AN ELECTRON DIFFRACTION STUDY OF THE CRYSTALLINE STRUCTURE OF THE LIPIDS IN THE PUPAL EXUVIAE OF CALLIPHORA ERYTHROCEPHALA

BY DR H. HURST, Department of Colloid Science, University of Cambridge

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(With Plates 4–6 and One Text-figure)

Electron diffraction techniques have been extensively used for the direct crystallographic examination of thin films of fatty materials, but they have hitherto not been applied to the study of natural membranes. These techniques provide more information than diffraction of X-rays about the atomic and molecular organization of surface structures. X-ray diffraction methods have been used successfully by Fraenkel & Rudall (1940, 1947) for elucidating the molecular organization of the chitin and protein components of the bulk cuticle framework in blowfly larvae; but the thin lipid-rich epicuticle constitutes a small proportion of the entire cuticle, and would not contribute effectively to the observed diffraction patterns.

Most membranes, including insect cuticle, are far too thick to permit penetration of the electron beam. The cuticle used as test material in the present paper is the thin membrane which covers the developing pupa of the blowfly larva, Calliphora erythrocephala. This pupal skin is relatively rich in waxy materials, which prevent excessive loss of water from the pupa in the same way as the waxy components exert a waterproofing action in more typical insect cuticles (Wigglesworth, 1945; Beament, 1945).

It is known that the waterproofing properties of the waxes of insect epicuticle are impaired at critical temperatures, which vary for different insects; and it has been suggested that each critical temperature corresponds to the transition point at which the molecules of crystalline wax become more mobile and assume a hexagonal type of packing, which is associated with an increase in intermolecular space (Beament, 1945).

It is the object of the present paper to characterize the structure of the lipids in the Calliphora pupal skin by comparison with artificial collodion membranes containing waxy materials of known crystalline structure; and to compare the changes in crystalline structure which occur when the membranes are exposed to temperatures at which the original crystalline organization breaks down.

MATERIAL AND METHODS

Collodion-wax membranes

The stock solution used was a 1% solution of collodion in amyl acetate; the fatty materials were dissolved in different samples of the stock solution in concentrations
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of c. 3–5%. The best results were obtained when the solutions were almost saturated. The waxy materials consisted of \( n \)-fatty acids, unsaturated fatty acids, and \( n \)-paraffins.

The technique used for the formation of the membranes was essentially similar to that which is in general use for the preparation of thin collodion supporting bases for the examination of specimens in the electron microscope. A drop of collodion solution spreads rapidly on a clean water surface to form a thin lens; after the solvent has evaporated a thin collodion membrane (c. 200 A. thick) is left floating on the surface.

If a long chain capillary active substance, such as a fatty acid, is present in the collodion solution, adsorption and orientation of a monolayer of fatty acid occurs at the amyl acetate-water interface during spreading and lens formation. Within the bulk phase of the lens, the fatty acid molecules are distributed at random, since there is no interface to attract the polar groups of the molecules (Text-fig. 1a). As the solvent evaporates, crystallization of the fatty acid occurs within the lens. At the same time the collodion membrane is laid down and acts as a support for the fatty acid crystals.

When the waxy component in the membrane-forming solution is a long chain paraffin, no monolayer of paraffin molecules is formed at the amyl acetate-water interface (Text-fig. 1d), for the molecules of paraffin are non-polar, but crystallization can occur in the bulk phase of the lens during membrane formation.

If there is a random orientation of fatty acid, or paraffin crystals in a collodion membrane, a relatively weak diffraction pattern will be obtained by transmission of the electron beam. The diffraction pattern will be more intense if the crystals show a common alignment of the molecular axes in relation to the basal plane of the membrane. The single layer of fatty acid molecules adsorbed at the amyl acetate-water interface during membrane formation will not contribute effectively to the diffraction of the electron beam, for the main scattering points are located in the thicker framework of the membrane. The purpose of comparing the diffraction patterns of collodion-fatty acid and collodion-paraffin membranes is to decide whether the orientation of the crystals in the bulk membrane frameworks is influenced by the original fatty acid monolayer. If the hydrocarbon chains of the molecules in the monolayer provide a basis on which the crystals in the membrane are deposited, it would be expected that a more intense pattern should be given by the collodion-fatty acid than by the collodion-paraffin membrane. This difference should disappear after the membranes have been exposed to high electron beam currents when the crystals melt or undergo transformation to a polymorphic crystalline modification. When the specimens are re-examined at the original beam currents, there will be no mobile fatty acid monolayer at the surface of the collodion-fatty acid membrane, and crystallization should occur at random in the collodion framework, as with the collodion-paraffin membrane (Text-fig. 1b, c). If this takes place, there will be an irreversible fading of the diffraction patterns from the collodion-fatty acid membranes, which will be independent of any change in crystalline structure; but the original random orientation of paraffin crystals in the collodion membrane should give a
pattern which is substantially unchanged in intensity after melting and recrystal-
lation has occurred (Text-fig. 1e).

Text-fig. 1. Stages in artificial membrane formation. (a) Lens formation by drop of collodion
solution spread on water surface, showing adsorbed monolayer of fatty acid at amyl acetate-water
interface, and random distribution of fatty acid molecules in bulk phase of lens. (b) Distribution
of fatty acid crystals in supporting collodion membrane after evaporation of amyl acetate. The
molecular axes of the crystals are normal to the basal plane of the membrane. (c) Random
distribution of fatty acid crystals in membrane after thermal disorientation at high electron beam
currents. (d) Lens formation by drop of collodion solution spread on water surface, showing
random distribution of paraffin molecules in bulk phase of lens. (e) Random distribution of
paraffin crystals in membrane after evaporation of amyl acetate.

Calliphora pupal cuticle

The thin pupal exuviae of Calliphora vary in thickness from 1·5 to 3·0 μ. Beament
(1945) has calculated that the fat soluble waxes extracted by treatment in boiling
chloroform can form a superficial layer constituting 5·8 % of the thickness of the
pupal skin. The skins were obtained from the puparia after the adults had emerged.
The outer surface of each skin is hydrophobic, but the inner surface is hydrophilic.
 Portions of the membranes were floated on water with the hydrophilic surface in
contact with the water. They were mounted on circular grids adapted for use with
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the R.C.A. electron microscope and diffraction unit. A similar technique was used with the collodion-wax membranes.

The changes in diffraction patterns given by the pupal skins were observed after: (i) heating at high electron beam currents, (ii) extraction of the fat soluble waxes by chloroform, and (iii) disruption of the lipo-protein organization of the epicuticle by treatment in strong alkali.

Calculation of side spacings corresponding to Debye-Scherrer rings in diffraction patterns

Gold was volatilized in vacuo and deposited in a micro-crystalline film on to collodion bases mounted in grids. The crystals were of the face-centred cubic type \((a = 4.07 \text{ Å})\), and were orientated at random on the collodion bases. Each grid was inserted into the diffraction apparatus so that the gold film faced downwards. Photographs of the diffraction patterns were taken at constant voltage \((50 \text{ kV})\) across the electron gun, using a beam current of 200 \(\mu\text{A}\). The side spacings represented by the Debye-Scherrer rings are inversely proportional to the ring diameters (Bragg’s Law), the distance from the photographic plate to the specimen also remaining constant. For the four inner rings in a typical transmission pattern (Pl. 4, fig. 1), the side spacings may be calculated from the relationships in Table 1.

Table 1. Calculation of spacing of planes from electron diffraction transmission pattern of micro-crystalline gold film deposited on collodion base. The crystals are of the face-centred cubic type \((a = 4.07 \text{ Å})\)

<table>
<thead>
<tr>
<th>Laue indices ((hkl))</th>
<th>Relative ring diameters (\sqrt{(h^2+k^2+l^2)})</th>
<th>Spacing of planes ((\text{Å})) (d/\sqrt{(h^2+k^2+l^2)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>(\sqrt{3})</td>
<td>2.348</td>
</tr>
<tr>
<td>200</td>
<td>(\sqrt{4})</td>
<td>2.035</td>
</tr>
<tr>
<td>220</td>
<td>(\sqrt{8})</td>
<td>1.441</td>
</tr>
<tr>
<td>311</td>
<td>(\sqrt{11})</td>
<td>1.225</td>
</tr>
</tbody>
</table>

In order to calculate the side spacings corresponding to the rings in the diffraction patterns from the collodion-wax membranes and insect cuticle, any particular ring in the gold diffraction pattern may be selected as a standard. The diameter of this ring is compared with those of the ‘unknown’ patterns, and the side spacings calculated in a manner similar to that used for the rings in the gold diffraction pattern.

It was observed that a slight distortion of the rings in the patterns sometimes occurred, owing to partial ‘buckling’ of the membranes on the grids. This introduced an experimental error in the measurement of ring diameters on the photographs, but was not of significance, since the crystalline structure of the fatty components could be characterized by relative ring diameters measured in the zones where the distortion was at a minimum.
RESULTS

Collodion-fatty acid membranes

Preliminary tests showed that the diffraction patterns faded rapidly at high beam currents (50 kV., 400 μA.) owing to excessive heating and melting of the waxy components in the electron beam. This fading was irreversible, but clearer patterns were obtained at lower beam currents (100-200 μA.) which could be photographed.

Myristic acid (C₁₄) gave a faint ring pattern, which faded rapidly even at low beam currents, but sharp patterns were obtained with the unsaturated elaidic (C₁₈) and brassidic (C₂₂) acids (Pl. 5, figs. 3, 5). With increase in chain length of the fatty acids, the diffraction patterns were more persistent to prolonged exposure to the electron beam. Octacosanoic acid (C₂₈) gave a pattern of continuous rings, but with the higher homologue, tetacontanoic acid (C₃₄) the rings were divided into discrete spots, showing that the membrane contained relatively larger and more isolated crystals (Pl. 5, figs. 1, 2).

Collodion-paraffin membranes

Somewhat irregular rings made up of isolated spots were apparent in the diffraction patterns of dotriacontane (C₃₂) and hexatriacontane (C₃₆) (Pl. 5, figs. 6, 7).

Crystalline structure

Brummage (1947) observed that the spacings given by thin films of the n-paraffins, tetracosane (C₂₄), triacontane (C₃₀), and tetacontane (C₃₄) agreed with the calculated spacings from an orthorhombic crystal structure with lattice constants \( a = 7.45 \text{ A.}, \ b = 4.97 \text{ A.}, \ c = \text{twice the length of the molecule (Müller, 1928).} \) For crystals orientated in the \((hko)\) plane, the ring dimensions are given by the relationship

\[
\rho = \lambda L \sqrt{\frac{h^2}{a^2} + \frac{k^2}{b^2}},
\]

where \( \rho \) is the ring radius, \( \lambda \) the wave-length of the electron beam, \( L \) the distance between the specimen and the photographic plate, and \( h \) and \( k \) are Laue indices relating to the \( a \) and \( b \) axes, respectively. Since \( \lambda \) and \( L \) are constants, the ring radii are proportional to \( \sqrt{[(h^2/a^2) + (k^2/b^2)]} \); the spacings represented by the rings can be calculated theoretically by substituting the appropriate values for \( h, k, a \) and \( b \) in the expression.

The relative ring dimensions in the diffraction patterns of the fatty acid and paraffin membranes are in agreement with the theoretical dimensions obtained from the above relationship; and the absolute dimensions, determined by comparison with the standard gold preparation, confirm the orthorhombic crystalline structure of the fatty materials in the collodion membranes. In Table 2, the theoretical values are based on an orthorhombic hydrocarbon chain lattice \((a = 7.45 \text{ A.}; \ b = 4.97 \text{ A.})\) adopted as a standard for comparison by Brummage (1947). The rings in the patterns
represent successive orders of diffraction by alternate carbon-carbon atom spacings of 2.54 Å in the zigzag hydrocarbon chains of the four molecules in the cross-section of the orthorhombic unit cell, when the molecular axes of the crystals are normal to the supporting membrane surface, but are otherwise distributed at random in the plane of the membrane.

Table 2. Relationships between calculated spacings for an orthorhombic hydrocarbon chain lattice ($a = 7.45$ Å; $b = 4.97$ Å) and observed spacings for fatty acids and n-paraffins

<table>
<thead>
<tr>
<th>Laue indices (hko)</th>
<th>Calculated</th>
<th>Elaidic acid (C_{18})</th>
<th>Brassidic acid (C_{18})</th>
<th>n-acid (C_{18})</th>
<th>n-acid (C_{18})</th>
<th>n-par. (C_{18})</th>
<th>n-par. (C_{18})</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.45</td>
<td>7.41</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>010</td>
<td>4.97</td>
<td>4.95</td>
<td>4.15</td>
<td>4.12</td>
<td>4.14</td>
<td>4.12</td>
<td>4.10</td>
</tr>
<tr>
<td>110</td>
<td>4.13</td>
<td>4.15</td>
<td>3.68</td>
<td>3.71</td>
<td>3.68</td>
<td>3.70</td>
<td>3.72</td>
</tr>
<tr>
<td>200</td>
<td>3.73</td>
<td>3.65</td>
<td>2.96</td>
<td>2.94</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>210</td>
<td>2.98</td>
<td>2.94</td>
<td>2.47</td>
<td>2.43</td>
<td>2.42</td>
<td>2.46</td>
<td>—</td>
</tr>
<tr>
<td>220</td>
<td>2.48</td>
<td>2.44</td>
<td>2.34</td>
<td>2.31</td>
<td>2.20</td>
<td>2.01</td>
<td>2.02</td>
</tr>
<tr>
<td>230</td>
<td>2.22</td>
<td>2.18</td>
<td>2.03</td>
<td>2.01</td>
<td>2.00</td>
<td>2.02</td>
<td>—</td>
</tr>
</tbody>
</table>

Apart from differences in ring intensity and continuity, the ratios of the ring diameters correspond closely to those obtained by other workers for orthorhombic crystals of long chain compounds (Garrido & Hengstenberg, 1932; Natta & Riganomonti, 1935; Schoon, 1938; Coumoulos & Rideal, 1941).

Changes in crystalline structure after thermal disorientation

Irreversible changes in diffraction pattern after exposure of the specimens to high beam currents may be due either to a reduction in the degree of preferred orientation of the crystals in a particular plane relative to the incident electron beam, or to a change in crystalline structure. If the nature of the patterns remains unchanged, but there is a reduction in intensity, it may be concluded that there has been no polymorphic crystalline change, but only a reduction in the degree of preferred orientation, or alinement of the molecular axes of the crystals normal to the plane of the collodion membranes. On the other hand, partial fading of the patterns, together with the persistence of rings characteristic of other crystalline modifications, may indicate that apart from a change in preferred orientation, molecular reorganization of the molecules has occurred.

In the homologous series of aliphatic fatty acids, the melting-points become progressively higher with increase in chain length, and this introduced a difficulty in ensuring that all the specimens were sufficiently heated to permit changes in
crystalline structure to take place, especially with the fatty acids within the range of higher melting-points. Similarly, a certain amount of heating occurred even at the lower beam intensities (100–200 μA.) at which most of the diffraction patterns were photographed, and since the exposures varied from 5 to 10 sec., the patterns were examined on the fluorescent screen for 10–20 sec. before exposing the plates. For example, the pattern from myristic acid (C₁₄) faded so quickly that it was not possible to obtain a satisfactory photograph. When the plates were examined, only the diffuse ring pattern due to the collodion base itself could be seen. The sharp ring pattern given by elaidic acid (Pl. 5, fig. 3) could only be obtained by exposing the plate immediately after the specimen was inserted into the apparatus.

Preliminary tests showed that an exposure of 10 min. at 400 μA. was sufficient to melt or raise to the transition temperature the crystals of the longer chain derivatives; and this was adopted as a standard procedure in most cases. The specimens were first exposed at the lower beam currents, and photographs taken of the diffraction patterns. If these proved satisfactory, the beam current was raised to 400 μA. and maintained at this level for 10 min., after which the specimens were allowed to cool, and photographs of the diffraction patterns taken at the original beam intensity.

**Saturated fatty acids.** Pl. 6, fig. 1, shows the diffuse pattern given by the collodion base alone. After exposure to the high beam current, irreversible fading of the diffraction patterns of octacosanoic acid (C₂₈) and tetracontanoic acid (C₃₄) occurred, and only the inner ring corresponding to a side spacing of c. 4·12 A. remained in a diffuse form. But this ring showed hexagonal groups of spots or arcs in both patterns (Pl. 6, figs. 2, 3), suggesting that, apart from a reduction in the degree of preferred orientation of the crystals normal to the membrane surface, there was a change in crystalline structure from the orthorhombic to the hexagonal type of close packing in which the molecules are more mobile (Müller, 1932). Moreover, the change was irreversible, indicating that the original alinement of the c axes, or hydrocarbon chains of the crystals normal to the membrane surface was determined during the stage in membrane formation when the solubilized fatty acid was in contact with the adsorbed monolayer at the amyl acetate-water interface (Text-fig. 1, a).

**Unsaturated fatty acids.** The influence of thermal disorientation on the initial preferred orientation of the crystals is brought out clearly with elaidic acid (Pl. 6, fig. 4). Only a faint inner ring (c. 4·12 A.) persists, showing a hexagonal array of spots, similar to those in the patterns given by the saturated acids after thermal disorientation (Pl. 6, figs. 2, 3). In this connexion, the original pattern of elaidic acid (Pl. 5, fig. 3) is typical of the arrangement of orthorhombic crystals in which the a and b axes of the crystals are not orientated at random in the plane of the membrane, as with brassidic acid (Pl. 5, fig. 5), but are mainly alined in one direction in this plane. In the diffraction pattern, the six bright spots of the inner group are distributed so that four spots are on the (110) ring, and two on the (200) ring; moreover, the relative dimensions of the rectangular grid of spots on the (110) ring correspond exactly with those of the cross-section of the orthorhombic unit cell.

After thermal disorientation, this arrangement of the crystals changed, and
the molecules became arranged in the hexagonal type of close packing, in which there is a single common side spacing between the molecules. The hexagonal array of spots on the single ring indicates that there was an alignment of crystals normal to the membrane surface and also in the plane of the membrane (Pl. 6, fig. 4). A random distribution of hexagonal crystals in the plane of the membrane would give rise to a single continuous Debye-Scherrer ring (c. 4.12 A.) as in the pattern of the collodion-brassicid acid membrane after thermal disorientation at high beam intensity (Pl. 6, fig. 5).

Paraffins. Pl. 6, fig. 6 shows the change which occurred in the molecular organization of the crystals of hexatriacontane (C₃₆) after thermal disorientation and re-crystallization. The original pattern consisted of isolated groups of small spots arranged in rings, the relative dimensions of which are consistent with an orthorhombic crystalline structure; the micro-crystals were distributed at random in the collodion framework, and the rings of spots were due to reflexion of the electron beam from planes in those crystals which were orientated perpendicular to the plane of the collodion supporting base (Pl. 5, fig. 7). The orthorhombic structure was maintained after the specimen was heated in the beam, but groups of bright larger spots appeared which could be resolved into three rectangular grids of spots on the (110) ring, each grid of which is associated with a single pair of spots on the (200) ring, representing an alignment of the \( a \) and \( b \) axes of the crystals in three directions in the plane of the membrane. Similar patterns have been described by Brummage (1947).

Calliphora pupal skin

The initial diffraction pattern of the Calliphora pupal skin was remarkably similar to that obtained with the fatty acids, especially with octacosanoic acid (Pl. 5, figs. 1, 4). After exposure to high beam currents, there was a partial fading of the ring pattern, and this change was irreversible, as with the artificial membranes (Pl. 6, figs. 2-5); but the persistence of a relatively intense ring corresponding to a spacing of c. 4.12 A. was similar to the change which occurred with brassicid acid (Pl. 6, figs. 5, 7), and suggested that a proportion of the thermo-labile lipids underwent a transition from an orthorhombic to a hexagonal lateral packing of the molecules.

The difference between the cuticle and the artificial membranes is seen in the persistence of an outer ring of c. 3.71 A. corresponding to the (200) spacing between the planes in orthorhombic crystals. Moreover, a rectangular grid of spots persisted on the inner (110) ring, and the appearance of a faint innermost ring (c. 4.92 A.) corresponded to the (010) spacing shown in the diffraction pattern of elaidic acid before thermal disorientation (Pl. 5, fig. 3). These changes are summarized in Table 3.

The main conclusion which may be drawn is that the thermo-labile lipids of the pupal skin are analogous to the fatty acid components in the artificial collodion membranes; but a proportion of the cuticle waxes, or lipids are relatively thermolabile. The thermo-labile lipids undergo a crystalline transformation from an ortho-
rhombic to a hexagonal arrangement of close packing of the molecules, and this change is irreversible, as shown by the fading of the outer rings in the original diffraction pattern. The diffraction pattern of the thermo-labile lipids after thermal disorientation is superimposed on the pattern of the thermo-stable lipids, which retain the orthorhombic structure, and also show an alinement of the $a$ and $b$ axes of the crystals in the plane of the cuticle, which gives rise to the rectangular grid of spots on the (110) ring in the pattern.

### Table 3. Orthorhombic crystalline structure of lipids in *Calliphora* pupal skin

<table>
<thead>
<tr>
<th>Laue indices $(hko)$</th>
<th>Calculated spacings (Å)</th>
<th>Original spacings (before heating)</th>
<th>Spacings after heating</th>
</tr>
</thead>
<tbody>
<tr>
<td>010</td>
<td>4'97</td>
<td>—</td>
<td>4'92</td>
</tr>
<tr>
<td>110</td>
<td>4'13</td>
<td>4'15</td>
<td>4'12</td>
</tr>
<tr>
<td>200</td>
<td>3'73</td>
<td>3'69</td>
<td>3'71</td>
</tr>
<tr>
<td>020</td>
<td>2'48</td>
<td>2'43</td>
<td>—</td>
</tr>
<tr>
<td>310</td>
<td>2'22</td>
<td>2'18</td>
<td>—</td>
</tr>
<tr>
<td>220</td>
<td>2'06</td>
<td>2'05</td>
<td>—</td>
</tr>
</tbody>
</table>

**Distinction between fat soluble and bound lipids.** *Calliphora* pupal skins were immersed in warm chloroform for 30 min., washed and mounted on grids in the usual way. It was assumed that this procedure resulted in the removal of the bulk of the thermo-labile, or fat soluble lipids. Pl. 6, fig. 8, shows that the residual lipids gave rise to a sharp ring pattern which could be related to the (110) and (200) spacings in the original pattern. The rings, however, were fainter, and a rectangular grid of spots on the (110) ring corresponded to a similar grid on the diffraction pattern after thermal disorientation. The pattern did not fade after the specimen was exposed to high beam currents, supporting the view that the thermo-labile lipids are fat soluble, whereas the thermo-stable lipids are 'bound' to the structural protein of the epicuticle layer in the pupal skin, and that this binding is responsible for the thermal stability.

**Action of alkali on bound lipids.** The bound lipids of the epicuticle are associated with the cuticulin layer, which is extremely resistant to the action of fat solvents, and cold concentrated mineral acids; but it is disrupted by hot alkali, or warm nitric acid saturated with potassium chlorate to liberate oily droplets (Kühnelt, 1928; Wigglesworth, 1947). The initial stages in the breakdown of the epicuticle framework were observed by immersing a pupal cast skin in 10% caustic potash for 15 min. The membrane was washed in distilled water and examined in the usual way. No ring pattern could be obtained, although the membrane was still intact. Similar negative results were obtained with preparations which had been treated first with chloroform to remove the fat soluble waxes. Clearly, the alkali destroys the organized structure of the epicuticle lipids before the membrane itself is broken down.

**Electron microscope examination of pupal skin**

Attempts to recognize a fine microscopic structure in the *Calliphora* pupal skin with the electron microscope proved unsuccessful. The cast skins were relatively trans-
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parent to the electron beam, and the structure revealed at a screen magnification of 20,000 times was homogeneous (Pl. 4, fig. 2). This simplifies to some extent the interpretation of the electron diffraction patterns; the changes in pattern described were due to changes in molecular organization of the lipid components, and not to gross modifications in membrane structure.

DISCUSSION

In comparing the artificial collodion-wax membranes with the Calliphora pupal skin, three aspects are of interest: (i) membrane formation, (ii) membrane structure, and (iii) membrane permeability.

Membrane formation

The membrane-forming material consists of a solution of nitrocellulose and fatty material in amyl acetate. Of these components, only the first two participate in the ultimate membrane. The nitrocellulose can be regarded as a fixed supporting framework which supports the crystals of waxy material after the solvent has evaporated, and in this respect the collodion fabric is analogous to the cuticulin layer of the epicuticle, which is associated with the fat soluble waterproofing waxes.

In the absence of nitrocellulose, a monolayer of fatty acid molecules would be formed at the amyl acetate-water interface, which could provide a starting-point for the deposition of crystals of fatty acid orientated with the molecular axes normal to the interface. Owing to the presence of the carboxyl group at the end of each molecule, association occurs between pairs of molecules, resulting in the formation of double-layer structures:

\[ \text{CH}_2 - \ldots \ldots \ldots - \text{C} = \text{O} \]

\[ \text{OH} \]

\[ \text{O} \text{C} - \ldots \ldots \ldots - \text{CH}_2 \]

The intermolecular binding is ionic, and the lateral binding between the molecules in the double layers is dependent on the residual van der Waals' forces between the hydrocarbon chains.

The nitrocellulose macromolecule, however, can also form an orientated monolayer at the amyl acetate-water interface during membrane formation; and the non-polar side chains of the polymer monolayer could also offer points of attachment for the crystals of fatty acid which are deposited in the bulk membrane framework. There is a third possibility that both the fatty acid and nitrocellulose form a mixed monolayer at the solvent-water interface, so that crystal deposition could proceed by association with the hydrocarbon chains of the fatty acid monolayer, or with the non-polar side chains of the nitrocellulose monolayer.

There is a final possibility that the nitrocellulose membrane does not act simply as a physical or mechanical support for the wax crystals, but exerts a stabilizing influence on their orientation, which may involve polar and non-polar association with the side chains of the nitrocellulose in the bulk membrane framework.

In considering which of these factors is of major importance in the formation of a crystalline system in which there is a high degree of preferred orientation of the
molecular axes normal to the nitrocellulose membrane surface, the first clue is provided by a comparison between the diffraction patterns of the collodion-fatty acid and collodion-paraffin membranes. The orthorhombic crystalline structure is common to the waxy components in both systems, which shows that this is mainly determined by the lateral van der Waals' association between the hydrocarbon chains during crystallization. The rings are much more sharply defined and intense in the diffraction transmission patterns of the collodion-fatty acid than of the collodion-paraffin membranes, and this is consistent with surface active nature of the fatty acid molecules, and with the non-polar nature of the paraffin molecules. The latter do not become orientated to form a monolayer at the solvent-water interface during membrane formation, and the ring patterns of isolated pairs of spots are those which would be expected from a random distribution of micro-crystals in which there is no common axis of alinement normal to the membrane surface.

Since a common feature of the formation of the collodion-fatty acid and the collodion-paraffin membranes is the deposition of the collodion framework, van der Waals' association between the fatty components and the non-polar side chains of the collodion fabric cannot be the main factor which influences the preferred orientation of the fatty acid crystals in the membrane, for this factor would also operate with the paraffin crystals. This leaves the possibilities that the orientation of the fatty acid crystals is determined by polar association with the polar side chains in the bulk collodion framework, or by non-polar association with the monolayers of collodion and fatty acid formed at the amyl acetate-water interface. The initial collodion monolayer can be regarded as an integral part of the fixed framework of the system, for it is incorporated in the ultimate membrane which is formed.

The irreversible fading of the diffraction patterns of the collodion-fatty acid membranes when these are exposed to high electron beam currents provides a further clue which restricts these possibilities to one in which the limiting factor is the mobile monolayer of fatty acid formed at the solvent-water interface. After thermal disorientation, the fatty acid molecules can still associate with the fixed collodion framework, but a solvent-water interface is no longer present, as the original adsorbed fatty acid monolayer is now incorporated in the bulk crystalline phase and is subject to similar thermal disorientation. This fatty acid monolayer is therefore a limiting factor in determining the common orientation of the molecular axes of the fatty acid crystals which are deposited in the collodion framework, and this acts mainly as a mechanical support for the three-dimensional crystalline system.

Membrane structure

The diffraction pattern of the collodion-C_{18} fatty acid membrane (Pl. 5, fig. 1) corresponds closely to that of the Calliphora pupal skin (Pl. 5, fig. 4). This observation is of particular interest in view of the conclusion reached by Beament (1945) that the fat soluble waxes extracted by chloroform from Calliphora pupal exuviae are sufficient to form a layer 0.18 μ thick on the surface of the epicuticle. The artificial collodion membranes were only about 150–200 A. thick. Adopting Bergmann's (1938) view as a basis for comparison that the average chain length of
Study of crystalline structure of lipids

the fat soluble epicuticular waxes from silkworm exuviae is about C_{30}, a layer of wax 0.18 μ thick would correspond roughly to forty single molecular layers, assuming that the hydrocarbon chains are orientated perpendicular to the surface of the layer, and that the vertical distance between two adjacent carbon atoms on the zigzag chains is about 1.43 Å.

Since forty molecular layers of wax cannot be accommodated within the collodion framework, it is likely that the micro-crystals project to a considerable degree above the surface of the dry collodion base to form a superficial layer of wax which is continuous with the underlying waxy components in the supporting collodion membrane. This is to be expected, for crystallization proceeds during membrane formation before the solvent has evaporated, and crystal growth is influenced by the original minute nuclei in the membrane-forming solution.

After the Calliphora pupal skin has been treated with chloroform, it still gives a well-defined diffraction pattern, which is thermo-stable. This introduces an experimental difficulty in correlating the artificial with the natural membranes, for chloroform may displace both the superficial waxes from the epicuticle surface, and also the waxes which impregnate the underlying cuticulin layer. The waterproofing waxes are secreted after the cuticulin layer is formed (Wigglesworth, 1945, 1947, 1948), and the essential condition for the formation of a superficial layer of crystals in which the hydrocarbon chains are perpendicular to the cuticulin surface is that the waxes should be mobile and in contact with a water surface. This condition would be fulfilled if the wax is solubilized by a more hydrophilic lipid, such as a phospholipid, as suggested by Beament (1945), during the process of secretion. A droplet of solubilized wax on the hydrated surface of the cuticulin layer would be analogous to a lens of a solution of wax in amyl acetate spread on a water surface (Text-fig. 1a), ignoring for the moment the presence of collodion. If the wax droplets spread or coalesce on the cuticulin surface an orientated monolayer of wax would be formed which could initiate the alinement of crystals in the superficial layer, although it is necessary to postulate that some change occurs in the solubilizing agent which leads to this crystallization. Here also, as with the artificial systems, crystals could extend inwards into the cuticulin framework, for this layer must necessarily be permeable to the solubilized wax during the process of secretion—a conclusion which is consistent with the absence of holes or 'pore canals' in the Calliphora pupal skin (Pl. 4, fig. 2).

While the electron diffraction evidence does not show whether the thermo-labile lipids of the pupal skin are entirely distributed on the surface or impregnate partially the underlying layer of the epicuticle, the difference in thermal stability between the fat soluble and 'bound' lipids suggests that the latter are linked in some way with the protein components of the epicuticle, although it is also possible that the distinction between the 'free' and 'bound' lipids is one of degree. The persistence of the orthorhombic crystalline structure of the bound lipids can be detected after the transition of the free lipids from the orthorhombic to the hexagonal structure when the pupal skin is exposed to high temperatures in the electron beam; and also after the free lipids are removed by chloroform. The presence of a rectangular grid of
spots on the (110) ring after such treatments suggests that there is an alinement of the \(a\) and \(b\) axes of the crystals of bound lipids in the plane of the epicuticle, and this may reflect a similar alinement of the protein fibrils in the epicuticle framework.

**Membrane permeability**

From the point of view of membrane permeability, the results are consistent with the findings of Wigglesworth (1945) that when insects are exposed for some time to high temperatures, the waterproofing properties of the waxes in the epicuticle are permanently impaired. The present results offer an explanation involving a crystalline transition from an orthorhombic to a hexagonal type of close packing. While this change is reversible with the long-chain paraffins (Müller, 1932), the long-chain fatty acids used in the formation of collodion-wax membranes in the present work are more typical of insect waxes, and the transformation in these systems, and in the *Calliphora* pupal skin is irreversible under the particular experimental conditions. As suggested by Beament (1945), such a change involves an increase in intermolecular space between the hydrocarbon chains of the crystalline wax molecules, and this would increase the permeability of the wax system to water. Müller (1932) has correlated the change from an orthorhombic to a hexagonal crystalline lattice with the anisotropic thermal expansion of the orthorhombic lattice in which the molecules behave as freely rotating cylinders before melting occurs. The expansion is greater along the \(a\) axis of the unit cell than along the \(b\) axis; the thermal expansion is much smaller along the \(c\) axis, owing to the strong binding force between the carbon atoms in the chains.

**SUMMARY**

A comparison is made between the known crystalline structures of long-chain fatty acids, paraffins, and the free and bound lipids in the epicuticle of *Calliphora* pupal exuviae.

Artificial collodion-wax membranes give electron diffraction patterns which are similar to those of the insect cuticle, indicating that the free, or thermo-labile waxes in the epicuticle consist of a three-dimensional arrangement of orthorhombic micro-crystals oriented perpendicular to the cuticle surface, but otherwise distributed at random.

The thermo-labile waxes of the epicuticle undergo irreversible disorientation when the pupal skin is exposed to high beam currents, and similar changes occur with the artificial collodion-fatty acid membranes. This is correlated with the mechanism of membrane formation from a solution of fatty acid and collodion in amyl acetate. When a drop of solution is allowed to spread on a water surface, a lens is formed and a monolayer of fatty acid adsorbed at the amyl acetate-water interface. This monolayer acts as a basis on which an alinement of crystals occurs in the bulk framework of the lens as the solvent evaporates. The crystals are immobilized in the membrane of collodion which is simultaneously deposited. When the dry membrane is heated no monolayer is present to act as an organizing factor in crystal
growth, which occurs at random in the bulk framework of the collodion membrane. Similar random crystallization occurs in collodion-paraffin membranes, where the waxy component is non-polar, and does not form a monolayer at the solvent-water interface during membrane formation.

Polymorphic crystalline modifications are observed after the collodion-fatty acid membranes and the *Calliphora* pupal skin are exposed to a more intense beam. Apart from a reduction in the degree of preferred orientation of the thermo-labile waxy components, there is a transition from an orthorhombic to a hexagonal type of lateral close crystalline packing. This is correlated with the irreversible increase in permeability of the cuticle to water at high temperatures.

After the thermo-labile lipids are removed from the pupal skin by chloroform, the residual lipids give a diffraction pattern which does not fade when the cuticle is heated. The pattern suggests that the bound lipids can form orthorhombic crystalline aggregates in the cuticulin layer, and that the thermo-stability is due to association between the lipids and the structural proteins of this layer.

The organized structure of the bound lipids is destroyed when the pupal skin is in contact with strong alkali before the membrane itself is disrupted.

The pupal skin is homogeneous when examined with the electron microscope at a screen magnification of 20,000 times.

The structural relationships between the free and bound lipids of the epicuticle are discussed.

The electron diffraction diagrams were taken with the R.C.A. electron microscope and diffraction unit in the Cavendish Laboratory, Cambridge, by kind permission of Dr V. E. Cosslett. I am indebted to Mr. R. G. Allen and Mr. J. W. Menter for helpful advice and discussions; and to Mr G. R. Crowe for technical assistance. The samples of pure chemicals used were kindly supplied by Dr J. H. Schulman.

REFERENCES

EXPLANATION OF PLATES

PLATE 4

Fig. 1. Electron diffraction transmission pattern of micro-crystalline gold film deposited on collodion base. Table 1 refers to the side-spacings represented by the four inner rings.

Fig. 2. Electron micrograph of Calliphora pupal skin, showing homogeneous structure.

PLATE 5

Electron diffraction transmission patterns given by collodion-fatty acid, collodion-paraffin membranes, and by Calliphora pupal skin before exposure to high electron beam currents.

Fig. 1. Octacosanoic acid (C₄₈).
Fig. 2. Tetracontanoic acid (C₅₂).
Fig. 3. Elaidic acid (trans) (C₁₈).
Fig. 4. Calliphora pupal skin.
Fig. 5. Brassidic acid (trans) (C₃₈).
Fig. 6. Dotriacontane (C₆₀).
Fig. 7. Hexatriacontane (C₇₈).

PLATE 6

Electron diffraction transmission patterns of collodion, collodion-fatty acid, collodion-paraffin membranes, and of Calliphora pupal skin after thermal disorientation at high beam intensity.

Fig. 1. Collodion. The diffuse Debye-Scherrer ring is due to the collodion base alone.
Fig. 2. Octacosanoic acid (C₄₈). There is a bright hexagonal array of diffuse spots and a weaker hexagonal pattern, representing two groups of crystals covered by the incident electron beam. In both the molecules are oriented perpendicular to the membrane surface, but the groups are not aligned in the plane of the membrane.
Fig. 3. Tetracontanoic acid (C₅₂). The pattern is weak, but essentially similar to that in fig. 2. Two groups of hexagonal crystals have been covered by the electron beam.
Fig. 4. Elaidic acid (C₁₈). A single weak hexagonal pattern can be recognized.
Fig. 5. Brassidic acid (C₃₈). The continuous Debye-Scherrer ring represents a random distribution of hexagonal micro-crystalline aggregates in the plane of the membrane.
Fig. 6. Hexatriacontane (C₇₈). The spots are distributed in two rings corresponding to the arrangement shown in the elaidic acid pattern (Pl. 5, fig. 3). The orthorhombic crystalline structure is maintained after melting and recrystallization. The electron beam has covered three groups of crystals. The rectangular grids of spots on the (110) ring can be identified from the corresponding pairs of bright spots on the (200) ring, showing that the crystals are aligned in three directions in the plane of the membrane.
Fig. 7. Calliphora pupal skin. The most intense ring is due to the presence of a random distribution of hexagonal crystals of thermo-labile lipids similar to those responsible for the diffraction pattern of brassidic acid (fig. 5). The inner and outer rings are due to the thermo-stable bound lipids.
Fig. 8. Calliphora pupal skin after treatment with chloroform. The diffraction pattern is due to the bound lipids. The (110) and (200) rings have sharpened. The rectangular grid of spots on the (110) ring corresponds to that in fig. 7.
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