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Structure and assembly of bacterial surface layers composed of regular arrays of subunits

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[Plates 54–57]

Regular arrays of subunits are observed on the surfaces of many bacteria, and the structure and function of such an array are being examined in a study of the Gram-negative bacterium *Acinetobacter* strain MJT/F5/199A. The subunits are on the surface of the outer membrane and are visible in electron micrographs of freeze-etched intact cells and of negatively stained preparations of isolated cell walls and outer membranes. The surface subunits can be detached from the membrane by various treatments and will then reassemble spontaneously to form the same regular pattern as that seen on the intact bacterium. The results of studies of the properties of the self-assembly system are described and its relevance to the formation of surface layers composed of regular arrays of subunits in intact bacteria discussed.

Introduction

The outermost layers of the cell envelopes of many bacteria are composed of regular arrays of subunits (see reviews by Glauert & Thornley 1969; Holt & Leadbetter 1969). These regular surface patterns were first observed in electron micrographs of metal-shadowed preparations and replicas of intact cells and cell envelopes. Subsequently the application of the negative-staining technique enabled the arrays to be studied at high resolution, while the recent development of the freeze-etching technique has made it possible to examine the arrays on the surfaces of intact cells. These studies have shown that regular surface patterns on bacterial surfaces are much more widespread than was previously realized.

Location and characteristics of the regular arrays of subunits

The regular arrays of subunits found in the cell walls of Gram-positive bacteria are located in the outermost layer of the wall as observed in electron micrographs of thin sections (figure 1). In *Bacillus polymyxa* (Nermut & Murray 1967) and other bacilli (Holt & Leadbetter 1969) this outer layer stains densely with osmium, and is missing from cells from which the regular array of subunits has been removed. Regular patterns have been seen on the surfaces of many Gram-positive bacteria, particularly those in the genera *Bacillus* and *Clostridium*, and many of these have tetragonal symmetry. However, hexagonal patterns have been found also; for instance, two otherwise very similar species of clostridia differ mainly in having either hexagonal or tetragonal arrangements of their surface subunits (Hollaus & Sleytr 1972; see also figures 2–8, plates 54 and 55). Some species of bacilli also have regular hexagonal patterns on the exosporium, and the outer surface of the spore coat may have a repeating pattern of regularly-spaced ridges (Holt & Leadbetter 1969).

In Gram-negative bacteria the majority of the regular arrays of subunits are on the surface

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of the outer membrane (figure 1). The array is sometimes only revealed in sections after special fixation techniques, and appears as faintly stained particles with a spacing of about 10 nm in *Spirillum serpens* (Murray 1963; Buckmire & Murray 1970), and as a dense layer with indications of a fine repeating structure in *Cardiobacterium hominis* (Reyn, Birch-Andersen & Murray 1971). Most of the patterns seen on Gram-negative bacteria have hexagonal symmetry, but several instances of tetragonal symmetry are known, including the surface of the *Acinetobacter* strain

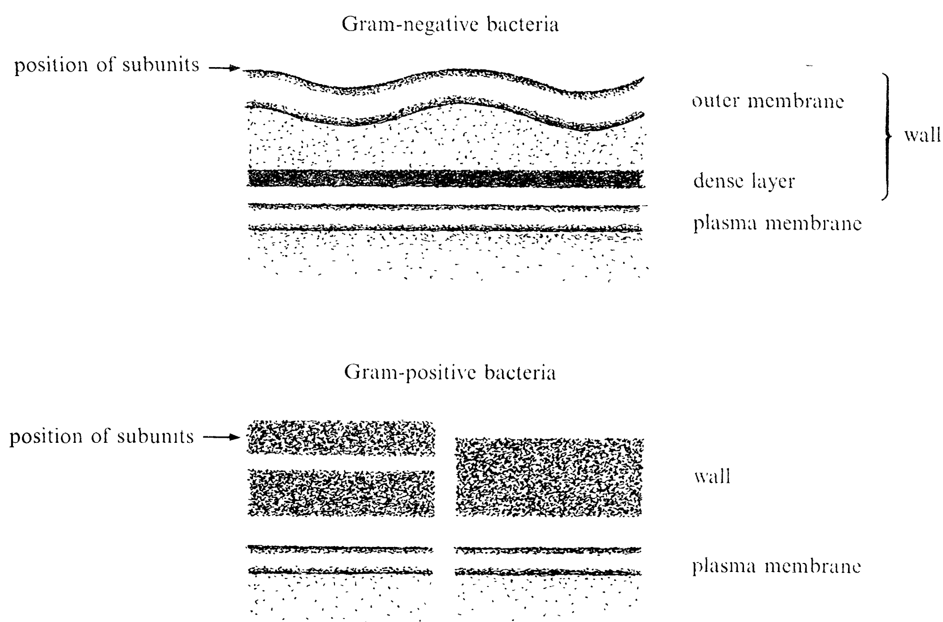

FIGURE 1. Diagrams illustrating the structure of bacterial cell envelopes and the positions of regular arrays of subunits in thin sections of Gram-negative and Gram-positive bacteria.

MJT/F5/199A studied by the present authors (Thornley, Glauert & Sleytr 1973; Sleytr & Thornley 1973), while the more complex cell wall of *Nitrosocystis oceanus* shows both types of pattern associated with different layers (Watson & Remsen 1970). Regular patterns are also found in additional outer layers in the envelopes of some Gram-negative bacteria (e.g. *Nitrosocystis oceanus*, Watson & Remsen 1970) and even in the external sheath which loosely surrounds groups of cells of *Lampropedia hyalina* (Chapman, Murray & Salton 1963; Pangborn & Starr 1966).

The location of the regular arrays of subunits on the outer surfaces of many bacteria has been

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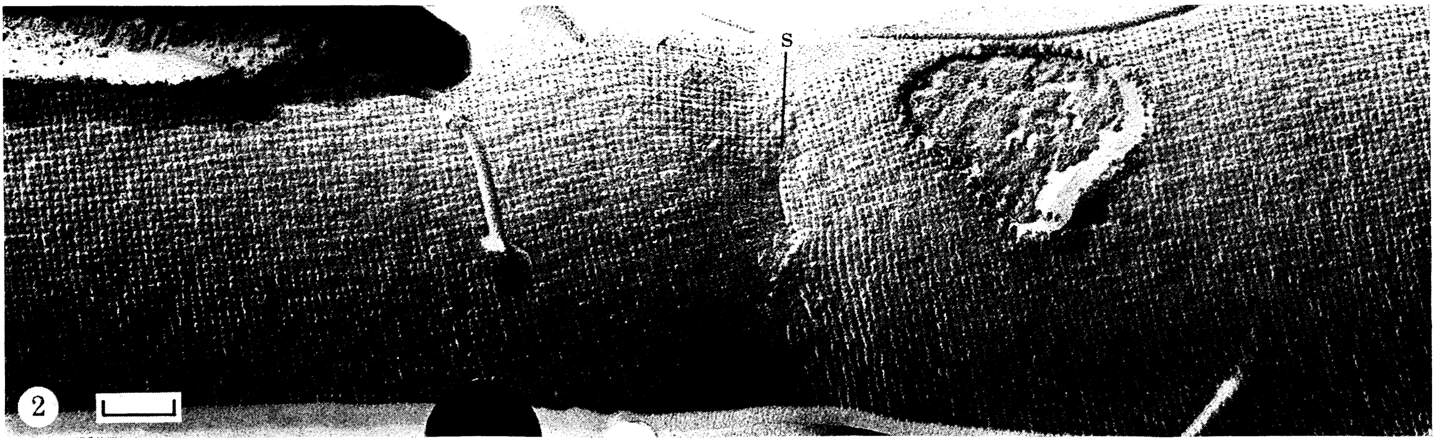
DESCRIPTION OF PLATE 54

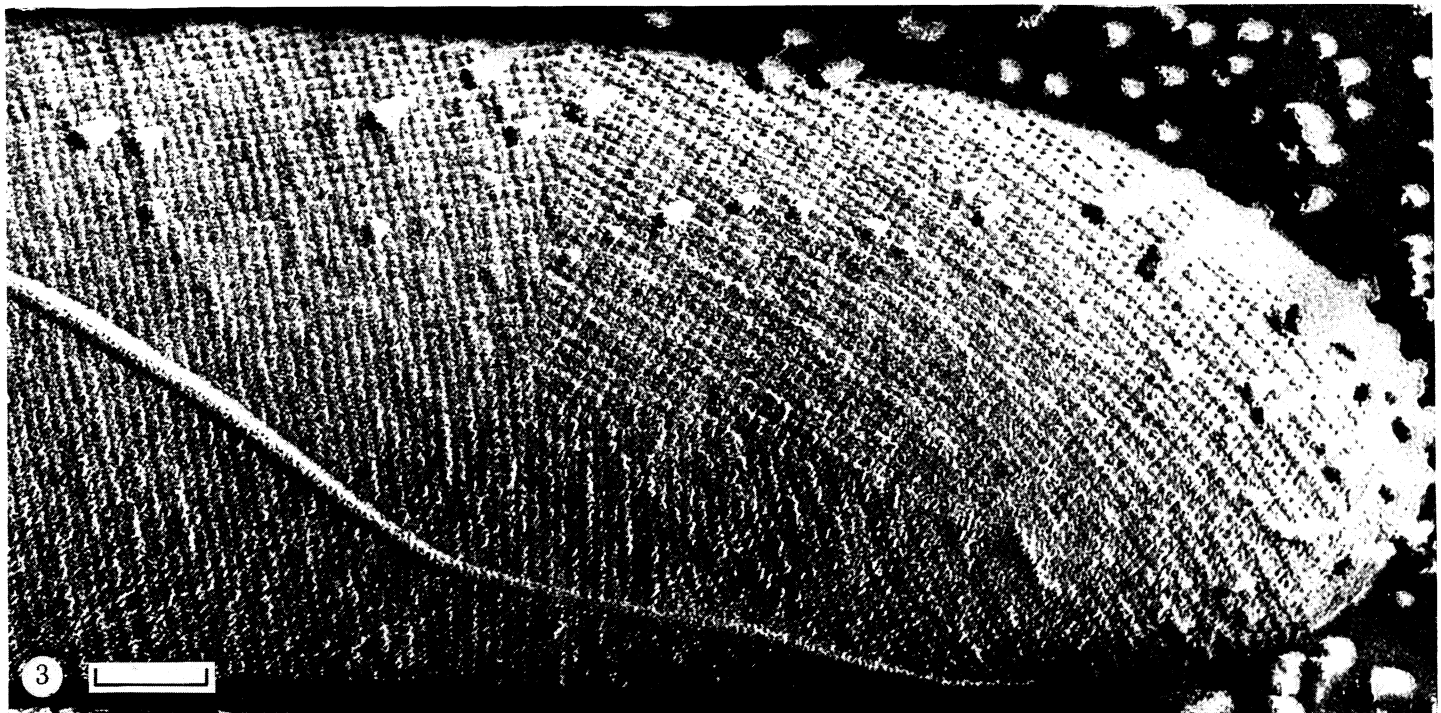
FIGURES 2–4. Electron micrographs of replicas of freeze-etched preparations of clostridia. The scale marks represent 0.1 μm.

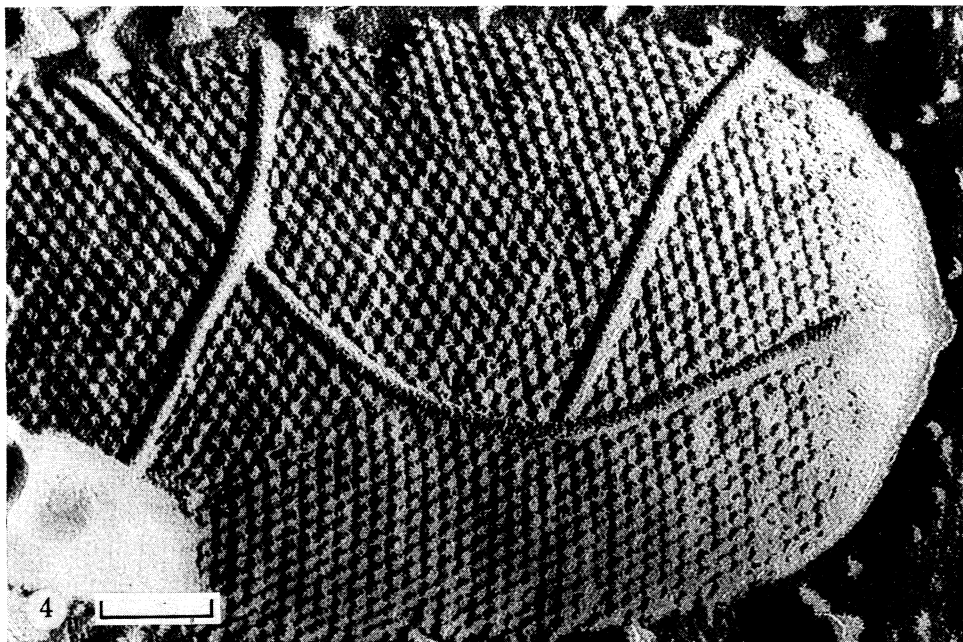
FIGURE 2. *Clostridium thermosaccharolyticum*. A large area of the surface of a dividing cell has been exposed by etching, apart from a small region removed by fracture. The surface is covered with a tetragonal array of subunits which is uniform, except at the site of septum formation (s). (Magn. × 100000.)

FIGURE 3. *C. thermosaccharolyticum*. Abrupt changes in the orientation of the regular tetragonal array of subunits are observed towards the pole of a cell. A flagellum is present on the surface of the bacterium. (Magn. × 150000.)

FIGURE 4. *Clostridium thermohydrosulfuricum*. The array of surface subunits has hexagonal symmetry and two areas with different orientations of the pattern are observed near the pole of the cell. Many flagella are present. (Magn. × 150000.)







FIGURES 2–4. For description see opposite

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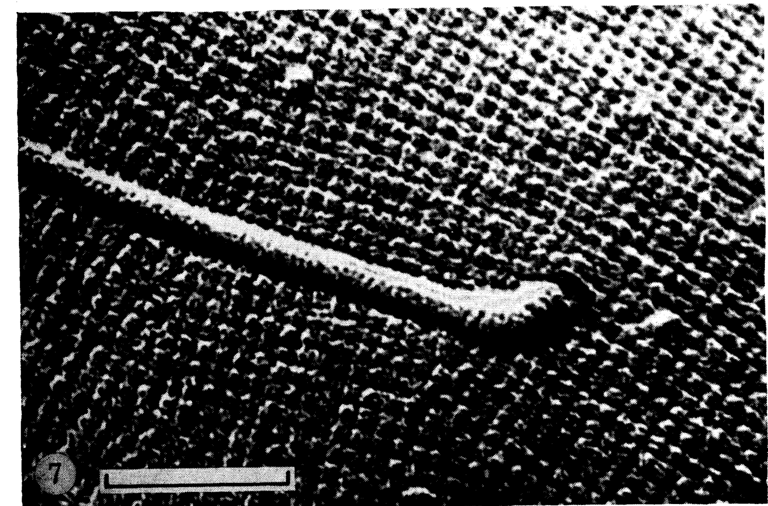

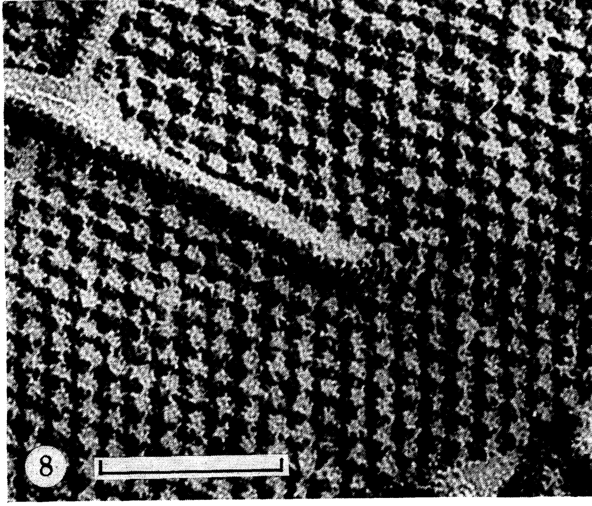

FIGURES 5–8. For description see opposite

confirmed by observations made with the freeze-etching technique (see review by Remsen & Watson 1972). Replicas of etched preparations provide views of large areas of the bacterial surface, as illustrated in figure 2, and suggest that the array of subunits usually covers the whole surface leaving no gaps.

Some irregularities in the pattern are necessary to cover shapes such as spheres or cylinders with rounded ends, and the distribution of these irregularities on two species of clostridia is illustrated in figures 2–6. In *Clostridium thermosaccharolyticum*, large areas of uniform tetragonal pattern cover the cylindrical part of the cell (figure 2), while regions near the poles, where the curvature alters, often show adjacent areas where the orientation of the pattern is different (figure 3). At the boundary between two areas of different orientation, some irregularities in the packing of the subunits is observed (figures 3, 4). These boundaries can be compared with grain boundaries between adjacent crystallites in a metal. At the central region of a dividing cell, many small 'crystallites' give the appearance of 'crazy paving' to the surface (figures 5, 6). As the septum grows inwards from this zone, and the part of the septum adjacent to the cell wall is split, new portions of the outer surface of each daughter cell must be revealed. It seems possible that the small areas of 'crazy paving' are a result of this process. Since this central zone eventually forms the poles of the daughter cells, with much fewer grain boundaries, it seems that the subunits must be capable of rearrangement. Presumably the regular array is the condition of least stress between adjacent subunits.

The regular pattern of subunits is also disturbed at regions of insertion of flagella (figure 7), although it is interesting to note that some flagella have about the same diameter as an individual subunit and thus appear to replace a subunit in the array without disturbing the regular pattern to any great extent (figure 8).

Fine structure of the regular arrays

In general, the subunits remain firmly attached to cell walls or envelopes during their isolation (see, for example, Thornley *et al.* 1973) and this enables the regular patterns to be studied at high resolution in negatively stained preparations. Two hexagonal patterns, on *Spirillum serpens* strain VHA and *Micrococcus radiodurans*, and two tetragonal patterns, on *Bacillus polymyxa* and *Acinetobacter* strain MJT/F5/199A, have been analysed in some detail.

The subunits of the hexagonal pattern on the surface of the outer membrane of the Gram-negative *Spirillum serpens* strain VHA (Murray 1963) are about 10 nm in diameter and appear polygonal with a central 'hole' that is penetrated by the negative stain. The subunits are linked by Y-shaped structural elements in a hexagonal array with a centre-to-centre spacing of about

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Description of plate 55

Figures 5–8. Electron micrographs of replicas of freeze-etched preparations of clostridia. The scale marks represent 0.1 μm.

Figures 5, 6. *Clostridium thermosaccharolyticum*. Near the site of septum formation the tetragonal pattern shows many small areas with the different orientation, giving the surface a 'crazy paving' appearance. (Magn. × 150000.)

Figure 7. At the site of insertion of a flagellum the tetragonal pattern on the surface of *C. thermosaccharolyticum* is disturbed. (Magn. × 240000.)

Figure 8. The diameter of the base of a flagellum on *C. thermohydrosulfuricum* has similar dimensions to the spacing of the regular hexagonal array of subunits on the bacterial surface, and the array is only slightly disturbed at the site of insertion of the flagellum. (Magn. × 240000.)

15 nm. In another strain of *Spirillum serpens* (MW 6) the subunits are linked directly and are more widely spaced than in strain VHA (Murray 1968).

The hexagonal array on the surface of the outer membrane of the Gram-variable *Micrococcus radiodurans* (Thornley, Horne & Glauert 1965; Work & Griffiths 1968) is similar to that on *Spirillum serpens* strain MW 6; the subunits appear as hollow rings about 12 nm in diameter and are linked directly with a centre-to-centre spacing of about 17.5 nm (figure, 9, plate 56). At folded edges of fragments of the cell wall the subunits appear as projecting 'pegs', each about 12 nm long.

Both the tetragonal patterns that have been analysed have much finer spacings than the hexagonal arrays just described, and consequently little detail is directly visible in the electron micrographs. It is necessary to apply optical diffraction and filtering techniques to obtain information on the fine structure of the subunits. Finch, Klug & Nermut (1967) investigated the tetragonal pattern on the surface of the cell wall of the Gram-positive *Bacillus polymyxa* and images from which the 'noise' had been filtered optically showed hollow, square morphological units about 7 nm in size, arranged in a square lattice of side 10 nm. The morphological units appeared to be composed of four smaller subunits, each 4 to 5 nm in diameter.

The tetragonal array of subunits on the surface of the outer membrane of the Gram-negative *Acinetobacter* strain MJT/F5/199A (figure 10, plate 56) has even smaller dimensions than the lattice of *Bacillus polymyxa* (Glauert & Thornley 1973). The unit cell is approximately 6 by 8 nm with axes at an angle of about 79°, as shown by analysis of electron micrographs of negatively stained preparations of isolated cell walls by Dr R. A. Crowther (of the M.R.C. Laboratory of Molecular Biology, Cambridge), using optical diffraction and computer filtering techniques. This analysis has not yet revealed much information on the shape of the subunits since the diffraction patterns show relatively few orders due to curvature in the arrays. The subunits appear as projecting 'pegs' at folded edges of the cell wall (Thornley & Glauert 1968; Thornley *et al.* 1973).

Composition of isolated subunits

Little is yet known about the mode of attachment of the subunits to the surface of the bacterial cell. Relatively few experiments have so far been done on a limited range of organisms, mostly with the aim of obtaining purified preparations for chemical analysis. The subunits have been removed from Gram-positive bacilli with sodium dodecyl sulphate (Goundry, Davison, Archibald & Baddiley 1967), by acidification (McNary, Carnahan & Brinton 1968; Brinton, McNary & Carnahan 1969; Howard & Tipper 1973) or with urea (Howard & Tipper 1973), and analyses showed them to consist mainly or entirely of protein, which in *B. sphaericus* made up

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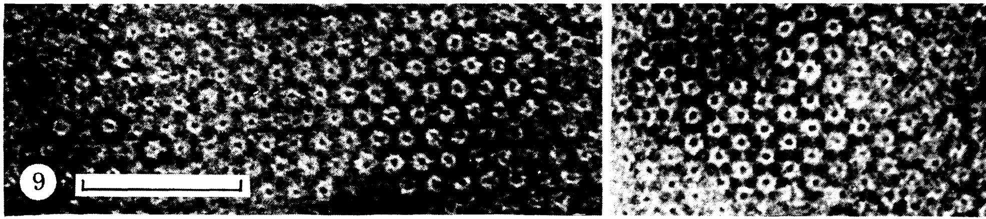
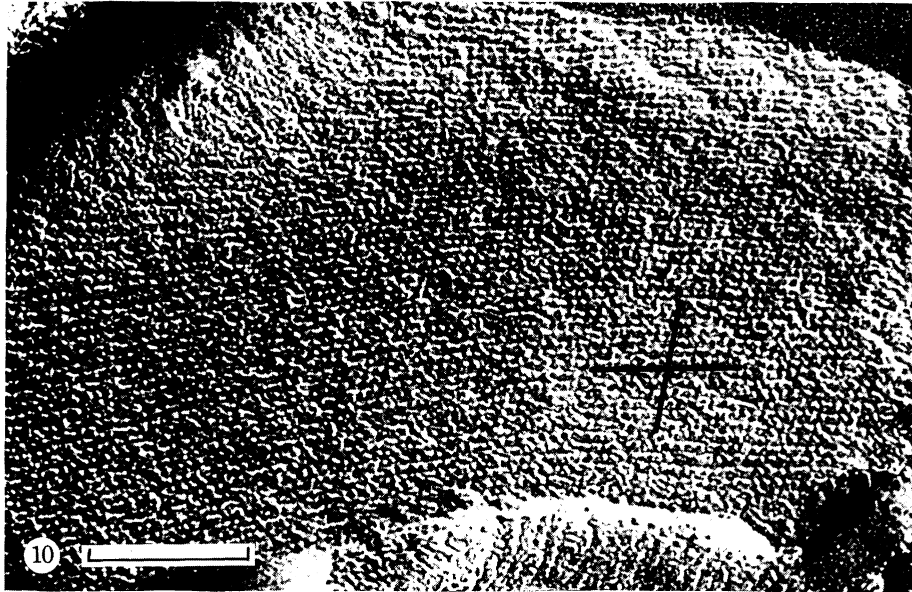
Description of plate 56

Figure 9. A negatively stained preparation of the hexagonal array of subunits from the surface of *Micrococcus radiodurans*. The subunits appear as hollow rings and are linked by fine spokes. (Magn. × 210 000)

Figure 10. The tetragonal array of subunits on the surface of *Acinetobacter* strain MJT/F5/199A is visible in a freeze-etched preparation. The subunits are arranged in rows in two directions at an angle of about 80° to each other. (Magn. × 210 000.)

Figure 11. A negatively stained preparation of fragments of the cell wall of *Acinetobacter* strain MJT/F5/199A, after ultrasonic treatment and a period for reassembly in the presence of 20 mM $MgCl_2$. The detached subunits have formed regular arrays independently of the fragments of cell wall. (Magn. × 210 000).





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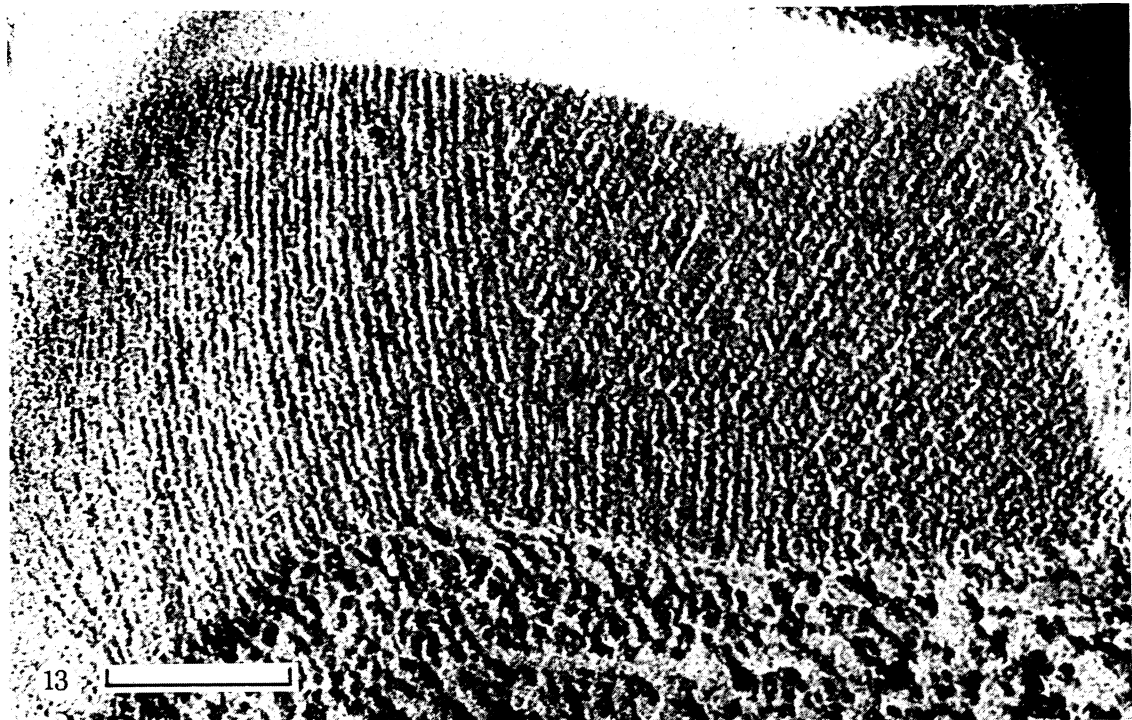
FIGURES 9–11. For description see opposite



FIGURES 12, 13. For description see opposite

16% of the total protein of the cell (Howard & Tipper 1973). The molecular mass was estimated to be 140000–150000 for the subunits from *Bacillus sphaericus* strain P-1 (Brinton *et al.* 1969; Howard & Tipper 1973), and to range from 86000 to 150000 for phage-resistant mutants of this strain (Howard & Tipper 1973).

The subunits on the surface of *Spirillum serpens* strain VHA have been isolated by heat treatment (60 °C for 2 h), which removes the subunits still attached to a basal layer containing lipopolysaccharide and phosphatidyl ethanolamine, followed by guanidine hydrochloride which removes the subunits from the basal layer (Buckmire & Murray 1970, 1973). The extract consisted of 98% protein and 2% carbohydrate and contained a single protein with a molecular mass of 125000–150000.

The subunits of *Acinetobacter* strain MJT/F5/199A are completely removed from cell walls by treatment with urea, EDTA or EGTA, or partially removed by ultrasonic treatment or freezing and thawing combined with washing in various salt solution. Preliminary results, obtained in collaboration with Dr K. J. I. Thorne, indicate that the protein which forms the pattern has a subunit molecular mass of 65000–70000 (Thornley, Thorne & Glauert 1974).

Assembly of isolated subunits

Under certain conditions the detached subunits have been found to possess the ability to reassemble into a regular array. The first report of such an *in vitro* assembly process was in an abstract by Brinton *et al.* (1969). They isolated the protein subunits of the tetragonal outer layer of *Bacillus sphaericus* strain P-1 (originally named *B. brevis*) by acidification and reported that this protein 'will assemble *in vitro* to form a square network with an appearance in electron micrographs identical to that of the network on the cell surface' on subsequent neutralization. It was also reported that the reassembled protein was cylindrically curved with a diameter equal to that of the bacteria from which it was isolated.

Subunits with the capacity to reassemble *in vitro* have also been obtained by Buckmire & Murray (1970, 1973) using a combination of heat, followed by guanidine hydrochloride, to release the subunits from the surface of intact cells of *Spirillum serpens* strain VHA. These subunits had the ability to reassemble into the original hexagonal array, but only if a template consisting of fragments of cell wall from which the subunits had previously been removed was present, and, in addition, calcium ions were necessary.

Our studies on reassembly of subunits from *Acinetobacter* strain MJT/F5/199A (Glauert & Thornley 1973) arose from observations on isolated cell wall or outer membrane preparations treated with ultrasonics. Subunits were detached and remained disaggregated in distilled water, but in various salt solutions had the ability to reassemble into regular arrays independently of the fragments of wall material (figure 11, plate 56). Analysis by optical diffraction and computer techniques (by Dr R. A. Crowther) showed that the arrays formed *in vitro* have similar

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Description of plate 57

Figure 12. Different orientations of the tetragonal pattern are seen in adjacent areas in a negatively stained preparation of an array of subunits of *Acinetobacter* strain MJT/F5/199A reassembled *in vitro*. (Magn. 240000.)

Figure 13. Similar discontinuities in the pattern of subunits are visible on the surface of an intact cell of *Acinetobacter* strain MJT/F5/199A in a freeze-etched preparation. (Magn. × 240000.)

dimensions to the arrays on the cell walls, suggesting that the information determining the pattern is contained in the subunits themselves and not in the underlying outer membrane of the cell wall.

The arrays are observed in preparations obtained by floating grids coated with collodion–carbon films face downwards on the surface of the sample, followed by negative staining, and it is thought probable that reassembly takes place at the air–liquid interface since moiré patterns, indicating the overlap of two arrays, are not observed. Indeed, the arrays contain grain boundaries, suggesting that reassembly begins from several different points and continues, in one plane, until the patterned areas are confluent (figure 12, plate 57). It seems likely that the air–liquid interface may provide a similar hydrophobic–hydrophilic environment to that at the surface of the bacterial cell. Similar grain boundaries have been observed, by freeze-etching, on intact bacteria (figure 13, plate 57).

A wide range of conditions allowed reassembly of ultrasonically detached subunits. These included incubation for periods of 30 min or longer at 20 °C in solutions of NaCl, $MgCl_2$, $CaCl_2$, $ZnCl_2$, ammonium bicarbonate or maleate, cacodylate or tris buffers, at pH values from 5.0–8.0 and at ionic strengths (of $MgCl_2$) between 0.015 and 0.6.

The process of reassembly of subunits from the *Acinetobacter* therefore differed from the process observed by Brinton *et al.* (1969) in that flat, rather than three-dimensional, arrays were formed, and from the system studied by Buckmire & Murray (1973) in that the underlying cell wall surface was not required as a template, and there was no requirement for specific cations. However, it is possible that the attachment of the subunits to the cell wall is mediated by particular cations in the *Acinetobacter*; this process has not yet been studied.

Biological functions of regular arrays of surface subunits

As yet little is known about the biological role of these regular arrays of subunits on the surfaces of bacteria, although the facts that they usually appear to cover the whole surface of the cell, and that they seem much commoner on free-living than on parasitic bacteria, suggest that they may have a protective function analogous to that of the protein coat of a virus. Apart from possible protection against environmental conditions, direct evidence for protection against a bacterial parasite has been provided by Buckmire (1971). He showed that removal of the patterned layer from strains of *Spirillum serpens* resistant to parasitism by *Bdellovibrio bacteriovorus* renders them susceptible to parasitism, and that reassembly of the layer onto the cell surface is accompanied by a return of resistance. This resistance of the cells is due to the inability of the parasite to attach, suggesting that the patterned layer may protect the bacteria by masking receptor sites for the attachment of *B. bacteriovorus*.

In a contrasting observation on *Bacillus sphaericus*, Howard & Tipper (1973) found evidence that the patterned layer provides receptor sites for a bacteriophage. They examined the subunits of the tetragonal layer from *B. sphaericus* strain P-1 and from phage-resistant mutants of this strain. The results suggested that the tetragonal layer in the wild type is probably the receptor for phage M. The subunits of the mutants have very different molecular masses and are apparently so modified that they no longer function as phage receptors.

To provide further evidence about the functions of these patterned surface layers, it is necessary to develop methods for removing them without affecting the viability of the bacteria, or to select mutants with and without the patterned layers.

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References (Thornley *et al.*)

Brinton, C. C., McNary, J. C. & Carnahan, J. 1969 Purification and *in vitro* assembly of a curved network of identical protein subunits from the outer surface of a *Bacillus*. *Bact. Proc.* p. 48.

Buckmire, F. L. A. 1971 A protective role for a cell wall protein layer of *Spirillum serpens* against infection by *Bdellovibrio bacteriovorus*. *Bact. Proc.* p. 43.

Buckmire, F. L. A. & Murray, R. G. E. 1970 Studies on the cell wall of *Spirillum serpens*. I. Isolation and partial purification of the outermost cell wall layer. *Can. J. Microbiol.* **10**, 1011–1022.

Buckmire, F. L. A. & Murray, R. G. E. 1973 Studies on the cell wall of *Spirillum serpens*. II. Chemical characterization of the outer structured layer. *Can. J. Microbiol.* **19**, 59–66.

Chapman, J. A., Murray, R. G. E. & Salton, M. R. J. 1963 The surface anatomy of *Lampropedia hyalina*. *Proc. R. Soc. Lond.* B **158**, 498–513.

Finch, J. T., Klug, A. & Nermut, M. V. 1967 The structure of the macromolecular units on the cell walls of *Bacillus polymyxa*. *J. Cell Sci.* **2**, 587–590.

Glauert, A. M. & Thornley, M. J. 1969 The topography of the bacterial cell wall. *A. Rev. Microbiol.* **23**, 159–198.

Glauert, A. M. & Thornley, M. J. 1973 Self-assembly of a surface component of a bacterial outer membrane. *John Innes Symp.* **1**, 297–305.

Goundry, J., Davison, A. L., Archibald, A. R. & Baddiley, J. 1967 The structure of the cell wall of *Bacillus polymyxa* (N.C.I.B. 4747). *Biochem. J.* **104**, 1 C–2 C.

Hollaus, F. & Sleytr, U. 1972 On the taxonomy and fine structure of some hyperthermophilic saccharolytic clostridia. *Arch. Mikrobiol.* **86**, 129–146.

Holt, S. C. & Leadbetter, E. R. 1969 Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. *Bact. Rev.* **33**, 346–378.

Howard, L. & Tipper, D. J. 1973 A polypeptide bacteriophage receptor: modified cell wall protein subunits in bacteriophage-resistant mutants of *Bacillus sphaericus* strain P-1. *J. Bact.* **113**, 1491–1504.

McNary, J. E., Carnahan, J. & Brinton, C. C. 1968 Ultrastructure of the cell envelope of a *Bacillus* species. *Bact. Proc.* p. 65.

Murray, R. G. E. 1963 On the cell wall structure of *Spirillum serpens*. *Can. J. Microbiol.* **9**, 381–392.

Murray, R. G. E. 1968 Bacterial cell wall anatomy in relation to the formation of spheroplasts and protoplasts. In *Microbial protoplasts, spheroplasts and L-forms* (ed. L. B. Guze), pp. 1–18. Baltimore: Williams and Wilkins.

Nermut, M. V. & Murray, R. G. E. 1967 Ultrastructure of the cell wall of *Bacillus polymyxa*. *J. Bact.* **93**, 1949–1965.

Pangborn, J. & Starr, M. P. 1966 Ultrastructure of *Lampropedia hyalina*. *J. Bact.* **91**, 2025–2030.

Remsen, C. C. & Watson, S. W. 1972 Freeze-etching of bacteria. *Int. Rev. Cytol.* **33**, 253–296.

Reyn, A., Birch-Andersen, A. & Murray, R. G. E. 1971 The fine structure of *Cardiobacterium hominis*. *Acta path. microbiol. Scand.* B **79**, 51–60.

Sleytr, U. B. & Thornley, M. J. 1973 Freeze-etching of the cell envelope of an *Acinetobacter* species which carries a regular array of surface subunits. *J. Bact.* **116**, 1383–1397.

Thornley, M. J. & Glauert, A. M. 1968 Fine structure and radiation resistance in *Acinetobacter*: studies on a resistant strain. *J. Cell Sci.* **3**, 273–294.

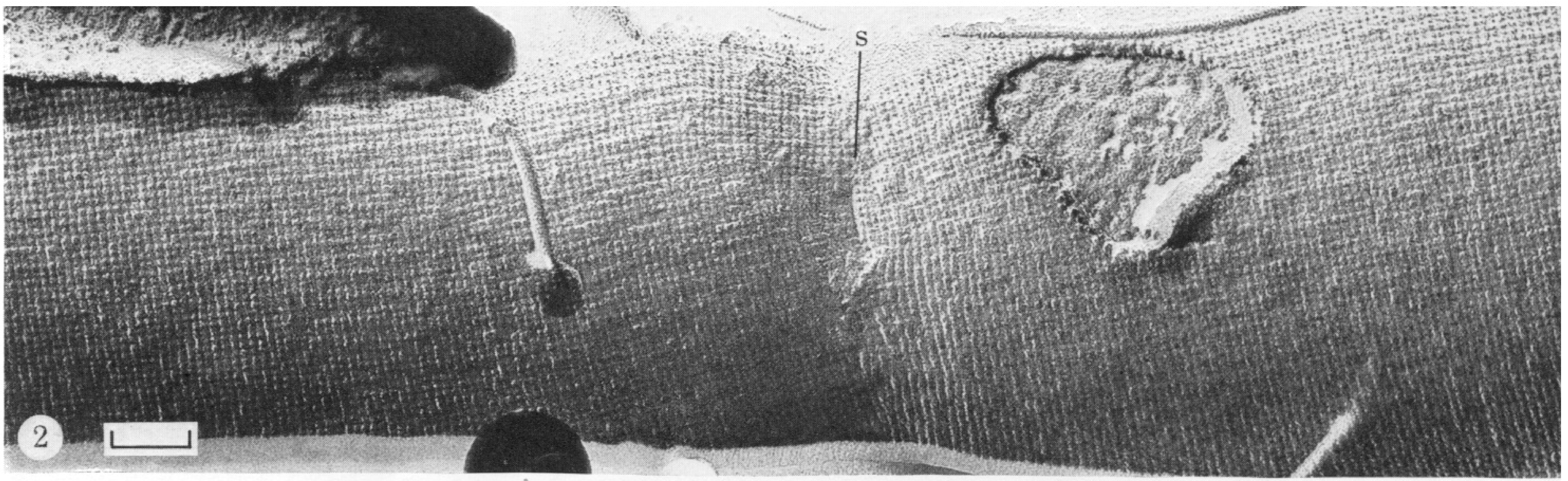
Thornley, M. J., Glauert, A. M. & Sleytr, U. B. 1973 The isolation of outer membranes with an ordered array of surface subunits from *Acinetobacter*. *J. Bact.* **114**, 1294–1308.

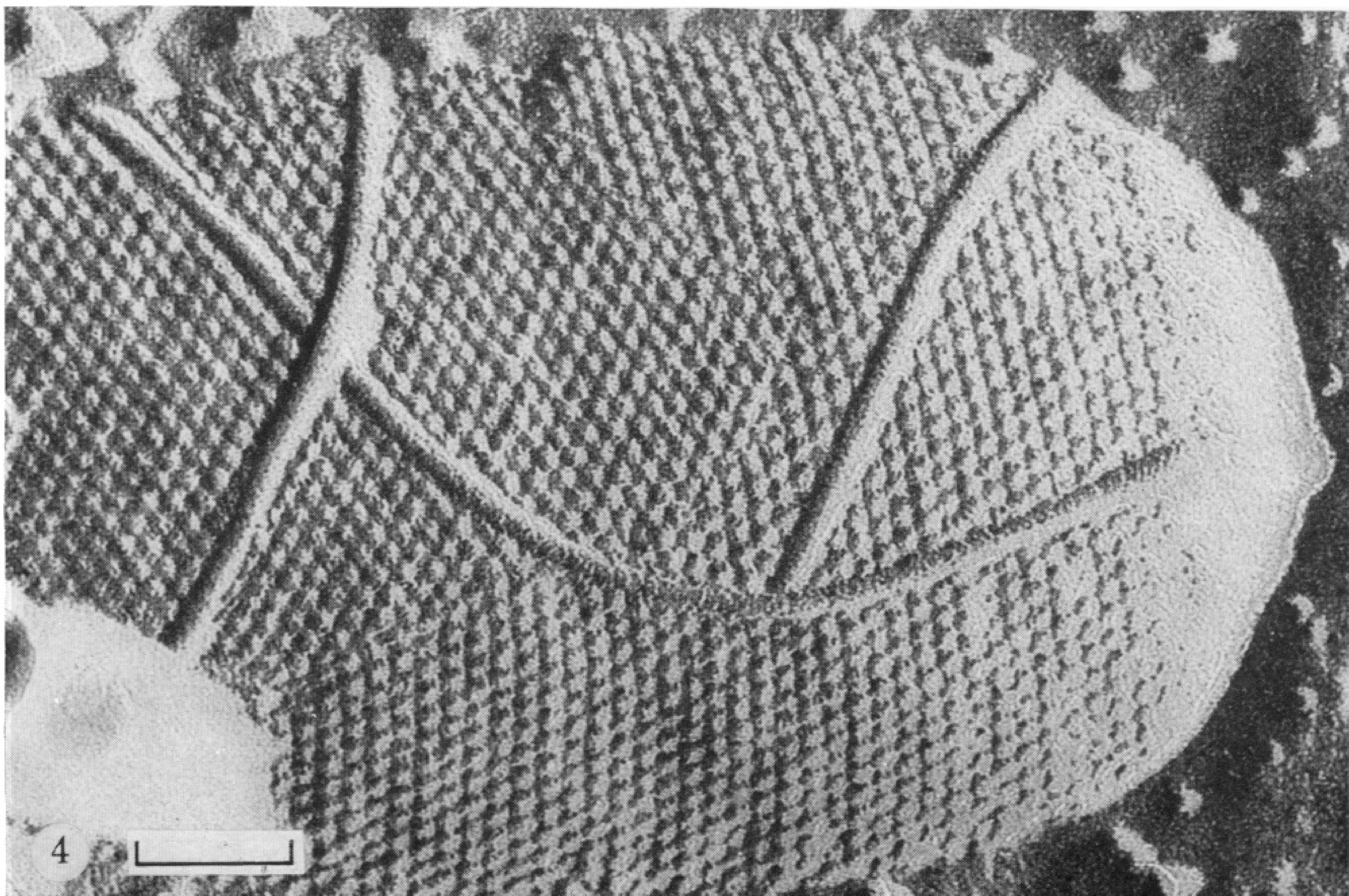
Thornley, M. J., Horne, R. W. & Glauert, A. M. 1965 The fine structure of *Micrococcus radiodurans*. *Arch. Mikrobiol.* **51**, 267–289.

Thornley, M. J., Thorne, K. J. I. & Glauert, A. M. 1974 The detachment and chemical characterization of the regularly arranged subunits from the surface of an *Acinetobacter*. *J. Bact.* **118**, in press.

Watson, S. W. & Remsen, C. C. 1970 Cell envelope of *Nitrosocystis oceanus*. *J. ultrastruct. Res.* **33**, 148–160.

Work, E. & Griffiths, H. 1968 Morphology and chemistry of cell walls of *Micrococcus radiodurans*. *J. Bact.* **95**, 641–657.





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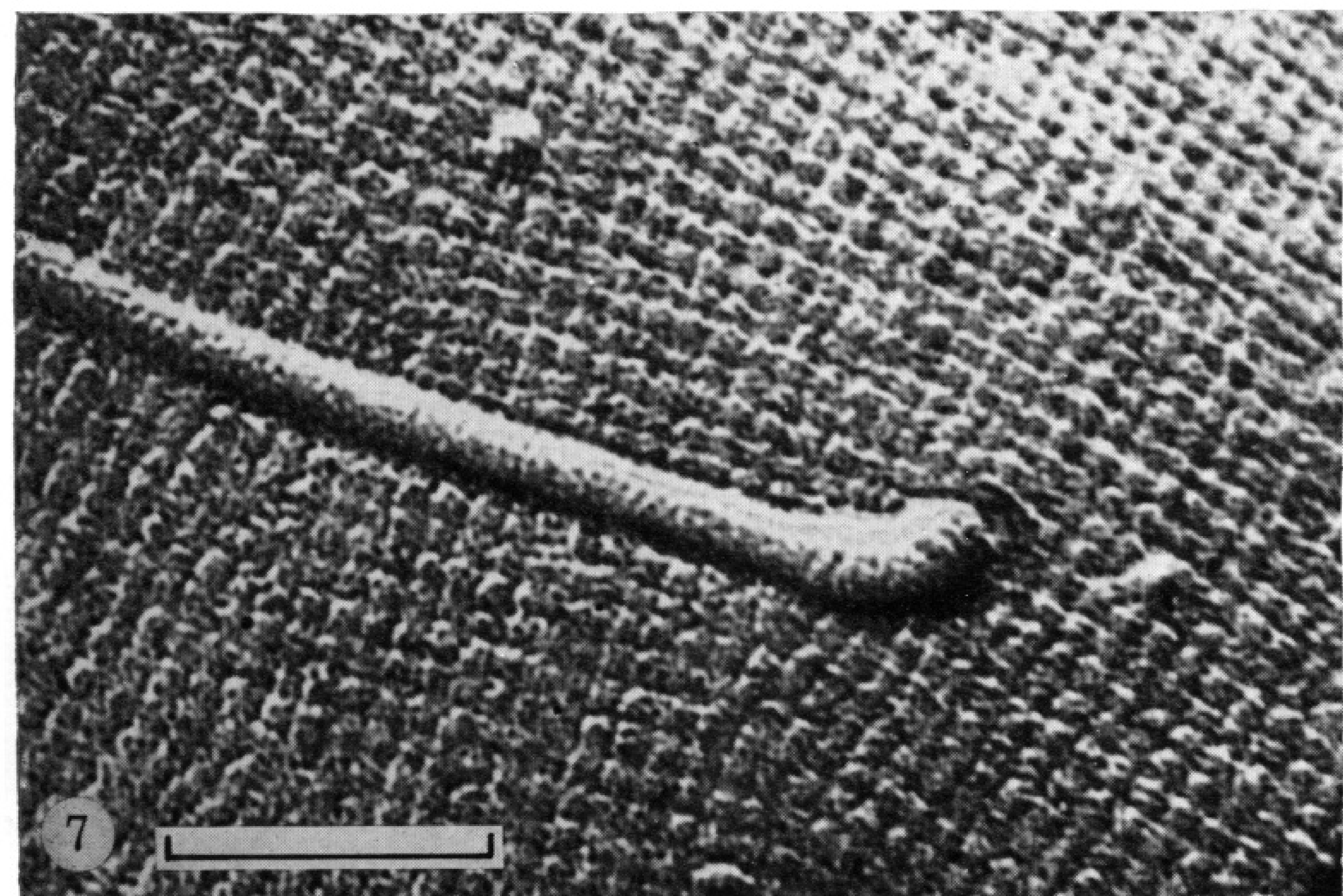

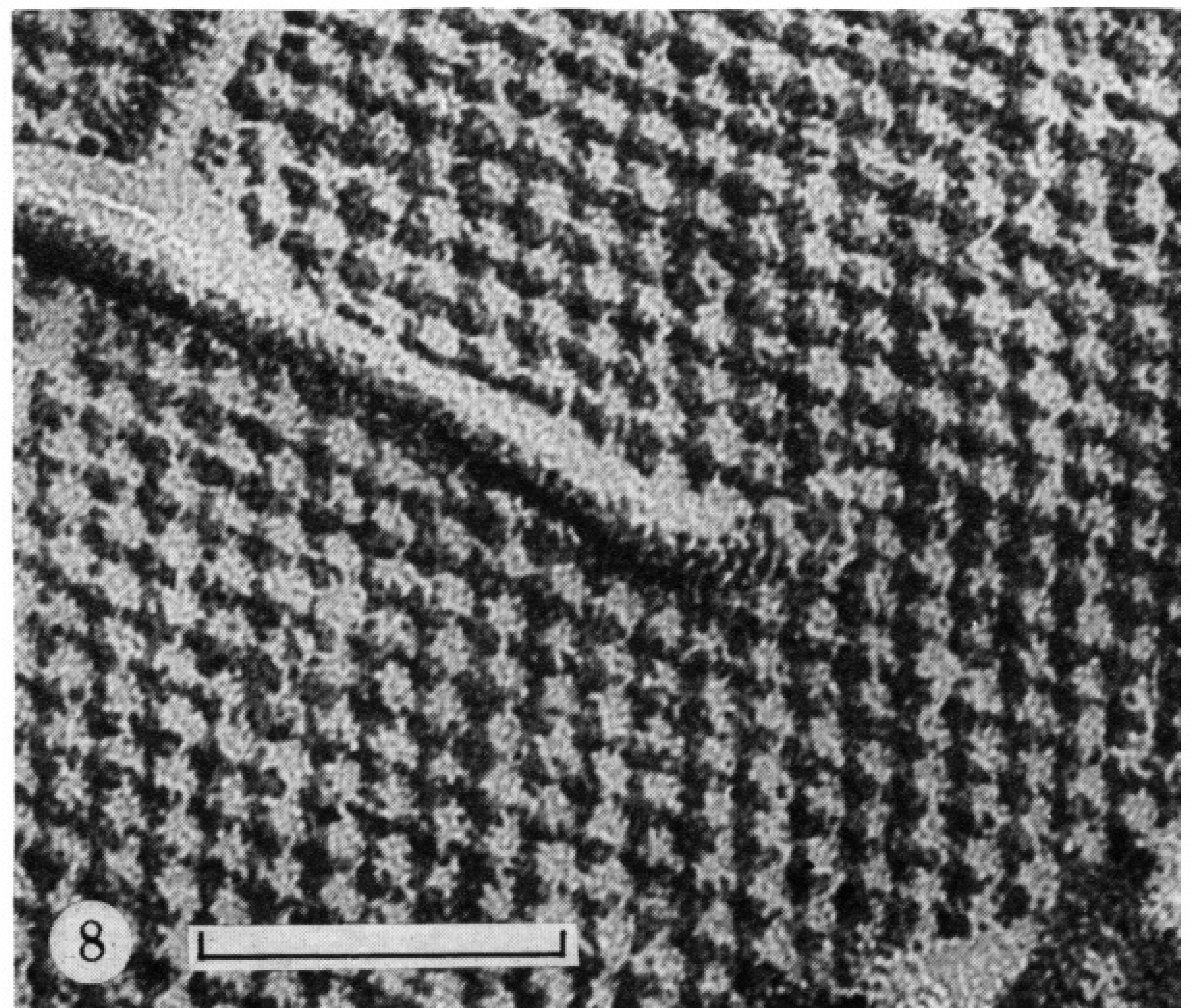

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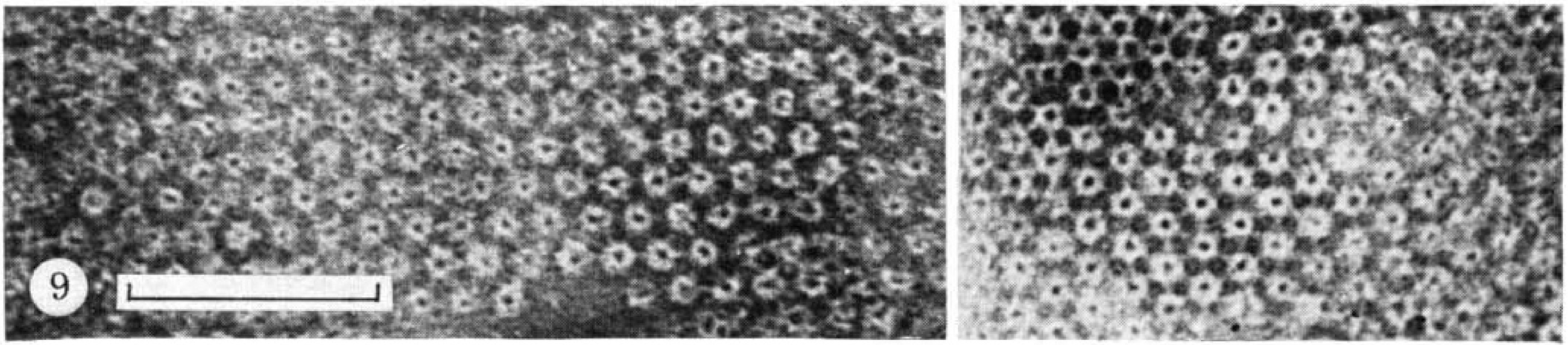

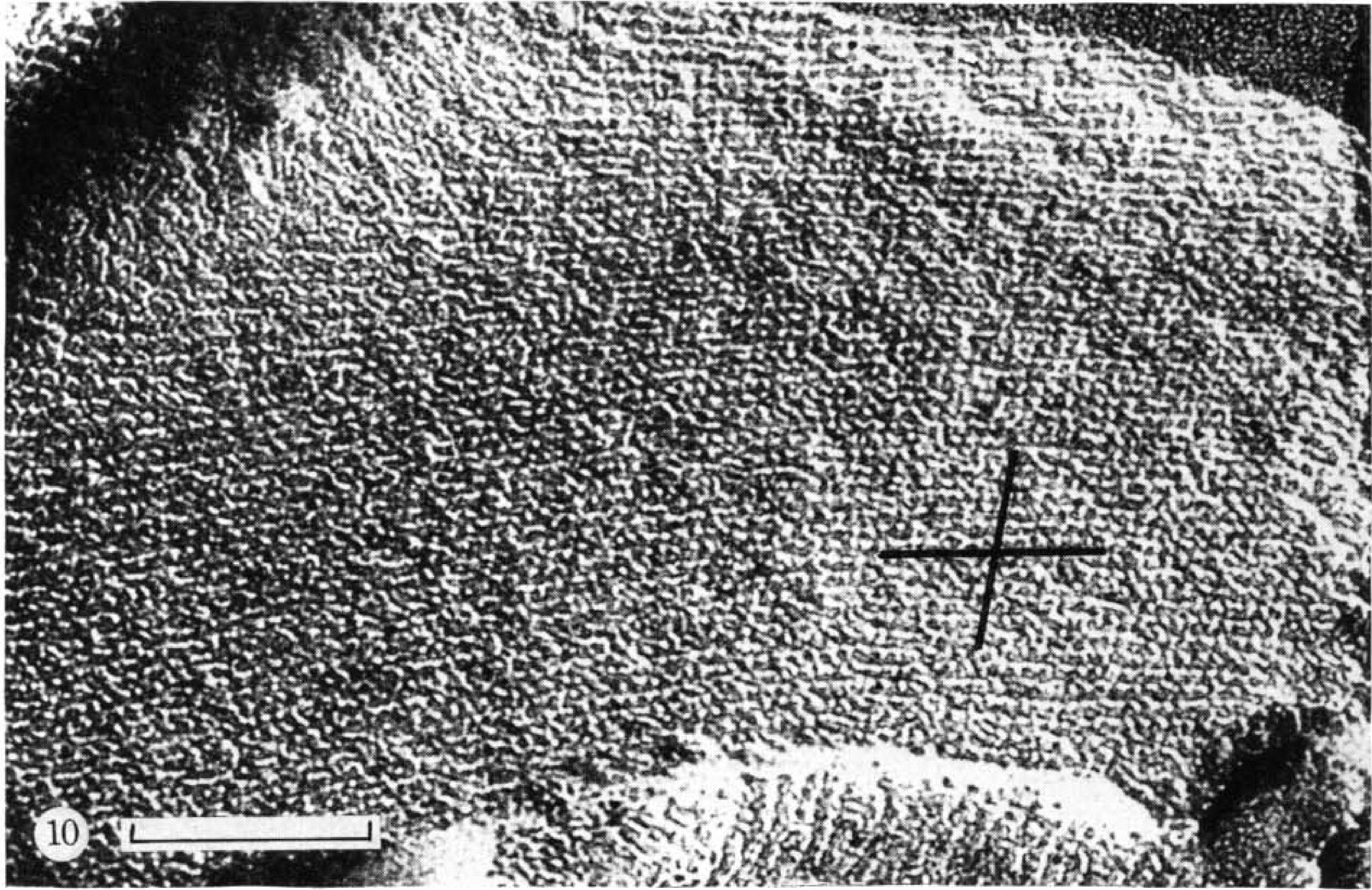

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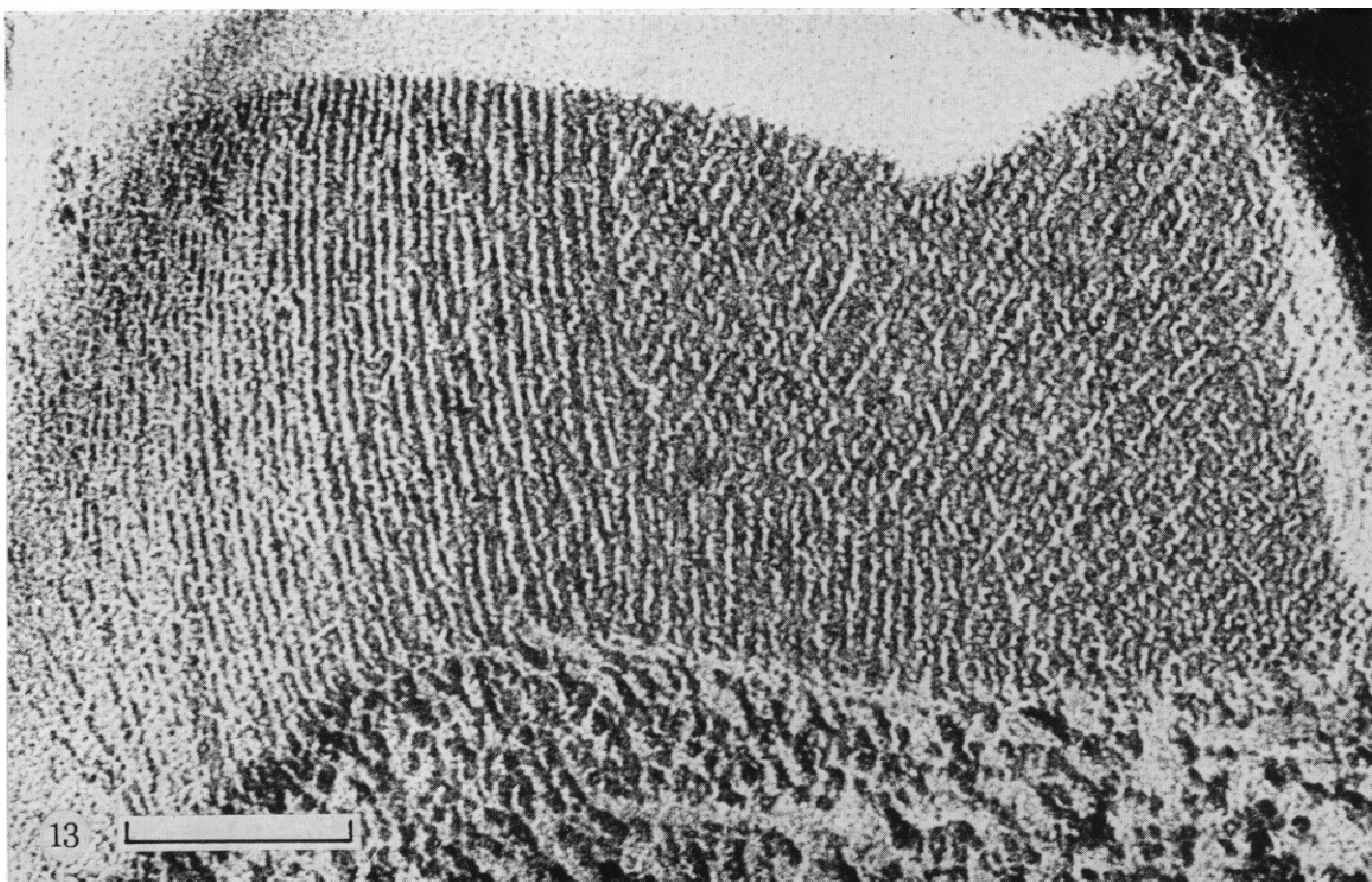

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