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
Montana State University

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Chemical, histological, and physiological work on the insect cuticle has indicated that this integument is composed of layers with discrete interfaces. The present work attempts to find the number and nature of these layers in the cuticle of the grasshopper, Melanoplus bivittatus, by purely physical means.

Methods attempted were; 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, 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 discussed as to their possible practicability. Micrographs of replicas obtained will be given in an addendum.

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Approved:

Arthur H. Johnson
In Charge of Major Work

Arthur H. Johnson
Chairman, Examining Committee

L. M. Nelson
Dean, Graduate Division

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ABSTRACT

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Methods attempted were:

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.

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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.

ACKNOWLEDGEMENTS

The author wishes to thank Prof. A. J. M. Johnson for his varied assistance during the course of this research; Dr. Roy V. Wiegand for his advice in the technical aspects of this problem; and Dr. Kurt Rothschild for his constructive criticisms of this work and its presentation. To the other members of the staff of the Department of Physics, and to the members of the Departments of Zoology and Entomology and of Chemistry, the author also wishes to express appreciation for many helpful discussions and timely suggestions.

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 resestant 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 falculata (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. falculata, 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 Tenebrio, 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 1.2×10^6 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

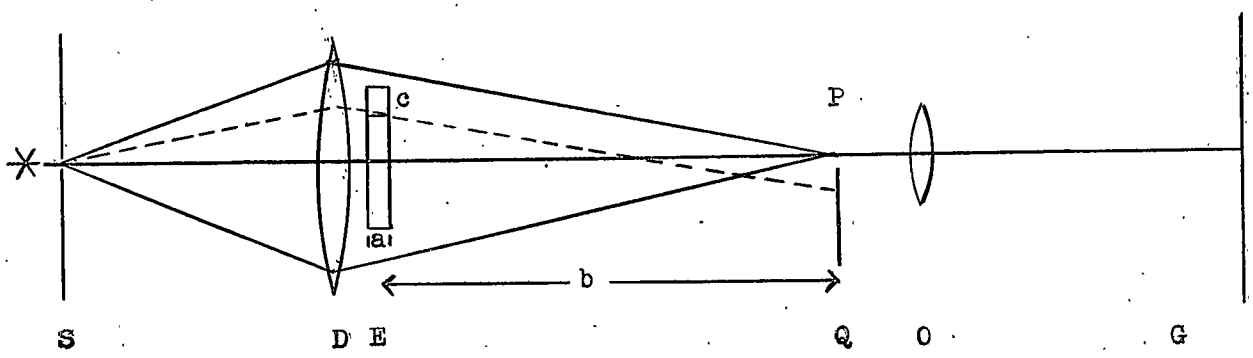

Figure 1. Elevation diagram of the schlieren apparatus.

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 undeviated 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 (1939) 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.

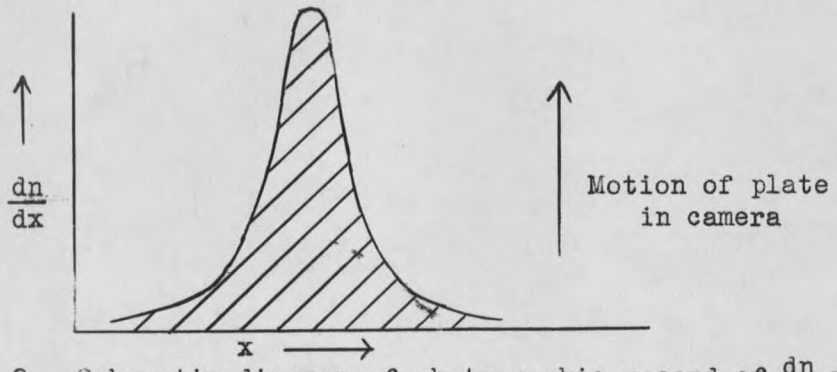

Figure 2. Schematic diagram of photographic record of $\frac{dn}{dx}$ versus x from schlieren apparatus.

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.

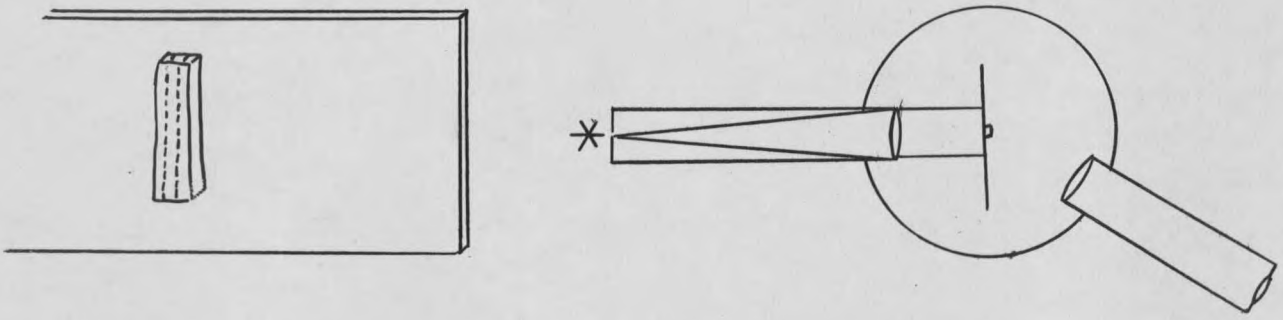

Figure 3.

a. Schematic diagram of cuticle section on slide.

b. Prepared slide mounted on spectrometer table.

Two sources of monochromatic radiation were tried: a sodium bulb (No. 23256, 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 A. 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 liik 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.

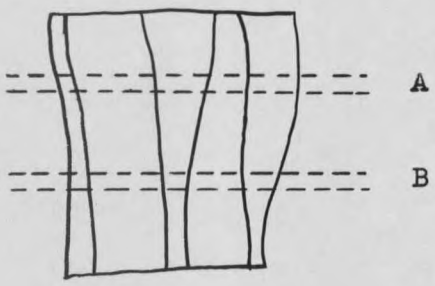

Figure 4. Diagram of possible variations in cuticle layers.

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 defition 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
λ = 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