Studies of the Development of the Imaginal Cuticle of Calliphora erythrocephala

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With two plates (figs. 1 and 2)

SUMMARY

The development of the imaginal cuticle has been studied with particular emphasis on the microtrichia and the pupal moulting fluid.

The microtrichia are formed from acidophil filaments of epidermal cytoplasm which remain as the cuticular pore canals after secretion of the endocuticle. Microtrichia at the base of the bristles are associated with nerves.

The cuticle before emergence consists of a single-layered epicuticle less than 1 μ thick and an endocuticle 3-5 μ thick. The epicuticle and the endocuticle of the sclerites are completely sclerotized after emergence.

The pupal moulting fluid was found to be a clear, salt-free, watery liquid containing protein and lipid and devoid of proteinases or chitinases. No evidence of dissolution was found in the pupal cuticle. The aqueous part of the moulting fluid is absorbed before emergence and this may be prevented by the addition of salts.

Evidence is presented for the formation of a denatured, hydrophobic, protein-lipoid film from the moulting fluid on the surface of the epicuticle after emergence.

Resistance to water loss develops after emergence and is not entirely dependent on cuticle darkening and hardening. A film of moulting fluid spread and dried on natural and artificial membranes lowers the rate of water permeation. Such films possibly are operative in regulation of water loss from the imaginal cuticle immediately after emergence. Waxy materials appear on the cuticle surface during the hardening phase. Their possible origin is discussed.

INTRODUCTION

Apart from studies of the formation of the bristles (Lees and Waddington, 1942; Lees and Picken, 1945; Schwenk, 1947), the imaginal integument in Diptera has received little study. In this paper the development of the imaginal cuticle of Calliphora erythrocephala Meigen has been studied with particular reference to the formation of the microtrichia and the properties of the pupal moulting fluid. The histological observations have been restricted mainly to the abdominal cuticle of the imago.

MATERIALS AND METHODS

Pupae of known age were obtained by isolating larvae at the white puparium stage from larval cultures and transferring them to an incubator at 24° C. for further development. Under these conditions the beginning of the true pupal [Quarterly Journal of Microscopical Science, Vol. 95, part 1, pp. 67-78, March 1954.]
period marked by the extrusion of the pupal horns and the appearance of an air space between the prepupal and pupal cuticles, occurred 24–25 hours after the white puparium stage.

Immersion of the puparium in water at 60° C. for 5–10 seconds greatly facilitated the removal of the pupa from the puparium. The pupae were then fixed in Carnoy-Lebrun or alcoholic Bouin. Peterfi's celluloid-paraffin embedding procedure after alcohol dehydration gave good results. The staining procedures used were Heidenhain's iron haematoxylin and Mallory's triple stain. Details of special procedures are given in the appropriate places in the text. Fine tungsten needles prepared by dissolving the metal in fused sodium nitrite were used for the preparation of peelings of the cuticle for examination under the electron microscope.

**Observations**

*The Epidermis*

The imaginal epidermis of the head and thorax develops from the peripheral cells of the imaginal disks, and in the abdomen from two pairs of histoblasts located dorsally and ventrally to the dorso-ventral muscles of the body-wall in each abdominal segment except the last of the third instar larva. These histoblasts are present in a quiescent state throughout the larval development and do not appear a few hours after puparium formation as stated by Bodenstein (1950) for *Drosophila* (fig. 1, A). The epidermis of the genital segment of the imago develops from genital disks located in the mid-ventral region of the last larval segment. During the prepupal period (period from the formation of the puparium to the extrusion of the pupal respiratory horns) the imaginal epidermal cells increase in size and number and after the formation of pupal cuticle they spread out on the outside of the larval epidermal cells and displace them into the body cavity of the pupa where they are phagocytosed. The imaginal epidermis is continuous over the abdomen 55–60 hours after puparium formation. The bristle-forming cells can be clearly distinguished at this stage by their large size. The first indication of the formation of the new imaginal cuticle occurs in the pupa 80 hours after puparium formation. The
epidermal cells have contracted away from the pupal cuticle, leaving a fluid-filled space between the pupal cuticle and the imaginal epidermis.

The formation of the microtrichia

A delicate layer staining with haematoxylin and acid fuchsin is secreted on the surface of the epidermal cells immediately after the contraction of the epidermis away from the pupal cuticle. At this time filamentous extensions are secreted by the epidermal cytoplasm. One of these extensions is produced by each cell and they become the microtrichia of the imaginal cuticle. After their initial formation they do not increase further in length and lie as flexible cell hairs against the newly deposited epicuticle (fig. 1, b). The microtrichia on the abdominal terga are 4–5 μ in length.

The cuticle in the 6-day-old puparium shows double staining with Mallory’s method. An outer layer, the epicuticle, less than 1 μ thick, stains pink and an inner layer, the endocuticle, 2–3 μ thick, stains blue. Fine acidophil filaments extend from the epidermal cells through the endocuticle and enter the microtrichia. Attempts to colour frozen sections of the imaginal cuticle with sudan black B were unsuccessful for two reasons; first, the masses of lipoidal material in the fluid pupal contents spread over the sections and masked any cuticle staining, and second, the imaginal cuticle at this stage is completely water-soluble. The imaginal cuticle from pupae 2 days before emergence, however, is not soluble in water and the epicuticle at this time colours with sudan black B. The endocuticle gives a positive chitosan test for chitin whereas the epicuticle and the microtrichia do not. The microtrichia of the ventral abdominal intersegmental region possess expanded bases which contain chitin. These are formed from folds which appear at the time the microtrichia are secreted. Endocuticular material is secreted into these folds but does not extend to the tips of the microtrichia. The chitin-protein complex of the endocuticle is secreted from the epidermal surface around the acidophil extensions into the microtrichia. The epicuticle does not increase in thickness during the formation of the endocuticle. No further cuticle deposition takes place in the imago after emergence. The thickness of the cuticle of the abdominal terga is approximately 5 μ; the intersegmental cuticle is 2–3 μ thicker.

Connexions between the epicuticle and the epidermal cells were only found in the microtrichia. Sections of the cuticle after emergence show no signs of pore canals.

Thin peelings of the endocuticle of newly emerged flies with their air sacs filled were examined under the electron microscope and revealed faint regularly distributed dark spots (fig. 1, c) which corresponded in number for a given area to microtrichia. They are interpreted as pore canals and represent filaments of cytoplasm which extend through the endocuticle to the microtrichia. They were clearly revealed as pink strands by staining the cuticle before emergence with Mallory’s stain (fig. 1, d). Microtrichia examined under the electron microscope from cuticle removed before emergence showed a well-defined core of dense material. This core was continuous with the pore
The canals in the endocuticle. The surrounding epicuticle appeared completely homogeneous.

The bristles

The development of the macro- and micro-chaetae and their sockets has been described in detail for *Drosophila* by Lees and Waddington (1942); Lees and Picken (1945); and Schwenk (1947). Fifty hours before emergence the chaetae have reached their full length and the tormogen and trichogen cells at this stage are reduced in size compared to that during active secretion. Cytoplasm extends into the lumen of the bristle shaft and stains intensely with acid dyes. When pieces of the body wall were treated with the Nadi reagent for demonstrating oxidase activity, a very strong positive purple reaction developed in the cytoplasm of the trichogen cells (fig. 1, F). The reaction was inhibited by KCN and by heating to 80° C. and was very much reduced below pH 5. The purple staining extended up the lumen of the bristles and in pupae 40 hours before emergence even the cuticle gave a positive reaction. The bristles began to darken between 40 and 50 hours before emergence, first becoming a pale reddish to tan in colour and then changing to a grey which increased in intensity until the bristles were completely black 24 hours before emergence. The natural melanization of the bristles occurred simultaneously with the appearance of oxidases in the cytoplasm of the trichogen cell and in the lumen of the bristles. The epidermal cells did not contain granules reacting positively to the Nadi reagent at the same time as the trichogen cells. They did, however, show numerous purple granules 6–12 hours before emergence. Cells underlying the intersegmental cuticle showed no difference in reaction from the cells underlying the segmental cuticle.

At the periphery of the socket of each bristle are located a group of 4–5 larger microtrichia which are formed like the others from epidermal cells but are associated here with sensory nerves (fig. 1, F). The function of these receptors is unknown. An association between bristles and nerves has been described by Stern in *Drosophila*. The whole bristle is regarded as a tangoreceptor. From the size and position of these sensory trichia they are more likely to be chemoreceptive.

The pupal moulting fluid

As soon as the outermost layer of the new imaginal cuticle is formed and the epidermal cells have contracted away from beneath the pupal cuticle, the space between the pupal cuticle and the newly developing imaginal cuticle becomes filled with a transparent, watery fluid devoid of cells, called the pupal moulting fluid. Passonneau and Williams (1951) found that a similar pupal fluid from the pupae of *Platysamia cecropia* L. was secreted by the epidermal cells as a transparent gel which did not at first attack the pupal cuticle. However, it later contained proteinases and chitinases that digested all but the sclerotized exocuticle and epicuticle of the pupal cuticle. The fluid was reabsorbed and replaced by air just before emergence. Isotopically labelled amino-
acids injected into the fluid were absorbed and incorporated into the proteins of the adult moth.

The pupal moulting fluid of *Calliphora* was extracted from puparia after the sixth day from puparium formation. A small incision was made through the pupal cuticle between the head and thorax after careful removal of a piece of puparial cuticle from between the respiratory horns. By gently pressing the posterior end of the puparium a drop of moulting fluid exuded through the pupal cuticle. By this method, 0.05 ml. of fluid could be extracted from twenty puparia. To obtain some data on its composition, nitrogen determinations were made by the micro-Kjeldahl technique and dry weight and ash determined. The results are shown in Table 1.

**Table 1. Composition of the pupal moulting fluid**

<table>
<thead>
<tr>
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<th>Percentage</th>
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<tbody>
<tr>
<td>Dry weight</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>Ash</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>Protein nitrogen (TCA ppt.)</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>Non-protein nitrogen</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Protein</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The protein N did not change significantly with age of the pupa.

The fluid reacted positively with all the protein colour tests and also to the Salkowski and Liebermann-Burchardt reactions for unsaturated sterols. It contained no sulphur, phosphorus, or reducing sugars. It reduced ammoniacal silver nitrate to a dark brown colour and decolorized iodine. Small pieces of fibrin stained in aniline blue, amaranth red, or congo red and soaked in distilled water until the excess dye was leached out were placed in a series of tubes and 0.01 ml. of pupal moulting fluid added to each with phosphate buffer. The tubes were incubated at 24°C for 24 hours. No liberation of the dye from the fibrin was observed either in acid or alkaline solution. It is concluded that no proteolytic enzymes are present in the fluid. Small cubes of endocuticle from larvae were placed in the fluid and when examined after 24 hours they showed no signs of dissolution. Chitinases therefore also appear to be absent. Histological observations revealed no change in the thickness of the pupal cuticle during the deposition of the imaginal cuticle. The pupal cuticle was not sclerotized and consisted of a thin outer lipoid layer and a chitin-protein layer 2–3 μ thick. Moulting fluid extracted from pupae 24 hours before emergence darkened on exposure to air and blackened on addition of catechol or 3,4 dihydroxyphenylalanine. The reaction was inhibited by heat, KCN, and sodium diethyl-dithiocarbamate. This indicated the presence of a polyphenol oxidase. The reaction was not given by the pupal moulting fluid removed from pupae 3 days before emergence.

When the moulting fluid was allowed to dry on a slide, it became viscous, gelated, and hardened to a tough, slightly brown plastic mass. Examination
under a binocular microscope revealed sparsely distributed brush-like batches of crystals within the gelated fluid. When the dried fluid was covered by a drop of water the protein dissolved, leaving crystals which floated to the surface. The amount of material was too small to perform satisfactory tests on it. It is thought that the moulting fluid contains wax-like or lipoidal materials held in solution by the hydrophilic protein of the moulting fluid. The dried fluid coloured intensely with sudan black B and was decomposed to a series of minute oily droplets on treatment with concentrated chlorinated nitric acid.

The gentle bubbling of air from a fine capillary through the fluid formed stable bubbles surrounded by a delicate protein film. The bubble surface dried rapidly and a series of brilliant interference colours were produced. After the fluid had been left for several days, the surface-film became water-insoluble and hydrophobic. Formation of a dried thin film of moulting fluid leads to the denaturation of the protein and the formation of interference patterns and a hydrophobic surface.

Water loss through the imaginal cuticle

The surface of the imaginal cuticle is completely wetted by the pupal moulting fluid. The fluid will also spread evenly over the extremely hydrophobe outer surface of the pupal cuticle. When flies 12 hours before emergence, before the moulting fluid is resorbed, were dissected from the pupal cuticle and allowed to dry, the unexpanded cuticle surface still remained hydrophil and the fly became shrunken and desiccated in a short time. The surface of the cuticle immediately after emergence, when the air sacs have just filled, is very hydrophobic and the resistance to desiccation is greatly increased. The rates of water loss of emerged and unemerged flies were determined by the method of Wigglesworth (1945) and the results are shown in Table 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Loss of weight %</th>
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<tbody>
<tr>
<td>Fly 12 hours before emergence, rinsed distilled water, dried on filter paper</td>
<td>39.0</td>
</tr>
<tr>
<td>Fly 12 hours before emergence, immersed in cold CHCl₃ for 3 minutes</td>
<td>60.8</td>
</tr>
<tr>
<td>Fly 12 hours before emergence, moulting fluid left on and allowed to dry for 10 minutes in oven at 36° C.</td>
<td>15.5</td>
</tr>
<tr>
<td>Fly just emerged, cuticle unexpanded</td>
<td>6.8</td>
</tr>
<tr>
<td>Fly just emerged, cuticle expanded but undarkened</td>
<td>4.0</td>
</tr>
<tr>
<td>Fly 1 day old, cuticle expanded and darkened</td>
<td>3.5</td>
</tr>
<tr>
<td>Fly 1 day old, immersed in CHCl₃ for 1 minute</td>
<td>64.0</td>
</tr>
<tr>
<td>Fly 1 day old, smeared with C99993 (cetyl ether of polyethylene glycol)</td>
<td>61.0</td>
</tr>
</tbody>
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The results show that resistance to desiccation occurs after emergence and that it is not entirely dependent on the darkening and hardening of the cuticle.
Imaginal Cuticle of Calliphora erythrocephala

The undarkened expanded cuticle is more resistant to water loss than the unexpanded cuticle. Chloroform and detergents markedly affect the rate of water loss.

In order to determine whether a thin film of dried moulting fluid affects water permeation, experiments were carried out with artificially prepared membranes by Beament's method (1945) (Table 3).

Table 3. The effect of dried films of moulting fluid on the rate of permeation of water through various systems at 20°C.

<table>
<thead>
<tr>
<th>System</th>
<th>Rate of permeation mg./cm.2/hr.</th>
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<tbody>
<tr>
<td>1. Water/1 mm. thick gelatin sheet/dry air</td>
<td>15.3</td>
</tr>
<tr>
<td>2. Water/1 mm. thick gelatin sheet/dried film of m.f./dry air</td>
<td>2.6</td>
</tr>
<tr>
<td>3. Water/CHCl₃ extracted scale-free Pieris wing membrane/dry air</td>
<td>33.7</td>
</tr>
<tr>
<td>4. Water/CHCl₃ extracted scale-free Pieris wing membrane/dried film of m.f./dry air</td>
<td>1.7</td>
</tr>
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</table>

The results show that a dried film of pupal moulting fluid can exercise a control over the water permeation through the extracted Pieris wing membrane.

The absorption of the moulting fluid

The imaginal cuticle at emergence is dry. The pupal moulting fluid disappears in the last hour before emergence and is replaced by air. At the time when the moulting fluid disappears, the volume of haemolymph in the imago increases and the ptilinum commences to expand. The ptilinum does not begin to expand unless the moulting fluid has been absorbed. Ligaturing the proboscis before the removal of the moulting fluid did not interfere with the removal of the fluid. The fluid is absorbed through the cuticle surface. This was shown by Fraenkel (1935), who regarded the unexpanded wing buds as the major cuticular region through which the pupal moulting fluid was absorbed. Fraenkel also found that the wing buds of pupae dissected from the pupal cuticle before emergence and immersed in distilled water, became swollen by the uptake of water. This experiment was repeated and confirmed. It was also found that if the flies were immersed in Ringer's solution, the swelling did not take place. The addition of 0.002 ml. of a 5 per cent. salt solution, pipetted from a micrometer pipette through a small opening in the pupal cuticle into the moulting fluid, prevented the absorption of the fluid by the imaginal cuticle. These experiments indicate that the aqueous part at least of the moulting fluid is probably absorbed through the cuticle surface by osmosis, and that this process is inhibited by hypertonic saline solutions.

In order to observe certain changes taking place during the absorption and immediately before emergence, puparia were fixed to a slide in such a way that normal emergence was prevented and a small window was cut in the puparial wall to the level of the pupal cuticle. The moulting fluid was observed
to become progressively more viscous as it decreased in volume, and an air space developed between the pupal and imaginal cuticles. When the fluid was completely removed, the ptilinum began to expand and contract for approximately 45 minutes, after which the cuticle began to darken. The air sacs remained unexpanded. The darkening of the cuticle is, therefore, not dependent upon the expansion of the air sacs. After the cuticle was melanized, the ptilinum became contracted and disappeared just as occurs in the normally emerged fly. The fly, however, became shrivelled up and died within a few hours. The cuticle does not darken uniformly when the flies are prevented from emerging nor does the cuticle surface show the iridescence, silvery lustre, or metallic colouring of the fly that has emerged, expanded, and darkened its cuticle naturally. When the inside of the pupal cuticle was examined from flies prevented from normal emergence and in which the cuticle has darkened, numerous black and brown spots were found. These spots were produced by drops of a gelated, tanned, and darkened protein adhering to the inside of the pupal cuticle. The spots lay immediately inside the pupal cuticle and the browning penetrated radially into the pupal cuticle just as if a substance had diffused from these spots into it. The explanation of the formation of these spots on the pupal cuticle is not certain but it is thought that they are produced by the protein from the pupal moulting fluid gelating between the pupal and unexpanded imaginal cuticle and into which the chromophoric substances which lead to the darkening of the imaginal cuticle have diffused. This observation suggested the possibility that the protein part of the moulting fluid was not absorbed but remained as a film on the surface of the imaginal cuticle, forming a thin additional layer. Protein and lipoid has been found in the moulting fluid up to the time when it is absorbed.

**A cuticle layer formed from the moulting fluid**

Additional evidence for the appearance of a protein-lipoid layer on the surface of the imaginal cuticle after emergence has been found from electron microscope studies of the epicuticle surface. A comparison of fig. 2, A and B, shows that a thin additional layer is present over the microtrichia after the absorption of the moulting fluid and before the darkening of the cuticle. This layer, it is thought, is formed from an unabsorbed film of protein and lipid remaining after the aqueous part of the moulting fluid has been absorbed.

The epicuticle surface examined under transmitted electron beam consistently showed ‘shadows’ of the microtrichia (fig. 2, C). These are shown only in

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**FIG. 2 (plate). A, electron micrograph of the microtrichia 24 hours before emergence. The core is clearly defined. Siemon E. M., kv. 90.**

**B, electron micrograph of the microtrichia from the epicuticle of the abdominal tergum immediately after emergence. The outermost layer is regarded as formed from the pupal moulting fluid. Siemon E. M., kv. 90.**

**C, electron micrograph of the microtrichia from the gena in surface view, showing the ‘shadows’ on cuticle surface. The shape of the microtrichia is clearly shown. The dark spots between the microtrichia may be cuticular sense organs. Siemon E. M., kv. 70.**
preparations of the cuticle from emerged flies with their air sacs filled. They are interpreted as representing thinner regions on the epicuticle surface. The microtrichia before emergence lie flat against the cuticle surface, but after emergence when the cuticle surface is becoming sclerotized, they lift away from it. If a deposit of protein from the pupal moulting fluid formed a mould round the microtrichia before they lifted from the cuticle surface, the ‘shadows’ are possibly explained. Examination of several preparations made it clear that the distribution of the ‘shadows’ was such that it could not have been produced by deflection or scattering of the electron beam by the uplifted microtrichia.

As the aqueous part of the moulting fluid was absorbed before emergence, the fluid became progressively more concentrated and with the appearance of an air space between the pupal and imaginal cuticles, it gelated. When the fly emerged the air sacs were filled, the wings expanded and the cuticle stretched to its final fully expanded condition. It is thought that the film of gelated moulting fluid stretches along with the expanding cuticle and forms a delicate surface layer over the epicuticle.

The imaginal cuticle after emergence

Fifteen minutes after emergence, provided the fly has extricated itself from its pupal environment, the air sacs were filled and the thorax and abdomen of the imago became fully expanded. The dorsal cuticle surface showed a silvery lustre as well as greenish interference colours. The wings extended and at first appeared a whitish opaque colour which changed within 30 minutes to a transparent hyalinated membrane, showing a shiny surface and series of iridescent colours on both the upper and lower surfaces. Darkening of the cuticle began after the first 20 minutes and increased gradually to an intense black on the thoracic and abdominal sclerites. Sagittal sections of the dorsal abdominal sclerites showed that the darkening began in the epicuticular and outer endocuticular layers. Just before this happened, the entire thickness of the cuticle was stainable by acid fuchsin. No exocuticle was found in the cuticle before emergence. In the fully darkened imago the entire endocuticle of the sclerites was sclerotized and melanized. The intersegmental conjunctiva were not darkened or hardened and the endocuticle remained soft and flexible. The exocuticle, defined as sclerotized cuticle, included both epicuticle and endocuticle and was completely absent from the intersegmental regions. Although the sclerites of the thorax and abdomen became completely melanized within 45 minutes after emergence, the hardening continued through the first 24 hours of imaginal life. Darkening and hardening occurred together but they were not completely interdependent. The hardening could continue although the cuticle appeared fully melanized.

Almost all regions of the cuticle showed silvery lustres depending on the direction of the incident light source. These lustres were produced by light scattering from the rounded tips of the microtrichia spaced one to two microns apart. The finer the surface units, the more perfect was the scattering of light
The microtrichia on the genae showed the most perfect lustre and it was in this region that the microtrichia were the finest and closest together. The microtrichia on the abdominal tergites were arranged in an extremely regular fashion, whereas on the dorsum of the thorax they were irregularly distributed. The long axis of the microtrichia on the abdominal tergites was at right angles to the anterior-posterior axis of the abdomen. The bases of the microtrichia were directed towards the dorsal median longitudinal axis. There was consequently a narrow median dorsal region where the microtrichia were not regularly aligned.

The surface of the imaginal cuticle became decidedly more waxy during the first 24 hours after emergence. The origin of the wax has not been determined. No specialized wax glands have been found in the epidermis, but this was not specifically studied. A point that may be significant is that the waxy materials appeared on the surface of the cuticle during the hardening phase. It is possible that cuticular, waxy materials are liberated during the orientation and dehydration that occurs as the hardening of the cuticle in the sclerotized regions progresses. When 1-day-old flies were immersed for 1 minute in chloroform, the interference colours on the abdominal sclerites underwent a slight shift towards the green, suggestive of a removal of a thin layer of material from the surface of the cuticle. The appearance and secretion of waxy materials on the surface of the cuticle of insects is a subject that requires much more intensive study. The surface of the imaginal cuticle of *Calliphora* is extremely hydrophobic. This is due to the combined effects of the microtrichia and a waxy surface.

**DISCUSSION**

Kroon, Veerkamp, and Loeven (1952), in a study of the process of extension of the butterfly wing, were of the opinion that the molecular changes observed within the wing during the extension of the cuticle provided an explanation of the permanent stiffness and hardness of the wing. The orientation of the chitin they regarded as not merely an accompanying phenomenon but an essential factor in hardening. It is significant that although melanization can be induced in *Calliphora* before the expansion of the air sacs, the cuticle does not become brittle or waterproof. The physical changes of the protein and chitin fibres upon stretching of the cuticle as the air sacs are expanded by muscular activity, appears essential for the process of hardening the cuticle.

The origin of cuticular waxes is still obscure. It is possible that the orientation of the protein and chitin fibres on the extension, dehydration, and sclerotization of the cuticle could lead to the exclusion of some of the weakly bound or labile lipoidal compounds from within the cuticle and to their appearance on the surface of the 'lipophil' epicuticle and there become oxidized to waxy materials. Qualitative observations indicated an increase in waxy materials on the cuticle surface after hardening. Epicuticle wax may arise in the following ways: (1) as a glandular secretion from the epidermis; (2) by liberation from
the cuticle during sclerotization; (3) from lipoid in the pupal moulting fluid. So far, special glands secreting wax or a wax precursor have not been observed in *Calliphora*.

It is realized that the hypothesis that the protein and lipoid present in the moulting fluid remain as a thin film on the surface of the imaginal cuticle, requires further study and confirmation in other cyclorrhaphous flies. The evidence for its existence is based on (1) the failure to demonstrate proteinases or chitinases in the fluid; (2) the absence of any sign of dissolution of the unsclerotized pupal cuticle; (3) the appearance of a new surface layer on the microtrichia after emergence; and (4) the appearance of a mould remaining after the microtrichia lift away from the epicuticle surface. The layer is too thin to be visible in histological sections of the cuticle and in the segmental cuticle it is probably sclerotized and melanized. It is thought that the contact with air of a film of gelated moulting fluid on the cuticle surface would lead to its denaturation and that this would be accelerated by the stretching and orientation occurring when the cuticle became fully expanded.

The cuticle before emergence consists of two layers, an epicuticle (cuticulin layer) less than one micron in thickness staining with acid fuchsin, haematoxylin, and sudan black and devoid of chitin, and an endocuticle 3-5μ thick staining with aniline blue and containing chitin. No dermal glands are present and a cement layer is absent. The endocuticle is perforated by pore canals extending into each microtrichium. After emergence, the epicuticle and endocuticle of the sclerites become completely sclerotized and melanized. No recognizable 'polyphenol' layer is present. Dennell and Malek (1953), in a comparative study of the epicuticle, do not recognize the polyphenol layer as a separate layer. In the sclerites of the imago of *Calliphora*, the precursor material responsible for hardening and darkening permeates the entire cuticle. Miller (1950) regards the cuticle of *Drosophila* as consisting of only epicuticle and exocuticle. The exocuticle, however, is formed by sclerotization of epicuticular, as well as endocuticular material, and the sclerites in *Calliphora* and *Drosophila* contain no endocuticle that has not been sclerotized to exocuticle. The arthrodial membranes, however, possess a well defined endocuticle and their staining reactions are similar to the entire cuticle just before emergence.

I wish to thank Prof. V. B. Wigglesworth, who suggested the research, for his valuable advice and criticism, and I also wish to acknowledge the help and encouragement given by Dr. J. W. L. Beament and Dr. M. G. M. Pryor. I am indebted to Dr. V. E. Cosslett for the provision of facilities to use the electron microscope.
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