The fine structure of the superficial layers of the tablet-forming micro-organism, *Lampropedia hyalina*, has been studied by several electron microscopical techniques. The outer envelope is distinct from the cell wall and surrounds not individual cells but groups of cells (tablets). This envelope appears to embody two complex layers, both possessing a regular-patterned structure. One layer (the perforate layer) is a honeycomb network of hexagonally distributed holes, each about 75 Å in diameter, and with a repeat spacing, from centre to centre, of 145 Å. The other, outermost (punctate) layer, which seems to be fragile and easily lost, is composed of outward-projecting spines, also in hexagonal array but with a repeat spacing of 260 Å. The probable structural relationship between the perforate and punctate layers is discussed. The structured envelope is separated from the cells by an amorphous zone (the intercalated zone) about 600 Å thick; this penetrates between the cells of a tablet and follows immediately behind the ingrowing septum in cell division. The intercalated zone is probably the important factor in cell cohesion; the significance of the structured envelope is uncertain but its function may be to separate the sheet into tablets by preventing inter-cell cohesion at certain places in what would otherwise be a continuous sheet of cells.

**Introduction**

In recent years much has been learned about the anatomy of the bacterial surface (see, for example, Salton 1960, 1961). A significant part of this knowledge results from the development of preparative techniques enabling the bacterial cell wall and other surface structures to be examined in the electron microscope. This paper is concerned with the application of such techniques to the study of the outer layers of *Lampropedia hyalina*.

The principal characteristic of *L. hyalina*, noted by Pringsheim (1955), is the cohesion of cells into regular rectangular arrays to form extensive sheets, one cell in thickness. The appearance and growth habit of the organism is described more fully by Murray (1963b). Pringsheim (1955), on the basis of light-optical observations, puts *Lampropedia* in the group of micro-organisms whose cells remain connected ‘without a special enveloping substance being recognizable’. A knowledge

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of the fine structure of the surface layers is clearly of importance in establishing the extent to which structural components are involved in maintaining the integrity and organization of a sheet. This problem has stimulated independent but complementary research in our two laboratories over a number of years; the work has been gathered together in one paper for convenience of expression.* A brief preliminary report of that part of the work carried out in Manchester has already been given (Chapman & Salton 1962).

Fine structure in bacterial cell walls has been studied by a number of investigators and in a few instances a fascinating macromolecular architecture has been revealed. Houwink (1953), who was the first to report this kind of structure, found that the cell wall of a large Spirillum species was multilayered, with one layer composed of hexagonally packed spherical macromolecules. Similar structures have been described in Halobacterium halobium (Houwink 1956) and in Rhodospirillum rubrum (Salton & Williams 1954). Macromolecular elements in rectangular array have been observed by Labaw & Mosley (1954) in the wall of an unidentified organism and by Baddiley, Boult & Davison (personal communication) in the wall of Bacillus polymyxa. These specimens were studied by simple metal-shadowing of disintegrated or complete organisms but sectioning and negative staining techniques have recently been used to study cell wall structure in Spirillum serpens (Murray 1963a) and in Micrococcus radiodurans (Glauner 1962; Thornley & Horne, personal communication). In all cases the repeat distance in the macromolecular pattern occurred in the range 100 to 150 Å.

It will be shown that, in Lampropedia hyalina, the outer envelope (which is distinct from the cell wall) also possesses a well-defined two-dimensional lattice structure. A repeat distance comparable with that in other organisms is present but the surface envelope is a more complex structure and appears to comprise two layers, one layer taking the form of a honeycomb network with a second layer of spines projecting outward from this.

**Materials and methods**

*Cultures and media*

A culture of *L. hyalina*, strain no. 1444–1 (I) or no. 1441–1 (II), were kindly provided by Professor Pringsheim from his algal collection at the Pflanzenphysiologisches Institut der Universität Göttlingen, Germany. The organism was grown on the media described by Pringsheim (1955); for most experiments either the beef extract, yeast extract, acetate medium (I) or a medium consisting of 0·3% Difco tryptone and 0·3% Difco yeast extract, with or without sodium acetate, at pH 7 (II) was used. The media were solidified with agar although in some cases fluid media were employed. Reasonably luxuriant, spreading, filmy growth on solid media was obtained after 1 to 2 days’ incubation at 20 to 25 °C; in liquid media the growing organism appeared as a fine pellicle. In both cases the organism showed all the morphological characteristics described by Pringsheim.

* In this paper ‘I’ and ‘II’ refer to the two laboratories in Manchester, England (J.A.C. and M.R.J.S.) and London, Ontario, Canada (R.G.E.M.), respectively.
Control preparations for optical microscopy were most conveniently made by spreading a small portion of culture in 7% Nigrosin and allowing this to dry on a coverslip. This was turned over and attached at each corner with wax to a microscope slide so that the preparation could be examined with an oil immersion lens through the back of the coverslip. The ‘negative’ image thus obtained compared favourably with a phase-contrast image.

Specimens for stained preparations were fixed in osmium tetroxide vapour or in Bouin’s fixative. Thionin was used as a cytoplasmic stain; cell-boundaries were stained by the Victoria blue or tannic acid—crystal violet procedures (Robinow & Murray 1953) used for showing the cell walls of many bacteria. A post-treatment in 5% mercuric chloride for a few minutes was sometimes given. Metachromatic staining of intercellular material with methylene blue or with Giemsa’s stain was carried out supravitally or directly after osmium vapour fixation.

Electron microscopy (I and II)

(a) Cell disintegration

For the purpose of examining wall fragments from disintegrated cells, either by shadow-casting or by negative staining, the cells were washed off suitable cultures with distilled water or saline and, after further washing, were subjected to disintegration in the Mickle apparatus under the conditions normally employed for cell-wall isolation (Salton & Horne 1951). The fraction usually designated as ‘cell-wall material’ was collected and washed by centrifugation. Such fractions were prepared for electron microscopy both before and after digestion with trypsin (2 h at 37 °C) and lysozyme (24 h at 37 °C), either singly or as combined enzymic treatments. Fractions were also subjected to treatment with 0.1N sodium hydroxide solution at room temperature for varying periods of time; optimum results were obtained after treatment for 10 min. Preparations were thoroughly washed with distilled water on the centrifuge.

In some instances, mild disintegration was obtained by brief exposure to ultrasonic vibration (II), using the larger (3 in. diameter) probe of the MSE ultrasonic disintegrator on 2 ml. of suspension for periods ranging from 10 s to 2 min. Such specimens were concentrated but not cleaned by centrifugation.

(b) Shadow-casting

Disintegrated specimens intended for shadow-casting were suspended in distilled water at such a dilution that the suspension appeared faintly opalescent under oblique illumination. A small drop was placed in the centre of a carbon-filmed grid, allowed to dry and shadowed in vacuo with tungsten oxide (II), with platinum—10% iridium, with pure platinum or, infrequently, with gold—40% palladium (I). Shadowing angles giving a shadow:height ratio of 4:1 or 5:1 were used. The mean thickness of the metal film was calculated to be 5 to 10 Å.
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(c) Negative staining

Specimens for negative staining were prepared in suspension in distilled water at higher concentrations than those used for shadowed specimens. Samples were mixed with roughly equal volumes of a 2% aqueous solution of phosphotungstic acid (PTA) adjusted to pH 7.0 with sodium hydroxide. A few drops of the mixture were placed in an all-glass nebulizer and sprayed (I) on to carbon-filmed grids in the manner described by Brenner & Horne (1959). Alternatively (II) a thin film was deposited on the surface of the grid from a platinum loop.

(d) Fixation, embedding and sectioning

For the electron microscopic examination of thin sections of complete organisms and groups of organisms, cells were washed from the agar media after growth for 2 to 7 days, using either the fixative (II) or saline or typtone growth medium (I) as the washing fluid. In the latter case the cells were deposited from suspension by centrifugation and, after decanting off the supernatant, the cell pellet was then dispersed in the fixative.

Various fixation procedures were employed:

(i) 1% osmium tetroxide in either distilled water or in M/40 tris buffer with M/100 calcium chloride for 1 h (II—early work).

(ii) The ‘standard’ osmium tetroxide—veronal acetate fixative (at pH 6.1) of Kellenberger, Ryter & Sechaud (1958). The duration of fixation was 16 to 20 h at room temperature and was followed, after suspension in agar, by post-fixation treatment for 2 h in uranyl acetate or lanthanum nitrate solution (I and II).

(iii) Potassium permanganate fixation, following the method of Luft (1956). Fixation was carried out for 24 h at 0 to 4 °C; 3 h fixation in permanganate was inadequate. No post-fixation with uranyl acetate or lanthanum nitrate was employed but in all other respects the procedure resembled the Kellenberger et al. technique (I).

Specimens were normally dehydrated in increasing concentrations of acetone, although alcohol was used after procedure (i). Embedding was in Araldite, as described by Glauert & Glauert (1958) but using longer impregnation times (I), or in Vestopal W (II), as described by Kellenberger et al. (1958). After fixation-procedure (i) methacrylate embedding, with polymerization by ultraviolet light, was used. Blocks were cut on a Huxley microtome (I) or on a Porter–Blum microtome (II) using glass knives. The sections, 500 Å or less in thickness, were picked up on carbon-filmed grids. Many of the osmium tetroxide-fixed specimens which had not already been ‘stained’ were treated by floating the grids on 1% lanthanum nitrate or uranyl acetate, or on 0.5 to 1.0% phosphomolybdic acid or phosphotungstic acid; these treatments provided a selective enhancement of the contrast of certain wall structures.

(e) Instruments

(I) In Manchester, specimens were examined in a Siemens Elmiskop I electron microscope using double condenser illumination and operating at 80 kV (figure 4 was taken at 100 kV). Exposures on Ilford lantern contrasty plates were made at
instrumental magnifications ranging from $\times 1000$ to $\times 80000$. (II) In London, Ontario, electron micrographs were taken with the Philips EM—100 A, modified by the installation of 1·8 mm objective pole-pieces, a Ladd anode, mechanical astigmatism correction, and accessory stabilization of input. An accelerating voltage of 60 kV was used. Exposures were made on Kodak fine-grain positive film at instrumental magnifications between $\times 6000$ and $\times 12000$.

**Observations**

*Light microscopy and low magnification electron microscopy*

*Lampropedia hyalina* grew on the surface of solid media or in the surface layers of fluid media in the form of large rumpled sheets, each sheet consisting of an extensive layer of cuboidal cells in rectangular array (see figures 1 and 2, plate 62, of a Nigrosin preparation). The cells were arranged in smaller square or rectangular groups of up to 128 cells, the groups being distinguishable by the slightly increased spacing between them compared with the normal inter-cell spacing. These features could also be observed in living organisms by phase-contrast microscopy. The sheets were readily broken up by mild mechanical disruption into the smaller square or rectangular groups; it will be convenient to refer to these smaller separable groups as 'tablets'. Tablets were one cell layer in thickness and usually contained between 16 and 128 cells with the maximum tablet width ranging from 5 to 20 microns ($\mu m$). Individual tablets were resistant to mild disruption and normally remained intact during the centrifugations and resuspensions involved in preparations for electron microscopy; most tablets also survived the dehydration and embedding procedures during the preparation of pellets for sectioning. This strong cohesion of cells into tablets is a remarkable feature of *Lampropedia* and does suggest the presence of some cementing substance between the cells.

The cells, approximately 1·0 to 1·5 $\mu m$ in diameter, were too small to be rewarding cytological objects in the light microscope. Cell boundaries delineated by the Victoria blue and tannic acid–crystal violet techniques (Robinow & Murray 1953) were those immediately enclosing the protoplast and these cell walls enclosed each of the individual cells within a group. Methods such as these failed to distinguish any particular boundaries to tablets or to a whole sheet. Some indication of the presence of an intercellular material was, however, obtained after staining with methylene blue or with Giemsa's stain; each tablet was surrounded by a pinkish metachromatic stain and this pink coloration extended between the cells (figure 3, plate 62).

Low magnification electron microscopy of complete tablets (figure 4, plate 62) confirms many of the morphological features noted by light microscopy. The spacing between cells is less clearly revealed, presumably because electron scattering in the interstitial material is comparable with that in the cell itself and also because some overlapping of the dried-down cells can be expected. Nevertheless, the boundaries of the protoplasts of several adjoining cells are distinguishable in figure 4. The thinned margins of the tablet suggest that some shrinkage has occurred.
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Electron microscopy of shadowed envelope fragments

Fine structure in an outer component of the surface envelope of *L. hyalina* was first observed in shadow-cast preparations of partially disintegrated organisms (figure 5, plate 63). A hexagonal pattern is evident in the fragments of the marginal layer projecting from the edges of the partially disintegrated cells. The pattern is observable, although with less clarity, in much of the surface over the bulk of the cells, indicating that the hexagonal structure is likely to be associated with the outermost component of the enveloping layer.

Detailed examinations in both laboratories of shadowed envelope fragments from more extensively disintegrated organisms have emphasized the remarkable regularity of the hexagonal structure (figures 6 to 8, plate 63). It was at first thought that the patterned wall consisted of spherical, or approximately spherical, particles in close-packed array and such an arrangement is suggested by a cursory examination of the envelope fragment in figure 6. Close study of a number of micrographs soon showed, however, that the structure was unlikely to be as simple as this and the appearance was more frequently that of a honeycomb network with holes or pits as the repeating units. This type of structure is apparent in figures 7 and 8. In figure 6 some degree of artifact may have been produced by heavy shadowing, but the appearance of the pattern may also depend on whether the shadowed surface is the inner or the outer aspect of the envelope. Attempts to study the fine structure of the outer surface of intact organisms by replica methods revealed the hexagonal pattern but with insufficient detail to provide additional information.

A specific direction in the two-dimensional hexagonal array is most conveniently indicated by defining a *principal axis* of the pattern as a line containing repeating units in closest packing. Thus in both figures 6 and 7 one of the principal axial directions is horizontal (i.e. parallel to the upper or lower edge of the page). In a given array three principal axial directions exist, mutually at 120°. In isolated envelope fragments, deviations up to 12° from the 120° mutual orientation are encountered, but such deviations are random from specimen to specimen and are presumed to be due to distortion of the layer during preparation. In shadowed specimens one (or two) of the principal axis directions will be emphasized at the expense of the other two (or one), depending on the direction of incidence of the shadowing metal film during evaporation.

The dimensions of the repeating structure were determined from micrographs of both shadowed and negative-stained specimens. The repeat distance along a principal axis (i.e. the centre-to-centre distance between nearest neighbours) was 145 (± 10) Å, taking the mean of measurements made in extensive fragments in micrographs from a number of separate preparations.

A conspicuous feature of many shadowed fragments is the 'castellated' edge (arrows in figures 6 to 8). This appearance is most readily observed when the edge is a principal axis and when the shadowing direction is such that the edge is in shadow and the angle between the edge and the incident-direction of the shadowing beam is small. The marked tendency for the structured layer to possess edges
which are also principal axes suggests that principal axial directions are directions of easy cleavage in the two-dimensional lattice. Fibrillar material (figures 8 and 9, plate 63) or spherical particles (figure 7) sometimes occurred in the vicinity of an envelope fragment, but the varying appearance and size of these elements suggested that they arose from contaminating cellular debris.

The honeycomb-structured envelope was detectable in cells from both the media described by Pringsheim (1955). Digestion of the isolated envelope preparations with trypsin and/or lysozome produced no apparent change in the structure. All the preparations, including those digested with enzymes, contained fragmented material in which no fine structure was detectable. Thus, in figure 9, although the castellated edge is visible around much of the fragment, the hexagonal pattern on the surface is obscured by a superincumbent structureless layer, presumed to be another component of the complex multi-layered envelope adhering to the patterned layer. The cleanest preparations were obtained after treatment with 0.1 N sodium hydroxide solution but even this failed to remove all the structureless material.

Negative staining of envelope fragments

Fine structure in the envelope was clearly revealed in specimens prepared by the 'negative staining' technique. Figure 10, plate 64, shows a typical envelope fragment from Mickle-disintegrated cells at the edge of a sprayed droplet; part of a similar fragment is shown at higher magnification in figure 11, plate 64. Dark regions indicate the presence of phosphotungstate so that the hexagonally distributed spot pattern must be interpreted as a pattern of holes (or, less likely, pits). There can be little doubt that this component of the envelope is a honeycomb network and not an array of close-packed spherical particles. The average diameter of the holes is 75 Å. It is difficult to decide their shape with any certainty; they could be circular but some of the indentations along the edge of the fragment in figure 12, plate 64, suggests a polygonal outline (arrow). Moreover, some holes in figure 11 do appear hexagonal in shape. No clear evidence of subunit structure in the framework of the honeycomb itself could be obtained although there are indications in figure 11 that the framework could be composed of three sets of fibrillar elements, mutually at 120°.

In a few instances overlap of two honeycomb-structured fragments gave rise to complex moiré patterns. These patterns occurred when the upper surface envelope of an incompletely disintegrated cell or tablet collapsed on to the lower surface.

The concept of the structured component of the envelope as a honeycomb network conforms satisfactorily with the picture presented by the shadow casting technique, both methods yielding the same repeat distance (145 Å) along a principal axis. Some interest attaches therefore to the later discovery (II) that the envelope possesses not one but two distinct structured layers. The second layer was first observed in negative-stained fragments obtained by exposing cells to brief ultra-sonic disintegration; the layer seemed to be easily lost and to avoid mechanical damage no subsequent purification or cleaning was attempted. Specimens prepared in this way occasionally displayed both structural components side by
side, as in figures 13 and 14, plate 65. The honeycomb network is less clearly resolved than hitherto, presumably due to the presence of contaminating debris, but its characteristic repeat distance is readily confirmed and at one place (arrow in figure 13) the castellated (‘stamp-paper’) edge of the honeycomb layer is discernible. The second layer is strikingly different, in both appearance and spacing, and takes the form of a hexagonal array of sharply defined units, linked together by fine filaments or ‘linkers’. The space remaining between the units and their linkers is permeable to phosphotungstate, although not perhaps completely so as the electron opacity of these regions tends to be less than that of the phosphotungstate surrounding the fragments. The repeat distance of the second layer (measured from unit to unit along principal axes) is 260 (±20) Å. The probable errors in both repeat distances are largely due to uncertainties in instrumental magnification. The relative spacing, taken as the ratio of the repeat distance in the second layer to that in the honeycomb layer, is free from this uncertainty when it is measured on micrographs showing the two layers side by side. The value of this relative spacing, determined from figures 13 and 14, is

\[
1.7 \pm 0.05 \quad \text{(figure 13),} \\
1.8 \pm 0.1 \quad \text{(figure 14).}
\]

The fine structure of the second layer has yet to be fully investigated but the existing evidence suggests that the repeating units are spines projecting outwards normally from the layer. In a few instances the edge of a negative stained fragment shows a side view of the spines, presumably resulting from folding of the layer on the supporting film (figures 15 and 16, plate 65). Some side views suggest the presence of a central cavity to each spine, but the side views indicate that this cavity does not continue right up to and emerge at the tip. Each spine appears to be a ribbed structure, possibly with the ribs occurring as continuations of the linkers.

It is convenient to refer to the layer of spines as the ‘punctate’ layer to distinguish it from the honeycomb or ‘perforate’ layer. This punctate layer was only observed in the London, Ontario, laboratory.

Thin sections of complete tablets

The spatial relationship between the structured layers and the cells can only be deduced from sectioned tablets. Satisfactory fixation and embedding of L. hyalina proved to be difficult and, with all the methods used, vacuolar expansion and disruption of cell contents were frequently encountered in sectioned material. Permanganate fixation resulted in particularly poor preservation of cell contents, but nevertheless provided useful information about the surface layers. Sections of osmium-fixed material were stained with dilute aqueous solutions of heavy metal salts; staining with lanthanum nitrate or, more profitably, with uranyl acetate, phosphomolybdic acid or phosphotungstic acid was found to give an increase in over-all contrast with marked enhancement of the electron opacity of cell walls of individual protoplasts. It was not necessary to stain permanganate-fixed specimens.
It has already been noted that tablets remained intact during the preparative
techniques for electron microscopy and it is therefore reasonable to assume that
the groups of cells observed in sectioned material are, in fact, sections of tablets in
various orientations. The shape of tablets and cells in section will clearly depend
upon the relative orientation of the plane of sectioning and the tablet. Sections
with the flat face of a tablet approximately parallel to the plane of sectioning
show the cells to be cuboidal in shape (figure 17, plate 66). Each protoplast is
closely surrounded by an intensely stained wall or membrane which can be re­solved as double at many places. There is in addition some indication of a differen­tiated plasma membrane at the protoplast surface where the protoplast has
retracted from the surrounding double wall. The plasma membrane of the proto­plast is shown more clearly in the Vestopal-embedded specimen of figure 20,
plate 67.

The cells in a tablet are separated from one another by an electron-transparent
zone of roughly constant width, usually about 500 Å from wall to wall in osmium-
fixed specimens. This thick inter-cell zone will henceforth be referred to as the
‘intercalated zone’. It could not be stained specifically by any metal salts, includ­ing
cupric chloride, but in osmium-fixed specimens faint fibrillar inter-connexions
between the cells could sometimes be distinguished (as in figure 17). In perman­ganate-fixed material the intercalated zone tended to be thicker (about 600 Å) and
fibrillar inter-connexions were never observed.

DESCRIPTION OF PLATE 62

All figures in plates 62 to 68 are electron micrographs, with the
exception of figures 1 to 3.

FIGURE 1. Low magnification photomicrograph of Lampropedia hyalina showing a large sheet
of cells. The negative image was obtained by spreading a small portion of the culture in
7 % Nigrosin, allowing this to dry on a coverslip and viewing by normal transmitted light.
(II, Magn. x 150.)

FIGURE 2. Photomicrograph of a Nigrosin preparation of part of a sheet of Lampropedia
hyalina at higher magnification. The cells are arranged in square or rectangular groups
of up to 64 (possibly 128) cells, the groups being distinguished by the slightly increased
spacing between them (as at arrows) compared with the normal spacing between cells. On
disruption of a sheet these groups survive as individual ‘tablets’. In an intact sheet,
such as that shown here, it is not always possible to decide where the boundaries of a
tablet lie; thus the group above and to the right of the arrows may consist of more than
one tablet. Some cells are undergoing division. (II, Magn. x 1800.)

FIGURE 3. Photomicrograph of OsO₄-fixed tablets taken in red light after staining with 0-001 %
toluidine blue for 5 min. Metachromatic material (appearing dark in the micrograph)
fills the spaces between the cells and surrounds the tablets. (II, Magn. x 1800.)

FIGURE 4. Low magnification electron micrograph of a tablet (or tablets) of Lampropedia
hyalina, showing the cuboidal shape of the cells. One or more electron-opaque granules
occur in individual cells. Dividing cells show greater over-all electron-opacity than non­dividing cells. This specimen was lightly shadowed with gold-palladium but the shadow­ing film is so thin that its contribution to contrast is negligible and the observed contrast
arises almost entirely from differential electron scattering in the specimen itself; the
effect of the shadowing film is just discernible at the dried-down edge of the tablet.
Direct print without an intermediate negative. (I, Magn. x 8000.)
Figure 5. The structured envelope of *Lampropedia hyalina* projecting from the edge of a partially disintegrated tablet. Shadowed with iridium-platinum. (I, Magn. × 50,000.)

Figures 6 to 9. Isolated fragments of the envelope. Figures 7 and 8 reveal the honeycomb-network nature of the structured layer (perforate layer). The centre-to-centre repeat distance averages 145 Å. The arrows indicate ‘castellated’ edges. In figure 9 a fragment shows a castellated edge but a structureless layer covers the surface. The specimens of figures 6 and 7 were digested with trypsin. Iridium-platinum shadowing. (I, Magns. (6) × 135000, (7) × 145000, (8) × 135000, (9) × 135000.)
Figures 10 to 12. Fragments of the honeycomb (perforate) layer of the envelope from Mickle-disintegrated and NaOH-treated cells, negative-stained with phosphotungstate, demonstrating the hexagonal distribution of holes. Some holes (arrows in figures 11, 12) show evidence of a hexagonal outline. There are indications in figure 11 that the layer is made up of three sets of parallel fibrils, each set at 120° to the other two. (I, Magns. (10) × 320,000, (11) × 560,000, (12) × 320,000.)
Figure 13. Envelope fragments from a specimen after mild ultrasonic disintegration, negative-stained with PTA. Two distinct structured layers, with different spacings, are present. The perforate layer (spacing 145 Å) is poorly resolved but the castellated edge can be seen (arrows). More prominent is the punctate layer of sharply defined units in hexagonal array with a repeat spacing, from unit to unit, of 260 Å. The units are linked together by fine filaments or 'linkers'; these form triads between three adjacent units.

Figures 14 to 16. Negative-stained fragments of the punctate layer. In figure 14 the perforate layer is also visible (arrows). The profile view of the folded edge in figures 15 and 16 shows that the units of the punctate layer are outward-projecting spines. (II, Magns. (14) x 120000, (15) x 120000, (16) x 120000.)
Figure 17. Central part of a sectioned tablet from a 72 h culture, fixed in OsO₄, embedded in methacrylate and stained with phosphomolybdic acid. The strongly stained double cell wall (w) and the electron-transparent intercalated zone (i) are shown. The protoplast membrane (m) is visible where the protoplast has retracted from the cell wall. (II, Magn. x 30 000.)

Figure 18. An oblique section through a tablet fixed in permanganate and embedded in Araldite. The structured envelope (e) forms the outermost border of the tablet and only occasionally penetrates between cells. It is separated from the cell walls (w) by the intercalated zone (i). (I, Magn. x 45 000.)

Figure 19. The structured envelope (e) in a permanganate-fixed, Araldite-embedded specimen appears as a magin of discrete dots. This is probably the perforate layer of the envelope. Beneath the envelope the intercalated zone (i) is wider than the zone separating cells and it possesses a complex layered structure. (I, Magn. x 130 000.)
Figure 20. Cells at the edge of an OsO₄-fixed, Vestopal-embedded tablet showing the intercalated zone (i), cell wall (w), protoplast membrane (m) and spines of the punctate layer of the structured envelope (s₁ and s₂). At the tablet edge (right-hand side of micrograph) the punctate layer lies roughly in the plane of sectioning and regular arrays of spines are visible (s₁). The spines are seen in profile (s₂) where the punctate layer has penetrated between the cells. Uranyl acetate post-fixation treatment. (II, Magn. x 90000.)
Figures 21 and 22. Sections of OsO₄-fixed, methacrylate-embedded tablets showing the punctate layer. The spines are arranged in regular arrays, usually with a hexagonal distribution but sometimes this is distorted into other arrangements; the spacing is variable and tends to be less than 260 Å, due to section compression and oblique viewing. Section stained with uranyl acetate. (II, Magn. (21) × 90000, (22) × 90000.)

Figure 23. An OsO₄-fixed, methacrylate-embedded section showing the spines in profile. The spines appear to be linked at their bases. Stained with PTA. (II, Magn. × 115000.)

Figure 24. A rare shadowed preparation showing the punctate layer. The centre-to-centre repeat spacing is 250 Å. Shadowed with tungsten oxide. (II, Magn. × 110000.)
Greatest interest centres on the *in situ* location of the periodic-structured components of the envelope. Figures 18 and 19, plate 66, are sections of permanganate fixed tablets (I) and it is immediately apparent that a structured layer forms the outermost border or envelope of a *complete tablet*. This envelope, which appears in permanganate sections as a margin of discrete dots, is separated from the cell adjacent to it by an intercalated zone slightly wider than that separating the individual cells of a tablet. Figure 18 is an oblique section through a tablet in which the structured envelope can be seen to follow the contours of the tablet surface; the distinct border of electron-opaque dots completely envelopes the tablet (except at regions where the layer lies in the plane of sectioning). The complex nature of that part of the intercalated zone lying between the structured envelope and the double wall of the protoplast is evident in this micrograph and also, at higher magnification, in figure 19. Thus the tablet is surrounded by a complex multi-layered structure with the patterned layer functioning as an outer envelope to the whole tablet and we are able to define a surface component which does not surround individual cells but surrounds *groups* of cells. Figure 25 is a simplified diagram of a transverse section through a tablet, illustrating the apparent relationships existing between the various marginal components.

**Figure 25.** A simplified diagram of a tablet in transverse section, showing the structured envelope (*e*), intercalated zone (*i*), cell wall (*w*) and protoplast membrane (*m*). Cell division (1) and tablet splitting (2) are illustrated.

A study of numbers of tablets has shown that, when cell division occurs in a tablet, the intercalated zone penetrates between the dividing cells immediately behind the ingrowing septum while the structured envelope remains at the surface of the tablet. In some instances (as in figure 20, plate 67) the structured envelope was observed to penetrate into a cleft between individual cells before looping back over the next cell. It is probable that this partial intrusion of the envelope between cells is associated with the splitting of a tablet into smaller tablets. (Cell and tablet division are shown diagrammatically in figure 25.)

The absence of spines and the close spacing of the dots suggest that the outer structured layer surrounding the permanganate-fixed tablets in figures 18 and 19, plate 66, may be identified as the perforate (honeycomb) layer of the envelope. Little information about the detailed structure of the layer can be deduced from these sections as the periodicity of the perforate layer (and also of the punctate layer) is less than the section thickness so that several repeating units must be superposed in a transverse section. Moreover, attempts to obtain positive staining of the perforate layer, both in sections and in fragmented material, met with very limited success (I).
The identification of the outer structured layer of figures 18 and 19 as the perforate layer is to some extent uncertain in view of its indefinite appearance and the difficulty in obtaining satisfactory positive staining of the perforate layer. Nevertheless, it is improbable that this outer structured layer in these permanganate-fixed sections could be the punctate layer as the Manchester workers used preparative methods which would (judging from the results obtained in the London, Ontario, laboratory) have failed to preserve the punctate layer. Moreover, micrographs of shadow-cast specimens of partially intact tablets (figure 5, plate 63) suggest that the perforate layer is likely to be an outermost layer of a tablet rather than an inner component surrounding cells. In specimens of extensively disintegrated organisms some regular hexagonal-patterned fragments of the perforate layer extended over several microns and these could hardly have surrounded single cells. Thus all the available evidence supports the view that the perforate layer surrounds the tablet rather than individual cells within the tablet.

The punctate layer (of spines) is clearly resolved in many osmium-fixed specimens when care is taken to avoid mechanical damage (II). As in permanganate-fixed material the envelope encloses a complete tablet and rarely dips into the interstices between cells. After osmium fixation the spines appear as densely stained isolated units, 40 to 60 Å in diameter, seen in parallel (or nearly parallel) section to be arranged in regular arrays (figure 20, plate 67 and figures 21 and 22, plate 68). Although a hexagonal distribution of the units is manifest in most instances, the units are sometimes arranged in squared arrays or in parallel strings. Such abnormal arrangements may result from compression (and possibly shear) of the section during cutting and also by oblique viewing of the arrays; shrinkage of the methacrylate during polymerization may also occur. Thus little significance can be attached to variations in the arrangement and reductions in the dimensions of the sectioned arrays. The observed patterns and repeat distances are all consistent with the previously described model of the punctate layer as a two-dimensional hexagonal lattice with a repeat distance of approximately 260 Å.

The spines of the punctate layer are most readily identified when the plane of sectioning is roughly normal to the envelope, as in figure 23, plate 68. The appearance of the layer is not markedly changed by staining and the units are still apparent in unstained sections showing relatively low over-all contrast elsewhere. It must be presumed that the spines are osmiophilic.

The perforate layer cannot be distinguished with any certainty in the osmium-fixed tablets and all our observations suggest that this layer, unlike the punctate layer, is not osmiophilic. It has already been noted that it is not readily stainable. Its precise location with respect to the punctate layer must therefore remain in doubt, although it seems certain that it must lie inside rather than outside the punctate layer as no structure is visible beyond the outward-projecting spines in both negative-stained and sectioned material. In osmium-fixed specimens stained with various metal salts there are indications of a very thin layer 80 Å or so inside the punctate layer but little structure can be detected therein. It seems more
probable that the two structured layers are very much closer together or even in contact and that the perforate layer is included in the unstained material supporting the spines.

Further observations

The shadowed preparations of mechanically disintegrated and cleaned organisms so far described (figures 5 to 8, plate 63) yield only the spacing corresponding to the perforate layer. Some attempts were made to demonstrate the punctate layer in shadowed fragments from organisms subjected to brief ultrasonic disintegration without cleaning (II) but only in a few instances could the longer spacing be found. Thus in figure 24, plate 68, the centre-to-centre repeat distance between the projections in hexagonal array is 250 Å. The apparent bluntness of the projections, compared with the usual sharpness of the spines in negative-stained preparations, may be due to the lateral thickness of the shadowing metal film.

No systematic investigation of the chemical nature of the surface structures has yet been undertaken, but the results of some preliminary observations should be mentioned. The interesting study of the localization of cellulose as the cementing substance in Sarcina ventriculi packets by Canale-Parola, Borasky & Wolfe (1961) raised the possibility that a component of the envelope or intercalated zone surrounding Lampropedia hyalina might be of a similar chemical nature. Tablets of L. hyalina were placed in Schweitzer's reagent (cupra-ammonium sulphate) and held overnight at room temperature. Under this treatment the tablets were broken down into what appeared to be single cells. The dissolution of the 'cementing' substance holding cells of L. hyalina together appeared to be even more complete than that observed in packets of Sarcina ventriculi treated with the same reagent (Canale-Parola et al. 1961). Isolated envelope fractions of Lampropedia hyalina showing the perforate layer (as illustrated in figures 5 to 8) were also treated in Schweitzer's reagent (overnight at room temperature). The insoluble residues were recovered by high-speed centrifugation (10000 g for 30 min), washed thoroughly with distilled water on the centrifuge and then examined in the electron microscope as shadowed preparations. Only amorphous fragments could be detected and extensive examination of the treated preparation failed to reveal any of the fine structure originally present.

This action of Schweitzer's reagent, the insolubility of the perforate layer in 0·1 N sodium hydroxide and the metachromasia of the material immediately surrounding the tablet and extending between the cells (figure 3, plate 62) strongly suggest that the intercalated zone and the perforate layer of L. hyalina are composed of a high-molecular-weight polysaccharide and that the sheet-forming habit of this organism may be related to the aggregation of cells in Sarcina ventriculi. Nothing resembling the usual microfibrils of cellulose has been observed, however, and further chemical and cytological studies are necessary before it can be concluded that material of a cellulose nature is responsible for cell adhesion in Lampropedia hyalina.
The surface anatomy of *L. hyalina* presents a number of remarkable features. An extracellular component appears to be responsible for the adhesion of cells into sheets or tablets; the outermost extracellular layer (or layers) takes the form of a surface envelope which surrounds not a single cell but a whole group of cells; moreover, this envelope possesses an unusual and complex structure.

Our observations indicate that the cells synthesize and secrete several specialized components which grow *in situ* to surround cells or groups of cells and that unification into rectangular tablets is due to the mode of cell division and the presence of a 'cementing substance' in the extracellular material. It is tempting to postulate that the structured surface envelope prevents the separation of cells after division and therefore plays the dominant role in sheet formation, but this explanation is rendered much less plausible by the observations of Murray (1963b) who shows that cells of a non-sheet-forming strain of *L. hyalina* are still surrounded by at least part of the structured envelope. The intercalated zone is, however, absent in the non-sheet strain and it is more probable, as suggested by Murray (1963b), that the intercalated zone in the sheet-forming strain is the important factor in cell cohesion.

If the material of the intercalated zone acts as the cementing substance or 'inter-cell glue', then, in the absence of an outer envelope, there would be nothing to stop the formation of one large continuous sheet of cells, with no subdivision into tablets. Thus the main function of the outer structured envelope may be to separate the sheet into smaller units (i.e. tablets) by preventing cohesion between adjoining cells at certain places in the sheet. The biological significance of this can only be surmised but the advantages conferred by a habit of colonization might well be lost if the colonies grew to such a size that they could not readily be dispersed.

The surface envelope has been shown to be a highly complex structure, apparently embodying two regular patterned layers. The inner perforate layer is a honeycomb network with holes arranged in a regular hexagonal array. The nearest-neighbour repeat distance between hole centres is $145 \pm 10 \text{Å}$ and each hole has a diameter of about $75 \text{Å}$. The shape of the holes is uncertain although there are some indications of a polygonal, rather than circular, outline (figures 11 and 12, plate 64). The holes are likely to possess hexagonal or, at least, trigonal symmetry and a possible arrangement, in which each hole is assumed to be a regular straight-sided hexagon, is shown diagrammatically in figure 26. The edge-structure of this arrangement accords fairly closely with that seen at the edge of the negative-stained fragment in figure 12 and with the castellated edge in shadow-cast specimens (figures 7 and 8, plate 63). The model also allows easy cleavage along a principal axial direction.

The striking regularity of the structure implies an assemblage of macromolecular subunits arranged in a two-dimensional crystal lattice, but evidence on the nature and mode of packing of these subunits is still lacking. Nevertheless, it is instructive to note that the arrangement of figure 26 could be built up from three sets of
fibrillar units, each set making angles of 120° with the other two and with the units interwoven to preserve a symmetrical arrangement as shown in figure 27. There are suggestions that the shadowed ridges in figures 7 and 8 exhibit the height variations that might be expected in such structure but, even so, the model must be viewed with caution as it is hard to visualize a system that would allow the synthesis of interwoven elements in a biological fabric. On a coarser scale Frei & Preston (1961) describe some irregular interweaving of bundles of cellulose microfibrils in certain plant cell walls (e.g. Chaetomorpha). The calculated width of the conjectured fibrillar subunits in Lampropedia hyalina is 42 ± 3 Å and this is too small for the subunits to be identified as cellulose microfibrils.

The punctate layer was only observed in fragments after mild disruption without cleaning by washing and centrifugation (II) and it seemed that the layer was fragile and easily lost. The Manchester workers (J.A.C. and M.R.J.S.) failed to observe the punctate layer at all; it was probably removed in the Mickle-disintegration or ultra-centrifugation techniques or by the enzymic or chemical cleaning procedures although the possibility remains that the strain used in Manchester was a mutant strain and did not, in fact, possess the punctate layer. Unfortunately we have not been able to check this point.

The relationship between the perforate layer and the punctate layer is particularly intriguing. It has not been possible to show that the two layers are separated by any significant distance; they could be in contact and may even be combined as a single two-component layer. The existence of a structural relationship implies that the number of holes must be integrally related to the number of spines; our results favour an arrangement with three holes to one spine as this demands a relative spacing (punctate spacing: perforate spacing) of 1.73, in reasonable accord with experimentally observed values. Figure 28 shows a possible model with the spines lying immediately above holes. In this model, a principal axis of one layer is at right angles to a principal axis of the other and there are some indications that this is the case in figure 13. When the triads of linkers between the spines are inserted in the model (figure 28), their junctions are seen to cover all the holes of the perforate layer. This may explain why it is difficult to see the perforate layer in negative-stained preparations when the two layers are in conjunction.

A topological point remains to be considered. The structural envelope has to embrace a curved surface so that the complex network of spines, linkers and holes
must possess some degree of elasticity. Even so, it is not possible to cover the total
surface area of a three-dimensional body with a hexagonal network without intro-
ducing faults and dislocations in the otherwise regular surface structure. Few
faults of this nature have been seen in disintegrated fragments but this is not
altogether surprising as a fault would be a region of weakness at which breakage
would tend to occur on disintegration. The appearance of shadow-cast specimens
of almost intact envelopes suggests that the orientation of the network changes
abruptly from place to place on the surface. Thus the over-all structure may be
mosaic in character.

![Figure 28. The suggested spatial relationship between the perforate layer (of hexagonal
holes) and the punctate layer (with spines represented as opaque dots). With three
holes to one spine, the relative spacing of the two layers is 1.73. The spines are shown
immediately above holes with triads of linkers between the spines, the linkers joining
above the remaining holes, but other arrangements (with lower symmetry) are possible.]

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