Chitin lamellogenesis in locust cuticle

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With 4 plates

Summary

The organization and functions of cuticular lamellae, a structural feature common to all arthropod skeletons, have been studied by growth experiments analysed with the polarizing light microscope. Use has been made of the daily incremental growth of locust solid endocuticle, in which morphogenesis of chitin lamellae (lamellogenesis) can be experimentally altered by varying light and temperature at the time of deposition. Locust endocuticle is all deposited after ecdysis and is composed of alternating night zones (lamellate) and day zones (non-lamellate), distinguishable morphologically, histochemically, mechanically, and morphogenetically. Chitosan tests, confirmed by chromatography, show that the lamellae contain chitin. Endocuticle grown in constant light at constant temperature is non-lamellate; it still contains the same proportion of chitin as normally lamellate cuticle, indicating that lamellogenesis is mainly a periodic process of permanent change in organization of chitin crystallites, occurring at deposition.

Lamellogenesis in solid cuticle is independent of chitin synthesis and of chitin pore-canal fibril formation. Swelling, mechanical, and form-birefringence tests combined with histological observations show that lamellate cuticle is structurally more highly organized but less well oriented than non-lamellate cuticle. One possible function of lamellae is in stabilizing the three-dimensional chitin framework. Normally in night conditions each chitin lamella is individually determined within about 30 min, and lamellae are continuous over large areas of cuticle. The structure and formation of chitin lamellae in locust solid and rubber-like cuticles are compared. Three-dimensional birefringence analysis of the locust hind tibia has revealed three chitin components: (1) chitin lamellae with the chitin crystallites oriented predominantly parallel to the plane of the cuticle surface; (2) chitin pore-canal fibrils, running radially through the cuticle; (3) chitin fibrils oriented longitudinally along the leg axis. Of these, the lamellae and longitudinal fibrils can be morphogenetically altered by suitable techniques.

Introduction

ALL arthropod skeletons are secreted by the distal borders of a single layer of epithelial cells, the epidermis or hypodermis. Epithelial cells have a marked tendency to adhere laterally to one another, so as to present a surface of contact to the internal and external media (Picken, 1960). Thus, when epithelia secrete external skeletons, these are usually stratified in planes parallel to the skeletogenic cells. It is thus not surprising that all the arthropod cuticles which have been examined are lamellated. Lamellae are found in the *solid cuticle* which forms the bulk of the arthropod skeleton, in *rubber-like cuticle* which forms some of the elastic thoracic ligaments in the insect flight system (Weis-Fogh, 1960; Neville, 1963*a*), and in the soft *arthrodial membranes*

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(Malek, 1958). So far, however, there has been no *experimental* analysis of the mode of formation of skeletal lamellae in any arthropod.

Locust skeletons grow by daily increments (Neville, 1963 *a*, *b*, *c*, *d*). They conveniently chronicle their progress by a sequence of pairs of daily growth layers, whose organization can be experimentally altered by subjecting the locusts to unusual environmental conditions during the period of deposition. Daily growth layers thus offer the possibility of a combined experimental and analytical investigation of the morphogenesis of lamellated cuticles. The results indicate that environmentally induced changes in organization at the tissue level probably reflect ultrastructural modifications.

Modern studies of insect cuticle formation, with the exception of those of Locke (1960, 1961, 1964) have focused largely upon the problem of bulk tanning of previously deposited exocuticle. But little is known about the way in which this cuticle is constructed prior to expansion and tanning, while endocuticle seems to have been neglected, largely because it does not undergo a phenolic tanning process. Again, in crustacea, attention has only recently been directed towards the mode of construction of the organic scaffolding in which mineralization occurs (Travis and Friberg, 1963 *a*, *b*; Travis, 1963). Pertinent questions therefore arise concerning the structural and ultrastructural architecture of untanned, uncalcified arthropod cuticle, its mode of organization, and its possible influence on subsequent tanning and mineral deposition. The control systems and rhythms governing this morphogenesis form another part of the problem and a preliminary analysis of the circadian rhythm influencing the organization of chitin lamellae in locusts is presented elsewhere (Neville, 1965, in preparation).

X-ray diffraction analysis shows that naturally occurring chitin displays preferred orientation of crystallites (Clark and Smith, 1936; Carlström, 1957; Rudall, 1963). The samples analysed by both X-ray and optical crystallographic methods have, however, nearly always been the more convenient ones, namely highly oriented bristles and tendons. The present birefringence analysis attempts to examine the preferred orientations of chitin crystallites in a sample of cuticle more typical of the skeleton in general.

Material, methods, and terminology

The work has been carried out mainly on the solid cuticle which forms the bulk of the exoskeleton of the adult desert locust (*Schistocerca gregaria*, Förskal, phasis *gregaria*). The hind leg tibia was selected as a convenient and typical sample. Material was fixed at 4° C for 48 h in neutral 4% formaldehyde, frozen-sectioned, and examined in glycerol or water by ordinary and polarization microscopy. All of the structures observed by this technique have also been confirmed in unfixed living material, hand-sectioned with razor blades; they are, therefore, not artefacts.

Adult locust cuticle possesses three advantages for birefringence analysis. (1) It is transparent, making possible the analysis of retardation interference colours. This would be difficult in amber-coloured cuticles. Pryor (1940)

records that interference colours of exocuticles may be obscured by amber-coloured sclerotizing substances of refractive index similar to that of chitin, which fills the spaces between the chitin crystallites. In locust nymphal cuticle the outer part of the exocuticle is locally melanized and the interference colours thus obscured. (2) The chitin fibril systems in the locust hind tibia lie predominantly at right angles to each other, thus simplifying optical interactions between them. (3) The orientation of the structures, and thus their form birefringences, can be changed by varying rearing conditions at the time of deposition.

Since temperature and light influence the structure of the cuticle being deposited at the time, the locusts were reared in specially constructed cages, consisting of refrigerators with ovens built inside them. In this way temperature could be changed rapidly in either direction. Air circulation was provided by a fan, and illumination by a cold circular fluorescent tube, so that temperature and light could be independently controlled, and preset by an electrical time-switch to follow a desired rhythm.

There is no single consistent terminology which is applicable to the cuticle of all arthropods. This is because cuticle differs from region to region in an individual, and from order to order. The nomenclature used in the present study is, for the major cuticle divisions, that of Campbell (1929), adopted by Richards (1951). The solid cuticle of the locust hind tibia comprises, (i) a non-chitinous epicuticle, (ii) a tanned but colourless exocuticle which is pre-ecdysial and very finely lamellate (the lamellae being spaced so closely together as to be just below the resolution of the light microscope), and (iii) a post-ecdysial endocuticle, with chitin lamellae which may be resolved in the light microscope. There is no mesocuticle between exocuticle and endocuticle, such as occurs in cockroaches (Malek, 1958). The endocuticle may be further divided into earlier and later deposits. The earlier ones are less birefringent in sections cut perpendicular to the cuticle surface than the later ones, which also have the lamellae crowded more closely together. These later deposits appear to be equivalent to the non-calcified membranous layer of crustacean cuticle (Travis, 1963).

The more detailed knowledge of the structure of the finer divisions of exocuticle and endocuticle made available by recent electron-microscope studies (Locke, 1960, 1961) has made necessary a more precise definition of the terms 'lamella' and 'lamina' (Locke, 1964). The term 'lamina' is now used to denote that part of a lamella in which the microfibres are oriented predominantly parallel to the surface of the cuticle. The term 'lamella' denotes the whole repeating unit, which may involve the parabolic microfibres which interconnect two laminae as defined above. Although these finer details were originally defined with respect to lamellae ranging from 0·1 to 1·0 μ thick as seen in the electron microscope (Locke, 1964), they have larger counterparts in crustacean cuticle from 10 to 20 μ thick (fig. 9), and even larger ones, up to 50 μ thick, in the tunicin of tunicates (Neville, unpublished). In the present account, the term *lamella* will be used throughout

to describe the repeating units, even though it is not yet known for locust cuticle whether these are composed of parabolic microfibres together with laminae, or merely of laminae. Locust exocuticle, then, contains several lamellae less than 0·5 μ thick. Endocuticle contains several pairs of daily growth layers, each pair comprising a day layer and a night layer. The combined thickness of one pair of layers is of the order of 10 μ, varying with location in the skeleton. The night layers themselves each contain several lamellae, whereas the day layers are non-lamellate.

Results

Structure of daily growth layers

Paradoxically, the clue to investigating the orienting mechanisms governing the ultrastructural organization of locust endocuticle lay at the tissue level. Low-power photomicrographs of transverse sections of the solid cuticle of locusts viewed between crossed polaroids show pairs of daily growth layers (fig. 1). Although at low magnification the alternate concentric layers of solid cuticle appear to be arranged with an orthogonal change in orientation, as in plywood and in the haversian systems of bone, higher magnification (fig. 2) reveals that they consist of lamellate layers alternating with non-lamellate layers. The lamellate layers, each composed of several lamellae, are normally formed during night conditions (25° C and dark), whereas the non-lamellate layers are formed during day conditions (38° C and light); the daily layering is driven by a circadian rhythm, which is normally phased to day/night environmental changes (Neville, 1965, in preparation). Daily zonation has been found to be due to alternation of lamellate and non-lamellate zones in tibiae, femora, thorax, wing veins, ovipositors, ocelli, and compound eyes.

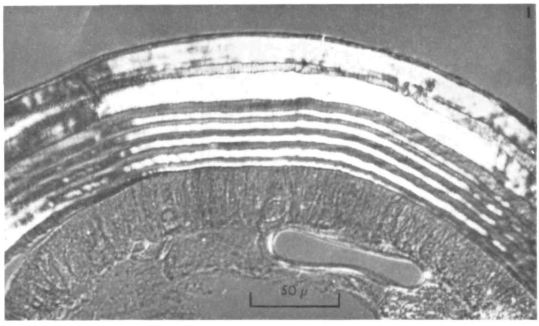
Chemical analysis of lamellae

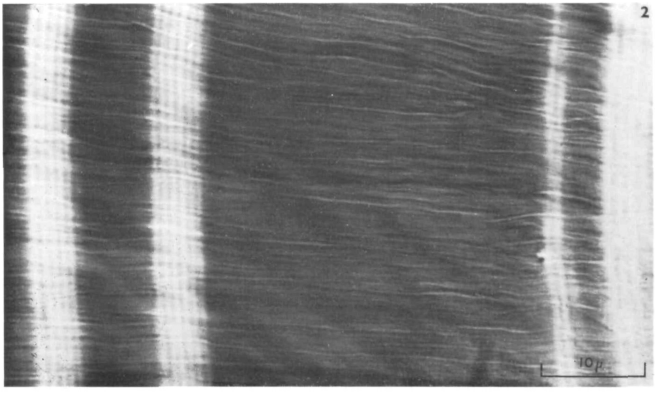
Resilin is a rubber-like protein (Weis-Fogh, 1960) and the daily growth zones in the resilin of locust rubber-like cuticle probably consist of variations in the degree of cross-linking of the protein network (Neville, 1963*a*). They do not involve the chitin lamellae which are also present in some samples of rubber-like cuticle. This proved not to be the case in solid cuticle, since removal of all significant amounts of protein (tanned as well as untanned), by boiling in open tubes for 1½ h in 20% NaOH at 160° C, left the

FIGS. 1 and 2. Transverse sections (20 μ thick) of adult locust hind tibiae photographed between crossed polaroids, 45° off extinction for maximum brightness of birefringence.

FIG. 1. Normal tibial cuticle from an adult killed 5 days after emergence seen at low magnification and showing 5 pairs of daily growth layers in the endocuticle.

FIG. 2. Higher magnification reveals that the pairs of daily growth zones consist of lamellate layers alternating with non-lamellate layers. Each lamellate layer consists of a group of birefringent lamellae (running from top to bottom in the photograph). Birefringent pore-canal components run from left to right. The experimental history of the zones photographed is, reading from left to right, 12 h day, 12 h night, 12 h day, 12 h night, 60 h prolonged day, inhibiting lamellation, 12 h night, 12 h day, 12 h night.





FIGS. 1 and 2

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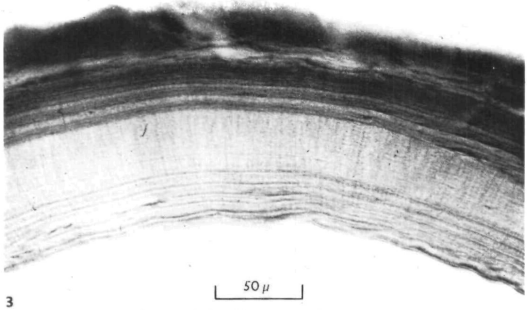

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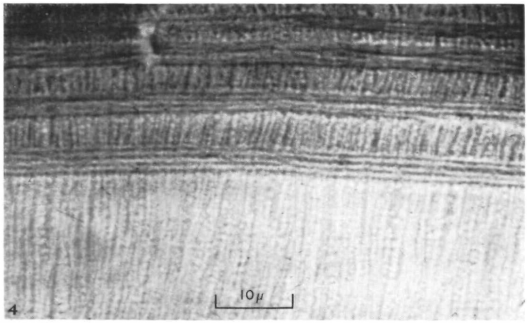

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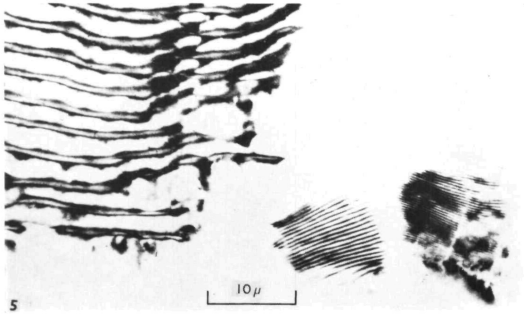

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FIGS. 3–5

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daily growth layers intact. This material was frozen-sectioned, and then treated with 0·2% iodine in potassium iodide solution and 1% sulphuric acid, when deacetylated chitin (chitosan) reacts to give a purple colour (Campbell, 1929). Cuticle which loses its protein shrinks, and so the frozen sections were pressed out gently to their original size. The result (figs. 3, 4, and 5) indicated that the lamellae which make up the night layers of locust endocuticle contain chitin. This was confirmed by fully hydrolysing the chitosan in 12N HCl for 24 h at 120° C in sealed tubes, and chromatogramming the hydrolysate. No significant amounts of amino-acids were present in the hydrolysate, indicating that all protein had been previously removed. According to Giles, Hassan, Laidlaw, and Subramanian (1958), chitin consists of polymerized glucosamine, with approximately 6 in every 7 residues acetylated. Chitosan test treatment deacetylates acetylglucosamine, so that the resulting full hydrolysate would be expected to contain only glucosamine; this was confirmed chromatographically.

There is a claim in the literature that the lamellae of cockroach cuticle disappear on heating with NaOH (Richards and Anderson, 1942), indicating that they do not contain stably oriented chitin. However, when the above observations were repeated on cockroach (*Periplaneta americana*) leg cuticle, the lamellae once again proved to contain chitin and were distributed in layers as in locusts.

It is concluded that the lamellae of locust solid endocuticle, which are normally formed in the night, contain chitin. Although removal of protein leaves the daily growth layers intact, the possible involvement of protein in the initial ordering of the chitin is not, of course, excluded.

Chitin pore-canal component

The fact that the whole of the structure visible in figs. 3, 4, and 5 survived boiling in concentrated alkali and reacted positively to the chitosan test indicates that chitin is not confined to the lamellate zones. Chitinous pore-canal fibrils are arranged perpendicularly to the plane of the chitin lamellae (fig. 4). They are present at all stages of cuticle deposition and may represent an important component of the three-dimensional chitin framework of cuticle. They are present in lobster cuticle (Drach, 1953), and Travis and Friberg (1963 *a*, *b*) have noted a similar radial and lamellate framework, important in ordering mineralization in a crayfish cuticle. Pore canals and hence also

FIGS. 3 to 5. Transverse sections of locust cuticle subjected to the chitosan–iodide test for chitin. All visible structure is due to chitin.

FIG. 3. A tibia from a locust reared after emergence in control conditions (4 lamellate and 4 non-lamellate zones), then with lamellation inhibited during a prolonged 60 h day, followed by 6 days and nights in control conditions (6 lamellate and 6 non-lamellate zones).

FIG. 4. The same at higher magnification. Both lamellae and pore-canal fibrils are made of chitin. In addition chitin fibrils which run longitudinally along the leg can be seen in section.

FIG. 5. Exocuticular lamellae (small) and endocuticular lamellae (large) from a median ocellus section subjected to chitosan–iodide test followed by gentle pressure. The chitin lamellae separate as entities from each other.

pore-canal fibrils are absent from locust rubber-like cuticle (Weis-Fogh, 1960; Neville, 1963*a*).

Lamellate and non-lamellate cuticle

The discovery that the structural organization of cuticle may be controlled by varying environmental conditions at the time of its deposition (Neville, 1965) makes possible experimental analysis of the details of chitin lamella formation. The process of organization of chitin lamellae will be called *chitin lamellogenesis*.

Locusts deposit their endocuticle after each ecdysis (Neville, 1963*c*), in contrast to *Podura* (Collembola) which deposits all of its endocuticle *before* ecdysis (Noble-Nesbitt, 1963*b*). In normal conditions when day alternates with night and sufficient food is available, the freshly emerged adult locust deposits pairs of daily growth layers (lamellate at night and non-lamellate during the day) in all regions of its solid skeleton until each part reaches maturity (as defined with respect to the cuticle). Thus the tibia will grow about 20 pairs of daily growth layers (in 38° C days with light, alternating with 25° C nights in darkness), representing 20 days' growth. If it is grown in artificially imposed constant day conditions, however, chitin lamellation is inhibited, and the result is a non-lamellate endocuticle (figs. 2, 3, 4, and 7). Once deposited, neither lamellate nor non-lamellate endocuticle changes with regard to the presence or absence of lamellae. Any combination of lamellate and non-lamellate layers can be produced to order.

Orientation

Since chitin is not restricted to lamellate layers, the question arose as to whether chitin lamellogenesis is mainly a process of permanent change in orientation of chitin crystallites occurring at deposition. Is chitin uniformly present throughout the endocuticle but organized differently in lamellate layers and non-lamellate ones?

Equally aged samples (4½ weeks after adult emergence) of lamellate and non-lamellate tibiae were each cut into four pieces to facilitate removal of epidermis, tracheae, and muscles. The two samples, each of 10 hind tibiae, were dried in evacuated desiccators over P_2O_5, and weighed. They were then deproteinized by 6 h hydrolysis at 96° C in 20% NaOH, washed carefully in distilled water, dried, counted, and reweighed.

Ten lamellate tibiae weighed 60·1 mg and of this 17·3 mg was chitin. Ten non-lamellate tibiae weighed 57·8 mg and contained 16·2 mg chitin. As a check that all protein had been removed, some of the deproteinized legs were subjected to 24 h further hydrolysis at 120° C in 12N HCl in sealed tubes. The resulting hydrolysate contained only glucosamine derived from chitin, and no amino-acids. (According to Hackman (1960) less strong hydrolysis in N NaOH for 60 h at 100° C removes all amino-acid residues except some histidine and aspartic acid, indicating that protein may be covalently linked to chitin via these amino-acids.) A further sample of deproteinized legs gave

a strong chitosan reaction with iodine and sulphuric acid, indicating that some of the chitin had been deacteylated.

The results show that lamellate tibiae contain 28·7% chitin, while non-lamellate tibiae contain 28% chitin. This indicates that chitin lamellogenesis is mainly a periodic process of permanent change in organization of chitin crystallites during deposition.

After the above deproteinization, sample tibial strips of lamellate and non-lamellate cuticle were placed in 3% acetic acid, which dissolves chitosan as chitosan acetate. The framework of the non-lamellate sample was swollen and dispersed much more quickly than that of the lamellate sample (fig. 6). The tibial spines (also lamellate and non-lamellate in the samples) showed similar differences in swelling and rate of solution. When chitosan preparations were subjected to gentle pressure (fig. 5) the lamellae were separated as entities from the less mechanically stable chitin between lamellae. Similarly, non-lamellate regions burst apart more easily than lamellate regions. This could be the morphological basis for cleavage of cuticle into laminae, which is facilitated by treatment with glycerol and slight hydrolysis (Schulze, 1926). The primary cleavage planes of endocuticle probably correspond to the daily growth layers. Preliminary experiments with the endocuticle of *Lethocerus* (Hemiptera: Heteroptera, Belostomatidae) have confirmed this. The persistence of lamellae in the exuvial space to form the ecdysial membrane (Kühn and Piepho, 1938; Richards, 1951; Passoneau and Williams, 1953; Wigglesworth, 1957; Malek, 1958) may be partly due to the greater mechanical stability of lamellar than of interlamellar material.

It is concluded that a lamellate chitin framwork is mechanically and chemically less easily disrupted than a non-lamellate framework; a possible function of lamellae is in structural stabilization of the three-dimensional chitin framework.

Time factors in chitin lamellogenesis

The time required for the organization of a single chitin lamella to be determined was measured experimentally. For this, locusts were reared in constant-day conditions for a week, giving rise to a non-lamellate tibial endocuticle. A short spell of night conditions was allowed in the middle of this period. This was most effectively carried out by moving some of the locusts out of a cage set for constant day conditions (38° C plus light) into one set for constant night conditions (25° C plus dark), so avoiding the delay of cooling down a hot cage. When a spell of night of 15 min duration was given, no lamellae were formed (fig. 7), the resulting endocuticle being non-lamellate throughout. With 30 min night, a single lamella was organized (fig. 8). Several conclusions may be deduced from this result, which was confirmed in at least a dozen individuals; (*a*) each chitin lamella, as defined in the polarizing light microscope, is a discrete unit and lamellae are formed periodically one by one; (*b*) chitin lamellae are formed in night conditions in solid cuticle; this confirms results from experiments in which locusts were

killed immediately after spells of night or day conditions and the most recently formed cuticle analysed in each case; (*c*) no lamellae were present after 15 min of night conditions, but one was present after 30 min; this shows that the organization of a chitin lamella can be determined in 30 min after the beginning of a night; (*d*) the fact that no lamellae were present after only 15 min night shows that lamellation is not merely triggered by light off or on stimuli; (*e*) night conditions can induce chitin lamellogenesis, but the process cannot be maintained in the presence of day conditions.

It is not yet known whether the organization of a chitin lamella is an all-or-none phenomenon as distinct from a graded orientation process. This might be pursued by electron-microscope studies of experimental material like that in fig. 8. Very thick lamellae like those of the freshwater crayfish *Astacus*, which are about 20 μ wide (figs. 9 and 10), also seem promising material for studies on the time course of formation of a single lamella.

Environmentally dependent and independent lamellogenesis

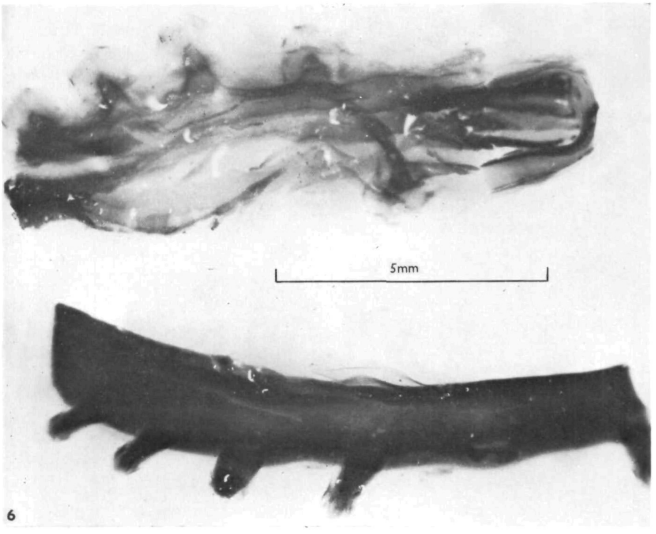
Day conditions inhibit lamellogenesis in many, but not all, regions of the skeleton, since they cancel out the effect of the circadian rhythm which normally governs the alternately lamellate and non-lamellate layers. The exceptions, which are always constant, may indicate regions where lamellae are functionally indispensable. Thus it has been found that chitin lamellogenesis cannot be inhibited in the following locations: (1) in the rubber-like cuticle of the prealar arm and wing hinge ligaments, where their function may be to constrain forces in directions parallel to the cuticle (Weis-Fogh, 1960); (2) in the median ocellus and compound eye cuticle; (3) at the ends of the tibiae and, (4) along the edges of wing veins, where they may serve strengthening functions; (5) in the innermost layer of endocuticle (this layer appears to be homologous with the non-calcified membranous layer of crustacean endocuticle; in the locust it can thus be distinguished morphogenetically as well as morphologically (see terminology section)); (6) in the exocuticle as a whole. The chitin framework of insect exocuticle must take on the full duty of skeleton during moulting and expansion prior to hardening, when the skeletal protein is temporarily plasticized out of action. Its organization must comply with the need to increase in surface area during expansion, whilst contributing towards differential expansion from region to region to preserve the required shape for the species. (The epicuticle may also play some part

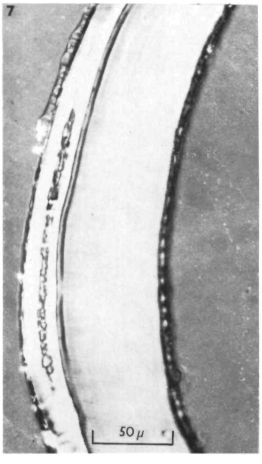
FIGS. 6 to 8. Morphogenetically altered locust cuticle.

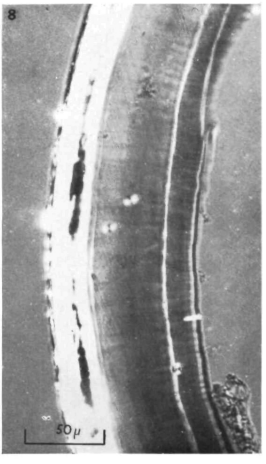
FIG. 6. Portions of experimentally produced non-lamellate tibia (above) and lamellate tibia (below), initially cut to the same size. The samples were deacetylated by hot alkali and swollen in 3% acetic acid. The lamellate sample is more resistant to swelling and dispersion than the non-lamellate sample.

FIG. 7. A sample of non-lamellate locust tibial endocuticle in transverse section, produced by rearing the locust in constant day conditions for a week following emergence, broken only by 15 min night in the middle of the week.

FIG. 8. As for fig. 7, but with 30 min night conditions allowed in the middle of the week of constant day. The organization of a lamella is determined during the 30 min night.







FIGS. 6–8

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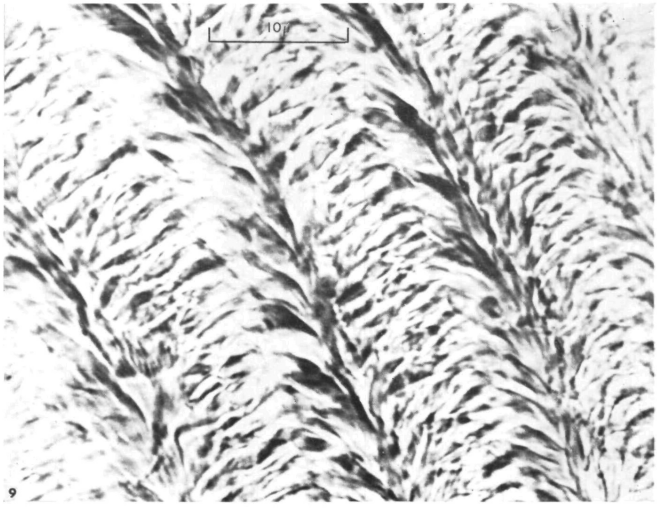

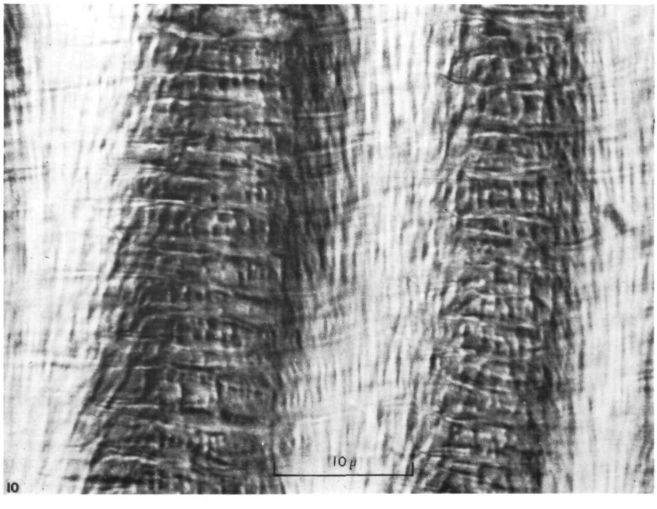

FIGS. 9 and 10

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in defining the size of an expanding insect; see Bennet-Clark, 1963.) In this connexion, therefore, it is interesting to note that the same environmental conditions which inhibit chitin lamellogenesis in the post-ecdysial locust skeleton do not affect pre-ecdysial lamellation. Since pre- and post-ecdysial cuticles are made by the same cells, there must be a change at ecdysis in their susceptibility to changes in external environment or to messages reflecting them. Moult and intermoult morphogenetic events may be under different control. This environmental independence is locally maintained in the cells whose post-ecdysial lamellae cannot be inhibited. Later all the cells once more become independent to secrete the innermost lamellated layer of the endocuticle. We may therefore postulate the existence of a coupling/uncoupling switch linking the secreting cells to morphogenetic information designed to reach them.

In blowflies there is a brief period in the imaginal instar when the emerging adult depends partially upon a hydrostatic skeleton and associated mode of locomotion (Cottrell, 1962 *a*, *b*). In all insects, the time of expansion and the time from expansion to hardening are critical, and damage by buckling at this stage would be made permanent by subsequent hardening. This further emphasizes the role played by the chitin framework at such times.

The chitin framework of locust cuticle

If, as the above results indicate, all the structural features of locust endocuticle and exocuticle visible in the polarizing microscope consist of chitin (figs. 3 and 4), what are these visible constructional units and how are they assembled? An approach to this was made by a three-dimensional birefringence examination of deproteinized sections of locust hind tibiae. Qualitatively, the results proved the same as similar observations on sections of whole unfixed locust cuticle.

In insect cuticle, chitin is arranged as micellae (crystallites) which are below the resolution of the light microscope. They are better regarded as regions of higher crystallinity merging into regions of lower crystallinity (Mark, 1943), the latter probably reflecting local decrease in hydrogen bonding between the molecular chains. Apart from some bound water molecules, which possibly replace an acetyl group on every seventh glucosamine residue along the chains (Giles *et al.*, 1958; Rudall, 1963), X-ray crystallography indicates that there is no further space for penetration of liquids into the crystallites (Carlström, 1957). Imbibed fluids can then only penetrate *between* the crystallites, and provided that (*a*) there is a difference in refractive

FIGS. 9 and 10. Details of the large lamellae of freshwater crayfish endocuticle.

FIG. 9. A chitosan–iodide preparation of a transverse section of *Astacus* leg cuticle. The wide laminae are composed of chitin fibrils oriented parallel to the cuticle surface and fanning out at angles up to 90°.

FIG. 10. A section of *Astacus* leg endocuticle between crossed polaroids, 45° off extinction for maximum brightness of birefringence. The highest birefringence is caused by the chitin fibrils of the laminae (3 shown in the photograph), which are oriented predominantly parallel to the cuticle surface.

index between the imbibed fluid and the chitin crystallites, and (*b*) the crystallites are anisodiametric and preferentially oriented in a given direction (usually either plane to the cuticle surface or along a fibril axis), then the structure will show form birefringence (Wiener, 1912). It is implicit in this theory that the imbibing fluid does not react with the crystallites either chemically or physically. Form birefringence is always positive along the direction of preferred orientation and can be seen in natural samples of chitin (e.g. Schmidt, 1934) as well as in artificially drawn chitin fibrils (Richards and Pipa, 1958). Chitin exhibits intrinsic birefringence due to molecular structure, but whether this is positive or negative with respect to the chain axis has always been a subject of controversy (Castle, 1936; Diehl and van Iterson, 1935; Frey-Wyssling, 1953). Since an imbibing fluid cannot penetrate the actual crystallite regions, intrinsic birefringence is not modified by change in refractive index of imbibing fluid. It thus remains as a residual birefringence after form birefringence has been balanced out by inbibing with a fluid of refractive index equal to that of chitin (Ambronn and Frey, 1926). However, for chitin, the measured value of the intrinsic birefringence *apparently* varies in practice according to the chemical composition and concentration of the imbibing fluid used. Thus xylol indicates a positive intrinsic birefringence, whereas increasingly strong mercuric potassium iodide solutions indicate increasingly large negative values, probably because some oriented imbibition occurs (Castle, 1936); also, some breakage of hydrogen bonds may involve differential swelling effects, with corresponding anomalies in refractive index. If the unknown value of the intrinsic birefringence were large compared with the form birefringence in a particular imbibing fluid, the form-birefringence method of analysing preferred orientations of crystallites in cuticle would not be justified. However, values from Castle's (1936) curves show that for water the retardation Γ for a given thickness of sample was 50 mμ, when the retardation due to intrinsic birefringence appeared to range from -7 mμ to $+15$ mμ. Since birefringence is equal to Γ/d, where d is sample thickness, then for the same thickness of sample two birefringences can be compared according to the ratio of the retardations produced. Thus estimates of the proportion of retardation measured in water contributed by intrinsic birefringence ranged from 7/50 to 15/50 (Castle, 1936) and 21/100 (Diehl and van Iterson, 1935). Measurements of retardation due to the chitin framework of cuticle in water may then be interpreted as being *mainly* an expression of positive form birefringence, and thus serve to indicate the main directions of orientation of crystallites. The following observations were therefore made on sections examined in water, which has a refractive index sufficiently different from that of chitin itself.

The interference colours of locust cuticle seen in sections 20 μ thick between crossed polaroids consist of first order greys and yellows (about 50 to 200 mμ retardations). Transverse sections of locust hind tibiae show the radially arranged chitin pore-canal fibrils, together with chitin lamellae arranged parallel to the plane of the cuticle surface (figs. 2 and 4). Being approximately

at right angles, these two systems of fibrils both extinguish simultaneously when parallel to the axes of crossed polaroids. Similarly, they are both maximally bright at 45° inclination to the polaroid axes. Both show marked parallel extinction, indicating good alignment of crystallites. Since lamellae are absent from normal day zones and from constant-day-reared, non-lamellate cuticle, cross-sections of these samples can be used to advantage to study the birefringence of the pore-canal fibrils. These show positive form birefringence along the fibrils.

Tangential sections of locust tibial cuticle or wing vein cuticle show marked positive birefringence with parallel extinction along the leg or wing vein axis in surface view. This was checked in tangential sections of isolated endocuticle. In such sections the pore-canal fibrils are seen in transverse section and cannot contribute significantly to birefringence, since viewing is then along the zigzag molecular chains (Carlström, 1957, 1962). The marked orientation along the leg axis could reside in either the lamellate or non-lamellate regions. Vertical sections of tibial cuticle cut along the leg axis showed that positive birefringence oriented along the leg axis was not restricted to lamellate regions. Further, it occurred throughout similar sections of experimentally produced non-lamellate cuticle. To check the inference that the chitin crystallites of the non-lamellate regions were oriented longitudinally along the leg, tangential sections of constant-day-reared, non-lamellate endocuticle were cut, and in surface view still showed marked parallel extinction, indicating good alignment along the leg axis.

Mechanical confirmation for this longitudinal orientation was obtained by teasing out non-lamellate endocuticle, when fibrils running along but not across the leg axis could be separated out. The direction of cleavage was parallel to the leg axis. This was also confirmed in the radius plus media wing vein. The transverse tibial sections which had been subjected to the chitosan iodide test showed some indication of these longitudinal fibrils in transverse section (fig. 4). X-ray diffraction evidence supports the finding of highly oriented crystallites along leg and wing axes in Orthoptera (Rudall, 1963).

Seen in transverse section, the longitudinal fibrils would be expected to show very little birefringence associated with the marked parallel extinction along the leg axis, and this was found to be so. As was expected, oblique transverse sections show some birefringence due to the longitudinal fibrils.

It is therefore concluded that there are three kinds of structural components of chitin in locust solid cuticle (fig. 11): (1) chitin lamellae, plane to the surface, and present in night deposits; (2) pore-canal fibrils, radial to the surface; these cut across the day and night deposits; (3) longitudinal fibrils, parallel to the leg and wing vein axes; these are present in day deposits and are similar to the balken fibres of beetle and bug cuticle. Of these three chitin components, two can be morphogenetically altered by different methods. Lamellae are inhibited by constant-day conditions during deposition. Longitudinal fibrils can be experimentally modified by growing implanted pieces of

leg inside the general haemocoel. Under such conditions, chitin lamellogenesis continues as normal, but the longitudinal fibrils need no longer be oriented perfectly along the leg axis (unpublished experiments).

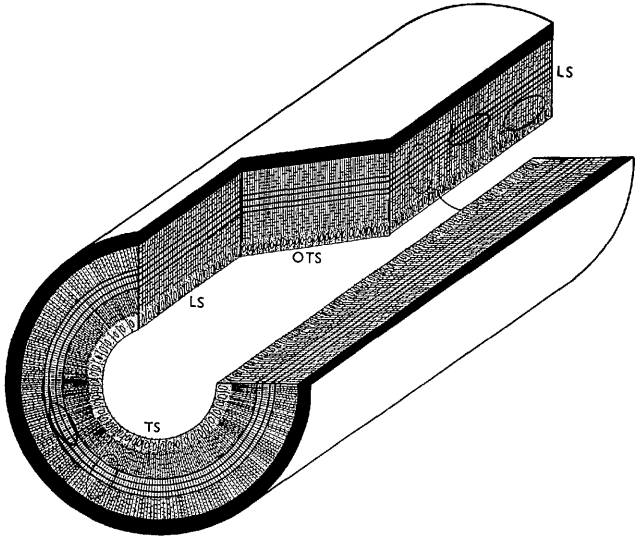

FIG. 11. Diagram of the chitin architecture of a locust hind tibia. The outermost layer, drawn black, is the exocuticle (covered by a very thin epicuticle); the innermost layer is the epithelium of epidermal cells. Between these two lies the endocuticle, in which the directions of maximum and minimum refractive indices for the various chitin fibril systems seen in various sections are indicated by biaxial indicatrices (drawn to the same line thickness as the relevant fibril system). *TS*, transverse section; *LS*, longitudinal section; *OTS*, oblique transverse section.

Discussion

Chitin architecture

The first electron micrographs indicating the apparently fibrillar nature of lamellae in insect cuticle (Locke, 1960) show that the microfibrils in *Calpodes* larvae (Lepidoptera: Hesperiidae) lie densely packed in each lamina, fanning out at angles up to 90° into the less well-ordered region between laminae. This type of arrangement has been confirmed in the larval cuticles of *Galleria* (Lepidoptera) and *Tenebrio* (Coleoptera) and in the adult cuticle of *Apis* (Hymenoptera) (Locke, 1961); in the egg-shell of *Melanoplus* (Orthoptera) (Slifer and Sekhon, 1963); in the arthrodial membrane at the base of setae in *Podura* (Collembola) (Noble-Nesbitt, 1963*a*); and in the cuticle of the crayfish *Orconectes* (Travis and Friberg, 1963*b*). Such an alignment with the

microfibrils in each lamina predominantly oriented parallel to the epidermis could account for the positive form-birefringence parallel to the cuticular surface (fig. 2). On a much larger scale, the lamellae of the freshwater crayfish *Astacus* are about 20 μ wide and are also composed of chitin (fig. 9). When observed in water they have a very similar pattern to that formed by the microfibrils in electron micrographs of the 0·5μ-wide lamellae (dehydrated) of *Calpodes* (Lepidoptera) larval cuticle (Locke, 1960, 1961). The appearance of *Astacus* endocuticular lamellae between crossed polaroids is shown in fig. 10. Considered together, figs. 9 and 10 show that the positive form birefringence of each lamina in *Astacus* is due to the orientation of chitin fibrils predominantly parallel to the plane of the epidermis. A similar conclusion has been reached for the laminae of the lobster *Homarus* (Drach, 1953).

Some early electron micrographs of the cuticle of the pronotum of the cockroach *Periplaneta americana* (Richards and Anderson, 1942) show groups of lamellae arranged in zones very closely resembling the growth zones seen in cockroaches by the present technique. The numbers of lamellae per zone, and the order of size (about 0·5 μ spacing, just at the limit of resolution of the light microscope) are again in agreement. It has been shown above that these cockroach lamellae are also constructed of chitin.

It is therefore reasonably certain that the microfibrils in published electron micrographs (Locke, 1960, 1961) are made of chitin. This could be checked by electron-diffraction methods. The longitudinal chitin fibrils in locust legs and wing veins are similar to the more highly crystalline balken fibres of beetles.

Chitin lamellae in solid and rubber-like cuticles

Chitin lamellae in solid and rubber-like cuticles differ in their pattern of formation. In rubber-like cuticle the lamellae are separated by layers of pure resilin (Weis-Fogh, 1960), and there is a periodic quantal synthesis of chitin (Neville, 1963*b*). Chitin deposition is turned on and off according to a frequency function which decreases with age. In solid cuticle, on the other hand, chitin is synthesized and deposited continuously, and it is the periodic process of change in organization of crystallites which gives rise to the chitin lamellae. There is evidence that in solid cuticle chitin fibrils may connect one lamella to another (Drach, 1953; Travis and Friberg, 1963 *a*, *b*). The lamellae of solid cuticle are therefore not such discrete entities as those of insect rubber-like cuticle. The chitin lamellae in crustacean rubber-like cuticle, on the other hand, do have interconnecting fibrils (unpublished observations). In locusts, these two kinds of cuticle differ further in that the chitin lamellae of solid cuticle are produced according to a circadian rhythm and thus occur in daily zones, whereas the chitin lamellae of rubber-like cuticle do not show any daily rhythm, even though the resilin fluorescence does. As a corollary of this, constant-day conditions inhibit chitin lamellation in solid cuticle, but leave those of rubber-like cuticle unaffected (Neville, 1965, in preparation).

The region of chitin lamellogenesis

One of the central arguments in the ultrastructure and morphogenesis of cuticles is the question whether organization of the visible structures takes place in contact with or away from the skeletogenic epithelium. Is organization determined in the zone of deposition adjacent to the cells, or is it controlled by post-secretory chemicals and stresses operating at a considerable distance from them (Richards, 1951; Picken, 1940, 1960)? It seems impossible to reach a generalized conclusion for all cuticles since examples supporting both cases now exist.

There are several claims in the literature of cuticular lamellae being formed at some distance from the epidermis, while separated from it by considerable thicknesses of intervening cuticle (e.g. exocuticle of *Sarcophaga* larvae (Dennel, 1946); *Cicada* eye lens cuticle (Verhoeff, 1926; Richards, 1951); organization of balken fibres in *Calandra* exocuticle (Reuter, 1937)). The appearance of interposed lamellae between pre-existing ones as evidenced by sections observed in the light microscope need not, however, necessarily involve an increase in number of lamellae. Lamellae previously too close together to be individually resolved by the light microscope could become visible simply as a result of cuticular swelling. The cocoon of the beetle *Donacia* is a specialized case, forming laminae after being secreted as a fluid while separated from the epidermis by intervening cuticle (Picken, Pryor, and Swann, 1947). In the exocuticle of the collembolan *Podura aquatica* lamellae are disorganized at a distance from the cells prior to hardening, whilst the intervening endocuticular lamellae remain intact (Noble-Nesbitt, 1963*b*).

The present results show that chitin lamellogenesis in locust cuticle occurs in parallel with deposition, that is to say, the lamellae are organized periodically and individually. Once formed, non-lamellate deposits remain non-lamellate (figs. 2, 4, 7, and 8). Similarly, lamellogenesis is an irreversible process. If a locust is induced to produce non-lamellate cuticle in constant-day conditions for several days, and then allowed sufficient night (e.g. 30 min) in which to organize a lamella before being killed, the last deposit is found to be the lamella, which lies closely apposed to the epidermis over its whole surface. Continuity of lamellae and their successive production in a one-by-one manner has also been established in *Podura* (Collembola) (Noble-Nesbitt, 1963*b*). In locust rubber-like cuticle (Neville, 1963*b*), as well as in solid cuticle, the most recently formed chitin lamella in animals killed at various ages during deposition is birefringent and thus crystalline. This evidence conflicts with the views of Richards and Anderson (1942) who regarded the cuticular laminae of cockroaches as being formed subsequent to secretion by a chemical phenomenon, and not as representing an unbroken sequence of deposition. They made this suggestion because Fraenkel and Rudall (1940) had shown that reorientation of chitin crystallites in blowfly puparia took place during subsequent reshaping and chemical tanning of the larval cuticle. The orientation of chitin crystallites in lepidopteran scales (Picken, 1949) and

in *Drosophila* bristles (Lees and Picken, 1945) is not a secondary process, being distinguishable in the polarizing microscope from the earliest stages of formation.

That organization of lamellae occurs in close contact with the cells has also been shown in electron micrographs of cuticles of *Galleria* larvae, *Tenebrio* pupae, and *Calpodes* pupae (Locke, 1961), in which the most recently formed region of cuticle (about 1 to 2 μ in thickness) is more coarsely granular and less well oriented than fully formed cuticle. These electron micrographs show that all of the fully formed lamellae have an appearance in cross-section like the much thicker ones of *Astacus* (figs. 9 and 10), whereas the granules which are to produce the most recent lamella at any stage are seen to be in the process of orienting into a similar pattern. These results allowed a correct interpretation (Locke, 1961) of the so-called subcuticular or Schmidt's layer (Schmidt, 1956), as the most recently forming layer at any age, not yet fully organized, rather than as a non-chitinous glue. The reason for the apparent absence of chitin from this zone is attributed to the removal of protein by boiling in concentrated NaOH during the deacetylation stage of the chitosan test. Those chitin granules which are not yet oriented and stabilized will be dispersed when the protein matrix disappears, whereas the stabilized chitin lamellae will remain. This is probably the main source of error connected with the controversial chitosan test, and bears out the comment that a negative result with this test need not necessarily indicate absence of chitin (Richards, 1951).

Although it is common for polymeric molecules which give rise to microscopic structures to aggregate spontaneously, even *in vitro*, chitin lamellogenesis, in several insects at least, appears to be under cellular control. Thus in locusts the process of lamellogenesis can be inhibited even though chitin secretion continues as normal: the two processes are clearly independent. This result would seem to indicate that chitin organization in locusts is not a purely physical process like the Schiller plane phenomenon, as suggested by Richards (1951). The cells are able to control the extracellular environment in which deposition and lamellogenesis occur. Figs. 2 and 4 indicate that pore-canal fibrils are still present in non-lamellate cuticle, showing that the formation of chitin pore-canal fibrils and chitin lamellogenesis are independent processes.

Chitin crystallizing from solution on a flat glass surface orients with the long axes of the molecular chains in the plane of the surface but otherwise at random (Clark and Smith, 1936). Since the chitin crystallites of each endocuticular lamina are predominantly arranged parallel to the plane of the cuticle, we may postulate a similar mechanism of orientation (with regard to this one plane) with the crystallites forming apposed to a pre-existing surface (i.e. the inner surface of the cuticle already present at a given stage). In this context the formation of a lamina might be regarded as a periodic turning off of some other orienting force which would otherwise have oriented the chitin crystallites differently (e.g. in parabolae between consecutive laminae, or axially along the leg).

What is the function of lamellae?

Simple mechanical and swelling tests show that inhibition of chitin lamellogenesis lowers the structural stability of the chitin framework of locust cuticle. This may be compared with the soft keratin in the stratum corneum of mammalian skin, where a lower degree of organization of fibrils gives a keratin which is less stable than the better oriented keratin of wool fibre (Mercer, 1961). That they contribute towards structural stability appears to be the first experimentally demonstrated mechanical function of lamellae in solid cuticle. Well-oriented samples of chitin such as the longitudinal fibrils along the tibiae are strong in the direction of orientation (ether linkages) but weak in other directions (hydrogen bonds and van der Waal's forces). The chitin crystallites in the lamellae do not share this same specific direction of weakness: a combination of the two orientations would then seem to possess advantages, especially during expansion of the exocuticle.

There is as yet no evidence to support the suggestion that lamellae provide for flexibility by sliding over one another (Ahrens, 1930). It has been suggested that the lamellar pattern seen in some electron micrographs might impart some elasticity, and allow for some stretching during an instar (Locke, 1960; Noble-Nesbitt, 1963*a*); it should be possible to investigate the supposed mechanical functions of chitin lamellae by comparing lamellate with non-lamellate samples in bending and point-loading tests. The daily layers in the resilin of rubber-like cuticle (Neville, 1963*b*) have no mechanical function, since resilin is mechanically isotropic (Weis-Fogh, 1960). The chitin lamellae of rubber-like cuticle constrain both swelling and mechanical deformation in the resilin present between them in directions parallel, but not normal, to the lamellae (Weis-Fogh, 1960). It is possible that the chitin lamellae prevent undue distortion of the pre-imaginal resilin during adult expansion.

Factors which directly influence the orientation of chitin crystallites may indirectly influence subsequent mineralization in crustacea. Thus, the lamellae of the branchial exoskeleton of the crayfish *Orconectes virilis* conform to the pattern described for *Calpodes* (Locke, 1960), but in addition to fanning out upwards and downwards, the fibrils also branch laterally (Travis, 1963). These lamellae influence the size and orientation of calcite crystals during mineralization. In the laminae, where the chitin crystallites are closely spaced and well ordered, the calcite crystals are restricted in size but well oriented, whereas larger calcite crystals grow in the pore canals and inter-laminar regions (Travis, 1963; Travis and Friberg, 1963*b*).

A molecular interlocking between chitin and protein in insect cuticle has been proposed on X-ray diffraction evidence (Fraenkel and Rudall, 1940, 1947), but has been criticized (Jensen and Weis-Fogh, 1961–2) on the basis that it would result in too brittle a material. Insect cuticle must be rigid enough to support the body and muscles, while remaining sufficiently flexible and elastic for efficient application of the locomotory muscles (Weis-Fogh, 1959, 1961). If the molecular chains of chitin and β-protein are covalently

linked via histidine and aspartic acid, as suggested by Hackman (1960), the orientations of the protein chains might be expected to vary with those of the chitin chains during experiments on chitin morphogenesis. Does the chitin orient the protein or vice versa?

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