticle to show the main features. The pore canals are smaller than in the pupa; there is an exocuticle, and the region with ramifying wax canals is much wider.

*ep*, epicuticle; *ex*, exocuticle; *end*, endocuticle.

(Osmium tetroxide, Epon). X 2,500.
Electron Micrographs of Calpodes Pupal and Tenebrio Larval Cuticle

Figure 15
Section of Calpodes pupal cuticle through the region between the cells and the endocuticle in the same plane as the axial filaments which extend across the unoriented layer and probably also within the cell. The arrows mark what may be the pore canal filament within the cell.
end, endocuticle; pm, plasma membrane.
(Osmium tetroxide, Araldite). X 50,000.

Figure 16
Slightly oblique tangential section of Tenebrio larval cuticle showing the finger-like processes of the cells on the right and the innermost, poorly ordered, granular layer of the cuticle on the left. Some of the cellular processes extend into this layer carrying the pore canal space with them.
psc, incipient pore canal space.
(Osmium tetroxide, Epon). X 42,000.

Figure 17
Tangential section just above that of Fig. 16 showing well defined pore canals with axial filaments and granular contents. Compare the dense homogeneous texture of this fully formed endocuticle with the granular precursor in Fig. 16.
pcf, pore canal filament.
(Osmium tetroxide, Epon). X 32,000.
Electron micrograph of a tangential section about 3/4 of the way through the cuticle from the epithelium; i.e. well above that of Fig. 17. In this example no pore canal filaments are present (cf. Fig. 26) but the pore canals contain numerous wax canal filaments. The pattern made by the pore canals and their shape in transverse section suggest that the lamellae of the endocuticle are made up as in Fig. 3, but the cuticle never appears fibrous as in Galleria (cf. Fig. 4). (Osmonic tetroxide, Epon). × 35,000.

Photomicrograph of a tangential section rather below that of Fig. 19, incubated in 5-bromoindoxyl acetate. The pore canals react intensely for esterase and all the density in this picture is due to the deposit of indigo. Phase contrast. × 2,600.

Electron micrograph. One pore canal from Fig. 18 enlarged to show the wax canal filaments. × 100,000.

Photomicrograph of a transverse section of cuticle from an intersegmental membrane incubated in 5-bromoindoxyl acetate. Nearly all the density in this picture is due to esterase. It is most marked in the cells, pore canals near the epicuticle, and at the base of the wedges of exocuticle. Phase contrast. × 1,500.

Photomicrograph of a transverse section of tergal cuticle treated as in Fig. 21 to show the presence of esterase. The reaction is most marked in the cells and throughout the length of the pore canals. Phase contrast. × 1,500.
Electron Micrographs of Tenebrio Larval Cuticle

FIGURE 4
Slightly oblique tangential section at the surface showing three regions of the epicuticle. On the left is the dense cuticulin layer. Below this is a region of the dense layer in which the wax canals align themselves perpendicular to the surface, and to the right the lower level of the dense layer where the wax canals ramify on emergence from the pore canals. The wax canals, seen as black dots in section, are present through even the outermost part of the cuticulin layer.
c, cuticulin; d, dense layer; wx, c., wax canals.
(Osmium tetroxide, Epon). × 52,000.

FIGURE 5
Tangential section just below that in Fig. 24 showing the junction between the lamellate cuticle with pore canals on the left and the innermost epicuticle with ramifying wax canals on the right. Just below the epicuticle the wax canal filaments are arranged in a ring around the periphery of each pore canal.
(Osmium tetroxide, Epon). × 24,000.

FIGURE 6
Tangential section just below the epicuticle, i.e. above that in Fig. 18, showing three pore canals in transverse section with the wax canal filaments arranged in a ring at the periphery and the pore canal filament in the center. The pore canal filament is not always present in this region (cf. Fig. 20).
(Osmium tetroxide, Epon). × 100,000.
the wax canals pass through the epicuticle, which has a thin, very dense outer cuticulin layer (Fig. 2, IV) and a broader, homogeneous, dense inner region (Fig. 2, V).

The distribution of esterase was similar to that of the Tenebrio larval cuticle described below.

The Tergal Cuticle in Tenebrio Larvae, 24 to 36 Hours after Moultting (Fig. 9)

The main features are shown in the survey picture (Fig. 14). The epicuticle is wider than in the pupa, due mainly to the thicker dense layer (V) containing the ramifying wax canals. The outer lamellate cuticle ( = exocuticle) is hard and dense, although with the light microscope it is not much darker in colour than the endocuticle. Details are shown in the slightly oblique tangential sections cut at different levels (Figs. 16-18, 20, 24-26).

The epithelium has many finger-like processes probably correlated with the secretion of endocuticle taking place at this time (Fig. 16). A few of the processes taper off and pass into Schmidt's layer surrounded by a pore canal space. In the lower endocuticle the pore canals have a denser lining and contain a single pore canal filament 150 to 200 A in diameter, together with small irregular granules (Fig. 17). As in Galleria the pore canal filament may extend throughout the length of the pore canal.

In about their distal third, the pore canals contain bundles of 4 to 20 wax canal filaments 100 to 130 A in diameter (Figs. 16, 20). In the lamellae just below the epicuticle, the wax canal filaments are arranged in a ring around the inside of the tube (Fig. 26). The pore canal stops at the junction with the epicuticle and the wax canals form an irregular feltwork (Fig. 25). Above the feltwork the wax canals are arranged perpendicular to the surface before passing through the thin dense layer of cuticulin (IV). Fig. 24 is the best evidence obtained that the cuticulin layer (IV) is penetrated by the wax canals. There is no loose-textured cuticle with intertwining wax canal filaments comparable to layer VI in Galleria. A cement layer is present but the oriented lipid layer (II and III) seen in Galleria could not be resolved.

The Shape of Pore Canals: In light microscope studies (Richards, 18), pore canals from several insects have been described as helically coiled. Helical pore canals were also reported in an electron microscope study of thick sections of cockroach cuticle (Richards and Anderson, 21), and in Rhodnius the crescentic holes seen in cross section were taken to confirm their helical nature (Locke, 11). However in the cuticles examined in this paper, another shape is more probable and the hypothesis that pore canals are usually helical may have to be reconsidered.

In many sections some pore canals have a circular outline (Fig. 26). The basic shape is probably that of a cylinder bent in a helix or in some other way. If the pore canals are indeed helical, the shape and the orientation of the holes seen in cross section will be influenced by two main considerations: (i) the phase relations of different helices, and (2) the plane of the section. The shape of the tube and the pitch and regularity of the helix do not affect the following conclusion.

(i) The Phase Relations of Different Helices: The orientation of the crescentic holes seen in section is influenced by their position relative to each lamella. In any one plane the outline of a pore canal has approximately the same orientation as that of any other pore canal in a similar position with respect to the lamella (Fig. 18). Whether or not they are helices, therefore, they are in phase, and the pattern repeats itself in each lamella.

(2) The Plane of the Section: If a number of helical tubes are cut in transverse section, then usually the crescentic holes observed would have a random orientation as in Fig. 23A. Even if they are all in phase, the crescents will have all orientations except in the rare sections exactly in the plane of the lamella, or, when all the pore canals...
are equidistant from one another, in sections cut in the set of planes parallel to the pitch of the helix. Many sections have been cut in all planes but the orientation expected for helices has not been seen. The usual orientation is as in Fig. 23B.

The most probable explanation is that the pore canals follow the arrangement of fibres in the lamellae, the hole taking a curved course repeated in the same plane in each lamella. The shape and orientation of the pore canals seen in some sections can only be explained in this way. In some sections the holes are symmetrical ellipses (Fig. 35), the long axis of each hole being parallel to its neighbours.

Helical canals, if they occur, can readily be explained by this observation that a pore canal follows the fibre pattern of the lamellae. A helical pore canal would result if the fibre pattern were to rotate about an axis normal to the surface, from one lamella to the next. This pattern has not been seen in the insects studied but it may occur.

The Distribution of Esterase: In unstained cuticle the pore canals are difficult to make out even with phase contrast, but after the esterase reaction they show up clearly throughout their length (Figs. 19, 22). In the intersegmental membranes the endocuticle is irregularly hardened in wedges in much the same way as in Galleria, and as in Galleria the esterase is very marked in the pore canals just below the epicuticle (Fig. 21). The epicuticle itself does not color. This could be due to lack of penetration rather than absence of esterase, as the loose textured epicuticle (VI) seen in Galleria is absent.

Honey Bee Wax-Secreting Cuticle (Fig. 27)

On the sternites of abdominal segments 4 to 7 are paired polygonal areas of thin, clear, light yellow cuticle through which the wax is secreted as a viscous fluid, hardening on the outside as a thick scale which is later removed and mandibulated to form the comb (Dreyling, 8).

Figs. 28 and 34 show the main features of this cuticle including the outer part of the epithelium. There are no dermal glands in adult bees (Schnelle, 23) and certainly none in the wax-secreting
Honey-Bee Wax-Secreting Cuticle

Figure 28
Electron micrograph of an oblique section showing the cuticle on the right and part of a cell on the left. The obliquely cut pore canals are filled with dense contents. Between the plasma membrane of the cell and the cuticle is a layer of ramifying wax canal filaments which project into the cell in bundles. pc, pore canal; wx.f., wax canal filaments; b, bundles of wax canal filaments; pm, plasma membrane; m, mitochondria. (Permanganate, Araldite). X 36,000.

Figure 29
Photomicrograph of transverse 1 μ section of cuticle and epithelium at the height of wax secretory activity. The pore canals show up as dark lines through the cuticle below which there is a distinct layer before the cell membrane. The bundles of wax canal filaments extend deeply into the cell and between the cell membranes. n, nucleus; c, cuticle; b, bundles of wax canal filaments; w, wax canal filament layer; l, layer of wax canal filaments between cells. (Osmium tetroxide and ethyl gallate). Phase contrast. X 1,200.

Figure 30
Photomicrograph of a 1 μ tangential section of the cells just below the cuticle at a stage similar to Fig. 29. The bundles of wax canal filaments between and projecting into the cells show up darkly. (Osmium tetroxide and ethyl gallate). Phase contrast. X 1,500.

Figure 31
Photomicrograph of a 1 μ transverse section of cuticle and epithelium not at the height of secretion incubated with 5-bromoindoxyl acetate. The esterase is located throughout the cell and to some extent on the inner face of the cuticle. Phase contrast. X 1,500.
**Electron Micrographs of Honey Bee Wax-Secreting Cuticle**

**FIGURE 32**
Tangential section through part of a cell showing bundles of wax canal filaments surrounded by an envelope of plasma membrane and desmosomes between two cells. b, bundles of wax canal filaments; m, mitochondria; tr, tracheole; d, desmosomes; n, nucleus.
(Osmium tetroxide, Epon). × 44,000.

**FIGURE 33**
Enlargement of three bundles of wax canal filaments from Fig. 28. The filaments appear tubular.
(Permanganate, Araldite). × 100,000.

**FIGURE 34**
Transverse section through the outer part of the cuticle showing the outermost oriented lipid layer, the cuticulin layer and wax canal filaments within pore canals in longitudinal section.
ol, oriented lipid layer; c, cuticulin.
(Osmium tetroxide, Araldite). × 67,000.

**FIGURE 35**
Pore canals in transverse section. The pore canals are filled with dense contents, some of which seem to be the cross sections of tubes with about the same dimensions as the wax canal filaments seen in the cells.
(Osmium tetroxide, Epon). × 68,000.
TABLE I

<table>
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<th>Insect</th>
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</tbody>
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cuticle. In agreement with this, no cement layer can be distinguished although there is some fuzziness at the surface. This may be the remains of surface wax, for the wax scales take up a small amount of osmium tetroxide although much less than comb wax. The oriented lipid layer (II and III) and the cuticulin (IV) appear much as in Galleria. And as in Galleria layer IV, but not layers II and III, is penetrated by the wax canals. Below this are about 7 lamellae with the dense homogeneous appearance of endocuticle hardened to become exocuticle. The cuticle is penetrated by numerous pore canals which pass right up to the cuticulin (IV). This cuticle differs most strikingly from those previously mentioned in that the pore canals are tightly packed with filaments, 100 to 130 A in diameter, (Fig. 35) resembling the wax canal filaments, for example, of Tenebrio larval cuticle (Fig. 20). They form a distinct layer between the cuticle and the epithelium and sometimes appear tubular, particularly after permanganate fixation (Figs. 28, 33). Whorled or twisted hanks of these filaments are found in pockets of the cell membrane, and transverse sections of such bundles are seen (Figs. 28, 32) lying between the mitochondria, fat droplets and nuclei, where their truly extracellular nature may be inferred from the plasma membrane profile limiting each bundle. They are readily distinguishable with the light microscope in osmium tetroxide: ethyl gallate preparations (Figs. 29, 30).

DISCUSSION

1. The Orientation of Fibres in the Endocuticle

Although Schmidt's layer is much coarser in texture than the endocuticle, the orientation of its components is probably the same. The coarse granules have a similar curved arrangement which is also followed by the pore canal spaces. Some of the orderliness of the endocuticular fibres probably already exists in Schmidt's layer. Schmidt thought of it as a glue to keep the epithelium and endocuticle together and called it the subcuticular layer, but it seems more probable that it is the innermost layer of endocuticle in the process of formation. It remains obscure how the fingered secretory edge of the epithelium can bring about this orderliness with a constant polarity, but it would be interesting if it could be correlated with the polarity and gradient behaviour described in other insect epithelia (Locke, 12, 13, 15).

2. The Pore Canal Filament

A number of processes occur at the surface of insect cuticle: secretion and repair of wax layers, tanning of the endocuticle to form exocuticle, the repair of surface cuticle after abrasion (Dennell, 7, Wigglesworth, 32). For the cells to participate in these processes, they must maintain some route across the cuticle, either through the loosely knit endocuticle as in some caterpillars, or by way of pore canals in harder cuticles. The pore canal filament may be the mechanism by which the cell keeps a hole in the newly secreted cuticle until the hardening is complete and the canal is permanent. It might accomplish this by inhibiting fibre formation in its immediate vicinity. The pore canal filaments might also function as anchors to stick the epithelium to the endocuticle.
Stylised diagram of cuticle structure and the distribution of cuticular esterase. The regions where an esterase has been detected are indicated by cross-hatching. There is an approximate correspondence between the distribution of the esterase and the region where the wax canal filaments arise. The wax canal filaments are presumed to be the precursor of the surface wax.
3. Surface Lipids

The relatively thick lipid layer on the surface of most cuticles does not survive in sections prepared for the electron microscope. On the other hand, the very thin layer and space below it which has been referred to as the oriented lipid layer (II and III) is well preserved. There are therefore two lipid layers in the epicuticle, a thick outer one probably protected and supported by the cement when it is present, and a monolayer or bimolecular layer perhaps in association with protein as in a cell membrane. This structure is in agreement with what is known of the permeability of the cuticle, for there is good physiological evidence (Beament, 3) for two lipid layers at the surface of the cuticle with different properties as barriers to water.

4. Pore Canals and the Epicuticle in Different Insects

A common structural plan can be seen emerging from this study of insect cuticles. Table I shows the general similarity in the layers of the epicuticle. The missing oriented lipid layer in Calpodes and Tenebrio may be due respectively to methacrylate embedding and the difficulty experienced in cutting sections of hard cuticle. The dense layer may be present in Apis, but there is little to distinguish it from the rest of the lamellate endocuticle. There can be little doubt that the structures termed wax canal filaments are the same in each insect. They have similar dimensions and the same high contrast although they vary somewhat in position. Fig. 36 shows the distribution of the wax canal filaments and esterase in diagrammatic form. If the wax canal filaments are mainly made up of wax, the esterase has the distribution which would be expected if it were concerned in the final stage of wax synthesis. The insolubility and inertness of wax presumably make it necessary to synthesize it as close to the site of deposition as possible, and the necessity for an endocuticle forces the cells to delegate at least some of their synthetic mechanisms to structures away from their surface. In this connection it is of interest that the epicuticle of Sarcophaga has a high ash content (Richards, 19). The precise localization of the esterase with the electron microscope should prove interesting.

Fig. 36 shows how the structures in Calpodes and Apis might have evolved from the more typical condition in Galleria and Tenebrio. The cuticle in caterpillars is greatly stretched in growth during an instar, and it might be difficult under these conditions to maintain the continuity of the pore canals. It may be for this reason that they are absent in Calpodes, in which the wax-secreting gland is little modified from that of normal cuticle and probably of recent evolutionary origin. In the honey bee, wax secreted in bulk is exceedingly important, and the structure of the cuticle is completely subservient to this demand. Transport problems are reduced by making the cuticle very thin and penetrated by numerous pore canals. The process of wax manufacture can thus be brought back close to the cells again.

5. The Nature of the Wax Canals and Their Filaments

The nature of the wax canal filaments is crucial to hypotheses relating fine structure to wax synthesis and transport, but several interpretations are possible.

Their appearance as tubes in some preparations could be due to the deposition of osmium or manganese compounds around a solid core and does not necessarily prove their tubular nature. The action of permanganate as a fixative is still uncertain but it may “unmask” phospholipid-protein and fix the unmasked protein to give rise to high contrast in membranes under the electron microscope (Bradbury and Meek, 4). The tubes seen after permanganate fixation suggest that the filaments are not homogeneous, and we may assume tentatively that there is a sheath of some sort around a core of different material. There can be little doubt that the filaments occupy spaces in the epicuticle because of the strong circumstantial evidence that in Calpodes wax is transported across the epicuticle by this route. Perhaps they also transport the bloom of wax appearing on the surface of Tenebrio pupae reported by Holdgate and Seal (10).

A possible interpretation would suppose that there are holes in the epicuticle by which the wax reaches the surface, and the dense filament which exactly fits the width of the hole but overlaps it in length is a wax micelle passing through to the outside. There are serious objections to this, for the cuticular lipids of insects are mostly saturated hydrocarbons or esters of long-chain fatty acids.
and alcohols (Richards, 18) which do not fix readily in osmium tetroxide. The layer of wax on the outside of most cuticles is relatively thick (0.1 to 1 μ, Beament, 2) but it does not survive in osmium-fixed material, nor are the wax scales of the honey bee made insoluble by osmium tetroxide however. It is, therefore, by no means certain that the contents of the holes in the epicuticle are the same as the surface wax. There are three possibilities: (a) The surface wax, even though unfixed when dry in bulk, might yet take up osmium when in a particular physical state, for in potassium linolenate emulsions the osmium that gives contrast in the microscope may be a deposit of reduced osmium between the hydrophilic surfaces of the layers (Stoeckenius, Schulman, and Prince, 25). (b) The contents of the holes may be an unsaturated precursor which undergoes a further chemical change at the surface. (c) The holes contain the surface wax mixed with an unsaturated lipid which is either volatile or similar to the thin lipid layer (II) on the outside of the epicuticle.

The distribution of esterase suggests that at least the final step in the synthesis of wax takes place in or near the spaces where the filaments are found. The crux of the problem is whether newly synthesized wax can form thread-like micelles spontaneously or whether there is a pre-existing protein/enzyme template shaped in the form of a tube which determines the shape of the wax and penetrates the epicuticle. The truth may be between these alternatives, the wax molecules aggregating in this manner under the particular conditions of solution of protein, enzymes, wax, and its precursors. This can only be determined by future in vitro experiments and by a study of the genesis of the epicuticle. For example, are the holes through the epicuticle formed by depositing cuticular material around pre-existing lipid filaments or does the hole form first and fill up later with wax?

The problem of movement of wax across the epicuticle remains. If the filaments are wax micelles freely ending below the inner surface of the epicuticle, there seems no cause for the wax to flow outwards.

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BIBLIOGRAPHY

34. Wigglesworth, V. B., A simple method for cutting sections in the 0.5 to 1 μ range, and for sections of chitin, *Quart. J. Micr. Sc.*, 1959, 100, 315.