ELECTRON MICROSCOPE STUDIES ON THE STRUCTURE OF EARTHWORM CUTICLES

by

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There are several reasons why studies on earthworm cuticles are of particular interest. Firstly, there is the relationship of the annelid to the arthropod cuticle. These two types have fundamentally different molecular structures, the annelid cuticle being composed principally of a collagen-type fibrous protein[1], while the arthropod cuticle is composed of a polysaccharide, chitin, and a β-protein, arthropodin[2]. A second interest is the problem of fibril orientation and layer formation in extracellular structures. The earthworm cuticle consists of a number of thin layers lying parallel to the surface, each layer being formed of fine parallel fibrils, with the fibril directions in alternate layers approximately at right angles[3]. If the cuticle is removed as a complete tubular skin and split at one edge, it tears naturally parallel to one of the fibril directions, i.e., along a spiral course running at 45° for the entire length of the worm. This disposition of fibrils, directed in alternating spiral paths in successive fibril layers, recalls the structure of the plant cell wall[4], and a similar structure is indicated in the surface of the mammalian sperm head[5]. Like others[4, 6] we have been interested in the manner of formation of these spiral and layer structures, and the earthworm cuticle is particularly suitable material in which to study them. The cuticle is easy to remove, and it is possible to carry out surgical operations and other experimental procedures very readily. Other problems concern the actual nature of the cuticular collagen-type fibrils secreted by the columnar epidermal cells, for they differ markedly from connective tissue collagen fibrils. Furthermore, an extracellular structure such as the earthworm cuticle offers convenient facilities for studying the processes involved in the formation of individual fibrils.

In the present paper we describe techniques which enable us to examine a whole series of levels in many types of structures. The methods do not involve extensive fragmentation or chemical degradation. They permit the study of delicate membranes and the interrelationship of units of structure over a wide area. They combine virtues of the procedures of both sectioning and microdissection.

Technique

The cuticle may be obtained in a number of ways. From worms anaesthetised in chlorotone the fresh cuticle may be stripped off with forceps, in which case many epithelial cells are also removed adhering to the cuticle. Cuticles may be obtained by blistering with liquid air or by macerating in saturated boric acid [7] or in 30–50% alcohol[3]. If necessary, adhering epithelial cells may be removed by a short period of digestion in trypsin at 18° C. Cleaned cuticles are washed in water and spread out on

References p. 18.

glass slides and allowed to dry; they decrease greatly in thickness but remain the same in surface area.

In preparing specimens for the electron microscope we employ what we call the layer-stripping technique. This consists of lifting the edge of a dried cuticle from the glass surface and stripping it off. On the glass surface there remains the lowest layer and often one or more of the superposed layers. We have applied a layer of collodion and lifted collodion plus specimen from the glass by wetting in water. This forms admirable material for examination by transmission in the electron microscope, and it can be 'stained' with heavy metal compounds.

Rather more interesting have been results using metal-shadowing[8]. In this case the stripped layer on the glass surface is first shadowed at a small angle with gold before applying the collodion. The slide is then placed first in pepsin and afterwards in trypsin digestion fluids, and the protein material is thus removed. The collodion film plus gold floats free, giving a shadow-cast replica of the fibril layer. This process, which has many applications, we refer to as the layer-digestion procedure.

In the following account of the structure of the earthworm cuticle we present information on three main topics: a) the structure of the 'middle' layers of the cuticle; b) the process of fibril formation; c) the remarkable corpuscular layer on the external surface. While a number of earthworms have been used, that found most convenient was *Allolobophora longa*, and most of the pictures illustrated here refer to this worm. All preparations were examined in the R.C.A. Type B instrument, using a 45 kV electron beam.

The formed fibril layers

Between the single external layer of corpuscles and the epithelial cells there are many layers of fibrils, all of which may be described as 'fully formed' except for the lowermost two or three in contact with the epithelial cells. In describing these layers we neglect for the present the pore canals which cause the fibril directions to be diverted from their regular parallel course. Similarly, we neglect phenomena associated with intersegmental sutures which have been occasionally observed in the present work. Fibril layers prepared by the layer-stripping, metal-shadowing, and layer-digestion techniques appear as in Figs. 1 and 2. The pictures are of nearly related regions of the same cuticle which was raised in 50 % alcohol but otherwise untreated. In Fig. 1 we see an average middle layer where many of its constituent fibrils, f, have been torn out adhering to the cleaved layer above. Fibrils not torn out can be recognised at F. All the fibrils appear to be bound together by a cementing material, and ridges of this matrix are seen at r, marking the form and direction of the fibrils in the next layer above. The pronounced ridges at R appear to be composed of the periphery of fibrils cemented together. Fig. 1 represents a very common appearance of the formed fibril layers that provides information about the size and direction of the fibrils and the presence of a matrix.

In a few cases, probably where washing-out of the matrix was promoted by an edge position, details of the fibril surfaces are revealed. Characteristic features are axes within the fibrils making a small angle with the main longitudinal axis. The angles vary but are of the order of 20°. The apparent microfibrils which lie parallel to these axes are 200–300 A wide. The presence of these oblique axes in fibrils has been found frequently in material lightly treated with trypsin. The more prolonged action of trypsin on the lower surface of the cuticle results in the removal of all cell residues and also the fibril

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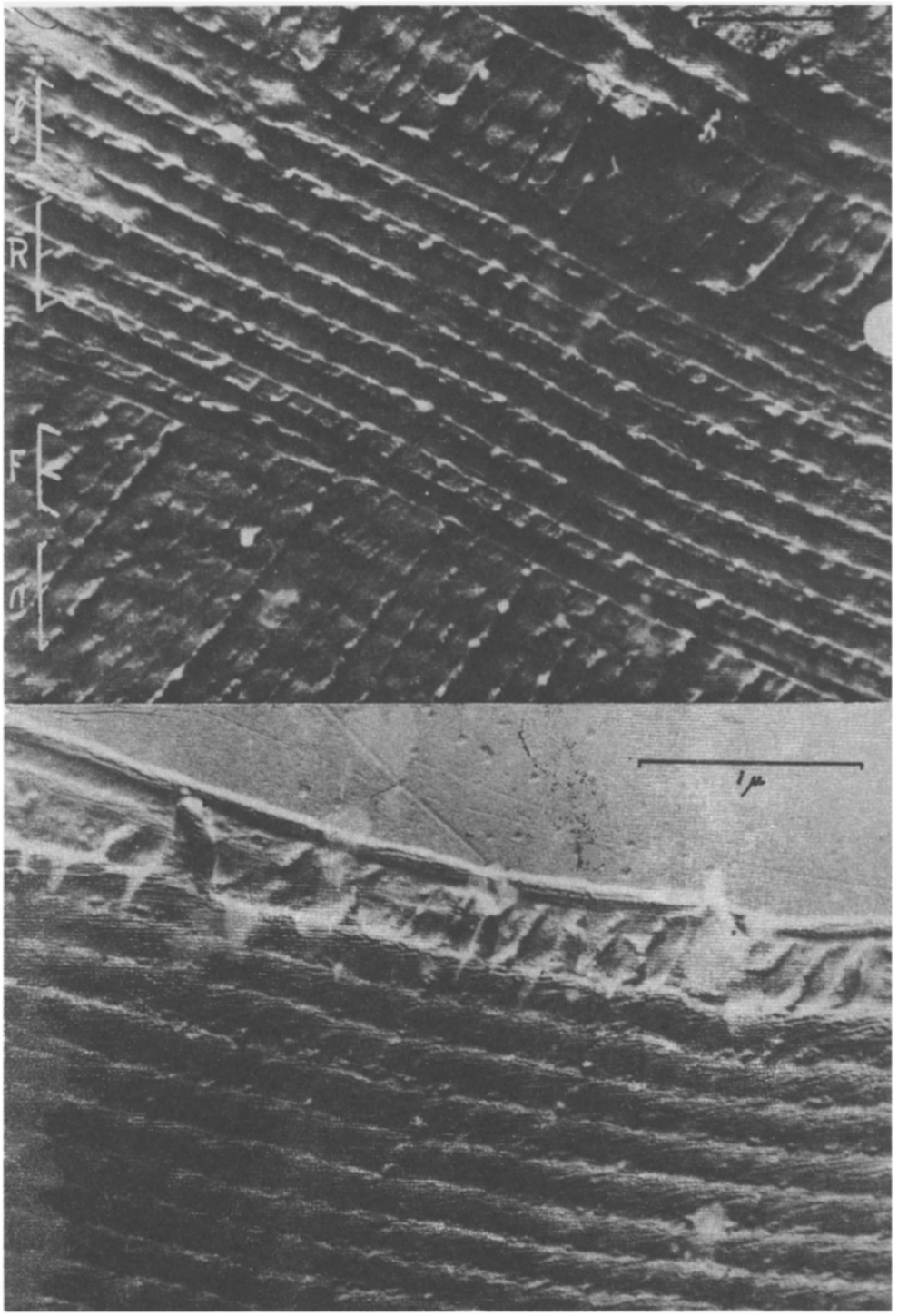

Fig. 1. (Top). Middle layers of earthworm cuticle
Fig. 2. (Bottom). Middle layers of earthworm cuticle with matrix removed

References p. 18.

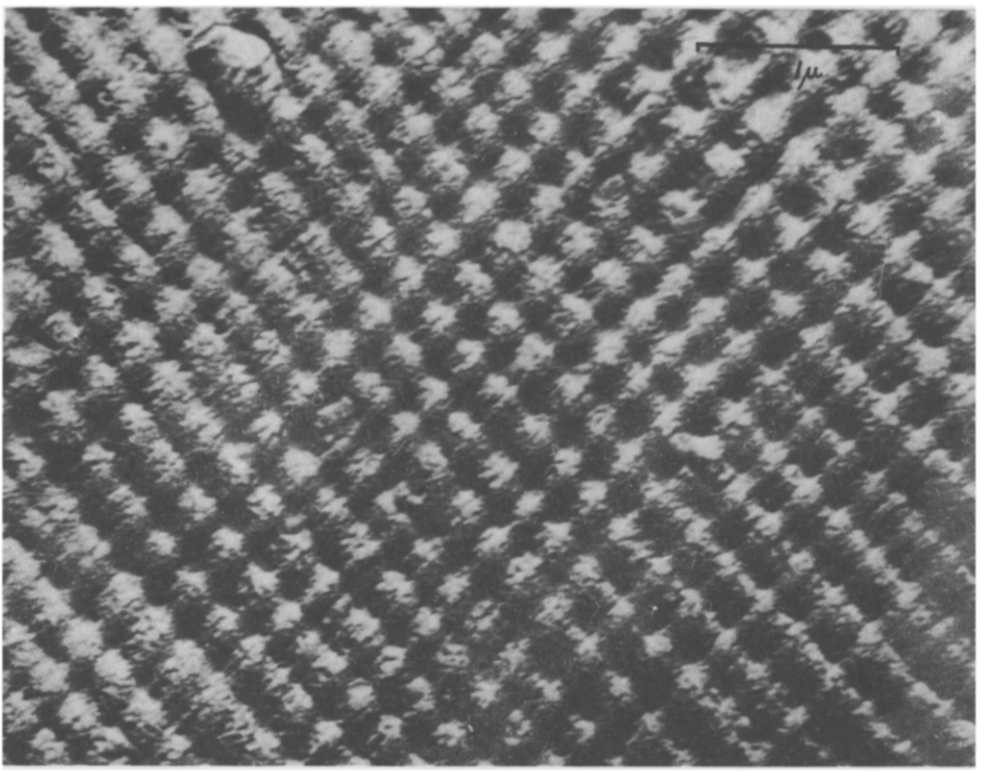

Fig. 3. Lower surface of earthworm cuticle treated with trypsin

References p. 18.

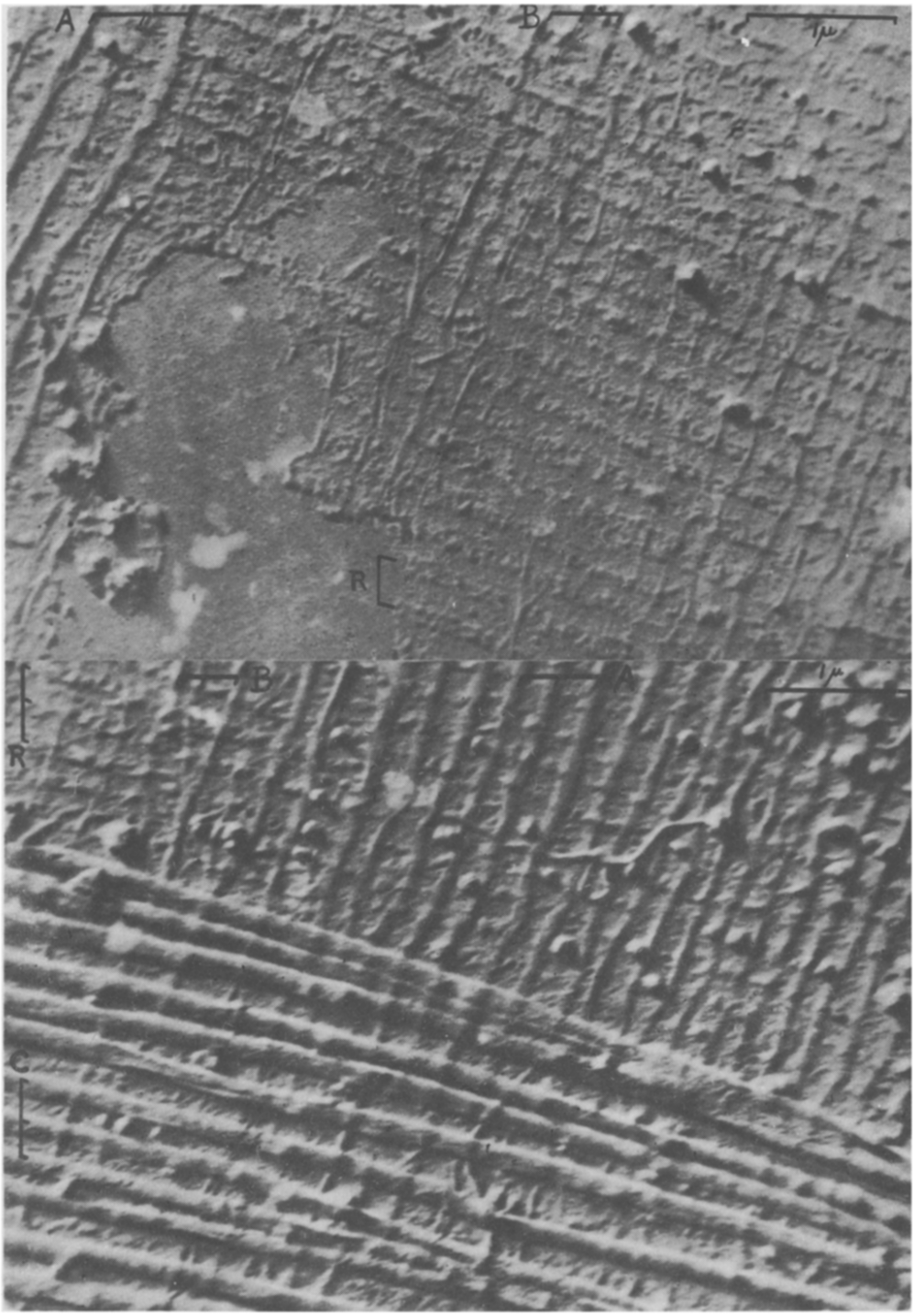

Fig. 4. (Top). Lowest layer of earthworm cuticle
Fig. 5. (Bottom). Earthworm cuticle. Lowest layer with the next two superposed layers.

References p. 18.

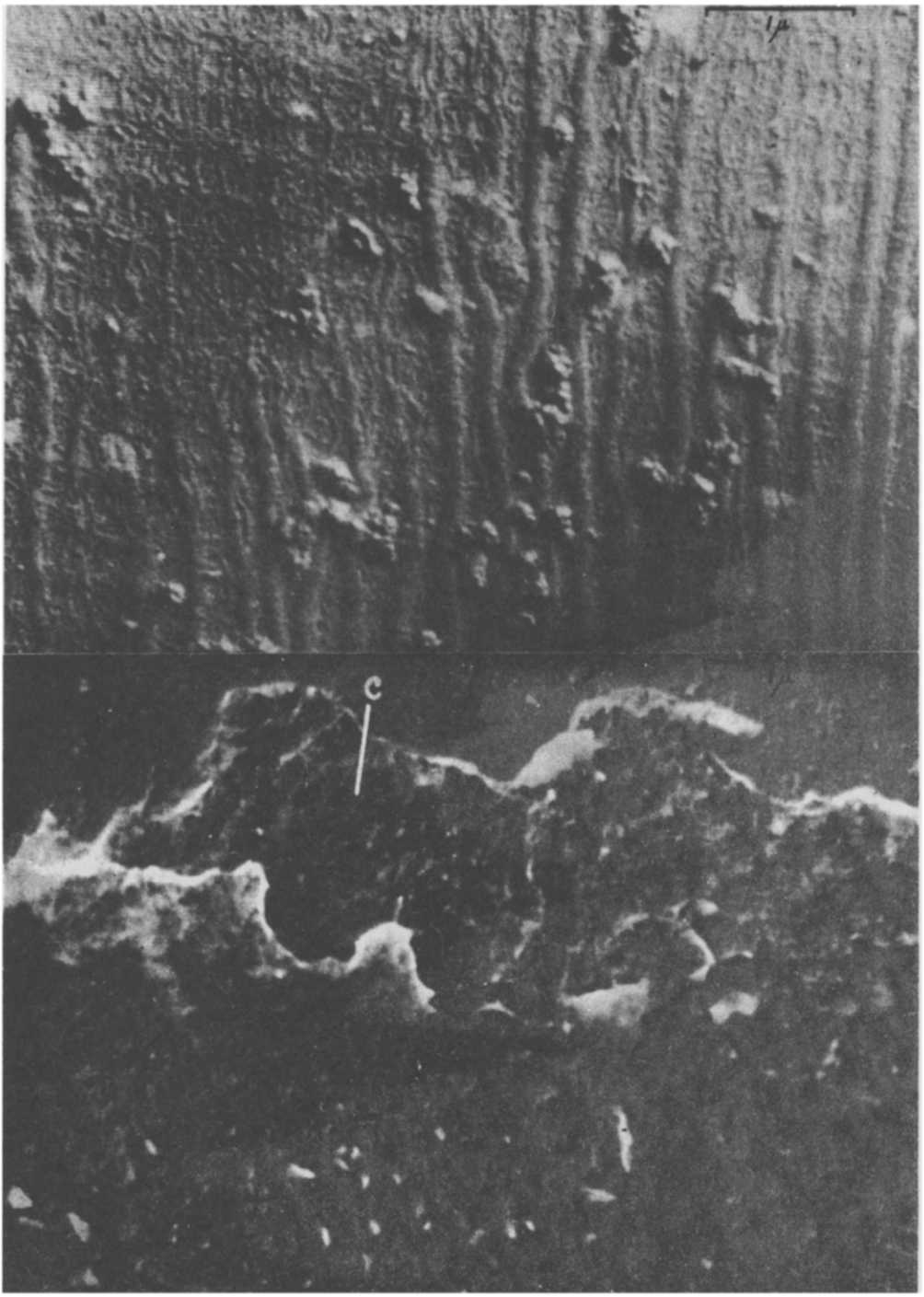

Fig. 6. (Top). Incomplete development of fibrils in lower layer of earthworm cuticle
Fig. 7. (Bottom). Periphery of the end face of an epithelial cell with lowest layer of the cuticle attached

References p. 18.

layers which are in process of formation. A typical picture of the lower surface of the cuticle after treatment in trypsin at p_H 8.6 for 2 hours at room temperature is shown in Fig. 3. The matrix between the fibrils has been removed and the surfaces of the fibrils have been etched and show a markedly rough or granular appearance. The superficial fibrils in Fig. 3 appear narrower and more separated than the next layer of fibrils below. The superficial layer is nearer the epithelial cells and the presence in it of narrower fibrils is in agreement with the results of the following section (cf. Fig. 5).

The designation of the fibrils as collagen-type is based on their wide-angle X-ray diffraction pattern, which is indistinguishable from that of gelatin and connective tissue collagens. However, cuticle collagen fibres show no sign of the cross banded structure studied by SCHMITT, HALL AND JAKUS[9] and by WOLPERS[10] in a wide range of collagen fibres. Cuticle collagen differs from mammalian collagen in its lesser stability in hot water. Cuticles shrink and dissolve in water between 40–50° C, yielding a solution which does not gel on cooling. The cuticle is also attacked fairly readily by trypsin. In these respects cuticle collagen resembles the dermal collagen of teleosts as described by GUSTAVSSON[11], and it will be of interest to compare the two forms in the electron microscope.

The process of fibril formation

We have not found any description of the manner of growth of the earthworm cuticle. It is easy to demonstrate that the cuticle will regenerate after removal, and electron microscope studies of the actively growing cuticle could be made on areas known to be regenerating. Here we present information on the fibril layers next to the epithelial cells as taken from normal adult worms in early summer, and it is to be presumed that the data correspond to growth stages in the formation of the fibril layers.

In the case of Figs. 4 and 5 the cuticle was untreated and stripped from an anaesthetised worm. The lower layers in contact with the epithelial cells were obtained by layer-stripping, and the casts were prepared by gold-shadowing and layer-digestion. Fig. 4 shows the lowest layer of the cuticle so far recognised. It has the appearance of a membrane with well-defined parallel ridges (R) running across it, the ridges being markedly granular or corpuscular. At places there are fibre-like elements running at right angles to the ridges on the membrane. These fibrils are larger and more continuous at A than at B. In Fig. 5 we see a nearly related part of the same preparation but with several fibril layers superposed. The lowest membrane with ridges R is recognisable, while there is also the first fibril layer with larger fibres A and smaller fibres B running at right angles to the ridges R. A second fibril layer C has larger constituent fibrils, and these run at right angles to the fibrils A and B but parallel to the ridges R. As far as can be judged, the fibre layer C may be described as fully-formed. The mere recognition of these stages is important; they can be examined in more detail in further studies. They suggest that the cuticle layers develop from the ridged membranous layer by the formation of small fibrils which increase in size, the organisation of material into fibrils being extracellular.

Another appearance of one of the lower layers is shown in Fig. 6. This was obtained from cuticle raised in 50 % alcohol. It shows well the phenomenon of fibres resting on a ridged membrane, and in one region the fibres appear to 'fade out' into the membrane. It is possible that this 'fade out' may represent the fibrils in process of development; or it may indicate that fibril formation is failing at this region and may never be completed. The clumps of particles on the fibre layer are often present where cuticles have been

References p. 18.

raised in alcohol; they probably represent broken-down and precipitated elements of the lower layers of the cuticle.

The lowest layers of the cuticle and their relation to the epidermal cells are illustrated in Fig. 7. Fresh cuticle with adhering epithelial cells was fixed in 5 % formaldehyde and prepared by the layer-stripping, gold-shadowing and layer-digestion procedures. The picture shows the periphery of the end face of a columnar epithelial cell, the end face being 25 μ in diameter. At the region C the lowest layer of the cuticle is torn away, thus revealing the texture of the cell surface or perhaps even a level slightly within the cell boundary. We are here approaching nearer the solution of the phenomena of fibre formation and orientation which seem to occur at the cell surface. The suggestion that the cuticle is formed by *transformation* of the cell surface[3] is not supported by the presence of any trace of cell outlines in the layers of the cuticle. It seems more likely that it is formed by *secretion* from the cell surface. The orientation may then be controlled by intrinsic orientation in the cell surface, as suggested by PRESTON[4] for the mechanism of cellulose fibre orientation in plant cell walls; or orientation may be due to molecular forces involved in the crystallisation of the fibres.

The superficial layer of corpuscles

If an area of cuticle is dried on a glass plate with its external surface uppermost, a gold shadow-cast of this outer surface can be obtained by the layer-digestion procedure. A typical picture of the external surface is shown in Fig. 8. It is remarkable for the presence of discrete and evenly distributed corpuscles which are often grouped in regular arrays, sometimes hexagonal and sometimes nearly cubic. The particles obviously vary in size, the larger being about 600 A in diameter. Surface particles of this nature have been found in all earthworms so far examined. Generally they cover the surface of the cuticle continuously as in Fig. 8, but on rare occasions an area of the surface some 10–20 μ in diameter will be without the layer of corpuscles, the fibrils of the main cuticle layers themselves being at the surface. The particles are not washed away in water or alcohol, nor are they removed by digestion for several hours in trypsin at room temperature. Between the corpuscles there appears to be a continuous membrane. The layer stripping procedure can be applied with sufficient success to isolate the superficial membrane with its corpuscles. Thus we can examine the membrane also from below, with the result shown in Fig. 9. At A we see the outer fibril layers of the cuticle, while at B is the underneath surface of the corpuscle layer — a rough surface which may be interpreted as a series of 'tails' or 'leaflets' attaching the corpuscle layer to the fibril layers. The interesting point at the moment is that we can examine both surfaces of a delicate membrane by means of layer-stripping. Equally it should be possible to examine the outer surface of the fibril layer when the corpuscle layer has been stripped away.

More information concerning the corpuscles was obtained by stripping off the layer and examining it directly without metal-shadowing or staining (Fig. 10). They are seen to vary in size and to be considerably opaque. Most remarkable are the obvious tail attachments which closely resemble those of the tailed bacteriophages[12]. Such tails may be a natural feature of the external corpuscles, or the tails plus the rough appearance of the under surface of the corpuscle layer (Fig. 9) may result simply from the tearing-away of the corpuscles from the fibril layers. We began this paper by stating our interest in the comparison of annelid and arthropod cuticles. The corpuscles which have been observed in the tracheal intima of the honey bee and cockroach[13], though less regularly

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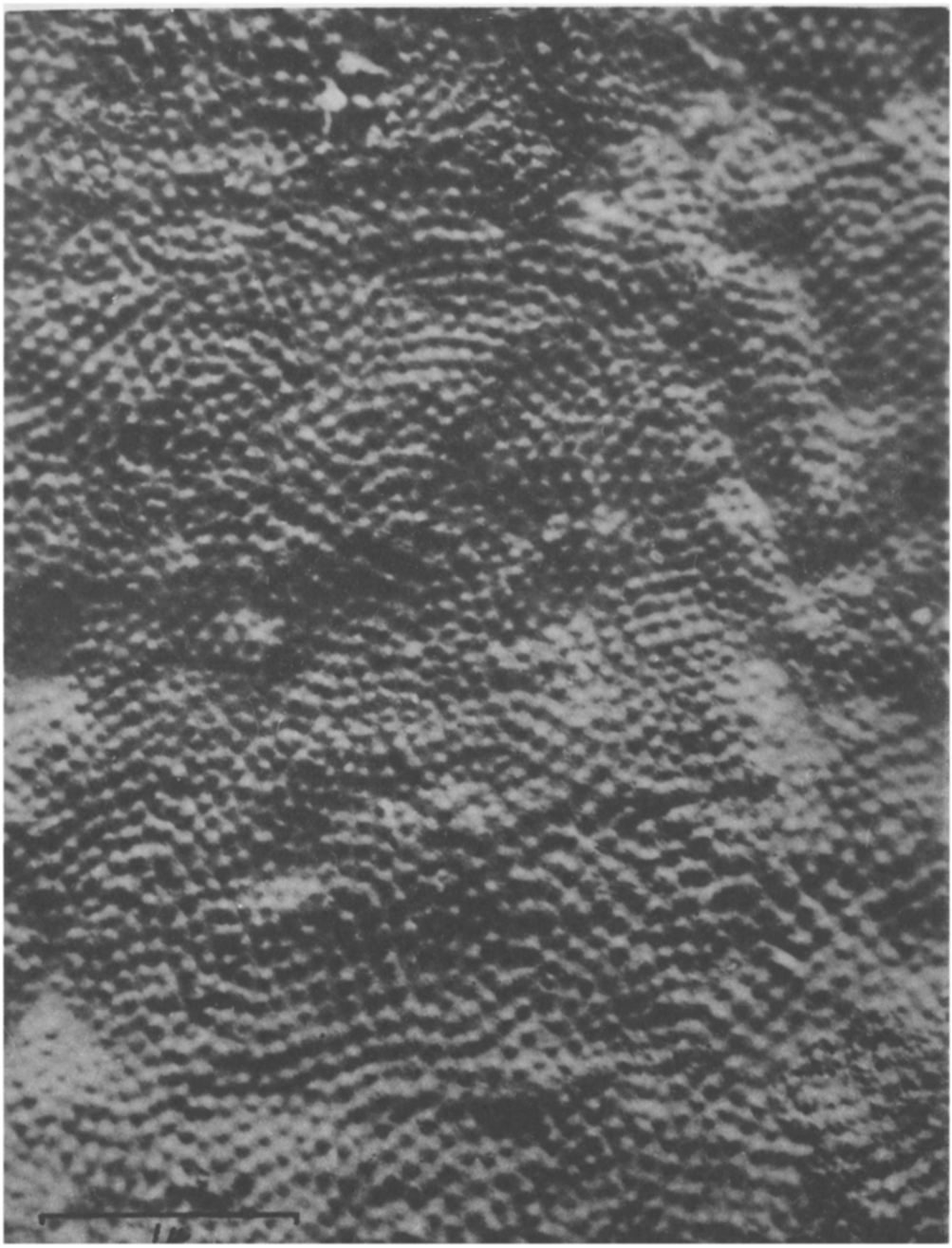

Fig. 8. The layer of corpuscles on the external surface of the earthworm cuticle

References p. 18.

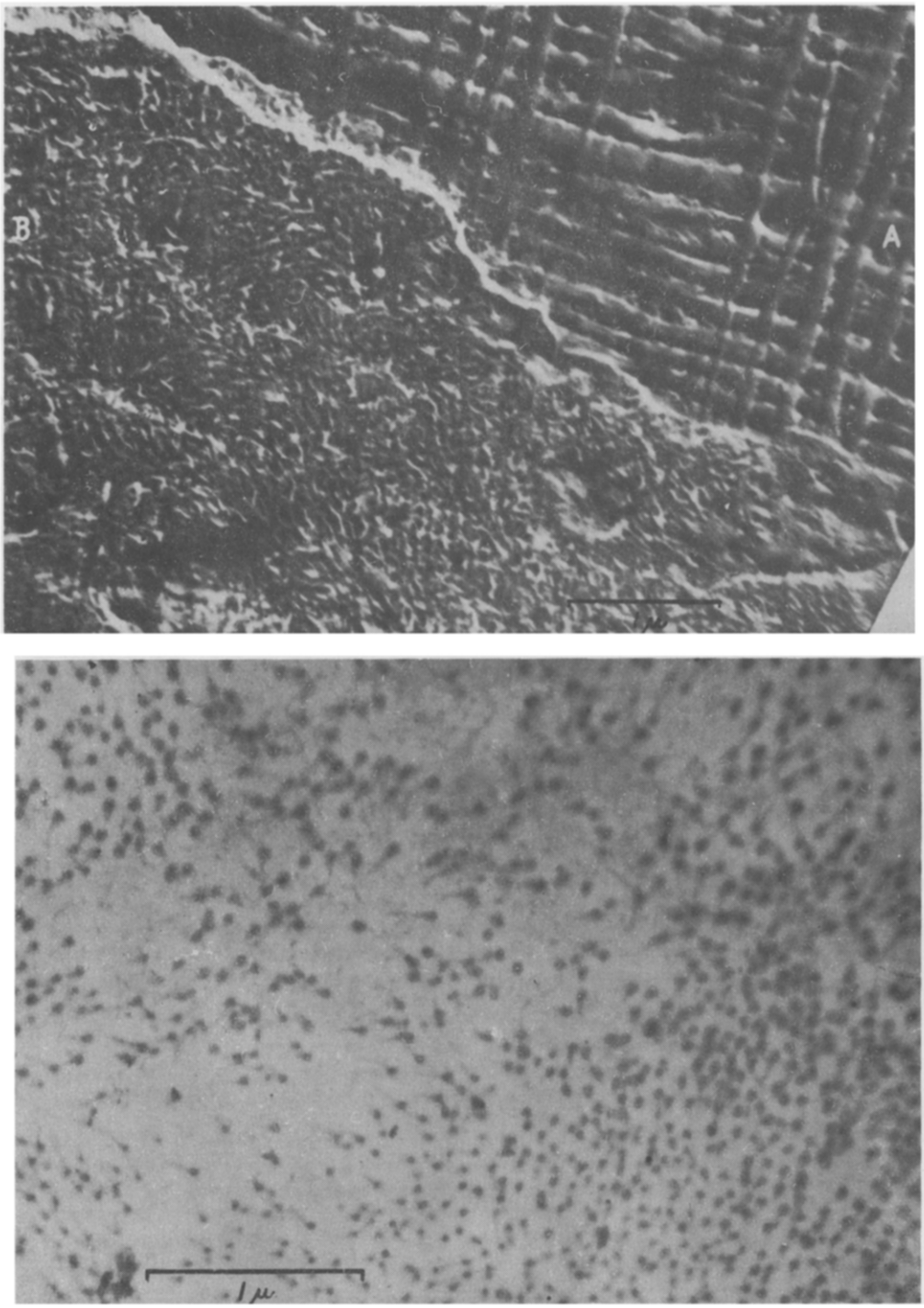

Fig. 9. (Top). Underneath surface of the external corpuscle layer of the earthworm cuticle
Fig. 10. (Bottom). Corpuscles from the external surface of the earthworm cuticle showing the "tails"

References p. 18.

arranged, are often of a similar size, and a possible counterpart of the 'tails' is present in the form of fine interconnecting fibrils.

Tests for the presence of nucleic acids in the surface corpuscles are being made to see if the latter may be definitely related to viruses and bacteriophages. Other studies in progress have to do with the replacement of the corpuscles when they are removed. From this group of studies it is already known that, after removal of the cuticle, the thin new cuticle regenerated for a period of 30 days has a complete covering of corpuscles on its surface.

At various points in this work a number of new experiments are required in order to reach more satisfactory interpretations of the many phenomena revealed by the layer-stripping and layer-digestion techniques.

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SUMMARY

1. The techniques of layer-stripping and layer-digestion are described. They make possible the study of many different levels in a biological structure. The organisation in both surfaces of very thin membranes can be examined.

2. These methods are applied in a study of the earthworm cuticle, which is seen to consist of parallel layers of fibrils embedded in a matrix. The fibrils are composed of a collagen-type fibrous protein, but they are not banded as are connective tissue collagen fibrils.

3. By obtaining in an undisturbed state those fibril layers immediately adjacent to the epithelial cells, we have been able to resolve some of the factors involved in the growth and orientation of the fibrils. The relation of the lowest cuticle layer to the epithelial cells is described.

4. The external surface of earthworms is covered by discrete, evenly distributed, and often regularly arranged corpuscles. When isolated these corpuscles show distinct 'tails'. In their size and in the possession of these 'tails' the surface corpuscles resemble certain bacteriophages.

RÉSUMÉ

1. Description des techniques d'enlèvement et de digestion des couches. Ces techniques rendent possible l'étude de nombreux niveaux dans les structures biologiques. Elles permettent d'examiner l'organisation des deux surfaces de membranes très minces.

2. Ces méthodes sont appliquées à l'étude de la cuticule du ver de terre, cuticule qui apparaît consister en couches parallèles de fibrilles enrobées dans une gaine. Les fibrilles sont constituées par une protéine fibreuse du type collagène, mais ne sont pas organisées comme le sont les fibrilles de collagène du tissu conjonctif.

3. L'obtention, sans modification de leur état, de ces couches de fibrilles immédiatement adjacentes aux cellules épithéliales, a permis de mettre en évidence quelques-uns des facteurs impliqués dans la croissance et l'orientation des fibrilles. Les auteurs décrivent la relation existant entre la couche inférieure de la cuticule et les cellules épithéliales.

4. La surface extérieure des vers de terre est couverte de corpuscules différenciés, régulièrement répartis et donnant lieu souvent à des arrangements réguliers. Une fois isolés, ces corpuscules montrent des "queues" distinctes. Par leur taille et par la présence de ces "queues", ces corpuscules de surface rappellent certains bactériophages.

ZUSAMMENFASSUNG

1. Die Methoden der Schichtabstreifung und Schichtverzehrung werden beschrieben. Sie ermöglichen die Untersuchung vieler verschiedener Niveaus in einer biologischen Struktur. Die Organisation in beiden Oberflächen sehr dünner Membranen kann untersucht werden.

2. Obengenannte Methoden werden in einer Untersuchung des Regenwurmhäutchens angewandt, das, wie dabei festgestellt wurde, aus parallelen Schichten von Fibrillen, die in einer Matrize eingebettet sind, besteht. Die Fibrillen sind aus einem kollagenartigen Fasereiweiss zusammengestellt, sie weisen aber keine Querstreifung auf wie die Kollagenfibrillen des Bindegewebes.

References p. 18.

3. Dadurch, dass wir diejenigen Fibrillenschichten, die sich unmittelbar an die Epithelzellen anschliessen, in unverändertem Zustand erhielten, war es uns möglich, einige der Faktoren, die bei dem Wachsen und der Orientierung der Fibrillen von Bedeutung sind, zu ermitteln. Die Beziehung der niedrigsten Häutchenschicht zu den Epithelzellen wird beschrieben.

4. Die äussere Oberfläche der Regenwürmer ist von diskreten, gleichemässig verteilten, und oft regelmässig angeordneten Körperchen bedeckt. Bei Isolierung zeigen diese Körperchen deutliche "Schwänze". Was ihre Grösse und den Besitz dieser "Schwänze" betrifft, ähneln die Oberflächenkörperchen gewissen Bakteriophagen.

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