A study of an insect cuticle: the larval cuticle of *Sarcophaga falculata* Pand. (Diptera)

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[Plate 8]

The rate of growth of the larval cuticle of *Sarcophaga* and the chemical composition of its layers are fully described.

The cuticle consists essentially of two fundamentally different layers. The outer layer—the epicuticle—is about 4 μ thick, and contains no chitin. It is basically composed of protein, having its isoelectric point at pH 5-1. At its surface a resistant lipoprotein complex forms a very thin membrane which may be separated intact from the remainder of the epicuticle which contains little or no lipid. The epicuticle is therefore a double structure.

The inner layer of the cuticle—the endocuticle—is much thicker than the epicuticle, and is a laminated chitin-protein complex of isoelectric point pH 3-5. Like the epicuticle it is a double structure. The outer zone of the endocuticle is secreted during early larval life, and is penetrated by pore canals which do not enter the epicuticle. The inner zone of the endocuticle is absent from the larval cuticle, but the outer endocuticle is destined to form the exocuticle of the puparium, the inner endocuticle remaining unchanged.

The pore canals at first contain cytoplasmic filaments, but these later become coiled and replaced by chitin.

I. INTRODUCTION

During the past fifteen years the claim made over a century ago (Odier 1823) that the insect cuticle contains protein and other substances in addition to chitin has been repeatedly confirmed (Kühnelt 1928a, b; Campbell 1929; Evans 1932, 1934, 1938;
Wigglesworth 1933; Trim 1941), and it has become abundantly clear that the cuticle is not a simple investment of chitin, but is of considerable chemical complexity. Pryor (1940a, b) has shown that the conversion of a soft white cuticle into the hard and dark armour of the puparium or mature insect is not the result of impregnation with 'incrusting substances' as had been previously suggested (Schulze 1922; Kühnelt 1928a), but is largely due to the 'tanning' of its proteins by the coloured oxidation products of a phenol secreted into the cuticle at the time of hardening. Moreover, Fraenkel & Rudall (1940) have shown that concurrently with this process pronounced orientation of chitin crystallites takes place within the protein phase of the cuticle, and this, together with loss of water and loss of solubility of the protein, results in the formation of a hard and dense structure. The changes involved in hardening affect only the outer zone of the cuticle, and result in the formation of a brown or black exocuticle, the inner zone persisting unchanged as a soft white endocuticle.

In addition to these two important constituent layers of the fully differentiated cuticle a thin resistant epicuticle overlies the exocuticle. It contains no chitin, and owes its protective qualities not only to the presence of fatty acids and cholesterol (Kühnelt 1928a), but also of paraffins of the order C_{27}–C_{31} (Bergmann 1938).

In spite of much fundamental work, however, many outstanding questions remain to be answered, since histological examination has not kept pace with advances made in the study of the chemistry of the cuticle. These advances have often been the result of work on whole cuticles or on extracts, and information on the location of the substances identified has therefore been lacking. The precise relation between the well-known facts that the enzyme tyrosinase is abundant in insect blood and other tissues, and that darkening of the cuticle involves enzyme activity, has not been demonstrated, although (following Dewitz 1902, 1905, 1916) it has been generally assumed that the relation is an intimate one. Furthermore, it has not been shown why darkening of the cuticle proceeds inwards from the epicuticle, although the oxygen consumed in the process is supplied not from the atmosphere directly, but from within the body by way of the tracheal system (Fraenkel 1935), and clearly the chromogen involved must also be supplied from within the body. The onset of pupation in Diptera, accompanied by the hardening and darkening of the cuticle of the third larval instar to form the puparium, has been shown to be due to the liberation of a hormone (Fraenkel 1935; Becker & Plagge 1939), the mode of action of which, however, is obscure. The fine pore canals which rise vertically through the cuticle (Leydig 1864; Holmgren 1902; Plotnikow 1904; Berlese 1909; Wigglesworth 1933) deserve further study, particularly with regard to the nature of their contents. And finally the importance of the epicuticle in controlling water loss through the cuticle and the permeability of the cuticle to oil-borne insecticides (Hurst 1943a, b; Alexander, Kitcener & Briscoe 1944a, b; Wigglesworth 1944a, b) points to the need for further examination of this layer.
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In an attempt to give at least a partial answer to these questions a detailed study has been made of the cuticle of the third instar larva, and of the puparium, of *Sarcophaga falculata*. The reasons for selecting this insect for study were that it is easily bred and provides a generous supply of larvae, the cuticle is thick yet readily handled and sectioned, and it provides admirable material for studying darkening and hardening of the cuticle. In addition it has, with *Calliphora erythrocephala*, already received considerable attention. Pryor (1940b) studied the formation of the puparium of *Calliphora*, and Fraenkel & Rudall (1940) have given an account of the physical and chemical properties of the larval cuticle of *Sarcophaga*. Trim (1941) has characterized the proteins obtained from larval cuticles of *Sarcophaga*. Earlier, Fraenkel (1935) had described experiments on *Calliphora* which showed a connexion between the liberation of a hormone and the formation of the puparium. And finally, dipterous larvae have already been the subject of work on tyrosinase and melanosis of the cuticle (Dewitz 1902, 1905, 1916; Gessard 1904; Graubard 1933). It was therefore hoped that a histological and histochemical study of the larval cuticle of *Sarcophaga* would lead to considerable integration of the information presented by these authors.

The present paper is devoted to an account of the larval cuticle, and in a later paper a detailed account will be given of the changes taking place during the formation of the puparium.

A preliminary note on the pore canals has already been published in *Nature* (Dennell 1943), and the events taking place at pupation have been outlined in a later account in the same journal (Dennell 1944).

II. Methods

It was essential throughout the work to have an abundant supply of larvae of known age reared under reproducible conditions. Flies were therefore kept in an illuminated cage maintained at 28° C and 65% r.h. in a large incubator, and on a diet of cane-sugar and water lived healthily. Addition of protein in suitable form is necessary, however, for mating and the deposition of larvae, and was originally supplied as horse-flesh. Later a proprietary dog food proved satisfactory, but ultimately moistened fish-meal, on which larvae are deposited freely and grow well, was used exclusively on account of its great convenience. The following procedure for obtaining larvae was adopted. Fish-meal was supplied to the flies until unsegmented eggs were shed, an indication that the production of active larva was imminent. The fish-meal was then removed, and after an interval of 1 or 2 days its re-presentation for a short period usually resulted in the deposition of a generous supply of larvae. The larvae were reared at 28° C in batches of fifty in 1 lb. jars containing 50 g. of fish-meal moistened with 50 c.c. of water, and ultimately allowed to pupate in dry sawdust. Their rate of growth under these conditions will be described in the following section.
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For histological work on the cuticle paraffin, frozen and hand-sections were used, and comparison of these formed a valuable check on the accuracy of observations. Dioxan (diethylene dioxide) over calcium oxide was used for dehydration in the preparation of paraffin sections, the hardening of the cuticle caused by the use of absolute alcohol being thus avoided. For sections cut by the freezing method pieces of cuticle were infiltrated successively with 12.5 and 25% gelatine solution at 37°C, and later embedded in 25% gelatine hardened in 10% formalin. Blocks of good consistency were obtained. They were frozen thoroughly after rinsing in distilled water, and sectioned while thawing. Sections of 15 μ were obtained, and after staining, usually with Sudan Black B (see Lison 1936) for fatty substances, were mounted in ‘Glychrogel’ or Apathy gum solution. Hand-sections of the fresh cuticle were obtained by rolling a sheet of cuticle and cutting the roll transversely while held against the thumb. These sections proved invaluable for work on the polyphenol oxidase of the cuticle and for experiments with various substrates.

Of many fixatives tried, Flemming without acetic, Gilson’s mercurio-nitric mixture and Carnoy-Lebrun gave the best results. For the cytoplasm of the pore canals of the young larva Flemming was unequalled, and penetration of the fixative was greatly improved by the addition of 0.9% NaCl.

Mallory’s triple stain proved admirable for the paraffin sections. Delafield’s haematoxylin and picro-indigo-carmine were also used, but to a less extent. The value of Mallory’s stain consisted in its perfect and constant differentiation of the epicuticle and in the clear distinction obtained between the cytoplasmic and cuticular contents of the pore canals. Other stains employed for comparison will be mentioned in passing.

Considerable use has been made of histochemical tests. In the hands of previous workers they have given valuable information on the nature of the constituents of the cuticle, but here, owing to the ease with which hand-sections of fresh larval cuticles may be obtained, it has been possible to extend their use to a study of the polyphenol oxidase of the epicuticle. The chemical tests employed will be mentioned appropriately in the text.

III. The growth of the larva

No significant differences in size have been observed between the many broods of larvae which have been reared during the course of this work. Under the conditions noted in the previous section the first and second larval instars are of only short duration, and the third and last instar begins at about 36 hr. after the larvae have been deposited. The three instars are easily distinguished, apart from the difference in size, by the form of the posterior spiracular openings. In the first instar larva each tracheal trunk opens by a simple pair of stigmatic slits, in the second by a similar but larger pair almost surrounded by a crescentic peritreme, and in the third by three slits surrounded by the peritreme. Just before the moult between second and third instars the characteristic tracheal armature of the third
instar is seen underlying that of the second, and a convenient datum point for assessing the beginning of the third instar is therefore available.

The third instar larva grows rapidly (figure 3) for about 2 days, during which the crop becomes greatly distended and shows through the transparent cuticle. When the larva has attained its full size the crop is progressively emptied, and after about a further 1½ days is no longer visible. The mature larva passes through a 'resting period' of about 1 day before pupation begins. The durations of the larval stages differ somewhat from those noted by Hafez (1940).

During larval growth there are therefore well-defined stages which make the assessment of age an easy matter. It will often be more convenient to refer to 'larva, crop full', 'larva, crop empty', and so on, than to quote the actual age of the larva. Where the term 'larva' is used without qualification the third instar larva is implied.

![Image](http://rspb.royalsocietypublishing.org/Downloaded from http://rspb.royalsocietypublishing.org/)

**Figure 1.** The larval cuticle at a little over 2 days, based on information from Mallory and chitosan preparations. e, epidermis; end. 1, outer endocuticle; end. 2, inner endocuticle; ep. 1, outer epicuticle; ep. 2, inner epicuticle; p.c., pore canal.

IV. The development and structure of the cuticle

The cuticle of the third instar larva of *Sarcophaga* consists of two primary layers, the epicuticle and the endocuticle. The epicuticle is secreted first in the development of the cuticle, before the cuticle of the second instar is shed, and soon attains its full thickness of about 4μ. The endocuticle, on the other hand, continues to be laid down throughout larval life, and even for a short time after pupation. There are two distinct phases in the secretion of the endocuticle, and distinct inner and outer zones of this layer are to be recognized. The first phase begins somewhat before the epicuticle has reached its full thickness and continues until a layer of endocuticle about 40μ thick has been produced (figure 1). This layer contains pore canals and, as will be seen later, is distinguishable in many ways from the inner layer, devoid of pore canals, which now begins to be added.
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Paraffin sections of larvae which bear the spiracular armature of the second instar overlying that of the third, stained in Mallory's triple stain, show the epicuticle as a deeply staining red layer, whereas the underlying endocuticle stains more lightly with aniline blue. The cuticle of the second instar overlies but is separated from that of the third, and consists of intact epicuticle and the almost completely digested remains of the endocuticle. The early growth of the third instar cuticle and the shedding of that of the second instar appear to take place in a manner similar to that described by Wigglesworth (1933) for *Rhodnius*. The new epicuticle appears to be secreted by the epidermis and not by special tegumental glands as in the 'cuticle' of *Homarus* (Yonge, 1932). No dermal glands for the secretion of a moulting fluid have been observed at this stage, but if they behave as in *Rhodnius* it is possible that by this time they have already degenerated. By the time the old cuticle is shed, the new cuticle of the third instar is about 10 μ thick, and during the next 1 \( \frac{1}{2} \) days it increases steadily in thickness to about 40 μ. It shows distinct horizontal striae suggesting a laminated structure.

While examining paraffin sections of third instar larvae about 2 days after deposition the pore canals were first noticed. They appear as red-staining irregular filaments, apparently cytoplasmic, projecting from the epidermis and rising some way into the endocuticle. Close examination showed that each filament is continued through the remainder of the endocuticle as far as the epicuticle as a delicate rod, which stains lightly with aniline blue and is therefore almost indistinguishable from the surrounding endocuticle (plate 8, figure 6). Tangential sections give further information. Both acidophil and basophil portions of the filaments appear as discrete structures occupying but not, apparently, entirely filling tubular canals in the endocuticle, so that a small annular space occurs between the wall of the canal and its contents. The appearance of the distal portions of the filaments suggests that they are composed of cuticular material. Numerous authors (for example, Leydig 1864; Tower 1906; Hass 1916; Poisson 1924; Wigglesworth 1933) have described cytoplasmic filaments extending from the epidermis into the cuticle in a number of insects, and apparently remaining in this condition throughout life, while Holmgren (1902), Plotnikow (1904), Berlese (1909) and Eder (1940) have expressed the view that these filaments are ultimately transformed into cuticular substance. A detailed study of the canals and their contents in *Sarcophaga* was therefore called for. It is important to notice, as Wigglesworth (1933) has pointed out, that the evidence adduced for the cuticularization of the pore canal contents is slender in the extreme, consisting merely of the similarity of the pink staining of the cuticle and the contents of the canals when haematoxylin and eosin have been used. But, on the other hand, the filling of the pore canals with air when a section is allowed to dry is itself only partially satisfactory evidence that the canals contain protoplasm. Wigglesworth (1933) makes no claim that it indicates more than that the canals are filled with protoplasm or with some fluid, but Richards & Anderson (1942) use similar evidence to support their contention that the canals are filled, not with protoplasm, but with a fluid, probably a salt solution,
in equilibrium with the underlying epidermal cells. As will be seen from the following account the statement that the canals are filled with cuticular substance is correct for Sarcophaga larvae at least.

In studying the pore canals portions of larvae were fixed at regular intervals in Flemming without acetic from the beginning of the third instar until pupation, and after sectioning were stained in Mallory's triple stain. At the same times the cuticles of other larvae of the same brood were heated to 160°C for 30 min. in potash solution saturated at room temperature. They were then washed, embedded in paraffin, and after sectioning were treated with 0·2% iodine solution followed by 1% sulphuric acid. This modification of the original van Wisselingh procedure gives a specific test for the presence of chitin, based on the conversion of this substance to chitosan (Campbell 1929). Its application here clearly revealed the presence of chitin in the pore canals of older larvae. Later, for demonstrating the presence and form of the canals rather than the composition of their contents, the procedure recommended by Schulze (1922, 1924) was found valuable and easier to apply than the chitosan test. In this procedure cuticles were first treated with diaphanol (chlorine dioxide in glacial acetic acid), sectioned, and then treated with a solution of zinc chloriodide. It is claimed that the violet colour given by chitin constitutes a specific reaction, but Kühnelt (1928b) denies this, and Campbell (1929) in his critical survey of the methods employed for the detection and estimation of chitin states that results of the test should be interpreted with caution. In the present experiments a chestnut brown instead of violet coloration was usually given by the contents of the pore canals of Sarcophaga larvae. The value of Schulze's test, however, lies in the fact that the epicuticle and epidermis remain intact, whereas in the Campbell method they are destroyed.

The information given by the Mallory and the chitin preparations is complementary and unambiguous. The Mallory-stained sections show the contents of the pore canals of the youngest larvae as delicate strands rising from the epidermal cells and passing into the endocuticle. Each strand soon breaks up into a tuft of fine filaments extending to but not entering the epicuticle (plate 8, figure 5). The filaments stain prominently with acid fuchsin, and closely resemble those illustrated by Plotnikow (1904) in the larva of the silkworm and of a Syrphid. In larvae a little older single cytoplasmic strands, apparently coiled, leave the epidermal cells and pass into the endocuticle, but fail to reach its outer border (plate 8, figure 6). In larvae yet older the strands are still shorter, and finally, in larvae about 3 days after deposition, when the cuticle is about 40µ thick, they are no longer visible.

The potash-treated sections, tested for the presence of chitosan, present further information. In the youngest larvae examined, which in Mallory-stained sections show the red-staining strands extending completely to the epicuticle, the endocuticle gave a rather pale but definitely positive reaction, but no indication of the pore canals was apparent. In older larvae only the outer ends of the canals gave a positive reaction, stronger than that of the endocuticle, and finally, in larvae
about 3 days old, the whole extent of the canals was revealed by a very deep violet coloration. In all larvae of the same age up to 3 days the sum of the extents of the canals as revealed by the two methods was equivalent to the thickness of the endocuticle. In larvae of about 3 days, however, it was often found that the chitinized pore canals did not extend fully to the inner border of the endocuticle (plate 8, figure 7). This was due not to the fact that withdrawal of the cytoplasmic filaments of the pore canals was incomplete, but that after their withdrawal the inner zone of endocuticle, lacking pore canals, began to be secreted. The growth of the inner endocuticle will be discussed later. The appearance of the cuticle when the contents of the canals are completely replaced by chitin and endocuticle growth has continued for a short time is seen in figure 1.

An indication that the red-staining contents of the canals seen in the Flemming-fixed sections are truly cytoplasm is given by an examination of cuticles which had been treated with 10% potash solution at 100° C for an hour before being sectioned and stained in Mallory. In all but the youngest larvae the distal ends of the canals are filled with chitinous plugs staining with aniline blue like the endocuticle itself. The basal ends, however, are completely empty, as would be expected if they had contained cytoplasm. More satisfactory evidence is given by boiling hand-sections of fresh cuticles in a 0.2% solution of ninhydrin (triketo-hydrindene hydrate) which in the presence of z-amino acids gives a deep blue coloration (Lison 1936). Over small extents of the cuticle the basal contents of the pore canals become prominently coloured by this treatment.

In general, the staining reaction of the cytoplasm of the canals with a variety of dyes is similar to that of the epicuticle, which, as will be seen later, is basically composed of protein. The significance of this similarity will be considered in discussing the possible functions of the pore canals.

Whether the cytoplasmic filaments of the canals are actually progressively withdrawn, secreting chitin from their tips and so leaving behind them strands of this substance, or whether they themselves become progressively chitinized from their distal ends inwards, is not clear. But the result of either process is the formation of chitinized plugs accurately moulded to the form of the canals, and, in chitosan preparations, revealing it with perfect clarity (figure 1 and plate 8, figure 7). Approximately the outermost third of each canal is relatively broad, and often splits into a number of branches. The central third is slender and closely coiled, often so closely that it resembles a more massive but uncoiled strand. The innermost third of the canal is again broader and uncoiled, but is always undivided.

The resemblance of these canals to those of the cockroach *Periplaneta americana*, described by Richards & Anderson (1942) as the result of a study with the electron microscope, is at once apparent. The pore canals of the cockroach also are coiled, but show no distal expansion or splitting. It is claimed that they are filled, not with cuticular substance, but with some fluid. In size the canals of the two insects are markedly different, those of the cockroach having an average diameter of 0.15 µ, and being coiled into a helix of pitch 0.25 µ, whereas those of *Sarcophaga*
have a diameter of about $1.0 \mu$ and a pitch of about $2.5 \mu$. This difference in size cannot be explained by a difference in the thickness of the cuticles of the two insects, for in both it is about $40 \mu$ thick.

Further indication of the solidity of the pore canal contents in *Sarcophaga* is provided by splitting horizontally the fresh cuticle of a larva about 3 days old. The pore canals of the two sheets so obtained are readily seen in surface view. Usually the outer sheet bears on its inner face the projecting stiff contents of the canals, while the inner sheet shows corresponding empty canals. The ease with which the contents are withdrawn from the canals suggests that there is no fusion between them and the surrounding endocuticle, and confirms the observation previously made that an annular space may exist between wall and contents of the canals. These fresh preparations give immediate information on the number of canals associated with each epidermal cell, for if the cuticle is handled carefully and examined in Ringer solution the cells remain in contact with the cuticle and their outlines are readily seen. The canals are arranged in clearly distinct fields corresponding to the distribution of the underlying cells, and as many as 50–70 canals are associated with each cell. Beneath the low rounded spines of the cuticle the canals are more closely crowded than in adjacent areas. The density of distribution of the canals is of the order of at least 15,000 per sq.mm., a low figure in contrast with that of 1,200,000 for the much finer canals of the cockroach.

The foregoing observations represent, apparently, the first unequivocal demonstration of the nature of the contents of the fully formed pore canals of any insect. The universal cuticularization of pore canals is, however, not necessarily implied, for the larval cuticle of *Sarcophaga*, being destined ultimately to be detached from the epidermis but retained as the puparium, may well show early specialization to this end. It seems reasonable to suppose that although during the early development of an insect cuticle the canals are always filled with cytoplasm, the later history of these structures may show considerable divergences in different insects.

With regard to the function of the pore canals it is generally agreed that, whatever their ultimate fate, they are concerned with the secretion of the cuticle. Wigglesworth (1933), referring to the endocuticle of *Rhodnius*, states: ‘The exact mechanism of its formation is not known, but it must evidently be separated from the cells in fluid form around the protoplasmic filaments that occupy the “pore canals’’, because there is no sign of any partitioning (“Feldung”) of the endocuticle to correspond with the underlying epithelium, such as occurs in those cases where the cuticle arises by a transformation of the superficial layers of the epidermal cells.’ But it must be remembered that endocuticular material may be secreted in the absence of pore canals; in the later development of the larval cuticle of *Sarcophaga* the inner endocuticle continues to grow after the pore canals are fully formed, chitinized, and detached from the epidermis. Even here there is no sign of the partitioning mentioned by Wigglesworth. Clearly the endocuticle is secreted in fluid form from the surface of the epidermis, and does not arise by transformation of superficial layers of these cells. In this connexion it is interesting to note that
Eder (1940) and Richards & Anderson (1942) report the absence of pore canals in soft larvae, and the latter authors, referring to the result of an investigation by Alexandrov (1935), state that ‘Presumably the species he used have no pore canals (fly larvae, probably similar to mosquito larvae, see below),...’. Lowne (1890–2), however, clearly refers to fine processes which pass from the epidermal cells into canals in the endostracum (endocuticle) of the larva of Calliphora erythrocephala. He furthermore describes the endocuticle as being divided by vertical planes into hexagonal prisms, but unfortunately in his illustrations the planes of separation seem to correspond with the pore canals. In the present work no cuticular prisms have been seen in either Calliphora or Sarcophaga, and Calliphora has pore canals like those of Sarcophaga.

Since endocuticle secretion may proceed in the absence of pore canals the suspicion arises that the canals may have functions unconnected with the production of this layer. It has been observed in Sarcophaga that chitinization of the distal contents of the pore canals does not begin until the epicuticle has attained its full thickness, and it seems possible that one function of the canals may be to assist in the production of this layer. As will be shown later the epicuticle is actually a double structure, consisting of an outer very thin resistant layer (figure 1, ep. 1), possibly corresponding to the epicuticle of other authors, and a much thicker inner layer consisting mainly of protein (figure 1, ep. 2). Richards & Anderson (1942) describe a similar double epicuticle in the cockroach. It is possible that the outer layer of the epicuticle in Sarcophaga is secreted by the surface of the epidermis, and that the inner layer is formed wholly or in part by secretion from the tips of the cytoplasmic filaments of the canals. Some support, admittedly slight, for this suggestion is given by the similarity often seen between the staining reactions of the inner epicuticle and the cytoplasmic filaments. In Carnoy-fixed sections stained with picro-indigo-carmine both the inner epicuticle and the filaments stain a distinct blue-green, but the cytoplasm of the epidermal cells is red. Similarly, in formol-fixed sections stained with Mallory the epicuticle and filaments are a clear red, but the epidermis is purple.

Other functions which have been ascribed to the pore canals are the transport of an oxidizing enzyme to the epicuticle (Wigglesworth 1938, 1939), and the transport soon after ecdysis of a protein and a phenol which react in the outer part of the cuticle to form the amber-coloured material of the exocuticle (Pryor 1940b). These suggestions will be examined in a later paper in describing the formation of the puparium of Sarcophaga.

The growth of the cuticle after the complete chitinization of the pore-canal contents is interesting. Not only does secretion of the inner layer of endocuticle begin when the canals are fully chitinized, but the outer layer of the endocuticle begins very rapidly to increase in thickness. The appearance of the cuticle at about 3 days has been shown in figure 1. The outer endocuticle then is about 40μ thick, and the inner endocuticle has already reached nearly 10μ. During the succeeding day the outer endocuticle increases to no less than about 140μ, while the inner
endocuticle has continued its growth and now forms a conspicuous layer over 40\(\mu\) thick. After the fourth day, however, no further growth of the outer endocuticle takes place, but the inner layer continues to grow, and immediately before pupation has reached a thickness of nearly 80\(\mu\), approximately a third of the total thickness of the cuticle (figure 2). It continues to grow for some time after the puparium has begun to form. These features of the growth of the cuticle are illustrated in figure 3.

**Figure 2.** The cuticle of the mature larva. Mallory and chitosan preparations. Abbreviations as in figure 1.

Profound differences between the two layers of endocuticle will be detailed in giving an account of the chemistry of the cuticle and in describing the formation of the puparium, but at present it is sufficient to state that while the outer endocuticle stains readily with aqueous methylene blue the inner stains only lightly or not at all; and that in addition to being devoid of pore canals the inner layer shows a rather coarser lamination than the outer.

Wigglesworth (1933) has described two clearly defined layers of endocuticle in the fourth nymph of *Rhodnius*, in which the outer half is laid down completely before moulting, and the inner half after the moult. The pore canals, however, penetrate both layers. Kühn & Pieho (1938) state that secretion of the endocuticle takes place at the same time as hardening and darkening of the cuticle in *Ephesia kuehniella*, a condition comparable with the continuation of growth of the endocuticle after the onset of puparium formation in *Sarcophaga*.
The growth of the outer endocuticle of the larva of *Sarcophaga* after chitinization of the pore canals is complete is clearly reflected in the change of form of these structures. At the third day the canals are helicoidal (figures 1, 3), but at the fourth day, when the outer endocuticle has increased in thickness more than threefold, they are drawn out and become almost straight (figures 2, 3). It is clear that an already existing layer of endocuticle expands, and is not increased in thickness merely by increment at its inner surface. The separation of the outer endocuticle from the epidermis by the intervention of the growing endocuticle renders this latter mode of growth improbable.

![Graph showing growth of larval cuticle thickness and length](image)

**Figure 3.** The rate of growth in thickness of the layers of the larval cuticle, and the growth in length of the larva. The cuticle is represented as inverted, the epicuticle lying along the abscissa. The extent of the chitinized pore canals is indicated by shading, and the extent of the cytoplasmic filaments by the unshaded area of the outer endocuticle (1).

Before leaving the morphology of the cuticle attention may be drawn to the figure of the larval cuticle of *Calliphora erythrocephala* given by Fraenkel & Rudall (1940) in which the epicuticle is indicated as the exocuticle. The formation of an exocuticle, however, is conditional on the process of hardening and darkening of the cuticle, which does not take place until the formation of the puparium. The
term exocuticle, denoting a brittle pigmented outer layer, was suggested by Mr R. E. Snodgrass (Campbell 1929), and Wigglesworth (1933) clearly defines the exocuticle as an outer pigmented layer of the endocuticle, present only in rigid portions, and containing chitin. As will be seen later, the layer identified as the exocuticle by Fraenkel & Rudall contains no chitin. Pryor (1940b) also mentions the exocuticle of the same larva, but it does not appear that he is confusing this layer with the epicuticle, as suggested by Richards & Anderson (1942), for he clearly refers to the fact that the epicuticle gives a positive argentaffin reaction whereas the exocuticle does not. His exocuticle may be the outer layer of the endocuticle. In his illustration (plate 17, figure 4) the layer giving the argentaffin reaction can only be the thick epicuticle.

V. THE RATE AND MODE OF GROWTH OF THE CUTICLE

In order to discover whether the rapid increase in thickness of the cuticle between the third and fourth days is due to an increase in the rate of cuticle secretion, or to other causes, a study has been made of the rate of increase in both thickness and weight of the cuticle. Measurements of cuticle thickness have been made at intervals on unstained hand-sections of fresh cuticles of a number of larvae. Errors due to contraction of the cuticle on fixation and preparation for sectioning have therefore been avoided, but it is possible that the hand-sections swell somewhat on mounting in water. The pore canals are readily seen in hand-sections as refringent rods in all but the youngest larvae, and the two layers of the endocuticle are therefore distinguished with ease. Measurements of cuticle thickness have been extended to the puparium, but consideration of their significance will be deferred to a later paper.

The results of measurements of cuticle thickness are shown in figure 3. The cuticle increases steadily in thickness during the first 1½ days of the third instar, and during this period the cytoplasm of the pore canals is progressively replaced by chitin. At about the third day, when the canals are fully chitinized, secretion of the inner endocuticle begins, and at the same time the outer endocuticle begins to increase rapidly in thickness. As a result the total thickness of the cuticle increases enormously between the third and fourth days of larval life. By the fourth day the outer endocuticle has reached its full thickness of about 140μ, but the inner endocuticle continues to grow and reaches a thickness of about 70μ immediately before the formation of the puparium. In figure 3 the point B indicates the thickness which would have been attained after 4 days if the cuticle had continued to thicken at its former rate: actually by this time it reaches a thickness of no less than about 180μ.

Observations have therefore been made on the increase in weight of the cuticle during larval life, and have been conveniently combined with quantitative estimates of the major constituents of the cuticle. The methods used have been those which Fraenkel & Rudall (1940) applied to a comparison of larval with pupal cuticles,
with the important exception that a different method of preparing the cuticles in the first place has been employed. Fraenkel & Rudall report considerable amounts of water-soluble material, chiefly protein, in the cuticle, and it was felt that their method of preparation, involving heating the larvae in water to 65°C and scraping the cuticles clean under water, might not only remove some of these materials but perhaps result in a changed water content. Larvae were therefore opened without being previously killed and the cuticles scraped clean under medicinal liquid paraffin. The paraffin did not obviously penetrate the cuticles and appeared to be completely removed when they were firmly pressed between filter papers before being weighed and dried. It is noteworthy that cuticles prepared in this way show a lower wet weight and therefore a lower water content than do cuticles cleaned under water. The dry weights of cuticles prepared by the two methods show no significant difference if the water-cleaned cuticles are immersed for only a short period, and it therefore appears that no paraffin is absorbed on the one hand, but that water is absorbed on the other hand.

Cuticles prepared under liquid paraffin consistently show a water content close to 55%, whereas Fraenkel & Rudall report a water content of about 70%. It is clear that in the estimation of the water content the results obtained vary with the methods adopted for the preparation of the cuticles. The chitin content of paraffin-prepared cuticles approximates to 20% of the wet weight, or about 42% of the dry weight. Fraenkel & Rudall (1940) originally recorded a chitin content of 60% of the dry weight in cuticles of *Sarcophaga* larvae, but now, in a private communication, they state that recent work with improved methods shows that chitin amounts to 52% of the dry weight (equivalent to 15.6% of the wet weight). Chitin contents of other cuticles have been recorded by Campbell (1929) for the cockroach *Periplaneta americana* (22–60% in different regions of the body), Kuwana (1933) for the silkworm (10–20% in exuviae), and Tauber (1934) for the cockroach *P. fuliginosa* (18–37%).

The primary object of the present study, however, is not to obtain information on the constituents of the cuticle but to throw light on the mode of increase in its weight during the period occupied by its rapid increase in thickness. From the foregoing it is evident that the quantitative study of cuticular constituents presents difficulties, and it is claimed only that the methods adopted in the present work give reproducible results and indicate the trend of cuticle growth during the period under review. With this in mind, a detailed quantitative comparison of the results of thickness and weight measurements will not be undertaken.

Figure 4a illustrates typically the mode of increase in weight of the cuticle of *Sarcophaga* during larval growth. Both wet and dry weights rise smoothly with some approach to a logarithmic mode of increase, and no sharp deviation from this mode capable of explaining the sudden expansion of the cuticle is apparent in the period 3–4 days. There is clearly no significant change in the water content, nor in the content of chitin and protein, in this period, and the possibility that the cuticle suddenly expands as a result of absorption of water is therefore excluded.
It necessarily appears that new cuticular substances in constant proportion must be added not only to the inner endocuticle, but to the outer endocuticle also. Owing to the separation of the outer endocuticle from the epidermis by the growing inner endocuticle these substances must pass though the inner endocuticle in soluble form. It is interesting to note that Trim (1941) has suggested that chitin may crystallize from a homogeneous fluid polysaccharide-protein complex secreted by the hypodermis.

![Figure 4](http://rspb.royalsocietypublishing.org/)  

**Figure 4.**  

*a.* The growth in weight of the larval cuticle.  

*b.* The increase in volume of the larval cuticle.

In view of the significance of these findings further observations have been made on cuticles scraped clean under water. These showed a lower dry weight, and an apparent water content of 70%, agreeing with the figure given by Fraenkel & Rudall, but again no deviation from the progressive mode of increase represented in figure 4a was noted.

The information resulting from a study of the changes in cuticle thickness on the one hand, and of weight increase on the other, remain irreconcilable until the growth of the cuticle is considered in relation to the growth of the larva as a whole.
Then the significance of the sudden increase in cuticle thickness becomes apparent, and it also becomes possible to suggest the cause of the helicoidal form of the pore canals.

As indicated in figure 3 the larva grows rapidly, and reaches its full size at about the end of the third day. During this rapid growth the crop becomes fully distended with food. No further change in size of the larva takes place until the pupal contraction, but during the later part of larval life the crop is progressively emptied.

During growth in length of the larva the area of the cuticle is greatly enlarged and little margin of cuticle secretion is available for increase in its thickness. When growth in length ceases, however, all secretion of cuticle is directed towards increase in thickness, and the full effect of the secretion of new cuticle is seen in sections as a sudden expansion of the cuticle which begins at the end of the third day. It is not necessary, therefore, to postulate any violent change in rate of cuticle secretion to account for this expansion, and as already shown none takes place.

Some reflexion of the rate of volume increase of the cuticle may be obtained by taking into account the length of the larva and the thickness of its cuticle at intervals during growth. In figure 4b is indicated the rate of volume increase expressed in arbitrary units obtained by multiplying the length of the larva by the thickness of its cuticle. The resulting product is, in the case of the youngest larva examined, expressed as unity, and later products proportionately. It is true that figures obtained in this way do not take into account the precise shape and proportions of the larva, and that they do not therefore give a strictly accurate indication of the volume of the cuticle, but they do serve to some extent as confirmation of the results of examination of the rate of increase in weight of the cuticle. As is to be expected, the curve expressing volume increase closely resembles that for weight increase, and again no sudden deviation is noted.

It is possible that the great distension of the crop during early life is the prime cause of the extremely rapid growth of the larva, and that during growth the cuticle may undergo mechanical stretching. Certainly a growing larva is much more turgid than an older one in which the crop is emptying, and the results of measurements on the pore canals indicate that stretching does occur. In larvae less than 2 days old the canals were noted, in longitudinal sections, to have an overall breadth at their branched distal ends of about 2μ. In larvae 3 days old the branched ends of the canals occupy about 5μ, indicating a stretching of the cuticle of the order of 250%. During this period the larvae actually increased in length from 9 to 20 mm., an increase of 220%.

The growing endocuticle therefore appears to be laid down under pressure from within transmitted by the body fluid. This pressure is not manifested in a reduction in thickness of the cuticle, for secretion takes place at such a rate that in spite of compression the cuticle continuously increases in thickness. The growth of the endocuticle and pore canals may therefore be pictured as taking place in the...
following manner. As new cuticular substance is added to the endocuticle it becomes compressed, and the cytoplasmic filaments of the pore canals, although undergoing an overall extension due to the thickening of the endocuticle, are necessarily involved in this compression and assume a helicoidal form. It is striking that at just that period (2 days, figure 3 A) when growth of the larva is proceeding most rapidly, and when the compression of the cuticle is presumably at its maximum, those parts of the filaments destined to form the coiled middle portions are still unchitinized. The uncoiled distal portions are chitinized early, before marked compression begins, and the uncoiled proximal portions are chitinized as growth in length is completed and compression ceases.

The expansion of the endocuticle when larval growth ceases is reflected in the great extension of the coiled strands of chitin filling the pore canals, which become pulled out like stretched springs. In one series of observations a larva about 3 days old, in which the outer endocuticle was about 50 μ thick, showed a pitch of 2–3 μ in the coiling of the canals. A later larva of the same brood, about 4 days old, had an outer endocuticle of about 150 μ, and its pore canals appeared sinuous, being actually extended coils with a pitch of 8–9 μ.

It should be emphasized that the growth of the cuticle as the crop is emptied after the third day does not appear to involve the expansion of previously compressed layers merely due to the relaxation of the pressure of the body fluid. In preparing cuticles for sectioning the body fluid is released when the larvae are cut open, but sections such as that illustrated in plate 8, figure 7 still show the coiled form of the pore canals.

After expansion the laminae of the outer endocuticle show no obvious difference in spacing from that seen earlier. They are therefore more numerous, and as they cannot be added to by the addition of layers at the surface of the epidermis, such as would be produced by rhythmic secretion, it seems probable that, as suggested by Richards & Anderson (1942), they really do represent distinct structural components of the cuticle, being perhaps chitin-protein polymers separated by layers of pure chitin. But it should be noted that whereas Richards & Anderson found that the laminae of the cockroach cuticle disappeared after treatment with alkali, those of Sarcophaga, even after the violent treatment demanded by the chitosan test, remain still apparent and are visible in the original photograph represented in plate 8, figure 7.

In conclusion, therefore, it seems that during early growth the outer endocuticle may first be added to by accretion. Its later expansion, after it has become separated from the hypodermis by the formation of the inner endocuticle, appears, on the other hand, to be due to growth by intussusception. There is no obstacle to the growth of the inner endocuticle by accretion.

The growth of the cuticle of the larva of Sarcophaga differs from that of the silkworm as reported by Kuwana (1933). In the latter a period of growth at the beginning of the fifth instar is separated from later growth by a comparatively quiescent period.
A study of an insect cuticle

No mitoses have been observed in the epidermis during the rapid growth of the larval cuticle of Sarcophaga. Trager (1935) found that during the growth of larvae of Lucilia sericata the epidermis increased in area not by cell division but by an increase in cell size.

VI. The chemical composition of the cuticle

Some of the staining reactions of the cuticle have already been noted in discussing the pore canals, and Mallory's triple stain has been referred to as providing a valuable means of differentiating between the epicuticle and the underlying endocuticle. With this stain the resemblance of the cuticle to that lining the foregut of the lobster (Yonge, 1932) is very marked, the epicuticle apparently corresponding to the 'cuticle', and the endocuticle to the 'chitin' of Homarus. As may be seen from table 1 this correspondence is emphasized when other stains are used, and is largely to be attributed to the fact that the isoelectric points of the constituent layers of the cuticle of Sarcophaga agree closely with those obtaining in Homarus. By using the methods employed by Yonge (1932) the isoelectric point of the epicuticle was found to lie at pH 5·3, and that of the endocuticle at pH 3·4. Whereas, however, the outer endocuticle consistently showed its isoelectric point at pH 3·4, that of the inner endocuticle proved to be somewhat variable, ranging in different parts of the same cuticle from pH 3·4 to as high as pH 4·3. As in Homarus, the differing isoelectric points will be seen to be a reflexion of the differing chemical constitution of the layers of the cuticle.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Homarus</th>
<th>Sarcophaga</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>epicuticle</td>
<td>endocuticle</td>
</tr>
<tr>
<td>safranin and light green</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Delafield's haematoxylin</td>
<td>blue</td>
<td>green</td>
</tr>
<tr>
<td>thionin</td>
<td>blue</td>
<td>almost</td>
</tr>
<tr>
<td>Mallory's triple stain</td>
<td>red</td>
<td>light blue</td>
</tr>
<tr>
<td>isoelectric point</td>
<td>5·1</td>
<td>3·5</td>
</tr>
<tr>
<td></td>
<td>5·3</td>
<td>3·4</td>
</tr>
</tbody>
</table>

Few observations on the isoelectric points of the layers of the Arthropod cuticles are available. Thomas (1944) describes the chitin and cuticle of Lepas anatifera as having respectively isoelectric points of pH 5·0 and 3·4–5, but it is evident that in the presentation of his results transposition of these values has occurred, for he remarks emphatically that his figures agree with those found by Yonge (1932) for Homarus. In a private communication Professor Yonge states that he agrees that the figures for Lepas are wrongly quoted. Browning (1942) describes the isoelectric point of the endocuticle of Tegenaria as lying between pH 5·0 and 5·6, but owing to its pigmentation he was not able to determine that of the exocuticle. In Tegenaria the endocuticle, in spite of its high isoelectric
point as compared with the chitin of *Homarus* and the endocuticle of *Sarcophaga*, still stains blue in Mallory’s triple stain.

In its possession of a thick epicuticle, then, staining red with Mallory but in general showing an avidity for basic dyes, the larva of *Sarcophaga* appears to agree more closely with *Homarus*, and perhaps with Crustacea in general, than it does with an insect such as *Rhodnius*, the epicuticle of which is stated to be less than 1 μ thick, not to stain with basic dyes or with lipoid stains, and to be of an amber colour (Wigglesworth 1933). But as will be seen from the results of chemical tests on the cuticle of *Sarcophaga* the epicuticle actually is a double layer, the outer being thin and generally resembling the epicuticle as described for *Rhodnius*, while it is the much thicker inner layer that resembles the ‘cuticle’ of *Homarus*. Pryor (1940b) affirms that the epicuticle of insects has much in common with the cuticle of Crustacea, but it does not appear that he recognized that the larvae of *Calliphora* and *Sarcophaga* possess a double epicuticle. It is probable that the available accounts of both the insect and the crustacean cuticle are incomplete with regard to the epicuticle, and that the cuticles of these two classes of Arthropods may resemble each other in fundamental structure even more closely than has been previously recognized.

The results of chemical tests on sections and pieces of cuticle (summarized in table 2) abundantly confirm the impression given by the different staining reactions and isolectric points, that the two layers of the cuticle are of different chemical constitution. They clearly reveal also that the epicuticle is actually a double layer.

### Table 2

<table>
<thead>
<tr>
<th>Test</th>
<th>outer epicuticle</th>
<th>inner epicuticle</th>
<th>outer endocuticle</th>
<th>inner endocuticle</th>
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</thead>
<tbody>
<tr>
<td>KOH saturated solution</td>
<td>remains</td>
<td>dissolves</td>
<td>remains</td>
<td>remains</td>
</tr>
<tr>
<td>HCl concentrated</td>
<td>remains</td>
<td>slowly dissolves</td>
<td>rapidly dissolves</td>
<td>dissolves</td>
</tr>
<tr>
<td>KOH 5% solution</td>
<td>remains</td>
<td>slowly dissolves</td>
<td>remains</td>
<td>remains</td>
</tr>
<tr>
<td>HCl 5% solution</td>
<td>remains</td>
<td>dissolves</td>
<td>slowly dissolves</td>
<td>slowly dissolves</td>
</tr>
<tr>
<td>xanthoproteic reaction</td>
<td>+</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>biuret reaction</td>
<td>-</td>
<td>+ + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Millon’s reagent</td>
<td>-</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>ninhydrin</td>
<td>-</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Mörner’s reagent</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>aldehyde reaction</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td>chitosan test (Campbell)</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td>chitin test (Schultze)</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>+ + +</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Schultz’s reaction</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>argentaffin reaction</td>
<td>+ + +</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ferric chloride test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>thionin</td>
<td>+</td>
<td>+</td>
<td>only in spines</td>
<td>-</td>
</tr>
<tr>
<td>methylene blue</td>
<td>+</td>
<td>+ +</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
A study of an insect cuticle

In their differential solubility in acids and alkalis the layers of the cuticle agree with those of Homarus (Yonge 1932). In boiling saturated potash solution the thick epicuticle dissolves, but the endocuticle remains, while in cold concentrated HCl the epicuticle is more resistant than the endocuticle. These differences are chiefly due to the distribution of protein and chitin in the cuticle. The same general results are given when more dilute solutions are used, but the greater resistance of the epicuticle seen with concentrated acid is not apparent with a dilute acid solution, the epicuticle actually dissolving more rapidly than the endocuticle.

The epicuticle

Valuable information has been obtained by subjecting pieces of cuticle to the action of dilute acids and alkalis, and after varying periods of treatment sectioning and staining them in Mallory. After treatment with dilute (5%) HCl at 95°C for 24 hr. the endocuticle was considerably macerated but still stained blue. The epicuticle was represented only by an exceedingly thin membrane detached from the remainder of the cuticle, and also staining blue. Cuticles treated similarly but for a shorter period had a similar general appearance, but although the thin blue membrane was present as before it was attached in places to the endocuticle by the remains of the red-staining epicuticle. It was at once apparent that what had until now been regarded as a single thick epicuticle possessed in fact a thin surface layer of different constitution. The thin outer layer of the epicuticle also resists treatment with potash solution, whether dilute or concentrated, and persists after the thicker inner epicuticle has disappeared.

Re-examination of the thin serial paraffin sections referred to in describing the pore canals revealed the outer layer of the epicuticle as a sharply defined very thin line forming the outer border of the main mass of the epicuticle (figures 1, 2, ep. 1 and ep. 2). The two epicuticular layers differ in staining reaction and are therefore quite distinct. In Mallory-stained preparations the outer layer is blue and the inner thick layer red; with safranin and light green the outer is a clear green and the inner red; and with thionin the outer layer stains deeply, but the inner only feebly or not at all except in the spines of the cuticle. It therefore seems justifiable to refer to the layers as the inner and outer epicuticles, and this procedure is supported, as will be seen later, by the results of chemical tests on the cuticle.

In no sections of normally fixed and treated cuticles was any separation of the two epicuticles encountered, but sometimes the epicuticle as a whole was detached from the endocuticle as a result of mechanical damage to the section, which then presented an appearance similar to that illustrated by Yonge (1932, figure 7). As in Homarus it presumably indicates that the epicuticle is in a state of tension. The outer epicuticle is considerably less than 1 µ thick, whereas the inner extends to about 4 µ.

In frozen, hand or paraffin sections stained with Sudan black B (see Lison 1936) the outer epicuticle stains deeply, but the inner becomes at most merely grey. This indication of the presence of lipoid substances in the outer epicuticle is
confirmed by application of Shultze’s modification of the Liebermann-Burchardt
reaction for cholesterol (Lison 1936). The outer epicuticle alone gives a positive
reaction. The persistence of the outer epicuticle in cuticles treated with hot con-
centrated potash solution is perhaps therefore to be explained by the presence in
it of unsaponifiable sterols, as well as by the oxidation and polymerization of its
lipoids (see later).

The destruction of all layers of the cuticle but the outer epicuticle by cold
concentrated HCl affords a ready means of isolating this layer. It remains as a
thin transparent membrane, and in spite of the presence of lipoids in it is not
dissolved by cold ethyl alcohol, ether, or chloroform. After these solvents it still
stains readily with Sudan black. It is therefore not surprising to find that
in paraffin sections, the preparation of which involves treatment with lipoid
solvents, the outer epicuticle is prominently stained with Sudan black or to
a less extent with the less effective Sudan III. The outer epicuticle of Calliphora
stains even more heavily than that of Sarcophaga, and appears to be rather
thicker.

Pryor (1940b) pictures the epicuticle, which he regards as a single layer, as
consisting of a tanned protein membrane impregnated with oxidized and polymerized
lipoids. Richards & Anderson (1942) have shown that the epicuticle of the cock-
roach is a double structure, the outer layer being thinner and more resistant to
treatment with acids than the inner. They suggest that an originally single protein
epicuticle has become differentiated into ‘a thin outer layer of polymerized lipo-
tanned protein and a thicker underlying layer of tanned protein without lipid’.
Kühnelt (1928a) detected the presence of cholesterol in the epicuticle and noted
the staining of this layer with Sudan III, but it may be recalled that over a century
ago Odier (1823) had recognized the presence of a superficial layer containing
fat-soluble material. Chibnall, Piper, Pollard, Williams & Sahai (1934) have
described the constitution of the primary alcohols, fatty acids and paraffins of
insect waxes, and Bergmann (1938) has identified in ether extracts of silkworm
exuviae the presence of two major components, first, a mixture of paraffins of the
order C_{27}–C_{31}, and secondly, a mixture of esters of n-alcohols and acids of the
probable order C_{25}–C_{30}. Cholesterol esters of fatty acids were recognized. Yonge
(1932) demonstrated the presence of lipoids in the cuticle of Homarus, and later
(1936) showed their importance in controlling the permeability of the cuticle.
Alexander et al. (1944a, b) have given valuable evidence, supporting the view of
Kühnelt (1928a), that the lipid epicuticle renders the insect cuticle largely
impermeable to water, and have shown experimentally the effect of a thin film of
beeswax in reducing the permeability to water of a thin celluloid sheet. Ramsay
(1935) had already shown the importance of an external film of fatty substance
in reducing the evaporation of water from the surface of the cuticle of Periplaneta.
Wigglesworth (1942, 1944a, b) refers to a layer of wax at the surface of, but outside,
the insect epicuticle. He does not, however, describe the constitution of the
epicuticle. Earlier (1933) he had described it in Rhodnius as being less than 1μ
thick, containing neither protein nor chitin, insoluble in fat solvents, and capable of being isolated by treatment with concentrated HNO₃. This description would appear to refer to a thin layer resembling the outer epicuticle of *Sarcophaga*. A thicker inner layer, if indeed present in *Rhodnius*, might well be masked, as it is in the puparium of *Sarcophaga*, by the formation of an exocuticle, or not revealed by the staining methods employed.

From the foregoing, then, it appears that lipoid substances may occur variously in the insect cuticle. They may form a relatively labile external layer of wax at the surface of the cuticle, they may be closely bound to the surface of the epicuticle so giving rise to a stable thin outer epicuticle, and they may impregnate the inner epicuticle when this layer is tanned. In the larva of *Sarcophaga* there is no appreciable external layer of wax, and the inner epicuticle, which is virtually untanned, is without lipoids. Hurst (1943d), as the result of work on the mode of insecticide penetration, draws a distinction between the ‘free’ and ‘bound’ lipoids of the cuticle, stating that ‘the protein and lipoid components of the insect cuticle are heterogeneous visco-elastic systems comprising chemically linked networks enclosing the more mobile or labile components which may be removed by solvent action’. This description would appear to apply to an insect in which the thick epicuticle is tanned and impregnated with lipoids.

In work on the hardening and darkening of the cuticle evidence pointing to the wide occurrence in insects of an epicuticle consisting of two layers was encountered. Puparia of *Sarcophaga* are completely softened and almost completely bleached by prolonged treatment with cold diaphanol. Mallory-stained sections of treated puparia completely resemble those of the larval cuticle in showing no exocuticle and in revealing a thin outer epicuticle and a thicker inner epicuticle staining with acid fuchsin. Sections of untreated puparia, on the other hand, show no sign of the epicuticular layers, and the amber-coloured exocuticle is unstained. Beneath the exocuticle the endocuticle stains with aniline blue, and between these layers occurs a red-staining zone which lies far within the cuticle and is not to be confused with the inner epicuticle. From a comparison of these sections it is clear that in hardening of the cuticle the protein of the inner epicuticle becomes heavily tanned and indistinguishable from the exocuticle; indeed, as will be described in a later paper, it is in the inner epicuticle that hardening of the cuticle commences owing to the presence of a phenol-oxidase. It is therefore to be suspected that hard cuticles generally may well possess a double epicuticle like that of *Sarcophaga*. Richards & Anderson (1942), it will be remembered, have already demonstrated such an epicuticle in the hard cuticle of the cockroach.

Terga and elytra of *Blaps mucronata*, *Tenebrio molitor*, *T. obscurus*, and *Dermestes vulpinus* have therefore been softened in diaphanol, sectioned, and stained in Mallory. All show, like the softened puparium of *Sarcophaga*, a thick red-staining inner epicuticle, but although a thin outer epicuticle appears also to be present it was not seen with the same clarity as in the much thicker cuticle of *Sarcophaga*. It seems possible therefore that the thin layer designated epicuticle by previous...
authors other than Pryor (1940b) and Richards & Anderson (1942) is actually the outer epicuticle.

Pryor (1940b) supports his view that the epicuticle consists basically of tanned protein by pointing to the fact that various tests indicate the presence of aromatic substances in this layer in larvae of Calliphora, Ephestia and Tenebrio. In his illustration (plate 17, figure 4) of the result of the argentaffin test on the larval cuticle of Calliphora the layer giving a positive reaction can only, on account of its thickness, be the inner epicuticle. The argentaffin test has been repeated on Calliphora in the present work, and the result obtained by Pryor confirmed, but the application of the test to Sarcophaga has yielded a strong positive result only in the outer epicuticle, the inner showing but little sign of blackening. The view that the inner epicuticle is tanned to a less extent than in Calliphora is confirmed by observations incidental to the performance of the xanthoproteic reaction on these cuticles. The action of concentrated HNO₃ on the cuticle of Sarcophaga produces a very slight dull green coloration in the inner epicuticle, but in Calliphora this layer becomes bright green. The colours are suggestive of those caused by the action of concentrated HNO₃ on catechol, and may well be due to the presence of an aromatic substance in the epicuticle. It should be noted, however, that in spite of these indications of the presence of aromatic substances, at no time before pupation does the ferric chloride test for dihydroxyphenols give positive results. The differences between the epicuticles of Sarcophaga and Calliphora will be referred to further in a later paper describing the hardening of the cuticle.

With reference to the nomenclature to be applied to the layers of the epicuticle, the outer epicuticle, rich in lipoids, may be referred to as the lipid epicuticle, and the inner, fundamentally a layer of protein, as the protein epicuticle. Even in those cuticles in which the inner epicuticle is secondarily hardened and impregnated with lipoids the terms may justifiably be employed, for it is likely that the manner of association of lipoids with the two layers differs fundamentally. Richards & Anderson (1942) have proposed the term ‘mediocuticle’ for the inner layer of the epicuticle.

Proteins

In examining the constitution of the larval cuticle of Sarcophaga, colour reactions indicative of the presence of proteins have been carried out either on pieces of cuticle which were later sectioned, or directly on hand-sections. The general results of these tests are expressed in table 2, and do not call for detailed comment. The positive response of the inner epicuticle to the xanthoproteic and biuret tests, and to the application of ninhydrin and Millon’s reagent, is to be particularly noted.

The use of Mörner’s reagent and the aldehyde reaction (Cole 1933) indicates the presence of tyrosine and tryptophane respectively in the outer endocuticle of the fully formed cuticle. These substances have not been recognized in other layers of the cuticle, and their presence here will be shown in a later paper to be directly related to the impending formation of a hard and dark exocuticle at pupation.
Similar results have been given by tests on the larval cuticle of Calliphora erythrocephala, but here tyrosine and tryptophane were noted also in the inner epicuticle of larvae with full crops. Older larvae which have emptied their crops show neither of these substances in the epicuticle. It is likely that just as tyrosine and tryptophane accumulate in the outer endocuticle of Sarcophaga prior to its conversion to exocuticle, they accumulate also in the inner epicuticle of Calliphora as a preliminary to the tanning of this layer which, as indicated also by the argentaffin test, takes place not at pupation but earlier. The role of tyrosine and tryptophane in hardening and darkening of the cuticle will not be discussed here, but it may be mentioned that Trim (1941) has recorded a decrease in the amount of tyrosine in the cuticles of a brood of Sarcophaga larvae on pupation.

The fact that the inner epicuticle of Sarcophaga gives a strong positive reaction with Millon’s reagent but responds neither to Mörner’s test nor to the aldehyde reaction, is perhaps due to the presence of aromatic substances responsible for the very slight tanning undergone by this layer.

Chitin

The chitin tests of Campbell (1929) and Schulze (1922, 1924) yield positive results in both layers of the endocuticle, but chitin is completely absent from the epicuticular layers (table 2). As described earlier these tests have revealed that the contents of the fully formed pore canals are composed of chitin. The differential solubility of the layers of the cuticle in acid and alkaline solutions is at least partially accounted for by the insolubility of chitin in alkaline solutions.

Although an exocuticle is lacking from the general extent of the soft white larval cuticle, the blunt spines possess hard tips of a pale amber colour. They are formed by the tanning of the epicuticular protein as noted by Pryor (1940b) in Calliphora, and unlike the remainder of the inner epicuticle give a negative result to the ninhydrin test. Schultz’s reaction for cholesterol, however, gives a stronger positive result than elsewhere, and they stain with Sudan black B and with thionin. It is interesting to note that these characteristics are shared with the outer epicuticle which appears also to consist basically of tanned protein.

In the spines of the cuticle and in the outer epicuticle is seen a close relation between tanning of the epicuticular protein and its association with lipoid substances. Pryor (1940b) has pointed out that the addition of aromatic groups is calculated to make the cuticle more lipophilic, but states that the exocuticle of the puparium of Calliphora does not contain lipoid substances. Wigglesworth (1942), however, suggests that lipoids may sometimes extend far into the exocuticle, and may perhaps occur in the endocuticle also. The presence of lipoids has not been recognized in the endocuticle of Sarcophaga larvae.

In concluding this account of the larval cuticle of Sarcophaga the structural and chemical differences between the inner and outer layers of the endocuticle may be summarized and emphasized. The outer layer possesses pore canals, and in the late larva is rich in tyrosine and tryptophane. It stains readily with methylene
blue. The inner layer possesses none of these qualities, and shows a less constant isoelectric point than the outer layer. It will be shown in a later paper dealing with the formation of the puparium that these differences are largely attributable to the fact that the hardening and darkening of the cuticle involves only the outer endocuticle, the inner remaining completely unchanged.

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Explanation of Plate 8

**Figure 5.** The cuticle of an early third instar larva (1⅓ days after deposition) of *Sarcophaga.* Flemming without acetic, in saline. Mallory. The pore canals are fully occupied with cytoplasm.

**Figure 6.** The larval cuticle at about 2⅓ days. Helly. Mallory. The distal portions of the filaments are now replaced by chitin.

**Figure 7.** The fully chitinized pore canals of the larval cuticle at about 3 days. KOH, iodine, and H₂SO₄.

ep. 2, inner epicuticle; end. 1, outer endocuticle; e, epidermis.

Figure 3 is reproduced with acknowledgements to the Editors of *Nature.*

Erratum

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Page 19, line 22. The acknowledgement should be to Mr H. A. Parker and not Mr H. M. Packer as printed.