Light and Electron Microscopy of the Sheath of a Blue-green Alga

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SUMMARY

Light microscopy observations of a *Nostoc* sp. of blue-green alga suggested that the sheath was comparable with the capsules of many bacteria. The sheath showed little internal differentiation under normal or phase-contrast illumination. It possessed a somewhat hazy outer margin from which portions sloughed off, it stained with Alcian blue and was probably polysaccharide. The sheath formed salt-like complexes with proteins at appropriate low pH values which rendered it visible by phase-contrast microscopy. The composition of the culture medium influenced sheath formation. Electron microscopy showed that the sheath consisted of a micro-fibrillar network containing small numbers of larger fibrils (about 200 Å in diameter) which was compressed into stria near to the cell wall and expanded into an open reticulum further out. It would appear to be formed by continuous secretion through the longitudinal walls of the trichomes, and as the outermost regions imbibed water the network structure expanded and finally sloughed off.

INTRODUCTION

One of the most conspicuous characters of the blue-green algae (Myxophyceae, Cyanophyceae) is the production of sheath substance of mucilaginous nature which is reminiscent of the capsules and mucoid substances produced by bacteria (Echlin & Morris, 1965). In the latter group these materials generally form homogenous structureless envelopes around the organisms by which they are secreted, although occasionally they possess a quite complex morphology (e.g. Labaw & Mosley, 1954; Tomcsik & Guex-Holzer, 1954). Among the blue-green algae there is a much greater variety of form and structure (see, for example, the review by Fritsch, 1945). The isolation of a heavily sheathed blue-green alga in this laboratory prompted an examination of this structure by using a variety of light and electron microscopy techniques, with a view to describing its nature and possible function in the ecology of this and other blue-green algae.

METHODS

Isolation and identification of organism. The algal isolate (FP23) was obtained from a green film of algal growth found lining a plastic plant pot when it was emptied. The organism was isolated (May 1966) from crude cultures prepared in liquid media A and B (see below). It was alternately cultured in solid and liquid versions of medium A and appeared to be a uni-algal, but not axenic, culture at the time of these observa-

tions. Subsequently single-colony isolates have been picked to render it undoubtedly uni-algal. It is regarded as a *Nostoc* sp. as it conforms to the descriptions of this genus given by Fritsch (1945) and Smith (1950).

Cultivation. Cultures were maintained in Knop medium (Pringsheim, 1949) and the medium given by Stanier, Doudoroff & Adelberg (1963, table 19-6) without a nitrogen source (referred to as medium A) or with NH_4Cl (medium B). Bulk cultures were prepared in 1 l. volumes in half-gallon wine bottles which were slowly rotated (8 rev./min.) about 12 to 15 in. (30 to 38 cm.) below 4×40 W white fluorescent tubes. Paddles affixed to the walls of the bottles agitated the medium, which was periodically gassed with a 7 to 10% (v/v) CO_2 -in-air mixture. Smaller quantities (25 ml.) were grown in 100 ml. Erlenmeyer flasks under similar illumination. All incubation was at room temperature (26° to 28° in the medium) for 7 to 14 days. Cultures were routinely handled aseptically in sterile containers although freshly prepared Knop medium for the bulk cultures was not always sterilized. No differences were observed in such cultures from those made in sterilized media. For plate cultures, 1.5% (w/v) agar was added to the liquid media.

Light microscopy. A Leitz Labolux microscope with phase-contrast optics was used routinely. Photomicrographs were made with a Leitz Ortholux microscope and Orthomat camera on Kodak Plus X film. All preparations were made without special fixation or drying to avoid artifacts due to distortion of organisms and sheaths. McKinney's (1953) capsule stain was used as described by her, but was subsequently replaced by mounting filaments directly in aqueous Alcian blue (0.5 to 1.0% w/v). Indian ink mounts were made with methods and precautions described by Duguid (1951). Non-specific protein staining of the sheath was achieved by Tomcsik & Guex-Holzer's (1954) method adapted as described below.

Electron microscopy. Fixation was by the Kellenberger-Ryter standard method (described by Glauert, 1965), and embedding was in Araldite. Some material was prefixed in glutaraldehyde and subsequently with osmium tetroxide (Glauert & Thornley, 1966). Sections were cut at a nominal 500 Å thickness (grey/silver colour) with an LKB Ultratome I ultramicrotome and mounted on carbon reinforced collodion membranes on copper grids. After staining with Karnofsky's lead hydroxide (Glauert, 1965) the preparations were examined in a JEM model T6 microscope, operating at 60 kV. Photomicrographs taken on Ilford NS50 plates at initial magnifications of $\times 5,000$ to $\times 12,000$ were printed at a further $\times 3$ to $\times 8$ magnification.

RESULTS

Growth

In the roll cultures (medium A) growth occurred as finely granular suspended dark-green clumps and attached to the walls as much lighter isolated pale-green jelly-like colonies, or as a nodular gelatinous sheet. The proportion of wall to suspended growth varied from bottle to bottle, even within batches put up at the same time. Cultures showing markedly gelatinous growth forms were studied in detail. Little or no sheath formation occurred in medium B.

Indian ink preparations

Teased portions of growth mounted carefully in Indian ink to avoid undue compression of the filaments possessed wide well-defined gelatinous sheaths around almost all trichomes (i.e. the chains of cells inside the sheaths), whether the cells were healthy or in various stages of degeneration (Pl. 1, fig. 1*a, b*). Sheaths averaged $18\ \mu$ wide (range 10 to $29\ \mu$). Healthy cells had a diameter of about $4\ \mu$. Degenerate cells were distorted and shrunken, some to mere specks. The outer limits of the sheath substance were not distinct and strands of sheath substance could be seen free and separating from the sheath surface around degenerate or lysed cells. Diffuse 'slime' layers were not seen.

Larger clumps of algal growth had the appearance of a completely transparent jelly in which were embedded long sinuous trichomes. In older cultures, the trichomes were often coiled in a loose spiral fashion within the sheath which itself retained a straighter course (Pl. 1, fig. 2). Possibly this spiralling within the sheath presaged degeneration because spiral coils of dead cells within wide sheaths were very common in old cultures. The sheaths were of uniform width except where heterocysts and akinetes had formed; at the former sites they narrowed to half size or less. Filaments frequently ruptured at heterocyst sites and the sheath rounded off at this point, leaving half the terminal heterocyst outside the sheath (Pl. 1, fig. 1*a*). The sheath also appeared to degenerate around akinetes in old filaments where it sloughed, became irregular in breadth, and partly infiltrated by the ink particles; ultimately it released individual akinetes.

Phase-contrast microscopy

The sheath was almost invisible under normal illumination, though its presence was sometimes betrayed by attached bacteria. Under phase-contrast microscopy visibility was generally no better, although the bacteria naturally became more distinct. Sometimes the features seen clearly with Alcian blue and protein could just be made out.

Alcian blue-stained preparations

Alcian blue is a stain widely used in histopathology as a specific stain for polysaccharides, and its use for the demonstration of bacterial (polysaccharide) capsules was developed by McKinney (1953). Her method worked with the alga FP23, but sheath stainability was weak, and phase microscopy of material mounted in dilute Alcian blue (about 0.5 to 1% w/v) was more satisfactory. Most stain was taken up at the sheath surface, the bulk of the sheath substance being only faintly stained. Internal striae which appeared to be condensations of intensely stained material could also be seen (Pl. 1, fig. 4). In some areas these striae appeared to split, expand and became less intensely stained. There were rarely more than three layers. Cross-striae in line with the cross-walls of underlying cells were frequently seen.

Filaments differed greatly in the extent to which they stained. Narrow sheaths and sheaths around old trichomes tended to stain most intensely (sometimes very deeply), while sheaths around apparently young vigorously growing trichomes took up very little stain. Staining was usually poor around akinetes and weaker or even absent in the narrow sheath zones next to heterocysts. Cell walls and undamaged cells were unstained. Broken cells stained readily.

Non-specific protein absorption by sheath

Tomcsik & Guex-Holzer (1954) showed the ability of capsules of various bacteria to 'stain' or absorb a variety of proteins and give a non-specific 'capsule swelling' reaction which made them visible under phase-contrast microscopy. It was thought that blue-green algal sheaths might react similarly. Growth from a two week culture of alga FP23 in Knop medium was washed twice with normal saline, resuspended in normal saline to give about 4.5 mg. dry weight/ml. and mixed with an equal volume of 0.1 % v/v bovine serum albumen (BSA) or about 3 % w/v sheep haemoglobin in distilled water. Volumes (0.5 ml.) of this mixture were then mixed with equal volumes of McIlvaine buffer and samples were examined mounted wet under coverslips by phase-contrast microscopy. Control preparations, examined in parallel with test preparations, consisted of algal growth treated exactly as above, but without added protein.

Optimum sheath visibility occurred at about pH 3.4 with both proteins, but values on either side of this showed protein uptake by the sheath. The sheaths in BSA-treated preparations, at pH 3.4, when washed with buffer at pH 7.2 became virtually invisible under phase-contrast microscopy, and this process could be followed microscopically with suitably mounted preparations (Pl. 1, fig. 3*a, b*). Examination of preparations at pH 3.4 in the presence of Indian ink to confirm the coincidence of the phase-contrast visible sheath and ink-outlined sheath resulted in coagulation of the ink. Observations with nigrosin resulted in some negative staining, but (unexpectedly) also in a marked positive staining of the protein-treated sheath. These observations will be recorded elsewhere.

The microscopic picture of the protein stained or protein + nigrosin stained sheaths was exactly comparable with that seen in Alcian blue preparations and (very faintly) in phase-contrast microscopy. Stain uptake was concentrated at the sheath surface and on longitudinal striations which tended to split, broaden and become less intensely stained. The sheath at heterocyst and akinete sites showed changes similar to those described above. The haemoglobin-treated sheaths were very intensely stained at values below pH 4.6 (note the much higher concentration of protein used) and sometimes gave the impression even of shrinkage.

Electron microscopy

The sheath substance was well preserved by the fixation techniques used, but intracellular details were sometimes poorly visible, especially when the sheath was thick (presumably fixative penetration was hindered). The internal structure of the cells will not be described here; but photosynthetic lamellae, α and β granules, ribosomes and nuclear material, and a wall structure (Pl. 2, fig. 6) now regarded as typical of the blue-green algae (Echlin & Morris, 1965) were observed.

Except that the outer investment and the innermost layers of the sheath seemed to be distinct structures, it has not been possible to describe the immediate cell-wall/sheath relationships in detail. This was partly because of lack of resolution, but mainly because these two structures became separated during processing, probably during dehydration. Variations in the dehydration procedure or solutions have so far not overcome this problem.

Cultures grown in medium B formed either a small thin almost electron-transparent sheath, or no sheath at all. In both osmium tetroxide and glutaraldehyde + osmium

tetroxide fixed material from cultures in Knop medium the sheath was readily seen in ultrathin sections. In young vigorously growing cultures it showed a dense striate structure next to the cells (Pl. 2, fig. 7; Pl. 3, fig. 9). Progressively further out from the cells the striae became looser and more open, they split lengthwise, expanded and became less densely stained. In the outermost regions the sheath structure comprised a fine micro-fibrillar network in which the meshes were elongated along the filament. The finest fibrils were estimated to have a diameter of about 50 to 70 Å. The number and density of striations close to the cell walls, and the amount of expansion in the outer layers, varied enormously from one filament to another. Sometimes the sheath was represented by numerous tightly packed dense layers with little or no fraying at the edges, and sometimes there were only one or two distinct layers and a much wider zone of the expanded network. The outer zones of adjacent filaments merged imperceptibly; sometimes sloughing of the outer zones was obvious. Filaments cut in cross-section showed the sheath as a ring of more or less tightly packed striations, expanding into a micro-fibrillar network at the periphery. Oblique sections showed that the lamellations themselves comprised aggregates of close-packed micro-fibrils, most of them oriented more or less parallel to the filament axis (Pl. 3, fig. 8). The extreme edges of these sheaths were probably never seen in sections, because the stainable electron-opaque material at this point was too sparse.

In the circumferential valleys formed at cell junctions (Pl. 2, fig. 7; Pl. 4, fig. 13), the sheath texture differed. Layering was absent, and this space was filled with even-meshed network of very fine (or sometimes less fine) micro-fibrils, reflecting differences in degrees of packing or staining. The unlayered appearance can be interpreted by considering the valley to be filled with small amounts of newly secreted sheath substance which expanded to fill the space created beneath older pre-existing sheath layers as the cells began to pull apart. Its open mesh would therefore be of the type found in the outermost unrestricted sheath layers.

Filaments from old cultures showing signs of degeneration lost their striate sheath structure and the sheath became a uniform homogenous mass of electron-transparent material. At the edges, the usual micro-fibrillar network could be seen, merging with the surrounding medium (Pl. 4, fig. 11). Sometimes empty sheaths were found whose cores were filled with a faint network.

The bacteria present in the cultures were very evident in sections; they were never found immediately adjacent to the algal cell walls in sheathed filaments, but they were sometimes found in the middle sheath layers, and they were very often found in the outer layers (Pl. 4, fig. 11). Large numbers occurred in the vicinity of degenerate cells and trichomes, smaller numbers around healthy trichomes. They were capable of colonizing empty sheaths, and small micro-colonies (presumably clones) were observed enclosed within a portion of some sheaths. The bacterial types involved could not be identified in the sections, though at least two morphological forms could be distinguished—small regular rods about $0.25 \times 0.5 \mu$, and larger more pleomorphic forms about 0.5μ diam. The smaller forms were sometimes deeply embedded in the sheath, but the larger forms were restricted to the outermost zones. Sometimes the texture of the sheath next to the embedded bacteria seemed different from portions remote from bacteria, but consistent changes were not noted. Bacteria were frequently found embedded in sheath material right in the centre of masses of algal growth and in the disintegrating sheaths around akinetes.

The sheath substance generally collapsed in metal-shadowed preparations, and only a faint 'halo', marked by the change in texture of the background, could be seen. At high magnification this appeared to be network of 40 to 70 Å diameter micro-fibrils in suitably oriented areas. In a few filaments the collapsed sheath contained numerous more or less parallel fibrils from 170 to 350 Å in diameter, mostly lying along the length of the filament (Pl. 5, fig. 14 to 16). Anastomoses and splitting of these macro-fibrils could be seen. Anastomoses made fibril length somewhat indeterminate, but some fibrils could be traced for 1.5 to 3 µ along the sheath; those running across reached about 0.8 µ in length. Between and overlying these macro-fibrils could be seen areas of the more usual micro-fibrillar network. The macro-fibrillar zone was comparatively narrow—even dried and flattened its width was only 2.5 to 4.5 µ, compared with the 17 µ measured in living Indian ink preparations, or the 6.5 to 10 µ of the narrowest sheaths measured by phase-contrast microscopy. Beyond this well-defined zone with sharp edges could be seen the faint electron-transparent zone described above. It has so far not been possible to identify these macro-fibrils in sections.

DISCUSSION

The mucilaginous structure described in this paper is generally referred to as a 'sheath' in standard works on the Myxophyceae (e.g. Smith, 1950; Fritsch, 1945; Desikachary, 1959). Is this structure comparable with sheath and capsular structures observed (less commonly) among the other procaryotic micro-organisms the bacteria? Bacterial sheaths and capsules are usually differentiated morphologically; the former is a hollow structure surrounding a trichome or chain of cells, where the latter is a more closely applied envelope with or without a well defined margin (e.g. Skerman, 1967). Empty sheaths, but not empty capsules, are frequently noted. Romano & Peloquin (1963) showed that the sheath of the bacterium *Sphaerotilus natans* is chemically distinct from the capsule; the former comprises a protein-polysaccharide-lipid complex and the latter a polysaccharide only.

The ready stainability with Alcian blue suggests that the algal sheath is largely polysaccharide (McKinney, 1953), and reported chemical analysis on other blue-green algae makes this likely. Polysaccharides of various compositions have been isolated from species of the genera *Rivularia*, *Calothrix*, *Nostoc* and *Anabaena* (O'Colla, 1962). Moore & Tischer (1964) found the 'capsule' polysaccharide (their terminology) of a *Nostoc* sp. to consist of arabinose, glucuronic acid and fucose, and in *Anabaena flos-aquae*, largely of glucose and xylose with minor amounts of glucuronic acid and ribose (Moore & Tischer, 1965). In this latter paper these authors also showed that the sheath polysaccharides were likely to be synthesized intracellularly, and then to diffuse out of the cell through the wall. If the rate of accumulation at the cell surface were to exceed the rate of absorption of water in the more superficial layers of the sheath, one might expect to find deeper layers more electron-dense than the outer layers. As more water was absorbed the outermost layers would slough and go into solution. (The amount of water taken up by the sheath varies with the medium; sheathed filaments from Knop medium can occupy up to 2½ times the packed-cell volume when transferred to distilled water; unpublished observations.) When the trichome dies, no further sheath material accumulates, and the existing deep layers would be expected slowly to absorb water, expand, become less electron-dense and lose their obvious

striated appearance. This seems to be what happens, because the sheath around old degenerate cells is almost uniformly homogenous and unstriated.

The increase in refractive index of the sheath on mixing cultures with protein solutions at low pH value seems to be in every way comparable with the observations of Tomcsik & Guex-Holzer (1954). These workers demonstrated the precipitation of a variety of proteins on, or within, the capsules of a number of bacteria at pH values near to the isoelectric points of the proteins. The reaction was interpreted as due to the formation of salt-like compounds. Changing the pH resolubilized the protein. In the serological sense these reactions can be interpreted as non-specific capsule-swelling reactions.

Marked sheath production occurred on Knop medium, very little in medium B. The main differences between these media are (a) nitrogen source, NO_3^- in Knop, and NH_4^+ in B, and (b) there is about three times as much SO_4^{2-} and six times as much PO_4^{3-} in medium B as in Knop medium. What influence the N source may have on capsular (sheath) polysaccharide production cannot be said, but it may be significant that Duguid & Wilkinson (1953) found that deficiency in phosphate or sulphate in the presence of an ample carbon source tended to increase capsule production in *Klebsiella aerogenes*; and with Nostoc FP23, sheath production was much better in the low phosphate medium.

The changes in the sheath next to heterocysts and akinetes are of interest. Both these cells derive from the transformation of single vegetative cells. During the vegetative cell stage incipient heterocysts would be expected to secrete sheath substance, but when they transform to heterocysts extensive changes occur in the cell's anatomy; in particular, the wall or coat becomes greatly thickened, birefringent and accepts Alcian blue (unpublished observations). Metabolic changes probably also occur (Fogg, 1949). Sheath formation ceases, and this would lead to the narrowing and loss of substance frequently seen in sheath adjacent to heterocysts. The function of heterocysts is unknown but in this species, as in many others, filament fragmentation occurs at heterocyst sites and the loss of sheath substance at this point would facilitate separation of the fragments.

Akinetes (spore cells) form by the separation and rounding up of individual vegetative cells. The wall or coat becomes birefringent (unpublished observations), and, as the culture ages, the spores become separated from the parent filament. Subsequently, they germinate, giving rise to new filaments, but, prior to being set free, the sheath substance can be seen to become very loose, frayed and often heavily infested by bacteria. Presumably sheath secretion by incipient akinetes also ceases, and sheath dissolution (aided by bacterial decomposition?) finally releases the spores. These morphological changes undoubtedly reflect switches in cell metabolism accompanying a major cell transformation.

Generally, bacterial capsules are electron-transparent structures, though opaque capsular material has been described in *Mycobacterium tuberculosis* by Knaysi, Hillier & Fabricant (1950). The sheath in Nostoc isolate FP23 is largely electron transparent except in areas where it has probably absorbed relatively little water, i.e. in the striated regions close to the cell wall.

The macro-fibrils noted in a few metal-shadowed filaments have not been identified in sections, but it is emphasized that they were found on only a few filaments, and in these they clearly constituted a small part of the total bulk. Possibly they represent

cellulose fibrils, as have been reported to occur in *Scytonema pseudogymanensi* (Singh, 1954) and *Nostoc* sp. (Frey-Wyssling & Stecher, 1954), but more evidence is needed to confirm this suggestion.

The macro-fibrils show a slightly spiral arrangement with reference to the filament axis, and in some oblique sections the micro-fibril orientation suggests a spiral winding around the trichome. One way such a spiral formation could occur would be for the trichome to move within the sheath. It was noted that trichomes could sometimes be seen to be spirally wound within the sheath (Pl. 1, fig. 2) and probably what has happened in these cases is that the cells have outgrown their sheath and been thrown into a spiral within the sheath. Sections, or metal-shadowed preparations of such a filament, would then show layers of sheath material at an angle to the main filament axis.

The bacteria present in the sections are worth comment. Blue-green algae are notoriously difficult to obtain in axenic cultures. The demonstration of bacteria within the sheath structure points the purely mechanical difficulty of separating the two micro-organisms, but these sections also suggest that there may be some ecological significance in the association. The two organisms are clearly very closely associated and could well exist in nature as a long-lasting partnership.

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A. A. TUFFERY

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EXPLANATION OF PLATES

PLATE 1

Fig. 1 to 4 are of unfixed filaments of *Nostoc* sp. isolate FP 23.

Fig. 1 *a, b*. Filaments mounted in Indian ink. Note the wide gelatinous fairly sharply delineated sheath around each trichome, which narrows and looses substance next to the heterocysts. Note the clear interval between heterocysts and vegetative cells formed by the degeneration of a number of vegetative cells. $\times 375$.

Fig. 2, 3 *a*. Portions of filaments in 0.1 % bovine serum albumin at pH 3.4. Note the intensification of the sheath structure, especially at the edges, and the internal striations. Phase-contrast illumination. Fig. 2 $\times 560$; fig. 3 *a* $\times 400$.

Fig. 3 *b*. Same field as Fig. 3 *a* but the staining with bovine serum albumin has been removed by flushing with buffer at pH 7.2. $\times 400$.

Fig. 4. Filaments mounted in 0.5 % Alcian blue, viewed by phase-contrast illumination. Stain has been taken up by the sheath material only, but neither evenly, nor to the same extent, by all sheaths. $\times 375$.

PLATE 2

Fig. 5 to 16 are of Kellenberger-fixed material cultured in Knop medium except where otherwise noted.

Fig. 5. *Nostoc* sp. Slightly oblique cross-section of a filament. Note compact (*a*) and less compact zones (*b*), showing a fine micro-fibrillar structure. $\times 26,100$.

Fig. 6. Wall structure of *Nostoc* sp. The outer investment is just resolved into a three-layered undulating structure. The inner investment (single dense layer) is visible but the cytoplasmic membrane is not demonstrated. Glutaraldehyde/uranium acetate fixation. $\times 76,000$.

Fig. 7. *Nostoc* sp. Note the compact striations in the sheath close to the cells (*a*) and the much more open network of the micro-fibrils in areas further out (*b*). There is also a much looser, open structure in the groove between separating cells (arrow). A cross-section of a bacterium can be seen embedded in the less compact part of the sheath. $\times 27,000$.

PLATE 3

Fig. 8. *Nostoc* sp. Oblique sections of filament. The more compact layers of the sheath can be seen to consist of fine micro-fibrillar elements. $\times 23,900$.

Fig. 9. *Nostoc* sp. Cross-section of filament, showing micro-fibrillar nature and variations in compactness of the sheath. $\times 19,500$.

Fig. 10. *Nostoc* sp. Cytoplasmic membrane (*cm*); inner investment (*i*) and outer investment (*o*) (poorly visualized with this fixation) of wall; sheath (*s*).

PLATE 4

Fig. 11. *Nostoc* sp. Portion of sheath and cell from an old culture. The sheath striations have completely disappeared, to be replaced by an almost homogenous matrix of sheath substance which became more and more dispersed away from the cell. Bacteria (arrows) are embedded in the outermost regions. Inner investment (*i*), outer investment (*o*), sheath (*s*). $\times 28,300$.

Fig. 12. *Nostoc* sp. Colonies of bacteria embedded in outermost zones of the algal sheaths. Arrows indicate electron-lucent zones of what may be bacterial capsule substance. Algal sheath substance (s). $\times 15,000$.

Fig. 13. *Nostoc* sp. The compact layered structure of the sheath gives way to a much looser, open structure in the circumferential grooves or valleys between dividing cells (arrow). $\times 28,300$.

PLATE 5

Fig. 14 and 15. *Nostoc* sp. Portions of trichomes in which the bulk of the sheath substance has dissolved, revealing a loose network of longitudinally orientated macro-fibrils suggestive of cellulose fibrils. Cr shadowed. Fig. 14, $\times 16,800$; fig. 15, $\times 29,900$.

Fig. 16. *Nostoc* sp. Enlargement of arrowed area in fig. 14 showing micro-fibrillar structure of the sheath matrix. Cr shadowed. $\times 69,000$.