A study of the structure of the grasshopper cuticle
by Patricia M Frith Chefurka

A THESIS Submitted to the Graduate Committee in partial fulfillment of the requirements for the
degree of Master of Science in Engineering Physics at Montana State College
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Abstract:
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The diffraction method, No diffraction pattern was obtained from a cross section of the cuticle, from
which it is concluded that the layers are either non-uniform or diffuse or both, III, The phase
microscope study, which indicated non-uniformity of the layers.

IV, The interference method, which also indicated non-uniformity.

V, The critical angle method, which gave strong evidence that interfaces between layers are not
discrete.

VI, The refraction method, which gave no conclusive results.

VII, The electron microscope studies. Several methods for making replicas were tried, and are
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addendum.
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PATRICIA M. (FRITH) CHEFURKA

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I. The schlieren method. It is concluded that this method requires a much larger "cell" than a cross section of the insect cuticle, and no feasible experimental set-up was found.

II. The diffraction method. No diffraction pattern was obtained from a cross section of the cuticle, from which it is concluded that the layers are either non-uniform or diffuse or both.

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VI. The refraction method, which gave no conclusive results.

VII. The electron microscope studies. Several methods for making replicas were tried, and are discussed as to their possible practicability. Micrographs of replicas obtained will be given in an addendum.
INTRODUCTION

The field of this research was the natural choice for a person with two main fields of interest, physics and entomology. The topic of the research, the structure of the insect cuticle, was suggested by Dr. J. H. Pepper of the Department of Zoology and Entomology, and Dr. Leon H. Johnson of the Department of Chemistry, Montana State College. The grasshopper, *Melanoplus bivittatus* Say, was chosen as the subject on which to work, in part because it is an insect of great economic importance in Canada and the United States, but mainly because it was hoped to correlate any results obtained with those obtained by the husband of the author in his studies on the permeability of the cuticle of this insect to water.

The cuticle is the layer covering the external surface of the body, the appendages, and the surfaces of the invaginated parts of arthropods. Much chemical and histological work has been done on the cuticles of many insects (see, for example, Wigglesworth 1933, 1947, and Dennell 1946), in which evidence has been found for certain layers and structures. However, very few experiments of a physical nature have been carried out, with the exception of Richards and Anderson's electron microscope studies of the cockroach cuticle (1942), Anderson and Richards' work on the structural colors of insects (1942), Fraenkel and Rudall's x-ray studies of the cuticle (1940, 1948), and Richards and Korda's work on variously treated cuticles (1948).

Thus it was that the study of the structure of the grasshopper cuticle was undertaken from the physical standpoint, in the hope of correlating results with the knowledge already gathered by other means, and of finding
something of the way in which parts of the cuticle are interrelated.
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The cooperation of Prof. Paul A. Anderson of the Department of Physics, State College of Washington, in taking the electron micrographs presented here is gratefully acknowledged, as well as his advice on methods of preparation of replicas for the electron microscope.

Lastly, I am greatly indebted to my husband, William, whose suggestions were valuable, who bore the brunt when methods were unsuccessful, and without whose confidence and inspiration this work would never have been undertaken.
REVIEW OF LITERATURE

Speaking generally, the cuticle of insects is divided into two regions:

1. the epicuticle, which is the outer region, and which contains no chitin. It is resistant to cold concentrated acids or bases. Its thickness varies from insect to insect, being 1 micron or less for Rhodnius prolixus (Wigglesworth 1933, 1947), and 4 microns for the larva of Sarcophaga fæculæ (Dennell 1946). In most insects the epicuticle is subdivided into a variable number of layers: four have already been described for R. prolixus and Tenebrio molitor (Wigglesworth 1945, 1947, 1948), while in the cockroach and S. fæculæ, for example, only two layers have thus far been detected (Richards and Anderson 1942; Dennell, 1946).

2. the endocuticle, composed mainly of chitin and protein, which lies below the epicuticle. It is in turn subdivided into (a) the outer endocuticle (Dennell 1946) or exocuticle (Campbell 1929), which is brittle and of variable thickness; and (b) the inner endocuticle (Dennell 1946) or endocuticle (Campbell 1929), which is thick and elastic.

Underneath the endocuticle lie the epidermal cells.

Wigglesworth (1945, 1948) has found the epicuticle of Rhodnius and Tenebrio to consist of (from inside outward):

1. the cuticulin layer, composed of condensed and polymerized lipoproteins. This layer is disrupted with the liberation of oily droplets by nitric acid saturated with potassium chlorate.

2. the polyphenol layer, composed of phenols. It is detected by its ability to reduce silver in an ammoniacal silver hydroxide solution.
3. the wax layer, probably composed of a mixture of alcohols, acids, paraffins, and esters (Chibnall et al. 1934). It is demonstrated by fat stains or chloroform extraction.

4. the cement layer, apparently composed of protein. It protects the wax from abrasion or extraction.

Other subdivisions have been found with different insects. For example, Richards and Anderson (1942) report the epicuticle of the cockroach to be divided into the outer lipid epicuticle, .02-.03 micron thick, and the protein epicuticle, 2 microns thick. Dennell (1946) divided the Sarcophaga epicuticle into two layers on the basis of staining reactions: the outer layer containing lipids of 1 micron thickness, and the inner layer containing proteins (lipids were not detected) approximately 4 microns thick.

In some instances the endocuticle has also been subdivided further than the two layers previously mentioned. Wigglesworth (1948), using Tenabrio, divides the exocuticle into outer and inner on the basis of staining.

Laminations occur in the endocuticle, running parallel to the cuticular surface. X-ray studies of Fraenkel and Rudall (1940, 1947) on the larval cuticle of Calliphora indicate that these laminations are alternate layers of chitin and protein. Richards and Anderson (1942) also found dense laminae to be present in both the outer and inner endocuticle in their electron microscope studies of the American cockroach. Composite dark bands were made up of three equally spaced bands .15 micron broad, and were separated by less dense bands 1.5 microns broad.

Richards and Korda (1948) showed that when cockroach tracheae were
treated with alkali chitin was present as a fibrous meshwork. However, the chitin might have become reoriented during and after the removal of the protein.

The endocuticle also contains vertical striations which are caused by the presence of pore canals. Wigglesworth (1933, 1947, 1948) showed that in *Rhodnius* and *Tenebrio* these canals extended from the epidermal cells through the endocuticle and penetrated the cuticulin layer. He suggested that they contained cytoplasmic filaments or some kind of fluid. Richards and Anderson (1942) in their studies of the pore canals of the American cockroach agree with Wigglesworth in the probable contents of the pore canals. However, in this insect the pore canals do not extend into the epicuticle. They were found to have a diameter of .4 micron at the basal end, tapering to .15 micron. They were helicoidal in form with a pitch of .25 micron. These authors also calculated that pore canals occurred at the rate of $1.2 \times 10^6$ per square millimeter of body surface.
EXPERIMENTAL METHODS

All of the methods tried use as their starting point the fact that in all insect cuticles studied by chemical means there have been found more or less discrete layers which differ in chemical composition. If this is so, then these layers should also differ in:

(a) index of refraction
(b) reaction to various solvents and reagents
(c) microstructure.

Several methods were considered and tried to test these basic assumptions.

In all experiments the abdominal portion of the grasshopper cuticle was studied, sections being taken perpendicular to the length of the abdomen. This portion was chosen because of the relative ease with which large areas of cuticle are obtained, and also because there is greater uniformity in the cuticle on this part of the body as compared to other parts.

After the insect was killed in a cyanide and ammonia killing bottle, the abdomen was separated from the thorax with a sharp razor blade, the tip of the abdomen removed, and an incision made the full length of one of the pleural folds. Viscera and fat body were then easily removed, and the cuticle was flattened, with as little pressure as possible, between pieces of filter paper.

When it was found necessary to dehydrate the cuticle, it was prepared as above and then placed in a laboratory oven at 40°C for one to two hours. This low drying temperature was used because it has been found that at approximately 42°C the lipoid material of the cuticle of this species begins to undergo some form of breakdown which is indicated by a sudden increase
in the rate of evaporation of water through the cuticle (Chefurka, unpublished work). Usual histological methods of dehydration were not used in order to eliminate changes in the cuticle due to contact with organic solvents.
I. THE SCHLIEREN METHOD

The schlieren (shadow) effect was first used by Foucault to test lenses for spherical and chromatic aberration*, and was then adopted by Toepler for detecting small differences in the refractive index of a medium (Toepler 1867)**. After about 1930 the use of this method for the observation of boundaries in the electrophoresis of proteins (Tiselius 1937; Abramson, Moyer, and Gorin 1942; Stern, Schein, and Wallerstein 1946) and in ultracentrifugal sedimentation (Lamm 1933; Tiselius et al 1937; Philpot 1938) became quite common, and many useful modifications were developed.

The theory as given here is essentially that of Longsworth (1939) and Longsworth and MacInnes (1939).

The horizontal slit, S (Figure 1), allows light from the source to fall on the long-focus schlieren lens, D, which forms an image of the slit at P. The schlieren diaphragm, Q, has a sharp upper edge in the plane of P, and is vertically moveable.

* cited by Edser 1915
** cited by Abramson, Moyer, and Gorin 1942
If, in the electrophoresis cell, \( E \), there is a boundary region, \( c \) (a region of continuously varying composition), then through this region there is a change in refractive index from \( n_1 \) above \( c \) to \( n_2 \) below. If \( x \) is used to indicate the distance from the bottom of the cell, then there will be a point in the boundary region where \( \frac{dn}{dx} \) has a maximum value, diminishing above and below this point in a normal Gaussian distribution.

The camera with objective, \( O \), and ground glass screen or photographic plate, \( G \), is focused on the cell. Any rays striking the boundary, \( c \), in the cell will be refracted downward as shown by the broken line, assuming that the substance on the bottom has the greater index of refraction. When the opaque diaphragm, \( Q \), is adjusted so that these refracted rays are just intercepted, then the cell, as seen by the photographic plate, will have a dark band across it. This band broadens as the diaphragm moves up and intercepts the less deviated rays. To elucidate this further, the entire cell will appear bright in the camera only if there is light coming from every portion of the cell. If, for some reason, the light coming from one particular part of the cell is cut off, then that part of the cell will appear dark.

The rays which are most refracted come from that part of the cell where \( \frac{dn}{dx} \) is greatest, and will be intercepted first by the upward-moving diaphragm, those from areas of smaller refraction gradients later. More concisely, the displacement of the diaphragm from the position of the un-deviated slit image is proportional to the refraction gradient, the position of this refraction gradient being that point in the cell which is conjugate to the edge of the dark schlieren band on the photographic plate.
The important technique introduced by Longsworth (1989) was a method for scanning, which allowed boundaries in the whole cell to be observed. The photographic plate was masked with a vertical slit, and the plate was driven past it horizontally at constant velocity. The plate and the moving diaphragm were actuated by the same mechanism, but moved at right angles. With this arrangement the most deviated rays from any boundary are intercepted first, the region of the cell containing the greatest refraction gradient showing up first as a narrow black horizontal band at the position of the photographic plate, but only that part falling on the vertical slit being recorded. As the screen, $Q$, moves up, the less deviated rays from the adjacent parts of the cell are also cut off, and the black band is broadened. The resultant photograph has a form typified by Figure 2, and is directly a graph of $\frac{dn}{dx}$ versus $x$. The area under the curve is proportional to the total change of index, $n_2 - n_1$, the constant of proportionality depending on the length, $b$, from the cell to the diaphragm (Figure 1), on the width, $a$, of the cell, on the magnification of the camera, and on the gearing between the plate and the diaphragm.

Figure 2. Schematic diagram of photographic record of $\frac{dn}{dx}$ versus $x$ from schlieren apparatus.
For the present problem the object of study was the grasshopper cuticle. If a cross section of this cuticle were made, it would be essentially the same as the electrophoresis cell, having horizontal boundaries between layers with different indices of refraction. The one great difference between the two cases is in overall size of the object studied. Whereas the distance between layers in the cell is measured in centimeters, in the cuticle it is measured in microns.

Because of this difference in size, it was proposed to study the deviated rays, rather than the conjugate dark bands on the object. This was to be done by using a moving slit at $Q$, rather than a moving diaphragm, which would also allow the observation of rays deviated both upward and downward. It was hoped that something could be learned of the sharpness of the boundaries in the grasshopper cuticle, as well as their number and positions.

This experiment was never set up, for two main reasons:

(a) If the work were to be done well, the specifications on the lenses had to be very stringent. One electrophoresis experiment required that the condenser lens be a 40 mm achromat with 100 mm focal length; the schlieren lens, a 4 inch achromat with 36 inch focal length; the camera objective a 2 inch achromat with 36 inch focal length (Longsworth 1939-40). This would have incurred too great an expense, especially in view of the second objection to the method.

(b) The theory, as formulated for the electrophoresis cell, used only geometrical optics. Even here, however, consideration had to be
given to such things as diffraction by the schlieren diaphragm, which made .1 mm the smallest practical source slit width (Longsworth 1939-40; Stern and Dubois 1940-41). In the insect cuticle, on the other hand, the overall length of the "cell" is of the order of one or two tenths of a millimeter, and, as given in the Review of Literature, some of the layers are around one or two microns.

Rather than obeying geometrical optical laws, then, one would expect it to behave as a diffraction grating of N unevenly spaced slits. This speculation led to the next method tried.
II. THE DIFFRACTION METHOD

Hand sections of fresh grasshopper cuticle were made and mounted on glass microscope slides, being held there by electrostatic attraction (Figure 3a). These slides were in turn mounted on a spectrometer table as shown in Figure 3b.

Two sources of monochromatic radiation were tried: a sodium bulb (No. 23266, Chicago Apparatus Company), and a mercury lamp (250 watt General Electric type A-H2) with a green filter to give light of wave length 5461 Å. The slit was made parallel to the specimen studied, both being perpendicular to the spectrometer table. Several different fresh specimens were used, and a 180° region around the specimen was scanned carefully with a completely dark-adapted eye for any evidence of diffracted radiation. However, no trace of diffracted light could be observed.

There are two possible reasons for the failure to find any diffraction pattern:

1. The layers of the cuticle fuse into one another so gradually that a section of the cuticle fails to act as a grating.
2. Since the work was done, not with a section of mechanically compiled layers, but with a section of biological origin, the layers are quite possibly irregular. They might look more as in Figure 4. If the boundaries through the length of the section were as they are shown in the region A, one diffraction pattern would be obtained. If they were as in B, a completely different pattern would be formed. According to this, then, it would be possible to get a different pattern from each micron length of the section, which would completely escape observation.

There has been evidence of something of this nature, modified greatly, with a poorly ruled grating. If such a grating is placed on a spectrometer, the cross hairs set on a prominent line, and the grating progressively covered with a piece of paper, the image will be found to undergo a number of changes. A typical succession of events might be a shading on one side of the line, gradually developing until a double line was seen, and finally the fading of the original line to leave only the displaced line (Bell 1888).

Supporting evidence for the second possibility was also obtained by the use of the phase microscope, as discussed in Method III.
III. THE PHASE MICROSCOPE STUDY

The phase microscope used for this portion of the work, made by Bausch and Lomb, required a filter to give roughly monochromatic light in the green region. For this microscope, the sections should be less than 10 microns thick, in order to make utmost use of its available magnifying power.

It was not found possible to obtain such thin sections of fresh material in the time available, but one 6 micron section was obtained of fixed tissue imbedded in hard paraffin. Using oil immersion, good definition was achieved at a magnification of 2400.

Definite longitudinal regions could be distinguished easily, each region being of non-uniform width along the section, thus bearing out the second hypothesis for the failure to obtain a diffraction pattern in Method II. No attempt will be made to describe the structure further, as only one section was observed, which section had been subjected to rather drastic treatment during fixation and imbedding. Furthermore, after it had undergone this treatment, it was left standing for a matter of months before it was sectioned and viewed.
IV. THE INTERFERENCE METHOD

If a film of material with index of refraction different from that of air is introduced into one light path of a properly adjusted interferometer, then a shift of fringes occurs due to the effective increase in one path length. This fringe shift could be determined by illuminating one half of the field with monochromatic light, the other half with white light. One could adjust to the central colored fringe, introduce the film, and then decrease the path length until the central colored fringe is again reached.

With some of the chemical techniques it is possible to remove from the cuticle all but one or two layers (Pryor 1940; Wigglesworth 1947, 1948), which layers could then be used in the interferometer. Knowing the refractive index of this remaining material, the thickness could be calculated from the relationship:

$$N = \frac{nd}{\lambda} - \frac{d}{\lambda} = \frac{d}{\lambda} (n - 1)$$

where

- $N =$ fringe shift
- $\lambda =$ wavelength of the monochromatic light in air
- $d =$ thickness of the film
- $n =$ index of refraction of material

The indices of some layers of the insect cuticle have been determined, as in Pryor's work (1940) where he found the index of the exocuticle (outer endocuticle) of the cockroach to be between 1.54 and 1.57. Similar determinations could be made on other parts, and even using approximations to the index useful information regarding thickness could be obtained.

To test the practicability of the above theory, a Michelson interferometer was adjusted to the central colored fringe of white light, and a portion of the cuticle introduced into the adjustable path. The abdominal
region of the cast skin from a fifth instar grasshopper was used, this being flattened carefully and passed through chloroform to remove the wax.

Although several attempts were made on each of three skins so treated, it was not found possible to observe the shifted colored fringes.

The explanation of this again would seem to lie in the non-uniformity of the layers. This method is, after all, sensitive to extremely small changes in thickness.

To check the validity of this explanation, artificial layers were compiled, using a glass microscope cover slip, Canada balsam, and cellophane, arranged as shown in Figure 5.

![Figure 5. Artificial layers used with the interferometer.](image)

White light fringes were observed through the regions a, b, and c. Through the cover slip the fringes were plainly visible, though narrower than in the original field, and they also displayed some irregularity of orientation, thus showing non-uniformity in the cover slip. Observation of the fringes through the region b revealed the same defects as through a, but greatly exaggerated. In the region c it was almost impossible to observe the fringes because they were so fine and so irregular.

The explanation offered, therefore, would appear to be borne out.
V. THE CRITICAL ANGLE METHOD

The theory underlying this experiment is again geometrical in nature. Nonetheless, some of the thicker layers of the cuticle (10 microns or greater) should obey geometrical optical laws.

The development of this theory is based upon the assumption that the cuticle is composed of layers with distinct interfaces. This is a logical starting point in view of the work already discussed in the foregoing review of the literature. The implications of this assumption can be found and tested to check on the accuracy of the assumption.

If these layers are discrete and have different indices of refraction, then at an interface between media with indices $n_1$ and $n_2$, $n_1 > n_2$, there will be a definite critical angle for the incident ray, beyond which it will be reflected from the interface rather than transmitted through it. The value of this critical angle is given by the relationship:

$$\sin \theta_c = \frac{n_2}{n_1}$$

where $\theta_c$ is the critical angle of the incident ray as measured from the normal to the interface. For the case where $n_1 < n_2$, all that is necessary is a reversal of the cuticle so that the light is incident on the opposite side of the boundary. These reflected rays should emerge from the surface or end of the specimen, as indicated in Figure 6. The apparatus described below was designed to observe these
rays.

The source, S (see Figure 7), was a flash light bulb, adjustably mounted in the barrel of a microscope, whose objective lens formed the condensing lens, C. The image of S was focused on the pinhole, P (diameter .034 cm), the light from which was collimated by D, another microscope objective lens. This arrangement gave a beam of light whose divergence was not greater than 2°.

![Figure 7. Arrangement of Critical Angle Apparatus.](image)

The grasshopper cuticle, G, was mounted in the beam in a manner to be described, and the emergent rays were examined with a Leitz research microscope. The whole was mounted on a two-meter optical bench; a sliding platform was built for the microscope.

In order to observe the emergent rays resulting from varying angles of incidence, it was necessary to be able to rotate the specimen without shifting it out of the field of view of the microscope. This was accomplished by the "rotaspec" (rota as in the Latin root for "rotate", spec from the Latin specere, the root of the word "specimen"), shown in Figure 8 a.

The base of this device was made 1" x 3", the size of a glass microscope slide, in order that the adjustments of the research microscope could be readily utilized. The bottom of the moveable section, A, was a portion
b. Alternative triangular mount.

of a cylinder with a radius of curvature of two inches. Down the centre of this surface was mounted a rack, R, with the same radius of curvature. This enabled A to be moved by turning H, the head of a pinion mounted in the base, B.

The clips, C, were made of microscope spring clips bent 90°, and were mounted on the side of A in the same manner as they are on a microscope stage. These clips held in place the glass mounting made of two microscope slides cemented at right angles as shown in Figure 8a. The upright slide was cut at the centre of curvature of the rack.

For very large angles of incidence a different specimen mount had to be made, since the end of the moveable section of the rotaspec interfered with the light beam at large angles using the above mount. This was simply done by using three microscope slides rather than two, and cementing them in the form of an isosceles triangle, as shown in Figure 8b. Two of the slides were shortened so that the vertex of the triangle would be at the
centre of curvature of the rack.

A fresh undried sample of grasshopper cuticle was mounted at the position indicated by G in Figure 8a or b, the upper edge of the sample flush with the end of the glass slide. The fluid from the cuticle was sufficient to hold it in position. The rotaspec with the mounted specimen was placed on the stage of the research microscope, and the microscope focused on the end of the sample. A magnification of approximately 900x was used. Observations of the end of the specimen were then made with a dark-adapted eye while the angle of the specimen was varied continuously. Various angles of the microscope were used also.

Although several specimens were used, and observations were made with the parallel light incident on first the outside and then the inside of the cuticle, nothing could be observed that could be identified as reflected rays, critically or otherwise. Interest grew when some colored fringes were observed along the edge of the cuticle, but they turned out to be identical with the colored fringes along the edge of the glass slide.

It may possibly be that, even with grazing incidence on the surface of the cuticle, the angle at some or all of the interfaces would not be large enough to give critical reflection from the interface. However, with all angles of approach there would have to be some reflection, which reflection would become greater as the critical angle was approached. If the original assumption of discrete interfaces was correct, this reflection should have been observed. Irregularity of the interfaces could hardly be used as an explanation for the negative results obtained, for even submicroscopic particles can be observed by the reflection of light. Smoke particles are
regularly observed for Brownian movement in this manner.

Hence there is supporting evidence for the first hypothesis advanced in Method II: that the layers fuse into one another, rather than being sharply delimited entities. However, the evidence of this method still does not eliminate the possibility that the layers are irregular as well as fused.
VI. THE REFRACTION METHOD

This method is closely associated with the previous one, both in theory and experimental technique. The problem here was to observe the prism-like deviations in light paths as they are represented, greatly magnified, in Figure 9.

![Figure 9.](image)

The source of light for this experiment was a shielded 40 watt show case bulb. This was used as a line source, and set parallel to the upper edge of the sample of grasshopper cuticle, again mounted on the rotaspec. The emergent rays were then carefully observed with a telescope for dispersed light while the specimen was rotated very slowly. Several positions of the telescope were used.

Again it was found impossible to observe these emergent rays which, in the ideal case, should have shown dispersion into spectra of the source, between which breaks might occur because of the layers of different index of refraction.

The negative results in this case could be attributed to any or all of a number of things:

(a) irregularities in the interfaces between layers, as mentioned before;

(b) roughness of the edge of the cuticle (It was found that no spectrum could be observed through a glass slide with a non-polished end,
the slide being in the same relative position in the system as the cuticle);

(c) insensitivity of the observing system.

It is felt that some information could still be obtained by this method if finer cutting tools and a very delicate method of observation were used.
Since the equipment and experience required for sectioning biological material for the electron microscope were not available, it was not attempted to observe actual sections of grasshopper cuticle. Furthermore, as this has already been done for the cockroach cuticle by Richards and Anderson (1942), and as much work has been done in the line of fixation and sectioning of other tissues, such as the work of Pease and Baker (1948) with rat liver, it was felt that little could be contributed in this field.

However, the opinion has been expressed by many that the conclusions drawn about cuticular membranes on the basis of the electron microscope studies done thus far may be invalid. The reason for this opinion is that nothing is known of the artifacts which may be introduced into such membranes by:

(a) fixation and dehydration techniques
(b) cutting into such very thin sections, with possible attendant oxidation of component substances
(c) subjection to the very high vacuum of the electron microscope system (.05 to .01 micron Hg)
(d) bombarding by the electron beam itself.

Hence it was proposed to bypass as many of these pitfalls as possible by developing suitable replica techniques, whereby the cuticle itself would never see the electron microscope nor the fixatives.

Only once did the work deviate from this path. It was thought that by use of certain reagents, sufficient of the cuticle could be dissolved away to leave a sheet thin enough to be examined by the microscope. The reagents
tried were:

(1) concentrated nitric acid
(2) thioglycollic acid (HS CH₂ COOH)
(3) pepsin in acid medium (a proteolytic enzyme),

which were efficient in dissolving the cuticle in the order listed.

The outer insoluble layer from the nitric acid treated cuticle was mounted on a specimen screen and introduced into the microscope, but, although it was the thinnest layer obtained from the three treatments, it was too thick for the observation of any of its structural detail whatsoever.

There was one interesting and curious feature of this specimen: the appearance at one edge of the membrane of an apparently perfect sphere which would be approximately 2 microns in diameter. Although it looked like a very small droplet of oil, this could hardly be the case, since in the high vacuum of the microscope almost any oily substances would have volatilized.

It is quite probable that hot concentrated nitric acid would have reduced the sheet thickness to a more observable value. However, this has already been done by Richards and Anderson (1942) for the cockroach cuticle, and is still subject to the criticism that with such drastic treatment it may be that the structure observed bears little or no resemblance to those of the untreated cuticle about which knowledge is desired.

METAL EVAPORATION APPARATUS

The metal evaporation apparatus is an essential part of any replica studies. The apparatus assembled for this study is pictured in Figure 10.

The vacuum system consisted of a metal two-stage oil diffusion pump
Figure 10. Metal evaporation apparatus.
(Distillation Products, Incorporated, model VMF-10, water cooled), in which was used silicone fluid DC 702 put out by Dow Corning Corporation. The high vacuum stack of this pump was sealed with soft solder through the centre of the 3/8 inch polished steel base of the vacuum chamber. The diffusion pump was backed by a Cenco Megavac pump with Cenco Hyvac oil. Rubber tubing was used to connect the Megavac with the forepressure stack of the diffusion pump. The jar used for the chamber was a vacuum dessicator, as this assured a better fit than any of the bell jars available.

For pressure measurements, a tipping McLeod gauge was used down to 1 micron of mercury; for pressures less than 1 micron, an ionization gauge was used (Distillation Products, Incorporated, type VG-2). The tubulation from the McLeod gauge was sealed into that of the ionization gauge, the latter being sealed through the base plate of the vacuum chamber with Cenco plicene cement.

The evaporation of metals and silica was accomplished in a spiral tungsten filament of 18 mil wire (Radio Corporation of America, CH513 coil). As this filament is very loosely coiled it was found necessary to put a small platform, turned up at the edges, in the bottom of this coil to prevent the minute amounts of material from falling through. This platform was made from molybdenum sheet.

The filament was mounted in the centre of the vacuum chamber, and to prevent foreign bodies from entering the high vacuum stack of the diffusion pump a platform of galvanized sheet iron was placed over the opening for this stack in the base plate.

The current was delivered to the spiral by an autotransformer (made by
Figure 11. Wiring diagram for metal evaporation apparatus.
The American Transformer Company; maximum brush current: 100 amps). One of the copper posts in the vacuum chamber to which this current was delivered was insulated through the plate; the other was merely supported in the base plate, a binding post on the side of the base plate acting as the other terminal for leads from the transformer. Over these posts fitted brass rings, held in place by set screws, which supported rods extending to the centre of the base plate. The tungsten spiral was mounted between the ends of these rods. With this method of supporting the filament, it was possible to adjust its position to obtain the proper angle for shadowing.

To make replicas, a stand was made which allowed the surface under study to be placed directly over the filament. The height of the supporting rings was adjustable, so that the distance of the surface from the filament could be varied; the distance usually used was 7 cm.

In Figure 11 is given the wiring diagram for the apparatus. The 0-30 amperes ammeter indicated in the tungsten spiral circuit was in reality two ammeters with a 15 ampere range each, placed in parallel, since the former was not available. The power supply for the ionization gauge had to be able to supply 10 milliamps at 200 volts, but other than this there were no specifications on it.

The operation of the system required the following steps:

1. the Megavac was started first.

2. when the Megavac had reduced the pressure to less than 100 microns (as indicated by the McLeod gauge), the diffusion pump was started.

3. when the pressure was approximately 1 micron (by the McLeod gauge)
the ionization gauge was put into operation, the pressure there­
after being obtained from the calibration of plate current with
pressure.

(4) evaporation was carried out at a pressure of approximately .5
micron. For silica a current of 28 amps for 60 seconds was
required, with the silica in the form of several small pieces
rather than one large one. The gold used for shadowing was .005
inch strip, and 20 amps for 30 seconds proved sufficient to
evaporate it.

(5) after evaporation, the ionization gauge was turned off, then the
diffusion pump, and when the latter had cooled somewhat the
Megavac was stopped. (Using silicone oil, the cooling of the
diffusion pump was not nearly so important as it would have been
had Octoil been used. Octoil is a product of Distillation
Products, Incorporated.)

(6) air was admitted to the system through a valve placed between the
Megavac and the diffusion pump.

REPLICA TECHNIQUES TRIED AND THEIR EVALUATION

This section will be subdivided into two parts:

A. methods for surface replicas, and

B. methods for cross section replicas.

A. Surface Replicas

1. A cuticle was placed in the evaporation apparatus and silica
deposited on the outer surface to a thickness of approximately 500 A. The
surface was scored into 1/8 inch squares, and it was then attempted to float these silica replicas off on a clean water surface*.

It is known that silica replicas cannot be made of oily surfaces. The fact that it was impossible to obtain replicas by this method might then in itself show that the outer surface of the grasshopper cuticle is oily, and hence that it is not further covered by what Wigglesworth (1945) has termed a "cement layer". However, it may also be that the nature of the surface of the cuticle was such that it bound the silica tightly, or again, that very minute pubescence on the surface caused the silica to adhere to it.

2. To check on the second possibility given in No. 1, a layer of 20 Å of NaCl was deposited on the outer surface of the cuticle before the silica was deposited (c.f. Williams and Wyckoff, 1946). It was still found impossible to float off the replicas, so from this, the explanation lies either in the oily surface, or the pubescence, or possibly a combination of the two.

3. It was thought to test the first possibility given in No. 1 by treating the cuticle in chloroform to remove the oil before the deposition of the silica. Replicas were not obtained. Neither were they obtained when a layer of salt was laid down first. Hence it would seem that pubescence is sufficient to prevent the film from floating off.

4. A drop of a .4% solution of collodium in n-amyl alcohol was put

* To get a clean water surface, the rim of a bowl was coated with paraffin; the bowl was filled to overflowing with water, the surface of which was then "swept" by running a waxed metal stip across the top of the bowl. The bowl used was black to aid in seeing the floating replicas.
on the abdominal surface of an intact dead grasshopper and allowed to evaporate. The collodion replica so formed was then stripped by the warm gelatin technique of Schaeffer (1942). This consisted of applying a 20% solution of gelatin in warm water to the surface at a rate of .5 drop per square centimeter. The water from the solution was evaporated by a stream of dry air, after which the gelatin split off or was cracked off, taking the replica film with it. The composite film was then placed gelatin side down in a Petri dish and washed with several changes of warm water to dissolve and remove the gelatin. The floating replica was picked up on a specimen screen by dropping the screen on it and picking up the screen with the replica by a strip of filter paper placed gently over it and then removed when the water started to come through.

From observation with the light microscope, this method appeared to produce a good replica.

5. An intact dead grasshopper was placed in chloroform for 15 minutes, and a replica was made of its abdominal surface in precisely the same manner as given in No. 4.

B. Cross Section Replicas

1. In the first effort to obtain a replica from a section of grasshopper cuticle, the dry cuticle was tightly clamped between two pieces of a lucite block as shown in Figure 14. The lucite block and the cuticle were shaved down with a sliding microtome until examination with a light microscope showed a smooth surface. The making of a silica replica was then attempted in the same fashion as described in No. 1 of Section A. The salt-silica method as given in No. 2 of Section A was also tried.
In neither case was it possible to float off the replica. Subsequent examination of the "smooth" lucite-cuticle surface in a metallurgical microscope at a magnification of 2500 showed great chasms, both in the lucite and at each side of the cuticle. This was, no doubt, responsible for the non-removal of the replicas.

2. An untreated cuticle was imbedded in Ward's Bioplastic by putting it into a single layer rather than between two layers as it is usually done. The polymerization of this plastic was carried out in an oven at 40°C rather than at the recommended temperature of 70°C. This was done in order to avoid changes in the cuticle due to raising its temperature above the previously mentioned "critical temperature".

Because of the experience with lucite, the sectioning of this plastic was not attempted. It was found that, with tapping, the cast broke in a clean fashion, so a break was induced across the imbedded cuticle.

The surface of this break was coated with a warm 15% gelatin solution which was then allowed to evaporate. This gelatin "negative" was removed from the cast and a .4% solution of collodion in amyl alcohol was applied and in turn allowed to evaporate. The gelatin was dissolved off as in No. 4 of Section A, and the positive collodion replica mounted on a specimen screen. This replica was shadowed with 10 Å of gold at a 5:1 angle (i.e. the perpendicular distance of the filament from the replica was five times the height of the filament above the replica; or, \(\tan \alpha = 1/5\)).
3. A dry cuticle was imbedded in paraffin with a melting point of 44 - 46°C. It was found necessary to allow approximately 15 minutes infiltration time in order to prevent bubbles from forming around the cuticle as the paraffin hardened. The infiltration was carried out in an oven at 42°C, the lowest temperature at which the paraffin would remain liquid on cooling.

Since this paraffin is quite soft at room temperature, it was kept at a temperature around 10°C while it was being handled. It was cut by hand with a microtome blade, the direction of motion of the blade being parallel to the line of the exposed cuticle. When a smooth paraffin-cuticle surface was obtained a 15% gelatin solution was applied to it as in No. 2 above, in an attempt to make a negative replica of this surface. This procedure was highly unsuccessful: miriads of small bubbles were formed between the paraffin and gelatin, especially along the exposed cuticle.

4. It was thought that with a different imbedding medium the gelatin replica might give better results, so a dry cuticle was imbedded in collodion. A 1:3 ether alcohol solution of collodion was used, and the hardening carried out in a chloroform atmosphere.*

The collodion block was kept flooded with alcohol while it was sectioned with a sliding microtome until a smooth collodion-cuticle surface was obtained. Again a 15% gelatin solution was applied to the surface to make a negative replica as in No. 2 of this section. However, the net results were the same as in No. 3.

* I am indebted to Norman Anderson of the Department of Zoology and Entomology for the collodion mounting.
5. The fact that paraffin is somewhat soluble in amyl alcohol ruled out the use of collodion in amyl alcohol for a replica of a paraffin-imbedded cuticle. Using methyl alcohol as a solvent for the collodion circumvents this difficulty.

A paraffin-cuticle surface was prepared as in No. 3, and a drop of a 0.5% solution of collodion in methyl alcohol was applied and allowed to evaporate. The warm gelatin stripping technique described in No. 4 of Section A was used. There was a tendency for some paraffin to adhere to the replica as it was removed, so the gelatin-collodion film was passed through two changes of xylol. The replica was then shadowed with 10 A of gold before the gelatin was dissolved off. The floating replica was mounted in the usual manner.

6. The technique of No. 5 was used on a cuticle which had been treated in chloroform for 15 minutes.

7. A chloroformed cuticle was imbedded in paraffin and cut as in No. 5. The exposed surface was immersed in concentrated nitric acid for 30 seconds and rinsed in distilled water. It was hoped to obtain an effect similar to the etching of metals. After the surface was dried a replica was made of it, again as in No. 5.

8. The positive replica technique of Schwartz, Austin, and Weber (1949) was tried on a paraffin-cuticle surface prepared as in No. 3. A drop of a 15% aqueous solution of polyvinyl alcohol (Du Pont's Elvanol A 51-05) was applied to the surface and the water evaporated. The same difficulty was encountered here as with gelatin --- masses of small bubbles formed between the plastic and the paraffin.
It was sought to eliminate the formation of these bubbles by drying the solution in a dessicator kept at a temperature around 100°C. This, however, did not provide for any better results.

**Note:** At the time of compilation of this thesis the electron micrographs of the replicas obtained were not yet made. It is hoped that these may be added in an addendum.

**IMBEDDING MEDIA**

Some further substances were checked as to their suitability for imbedding the grasshopper cuticle. These were:

(a) Carbowax 4000 and 6000. These compounds are manufactured by Carbide and Carbon Chemicals Corporation, and the 4000 compound was used by Richards, Anderson, and Hance (1942) for imbedding striated muscle to section it for the electron microscope. It was found that whether these waxes were solidified from the molten state, or whether from aqueous solution by removing the water in a dessicator, gross crystallinity resulted. It was also found impossible to get a silica replica of a carbowax surface.

(b) Gelatin. Undried cuticles were imbedded in various consistencies of gelatin, and attempts made to cut through the gelatin and the cuticle. With the methods available it was found that the strain on the gelatin from the pressure of the knife was sufficient to cause the gelatin to split away from the surfaces of the cuticle, especially the outer surface.

It is thought that a high speed rotary microtome might prevent this breaking away, and leave a surface suitable for study by collodion replicas. This would give results free of any distortion which might be attendant on the drying of the cuticle.
(c) Santomerse D. This detergent is a product of Monsanto Chemical Company, and has a melting point of approximately 40°C. It solidifies without any evidence of crystallinity, and is, from this standpoint, desirable. Its investigation was not carried further because of its possible drastic effects on the cuticle.
SUMMARY AND CONCLUSIONS

Literature pertinent to the structure of the insect cuticle has been reviewed. All of this literature supported the idea that the cuticle is composed of layers with well defined boundaries.

Experiments were designed and set up to find the number and thickness of these layers in the cuticle of the grasshopper Melanoplus bivittatus, and also the way in which they are interrelated.

The schlieren method is discussed in detail, but no feasible variation of the method could be found for application to this problem.

If a section of the cuticle could be regarded as N uniform slits of unequal widths, then it should have been possible to obtain a diffraction from it. Since no such pattern was obtained, it was concluded that the layers must either be of non-uniform thickness, or have diffuse boundaries, or both.

The phase microscope study and the interference method both supported the idea of non-uniformity of the layers, but from these methods no knowledge could be obtained about the diffuseness of the boundaries.

The critical angle method as described was independent of irregularity of the layers, but no critically reflected rays were observed. This is strong evidence that the layers fuse into one another, and do not have well defined boundaries between them.

An attempt was made to observe the prism-like refractions of paths of light rays striking the surface of a piece of the abdominal cuticle and emerging from the end of it. The failure of this attempt was attributed to the methods used, rather than to any inherent characteristic of the
The development of electron microscope replica techniques for the present and similar problems was then undertaken. Techniques were sought which subjected the cuticle to as few unnatural environments as possible, except where specific treatment was desired. The method which showed most promise for the abdominal cuticle surface was a collodion replica made from an amyl alcohol solution, stripped by the warm gelatin technique. The best technique for cross section replicas required imbedding the dried, unfixed cuticle in soft paraffin (m.p. 44-46°C), cutting the block at low temperatures, and making a collodion replica of the exposed surface from a methyl alcohol solution. The warm gelatin stripping was used here also.

A method which should have further study is the imbedding of a cuticle in a gelatin solution, and the sectioning of it for replication purposes.

Many of the replicas obtained were gold shadowed in the metal evaporation apparatus built for the purpose which is described in detail. This apparatus was also used for silica evaporation in the attempts to make silica replicas.

The electron micrographs of these replicas are not available at the time of compilation of this thesis, but will be added in an addendum.
LITERATURE CITED AND CONSULTED


Chefurka, W. Unpublished work.


A study of the structure of the grasshopper cuticle