JOURNAL OF BACTERIOLOGY, Feb. 1968, p. 641-657
Vol. 95, No. 2

Printed in U.S.A.

Morphology and Chemistry of Cell Walls of *Micrococcus radiodurans*

ELIZABETH WORK AND HILARY GRIFFITHS
Twyford Laboratories, Twyford Abbey Road, London, N.W. 10, England

Received for publication 10 October 1967

Walls of the pigmented strain of *Micrococcus radiodurans* showed several layers in the electron microscope. These layers include an outermost network structure removed by trypsin, a fragile soft layer containing hexagonally packed subunits, and a rigid layer penetrated by numerous holes. The two inner layers were separated by a process of autolysis, trypsin treatment, and gradient centrifugation. The hexagonally packed layer was less dense, pink in color, and it contained carotenoids, lipid, protein, and polysaccharide. The lipid consisted of odd-numbered as well as even-numbered fatty acids, and the polysaccharide contained rhamnose and mannose, but it did not contain heptose. The "holey" layer was white and was composed of a mucopeptide containing glucosamine, muramic acid, and four main amino acids (glutamic acid, alanine, glycine, and L-ornithine, in the ratios of 1:1.7:1.8:1.2, respectively). This layer also contained phosphorus, glucose, and a trace of *meso*- and LL-diaminopimelic acid. A white mutant, W_1, of *M. radiodurans* had no pigment or lipid in its walls, but it contained small amounts of the "hexagonal" layer. The holey layer, constituting the bulk of the wall, was similar in morphology and composition to that layer in the pigmented strain. Lysozyme did not remove the lipoprotein-polysaccharide component from the walls of the pigmented strains, and the hexagonally packed structure was not visibly affected, except for change in a minor structure. Most of the mucopeptide layer was solubilized by lysozyme, but a structureless bag-shaped residue was left. This residue contained phosphorus, carbohydrate, and limited amino acids, but it did not contain muramic acid, glucosamine, or ornithine. Aqueous phenol removed a lipoprotein component from strain R_1, which contained limited fatty acids. It also removed *meso*- and LL-diaminopimelic acid.

Micrococcus radiodurans is a gram-positive radiation-resistant coccus originally isolated by Anderson and co-workers (3, 13). The rose-pink organism usually grows in tetrad form. Its resistance to ionizing and ultraviolet radiations was studied by Moseley and others (7, 23, 24); colorless mutants (strains W) are also radiation-resistant (22). A morphologically similar pink species of *Micrococcus* (now referred to as *M. radiodurans* strain Sark) was independently isolated (R. G. E. Murray and C. F. Robinow, Abstr. 7th Intern. Congr. Microbiol., p. 427-428, 1958).

The walls of the pink species of *M. radiodurans* are not typical of gram-positive cocci; they contain lipoproteins, as well as a mucopeptide in which L-ornithine is the principal diamino acid instead of the more usual diaminopimelic acid or lysine (38). The walls were shown to be unusual morphologically, when examined in whole or broken cell preparations (14, 25, 35). Several characteristic structures were observed, such as hexagonal packing and regions penetrated by many holes.

The present investigation is a morphological and chemical study of wall preparations of pigmented and nonpigmented strains of *M. radiodurans*. Physical separation of the layers containing the hexagonal and perforated structures enabled us to determine the composition of these layers. The chemical and morphological effects of lysozyme and phenol were also studied.

MATERIALS AND METHODS

Organisms. M. radiodurans strains R_1 and Sark were obtained from R. G. E. Murray; a white mutant (W_1) of strain R_1 was kindly donated by B. E. B. Moseley. The cells were grown, unless otherwise stated, at 30 C for 24 hr in 2-liter conical flasks, containing 500 ml of medium, placed on a gyratory shaker. The medium used for strains R_1 and Sark contained (per liter): tryptone (Oxoid), 5 g; yeast

extract powder (Oxoid), 3 g; DL-methionine, 1 g; and glucose, 1 g (added after autoclaving). The medium was adjusted to *p*H 7.0. For strain W_1, 10 ml/liter of liver extract (Oxoid) was added. The harvested cells were washed twice with 0.9% NaCl and stored, if necessary, at −10 C.

Preparation of cell walls. The method used for the preparation of cell walls was essentially that described previously (38). Bacterial contamination during preparation was avoided by use of chloroform-saturated liquids throughout, except in the first step. Four stainless-steel capsules, each containing 30 ml of cell suspension [15 to 20 mg (dry weight)/ml], two drops of octanol, and 30 g of Ballotini beads, were shaken in the shaker head of a PR1 International Centrifuge (32) at 2 C. Shaking was continued until most of the cells were broken (30 to 50 min), and then the capsules were heated for 10 min at 60 C to inactivate lytic enzymes. The beads were removed by filtration, and the majority of unbroken cells were separated by centrifugation at 1,000 × *g* for 7 min. The walls were sedimented at 25,000 × *g* for 15 min, suspended in 200 ml of 0.1 M sodium phosphate buffer, *p*H 7.8, containing 1% crude trypsin (British Drug House, Ltd., Poole, Dorset, England) and a few milligrams of deoxyribonuclease, and incubated at 37 C until the optical density (OD) ceased to fall (6 to 18 hr). Any material subsequently sedimenting at 1,000 × *g* was discarded, and the walls in the supernatant fraction were sedimented at 20,000 to 25,000 × *g* for 15 to 20 min. These walls were then washed successively with three washes each of 0.9% NaCl, 0.1 M phosphate buffer, *p*H 7.0, and water. The preparations were lyophilized.

Density gradient centrifugation of walls. The technique (39) used for density gradient centrifugation of walls was described by Allsop and Work (2). This separation was usually applied to walls immediately after trypsin treatment. The sedimented walls were washed once with water and suspended in 1 M NaCl; 12 ml of this suspension was then layered onto a gradient of sucrose (0 to 40%, w/v, in 1 M NaCl) contained in a 250-ml glass centrifuge bottle. The gradients were made by the method of Ribi and Hoyer (28). After layering the walls on top of the gradients, the bottles were centrifuged for 30 to 60 min in a swing-out head at 1,400 × *g*. The individual bands were collected by pipette and were pooled and centrifuged. The pads were suspended in 1 M NaCl, centrifuged on fresh gradients until free from other fractions, dialyzed free from sucrose (negative Seliwanoff test for ketoses on 1 ml of washings), and lyophilized.

Lysozyme treatment of walls. Walls were suspended in 0.1 M sodium phosphate buffer, *p*H 6.8. Lysozyme, dissolved in the same buffer, was added to give a final concentration of 100 μg/ml, and the mixture was incubated at 37 C. At various times, the opacity of the suspension was measured, and samples were removed, cooled, and centrifuged at 2 C, washed once with water, treated with phosphotungstic acid, and examined in the electron microscope. Larger scale digestions were carried out as previously described (38).

Phenol treatment of walls. Walls (50 mg) were shaken for 4 hr at 18 C with water-saturated phenol (5 ml). Solids were separated by centrifugation, and the supernatant solution was freed from phenol by dialysis.

Ultrasonic treatment of walls. Walls suspended in 1 M NaCl in a test tube were subjected to mild ultrasonic vibrations by use of an ultrasonic generator, model T-40C1 (Ultrasonic Industries Inc., Albertson, L.I., N.Y.), for periods of up to 20 min.

Optical measurements. The opacity of suspensions was measured in the EEL portable colorimeter, model A (Evans Electroselenium Ltd., Halstead, Essex, England), using filter no. 623. Other measurements were made in Unicam Spectrophotometers SP500 and SP600.

Analyses. Extractable lipids were estimated by measuring the difference between the weights of dried material before and after two 0.5-hr extractions at room temperature with a mixture of equal parts (v/v) of chloroform and methanol. Fatty acid analyses were made by gas-liquid chromatography of fatty acid esters (19); phosphorus was determined by methods described elsewhere (1, 9). Amino acids and hexosamines were examined after hydrolysis in 4 N HCl at 100 C for 18 hr. These substances were then subjected to paper chromatography or electrophoresis and were developed with ninhydrin. Methanol-water-pyridine-12 N HCl (32:7:4:1, v/v) was used as a solvent to identify lysine, ornithine, and the isomers of diaminopimelic acid by their mobilities and by the characteristic colors produced by this solvent when the spots were developed with 0.1% ninhydrin in acetone (27, 29). Hexosamines were identified on paper previously washed with 0.1 M $BaCl_2$ irrigated for 48 hr with butanol-pyridine-water (6:4:3), or they were identified by paper electrophoresis (300 v) in 1 M acetic acid. Two-dimensional chromatograms in aqueous phenol (ammonia atmosphere) and butyl alcohol-acetic acid-water (63:10:27, v/v) separated other amino acids. Quantitative amino acid and hexosamine estimations were made automatically (34) by use of a Technicon analyzer (Technicon Co., Inc., Chauncey, N.Y.).

Total carbohydrate was estimated by the phenol-sulfuric acid method (11), with glucose as a standard, as described by Allsop and Work (2). Sugars were identified by paper chromatography after hydrolysis with 1 N H_2SO_4 for 4 hr at 105 C and subsequent neutralization with $Ba(OH)_2$. The solvents were butyl alcohol-acetic acid-water, as above; butyl alcohol-pyridine-water (6:4:3, v/v); and ethyl acetate-pyridine-water (5:2:7, v/v, top layer). Sugar spots were revealed by silver nitrate reagent (36). Glucose and galactose were estimated enzymatically by specific oxidases (16, 30) in the eluted neutral sugar fraction remaining after removal of hexosamines by paper electrophoresis. The delayed reaction with L-cysteine and sulfuric acid (10) was used as a test for heptose sugars in whole walls.

Electron microscopy. Specimens were examined in a Philips 100 microscope (M.E.L. Equipment Co.,

Ltd., Crawley, Sussex, England). Photographs were taken at instrumental magnification of 7,000 to 14,000 on Ilford Fine Grain Safety Positive 35-mm film, with an accelerating voltage of 60 kv.

Negative staining. One drop of bacterial wall suspension was mixed on a carbon-coated microscope grid with one drop of 2% (w/v) potassium phosphotungstate, adjusted to *p*H 7 with 1% potassium hydroxide. After approximately 1 min, most of the solution was drawn from the grid by a Pasteur pipette so that a thin film remained on the grid. This was left to dry before examination.

Thin sectioning. Whole cells and wall fractions were fixed in Veronal-buffered osmium tetroxide (18) and then mounted in agar and washed in 2% (w/v) buffered uranyl acetate. After dehydration in ethyl alcohol, the specimens were embedded in methacrylate or Epon 812 and sectioned with glass knives mounted in a Huxley microtome.

RESULTS

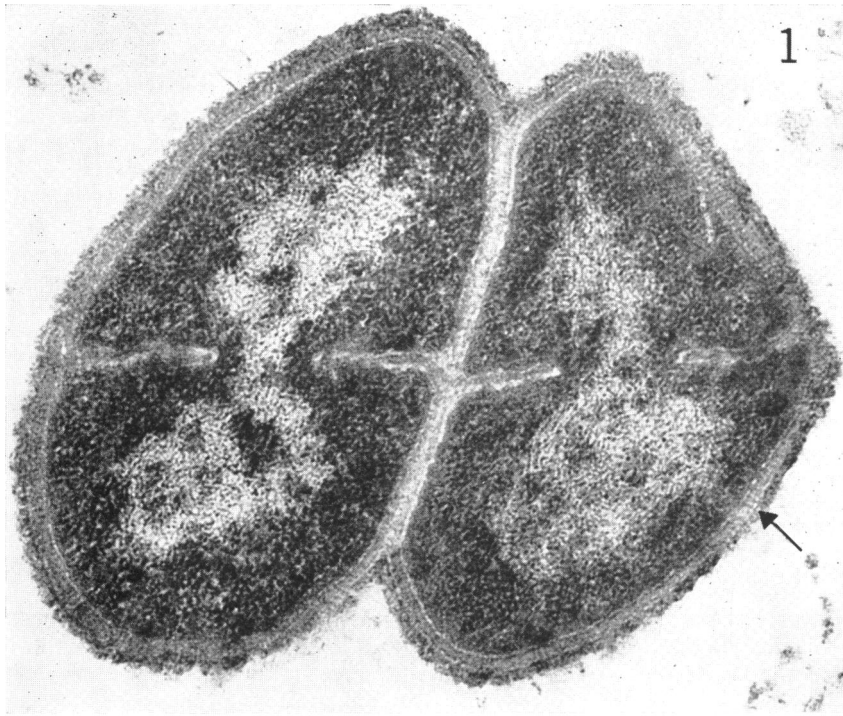
Thin sections of whole cells of M. radiodurans R_1 and W_1. Thin sections prepared from cells of *M. radiodurans* R_1 resembled those shown by Thornley et al. (35) with the exception that the cells were usually in groups of only 2 or 4. Figure 1 shows a stage in the development of a tetrad, with newly forming septa invaginating into two contiguous cells and with nuclear material in the process of separating into the daughter cells. An outer structureless layer, termed the sheath (35), did not form part of the internal septa but was continuous over the outer surface; the inner "wall" layer, together with cytoplasmic membrane, can be seen in the septa. Where the inner wall layer was cut radially, regular structures comprising alternating light and dark radiating bands are visible (arrow, Fig. 1). The thickness of this wall layer is about 160 A. The sheath varies in thickness (from 160 to 300 A), giving the bacterium an irregular convoluted outline.

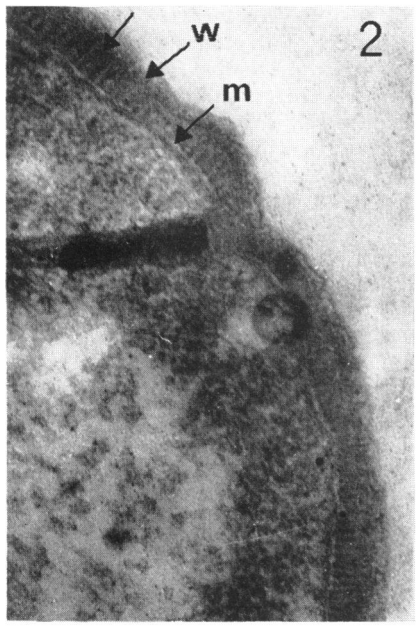
The cells of *M. radiodurans* W_1 grew in large flat groups, and individual cells were larger than *M. radiodurans* R_1 (1.9 μ diameter, as compared with 1.2 μ for R_1). The wall layer was thicker, up to 700 A (Fig. 2), and the light and dark radiating bands were again clearly visible in radial sections (arrow). The sheath was bounded by a 75 to 100 A convoluted membrane, and it had the same range of thickness as that of *M. radiodurans* R_1.

Morphology of walls of M. radiodurans. No significant morphological differences were observed between the walls of pink R_1 and Sark strains. The two strains will therefore be described indiscriminately. Walls prepared by the standard method resembled coccal cell walls in their shape but were larger (up to 2 μ diameter). They sometimes occurred as single cell envelopes with a visible equatorial plane of fracture; more frequently, pairs of envelopes were seen. There were two characteristic patterns in negatively stained preparations of the walls (Fig. 4), the most common being a spotted pattern previously termed "holey" (35), which usually covered most of the wall surface. The other, a hexagonal pattern, was seen in fragments of various sizes either covering portions of the wall surfaces or separated from them.

During certain preparations of walls (designated preparations 11 and 12), the materials were examined in the electron microscope at each stage of the preparation. The unsedimented broken cells, examined before or after the initial heat treatment, were often characterized by a structure which appeared as a network of curved double white lines surrounding darkly stained areas (in Fig. 5, the network is seen in profile at the folded edge of the wall, indicating that it was located on the outside of the wall). Three other types of structures were observed in these broken cell preparations, either the holey pattern (shown faintly in Fig. 5), the hexagonal pattern, or a composite pattern (Fig. 6). Treatment of the broken cells with trypsin removed the network structure; with minimal subsequent handling, whole walls completely covered with the composite pattern (as in Fig. 6) were occasionally obtained. Other such preparations showed various mixtures of holey and hexagonal patterns (Fig. 7a, 7b). Some preparations, examined immediately after trypsin treatment (i.e., before washing), showed a hexagonally packed layer as a hemisphere, which appeared to be either unfolding from around the spherical holey layer (Fig. 7a) or emerging from inside it (Fig. 7b). Subsequent washing processes usually resulted in the progressive fragmentation and gradual loss of hexagonal patterns.

Physical separation of two layers from walls. After trypsin treatment of one preparation (12) of walls of strain R_1, the pad sedimenting at 20,000 $\times$ *g* had an unusual appearance in the centrifuge tube. Instead of containing the usual homogeneous pink material, the pad consisted of two distinct regions, an opaque whitish lower region and a translucent pink upper band, which had the physical properties of a soft jelly. The components of the two regions were easily separated by density-gradient centrifugation. A narrow bright pink transparent band remained on top of the gradient, and a wide opaque creamy-white band was located almost immediately below this. In addition, a mixed pink and white deposit was separated from the bottom of the white band by a completely clear region occupying the bottom third of the gradient.





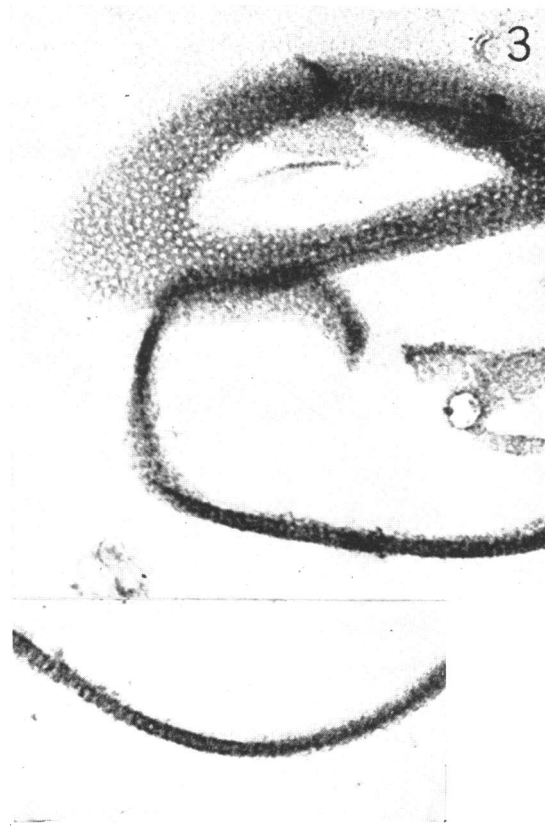
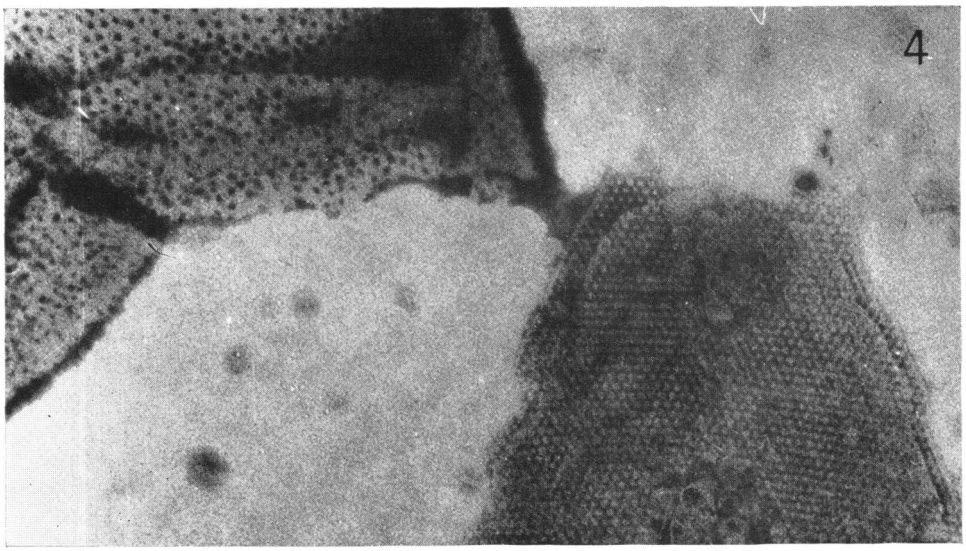
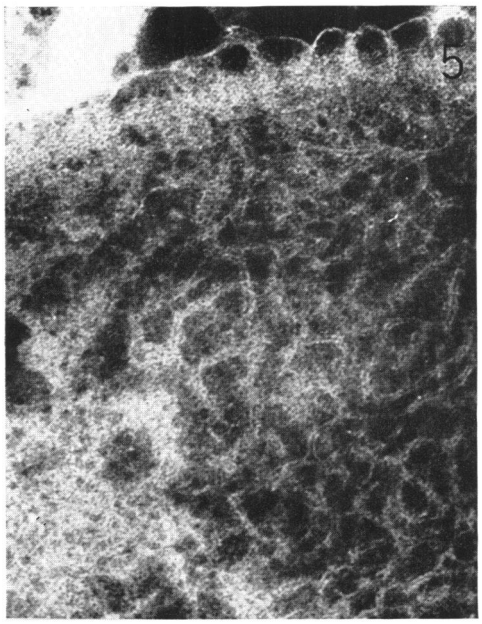


FIG. 1. *Thin section of Micrococcus radiodurans* R_1. *Stained with osmium tetroxide.* × *61,400.*

FIG. 2. *Portion of thin section of cell of Micrococcus radiodurans* W_1, *showing thick inner wall* (*w*) *with cross striations, under which the cytoplasmic membrane* (*m*) *is located. Stained with osmium tetroxide.* × *228,000.*

FIG. 3. *Sections* (*transverse and tangential*) *from purified white band of preparation 12 from walls of Micrococcus radiodurans* R_1. *Stained with osmium tetroxide.* × *83,200.*





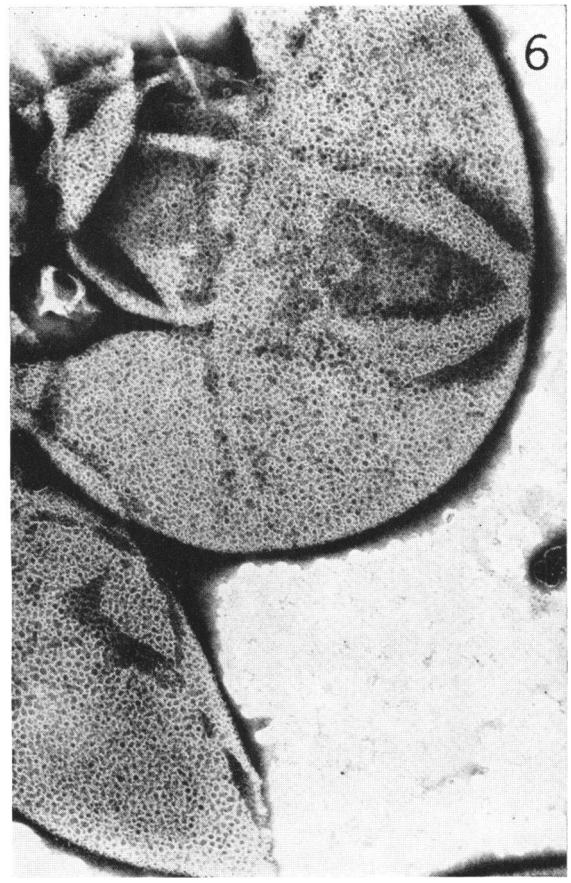


FIG. 4. *Part of preparation of wall from Micrococcus radiodurans (Sark). Two types of patterns are visible, holey and hexagonally packed. Negatively stained.* × *103,000.*

FIG. 5. *Surface of wall of Micrococcus radiodurans* R_1*, preparation 11. Cells were broken and heated as in Materials and Methods, then washed twice with saline and three times with water; no treatment with trypsin. "Network structure" and a region of holes can be seen. Negatively stained.* × *79,000.*

FIG. 6. *Walls of Micrococcus radiodurans* R_1*, preparation 11. Broken cells treated with trypsin as usual and examined without further processing. Surface is covered with "composite" pattern. Negatively stained.* × *36,000.*

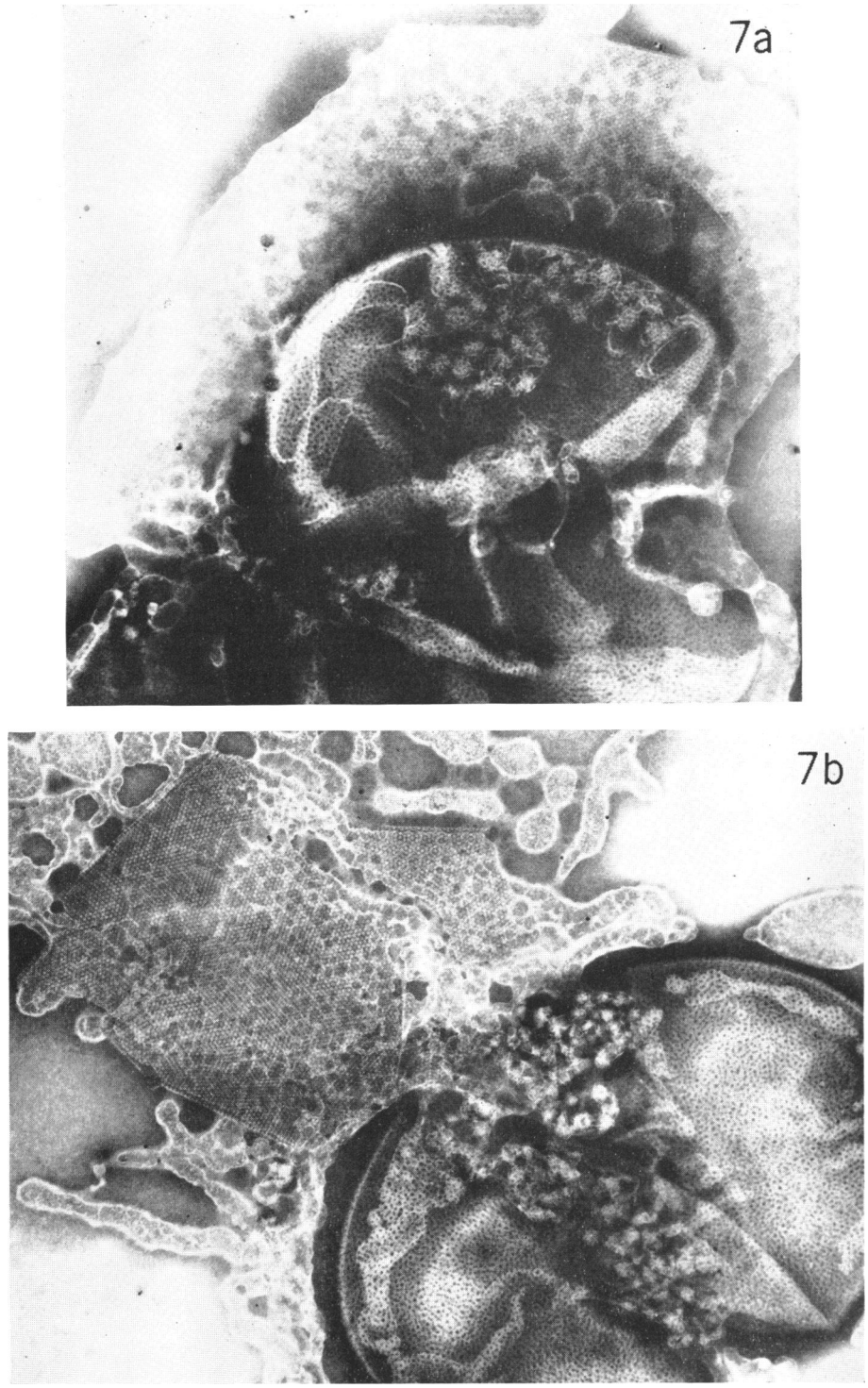


FIG. 7. *Walls of Micrococcus radiodurans* R_1, *preparation 12; examined immediately after trypsin treatment, negatively stained. (a) Walls have not been sedimented or washed and still contain some intracellular contents; the rigid holey layer is apparently surrounded by a sheet of hexagonally packed material which perhaps has just unfolded.* × *36,000. (b) The walls have been sedimented and the soft pink top region of the pad, containing a mixture of structures, was examined. Here the hexagonally packed material may be emerging from the spherical holey structure.* × *36,000.*

The top pink band consisted mainly of irregularly shaped masses of hexagonally packed material with some fragments of holey material, whereas the white band and deposit contained mostly holey material having the shape of whole cells, with a minor proportion of hexagonal structures. Reasonably homogeneous preparations of each of these bands were achieved after numerous gradient centrifugations and a further treatment of the white band with trypsin. The final preparation of the white band contained mainly unfragmented walls showing the holey structures (Fig. 8). The pink band consisted entirely of masses of irregularly shaped plates of hexagonally packed material interspersed with amorphous material (Fig. 9).

The experiments just described resulted in a physical separation of each of the two types of structures usually found together in the normal cell wall preparation of strain R_1 after trypsin treatment. The separation had not occurred before. Usually, after trypsin digestion, the pad was homogeneous in appearance, and density-gradient centrifugation did not produce the two discrete pink and white bands. Instead, there was a gradual merging from a dark pink opaque material at the top of the gradient to a wide orange-shaded opaque region which occupied most of the bottle. A dark pink deposit was always obtained. In attempts to repeat the separations, the walls were subjected, before heating or trypsin treatment, to ultrasonic disintegration for up to 20 min, a technique used for detaching a surface patterned layer from walls of *Lampropedia hyalina* (8). The ultrasonic-treated preparations were subsequently treated with trypsin and gradient centrifugation but did not separate into bands. Preparations digested with Pronase (0.25 mg per ml of 0.05 M phosphate buffer, *p*H 6.8) at 45 C for 4.5 hr did not separate into bands either.

Some success in separating hexagonal and holey structures was obtained if, immediately after breaking, the cells were held for some time at a temperature below 60 C. In preparation 14, the broken cells, still in the steel capsules in which they were broken, were incubated at 37 C for 4 hr prior to the usual heat treatment at 60 C. Subsequent separation of two layers occurred after trypsin treatment, and, after numerous density-gradient centrifugations and further trypsin treatment of the white bands, homogeneous pink and white bands were obtained.

A preparation (15) of walls of strain W_1, made in the usual way, separated on a density gradient into a deposit and two sharp bands, a very scanty colorless top band having the transparent appearance and jellylike consistency of the pink top band obtained from strain R_1, and a middle white cloudy band well separated from the top. Since there was very little in the top band (5 mg), the materials were not subjected to further gradient centrifugations but were washed and examined in the electron microscope. The top transparent band was morphologically identical to the crude pink band obtained from strain R_1, and it contained hexagonally packed pegs with occasional fragments of holey structures (Fig. 10). The lower cloudy band consisted mainly of complete walls covered with spotty patterns and a few hexagonal sheets. Other samples of walls from strain W_1 (e.g., preparation 17) did not separate into two bands on density gradients. The usual deposit was obtained, and the rest of the material formed a very wide single opaque white band each time it was subjected to a gradient.

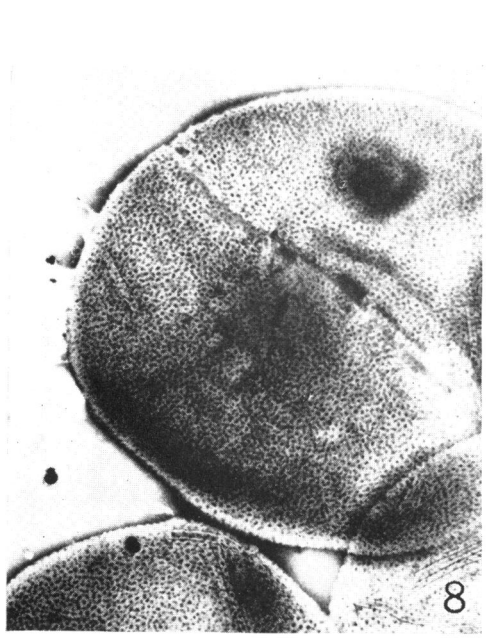
Fine structure of wall fractions. The hexagonally patterned material, which was isolated in the pink band from strain R_1 (Fig. 9), apparently fragmented easily and formed irregularly shaped masses bearing no relation to the shape of the whole bacterial wall. The pattern itself was very regular and distorted little during preparation. The margins of fragments frequently followed the line of the pattern so that fragments usually had some straight edges. The pattern in this and other negatively stained preparations was formed by lightly stained pegs arrayed in hexagonally packed rows (Fig. 15c,d); at the center of each peg there was usually a dark dot. Each peg was connected to its neighbors by lightly stained spokes. The dimensions of the pattern units are shown in Fig. 16.

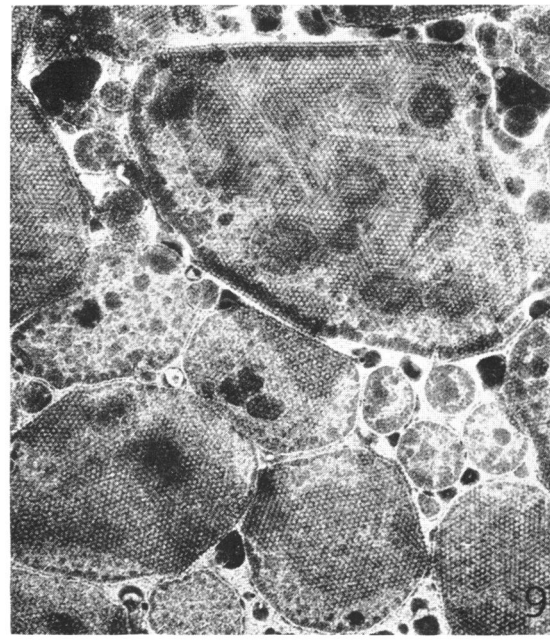
Two types of Moiré patterns were often seen superimposed on the basic pattern (Fig. 7b, 10, 13). The pattern with smaller periodicity (Fig. 14b) was observed more frequently than the larger pattern (Fig. 14c).

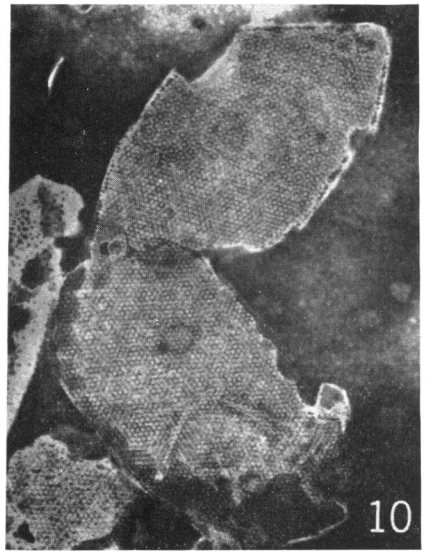
On sectioning, the material in the pink band showed no differential staining but appeared as homogeneous patches with no regular shape or thickness. The hexagonal layer could not therefore be recognized in sections.

The hexagonally packed structures seen in the scanty top transparent band from strain W_1 (Fig. 10) were similar to those from the pink band of strain R_1 walls. Similar structures were occasionally seen in routine wall preparations from strain W_1, so it must be concluded that they are normally present in this strain, although in small amounts.

The white band from strain R_1 contained patterned envelopes which retained the spherical shape of the individual pairs of bacterial cells







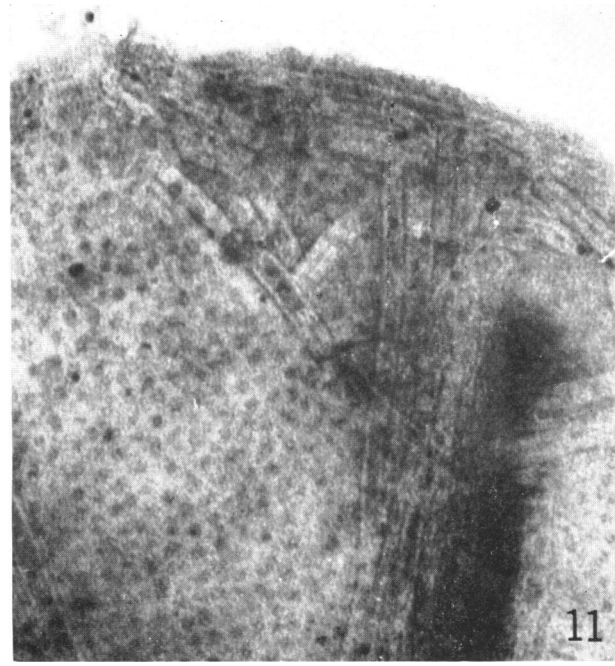
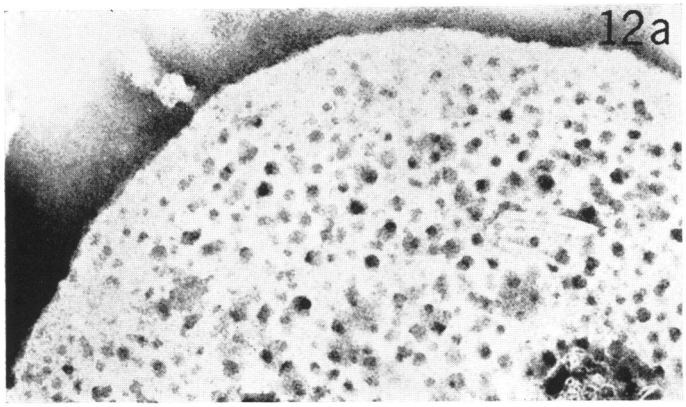


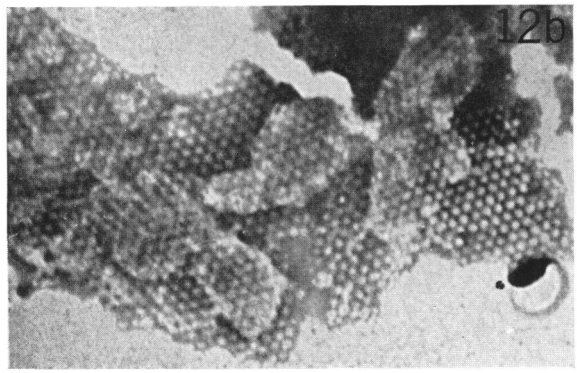
FIG. 8. *Walls from final fractionation of white band obtained by density gradient centrifugation of preparation 12 of Micrococcus radiodurans* R_1. *The spherical walls with visible planes of division are covered with holey pattern, and occasional sheaves are visible.* × *39,500.*

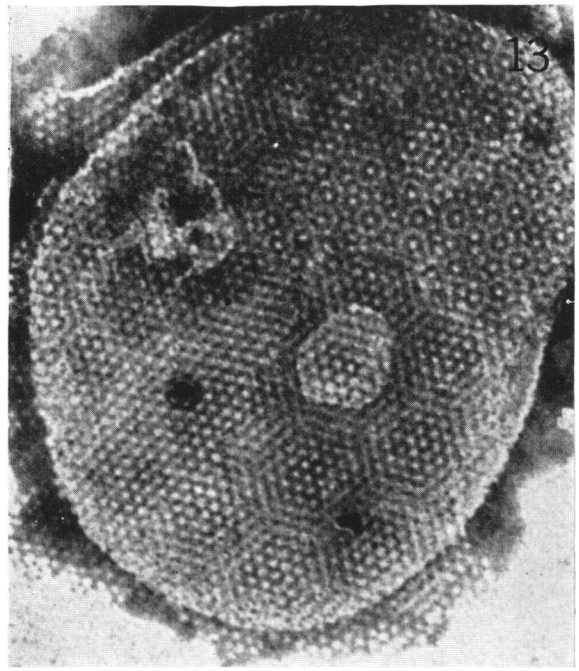
FIG. 9. *Portion of refractionated top pink band from density gradient centrifugation of Micrococcus radiodurans* R_1 *wall preparation 12. Collection of sheets or bags with hexagonal packing, some straight edges, and Moiré patterns, also spheres of unpatterned material.* × *64,000.*

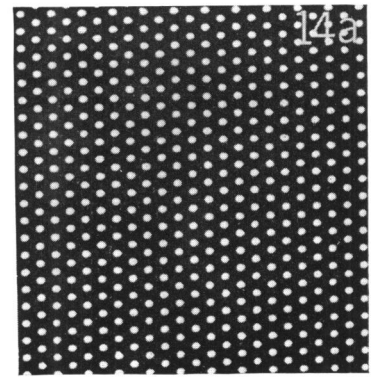
FIG. 10. *Part of unfractionated top transparent band from Micrococcus radiodurans* W_1 *preparation 15, showing typical hexagonally packed structure with fragment of holey material.* × *34,800.*

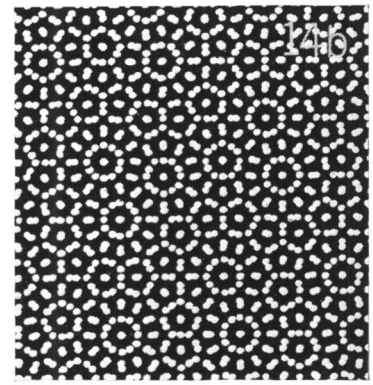
FIG. 11. *Portion of a wall of Micrococcus radiodurans* R_1 *present in white band obtained from density gradient centrifugation of preparation 14. A collection of sheaves is seen.* × *133,000.*











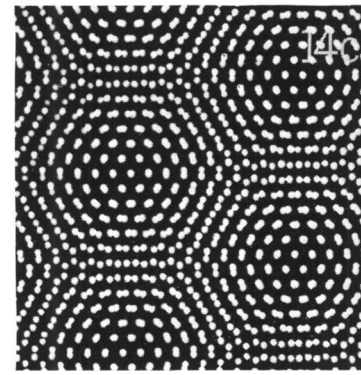


FIG. 12. *Effect of lysozyme on unfractionated wall preparation 17 of Micrococcus radiodurans* R_1. (*a*) *Holey portion, after 5 min with lysozyme, showing disappearance and deformation of holes.* × *84,240.* (*b*) *Hexagonally packed structure after 30 min with lysozyme; pegs and spokes clearly visible, but no dots remain at center of pegs.* × *80,400.*

FIG. 13. *Detail of a fragment of hexagonally packed material from walls of Micrococcus radiodurans strain Sark showing two types of Moiré patterns.* × *87,200.*

FIG. 14. *Diagrammatic illustration of origin of Moiré patterns in two hexagonally packed transparent layers.* (*a*) *Patterns perfectly aligned.* (*b*) *One pattern displaced 5°.* (*c*) *One pattern displaced 30°.*

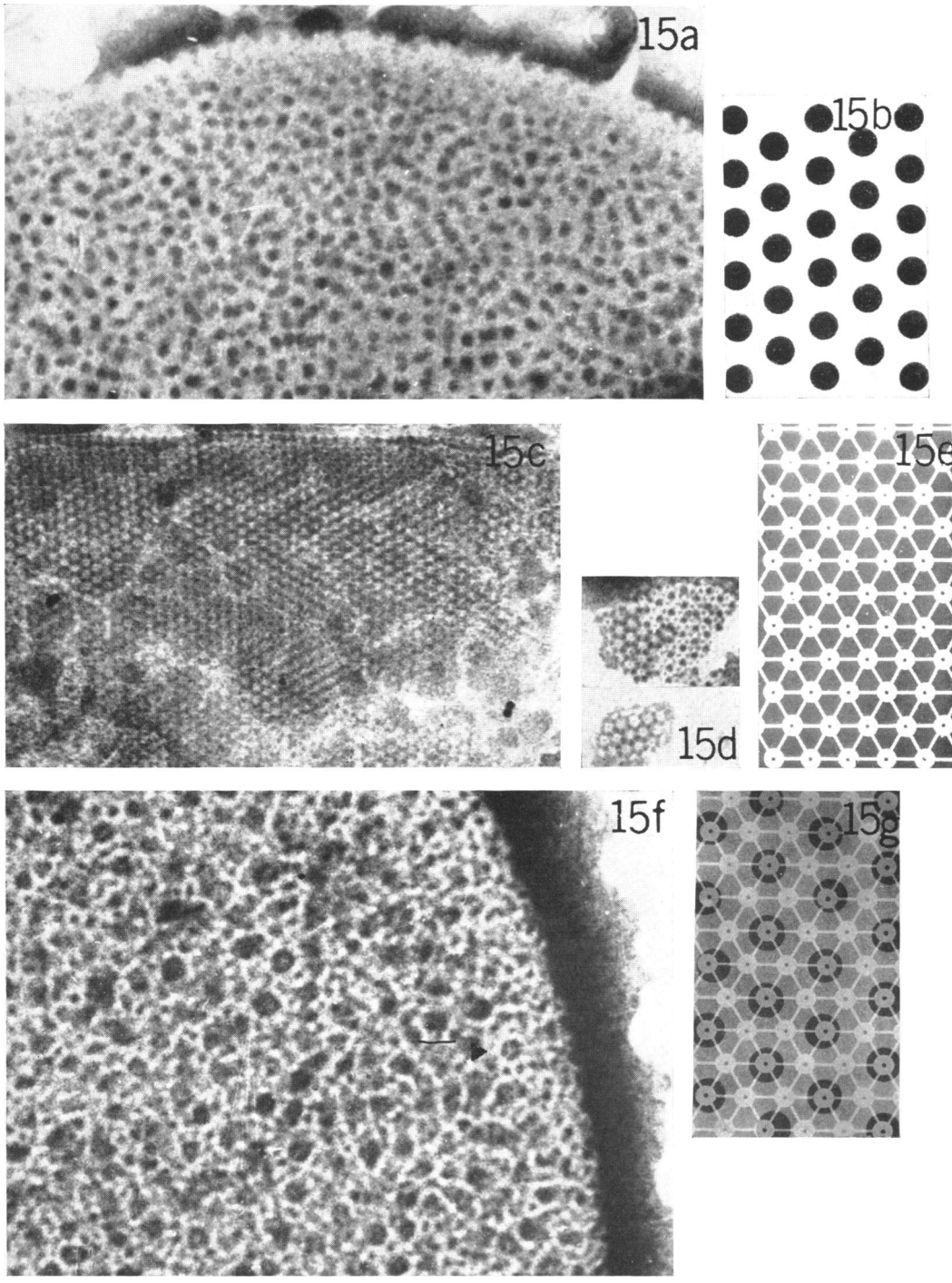


FIG. 15. *Detailed morphology and relationship of the three types of patterns seen in trypsin-treated walls of Micrococcus radiodurans. (a) Holey pattern in preparation 14 of strain* R_1. *The holes can be seen in profile at the folded edge of the wall.* × *133,000. (b) Diagram of distribution of holes. (c) Hexagonal pattern in an enlarged portion of Fig. 7b.* × *93,600. (d) Fragments from wall preparation 17 from strain* R_1 *after 5 min digestion with lysozyme. All parts of the pattern are clearly visible. Short digestion has rendered the pattern sharper but has not yet removed the central dots.* × *101,200. (e) Diagram of hexagonal pattern. (f) Composite pattern in an enlarged portion of Fig. 6. Note light pegs in dark areas with spokes radiating from them (arrow).* × *150,000. (g) Diagram of composite pattern which resulted from the superimposition of Fig. 15b and e.*

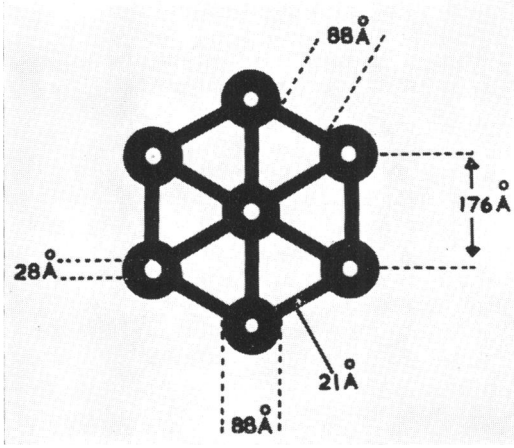


FIG. 16. *Dimensions of "hexagonal" patterns.*

(Fig. 8). In negatively stained preparations, the pattern which covered the whole surfaces of the spheres appeared as scattered, darkly stained, roughly circular spots on a light background. In profile (Fig. 15a), the spots were seen to be holes filled with phosphotungstate. Sometimes the holes occurred in rows alternating with those immediately above and below. However, it was usually difficult to detect any such regularity, which suggests (Murray, *personal communication*) that the layer became distorted during preparation. The holes varied in size and shape, having diameters of 80 to 150 A. In certain preparations from strain R_1 which had been put on several gradients, there were also short ribbonlike inclusions (sheaves) lying across the holes (Fig. 8, 11), sometimes singly but more often in bundles.

When fixed in osmium tetroxide and sectioned, the holey layer showed up well as a single morphological unit in both transverse and tangential sections (Fig. 3). The holes were similar in size and arrangement to the black spots observed in negatively stained preparations. In this case, however, the holes were white, whereas the background was stained. The holes had an average diameter of 100 A (range 80 to 120 A), and the layer was about 240 A thick. In profile, the holes were occasionally seen to extend right through the layer; more frequently, the holes were cut obliquely and appeared as depressions at one of the boundaries.

Origins of Moiré and "composite" patterns. The Moiré patterns in the hexagonal layer can be shown to originate from slight dislocations of two or more superimposed identical hexagonally packed layers, as shown diagrammatically in Fig. 14a–c. At least two layers can be seen in various fragments of the hexagonal layer. Sometimes one layer overlaps or folds over a smaller fragment of a different shape (Fig. 4, 13), or, more frequently, the two layers have almost identical shapes and are distinguishable only by the fact that a single row of pegs of one layer is visible outside the boundaries of the other layer (Fig. 7b, 9, 10, 15c). Sometimes one fragment shows two Moiré patterns of different periodicities, as well as the basal hexagonal pattern (Fig. 10, 13). At other times, a single Moiré pattern is apparent (Fig. 7b, 9), but usually the two layers are in perfect register and only the hexagonal pattern is seen (Fig. 4, 9, 15c). Slight dislocations of the two layers, resulting in a displacement of about 5°, would create the pattern with the small period (Fig. 14b). Rarer, larger displacements (up to 30°) would produce patterns with larger periods, one of which is shown in Fig. 14c.

The composite pattern is more complex and was observed infrequently. Its possible origin is illustrated in Fig. 15a–f. Scale models (Fig. 15b,e) of the holey and hexagonal patterns (Fig. 15a,c,d) were photographed. A print (Fig. 15g) was made from the two negatives superimposed so that each black spot of the holey pattern was over a peg of the hexagonal pattern. A new pattern emerged, consisting of darkly stained areas surrounded by white rings of pegs. Each dark spot contained one peg and its attendant radiating spokes. An enlargement of a portion of a wall containing the composite pattern (Fig. 15f) showed the presence of all these features. Darkly stained areas of various sizes were surrounded by white rings of unstained pegs. In some of the dark areas a lighter stained peg and its spokes were visible, and the central dark spot in the unstained pegs could sometimes be seen (the arrow points to both structures). The pattern was much less regular than that of the model, owing no doubt to the distortion of the holey pattern.

Chemistry of strair R_1 crude walls. Different preparations of crude unfractionated walls of strain R_1 gave variable analytical results, probably because variable proportions of the fragile pink layer were retained during preparation. Figures are therefore given for one preparation of strain R_1 only (Table 1). This preparation contained 4.3% extractable lipid, 1% phosphorus, 3.3% carbohydrate consisting mainly of D-galactose (2.6%) and D-glucose (0.5%), and smaller amounts of rhamnose and mannose. A minor amount of an unidentified reducing substance was found. This unidentified substance was found in the neutral fraction after separation of glucosamine by electrophoresis and had the

following chromatographic mobilities relative to glucose: butyl alcohol-acetic acid-water, 0.66; butyl alcohol-pyridine-water, 0.78; and ethyl acetate-pyridine-water, 0.65. It was not further investigated. There was no reaction for heptose (a constituent of lipopolysaccharides from gram-negative bacteria). Trichloroacetic acid (10%, w/v) extraction for 48 hr at 2 C did not remove any phosphorus-containing material, suggesting the absence of teichoic acids. The amino acids present in whole walls, consisting of all the protein amino acids and L-ornithine, were described elsewhere (38).

Chemistry of strain R_1 pink band. The materials from the top pink band from R_1 (preparations 12 and 14) contained carotenoids, lipids, carbohydrates, and proteins. Approximately 25% of the material was extractable into lipid solvents (Table 1). After extraction of lipids, the remaining white material contained 17 to 20% of carbohydrate, the main reducing sugars being glucose, galactose, rhamnose, and mannose. There was insufficient material from the lipid-free residues for quantitative phosphorus and amino acid analysis, but paper chromatography showed an amino acid pattern typical of a protein hydrolysate with little or no proline. Glucosamine was present, but no ornithine or muramic acid was detected (indicating absence of mucopeptide constituents). The fatty acid analysis of the extractable lipids of the pink layers is shown in Table 2. The main components were palmitic, hexadecenoic, and octadecenoic acids, but there were also significant quantities of odd-numbered saturated and mono-unsaturated straight-chain fatty acids, which are not common components of bacteria. Branched chain and cyclopropane fatty acids were not observed. These results are similar to those reported for whole cells of *M. radiodurans* R_1 (19).

Chemistry of strain R_1 white fractions. The materials from the white band and deposit were similar in composition (Table 1) and were mainly mucopeptides with ornithine as the diamino acid. When compared with the pink layer, the white fractions contained much less lipid (1 to 2%) and carbohydrate (2%) (Table 1). In addition, no rhamnose or mannose was found, and the main reducing substances, besides the hexosamines, were glucose or galactose or both, and a small amount of the unidentified neutral reducing substance referred to earlier. The main amino acids and amino sugars were glutamic acid, glycine, alanine, ornithine, glucosamine, and muramic acid (Tables 3, 4). Material from the white band had the following molar ratios relative to glutamic acid 1: glycine, 1:8, alanine, 1:7, ornithine, 1:2, glucosamine, 0:97, and muramic acid, 1:1. All other amino acids occurred in concentrations of less than 10% of those of the main four amino acids. Traces (too small to be estimated) of diaminopimelic acid and galactosamine were also detected.

Chemistry of strain W_1. Unlike strain R_1, W_1 had no detectable protein or extractable lipid in its unfractionated walls. The small amount of hexagonally packed material in the top band (obtained by gradient centrifugation, prepara-

TABLE 1. *Composition of walls and fractions from M. radiodurans R_1 and W_1*

Component	Crude walls		Pink band	White band		White deposit	Lysozyme residue from crude walls	Phenol extract from crude walls
	R_1 prepn 11	W_1 prepn 16	R_1 prepn 12 and 14	R_1 prepn 12	W_1 prepn 15	R_1 prepn 12	R_1	R_1
Lipid (%)	4.3	—[a]	26, 24	1	—	2.0	15	12
Carbohydrate (%)	3.3	6.1	15, 13	2.1	3.5	2.2	6.2	6.1
P (%)	1.0	0.6	ND[b]	ND	ND	1.0	1.6	1.3
Protein[c]	+	—	+	—	—	—	+	+
Ornithine[c]	+	+	—	+	+	+	—	—
D-Galactose (%)	2.6	1.6	ND	ND	ND	ND	ND	ND
D-Glucose (%)	0.5	1.1	+	+	+	+	ND	ND
Mannose[c]	+	—	+	—	—	ND	ND	ND
Rhamnose[c]	tr[d]	—	+	—	—	ND	ND	ND
Unknown neutral sugar	+	+	ND	tr	ND	ND	ND	ND
Glucosamine[c]	+	+	tr	+	+	+	—	—
Muramic acid[c]	+	+	—	+	+	+	—	—

[a] Not found.
[b] Not looked for.
[c] Identified by paper chromatography or paper electrophoresis.
[d] Trace.

TABLE 2. *Fatty acids of fractions from walls of Micrococcus radiodurans* R_1 (%, *w/w, of total fatty acid methyl esters*)

Fatty acid carbon no.	Pink band		Phenol extract
	Prepn 12	Prepn 14	
12:0[a]	0.7	0.4	1.0
14:0[a]	0.8	0.9	3.2
15:0[a]	1.2	1.2	4.5
16:0[a]	28.5	34.4	46.2
17:0[a]	3.25	4.8	13.6
18:0[a]	3.0	12.2	7.5
14:1[b]	0.3	0	0
15:1[b]	1.2	1.2	0
16:1[b]	45.0	22.1	1.3
17:1[b]	7.45	10.1	5.3
18:1[b]	7.45	13.2	0
branched 15			5.5
17			5.2

[a] Saturated straight chain acids.
[b] Monoenoic acids destroyed by bromination.

tion 15) was colorless and also contained no extractable lipids. There was insufficient material for a full analysis of the top band, but it appeared to consist mainly of carbohydrates. The mucopeptide constituents of purified wall preparation 15 from strain W_1 (the deposit was removed by gradient centrifugation) showed very similar ratios (Table 4) to those found in the crude unfractionated walls (preparation 16), but the concentration of mucopeptide constituents was higher in preparation 15. Both of the preparations resembled the white band from strain R_1, and they contained no trichloroacetic acid-extractable phosphorus.

Effect of lysozyme on wall structure and chemistry. When unfractionated walls of strain R_1 were incubated with lysozyme, there was a rapid change in morphology concomitant with a fall in OD. After 5 min, when the OD of the suspension had decreased slightly, the black spots in the "holey" layer became larger, irregular, and less well defined (Fig. 12a), whereas the hexagonal patterns were more clearly delineated (Fig. 15d). With longer digestion, the black spots gradually became more diffuse and rare. After 2 hr (33% fall in OD), the whole structure, although still rigid and of the same shape as the original cell, lost its characteristic appearance and showed little ultrastructure. The dots in the middle of the pegs of the hexagonal structures disappeared after 30 min (Fig. 12b), but the connecting spokes, although difficult to detect, were always present. Even digestion for 21 hr did not alter the exact alignment of the pegs, and Moiré patterns could still be detected. However, the outlines of the structures were no longer clear-cut and a lot of structureless background was present. Some sheaves were still visible in portions of residues that retained the cell shape.

Between 80 and 90% of the wall weight was solubilized by incubation with lysozyme for 21 hr, but no color went into solution. The residue was pink and was apparently composed mainly of lipoprotein-polysaccharide (Table 1); it con-

TABLE 3. *Amino acid and hexosamine composition of Micrococcus radiodurans wall fractions (moles per* 10^4 *g)*

Amino acid	R_1 white band (prepn 12)	W_1 crude walls (prepn 16)	W_1 purified walls[a] (prepn 17)	Sark muco-peptide[b]
Aspartic acid	0.88	0.24	0.24	0.90
Threonine	0.48	0.19	0.23	trace
Serine	0.43	0.61	0.22	0.75
Glutamic acid	6.98	4.56	7.62	8.06
Proline	0.24	0	trace	0
Glycine	12.65	9.83	14.80	19.85
Alanine	11.55	7.66	13.05	14.90
Half-cystine	0	0	trace	trace
Valine	0.48	trace	0.22	trace
Diaminopimelic acid	trace	trace	trace	0.42
Methionine	0.24	0.17	0.28	0.12
Isoleucine	0.24	trace	0.10	trace
Leucine	0.56	0.11	0.34	trace
Tyrosine	0.16	trace	trace	0
Phenylalanine	0	0	0	0
Lysine	0.44	0.60	0.39	0
Ornithine	8.17	5.08	9.60	11.6
Histidine	0.09	0	0	0
Arginine	0.32	trace	0	trace
Glucosamine	6.80	4.60	7.50	6.94
Galactosamine	0.32	0.09	trace	1.34
Muramic acid	7.78	5.52	8.85	8.81
Nitrogen (%)	8.8	6.0	7.3	12.8

[a] Density gradient centrifugation, deposit removed.
[b] Nonprotein, nondialyzable fraction from lysozyme digest (38).

TABLE 4. *Molar ratios of mucopeptide constituents*

Constituent	R_1 white band	Sark muco-peptide	W_1 crude walls	W_1 purified walls[a]
Glutamic acid	1.0	1.0	1.0	1.0
Glycine	1.8	2.5	2.1	1.9
Alanine	1.7	1.9	1.7	1.7
Ornithine	1.2	1.4	1.1	1.2
Glucosamine	0.97	0.93	0.96	0.98
Muramic acid	1.1	1.1	1.2	1.2

[a] See Table 3.

tained 15% extractable lipids, 6.3% carbohydrate, and only the amino acids of proteins. No muramic acid or ornithine was present, indicating that the mucopeptide component had been removed.

The white holey layers from fractionated walls of strains R_1 and W_1 (preparations 12 and 15) were not completely solubilized by lysozyme. Very small resistant residues (less than 5% of starting weight) remained. These residues contained no muramic acid and only traces of glucosamine, but their other sugar components were the same as those of the original wall fraction. A change in amino acid composition from the typical mucopeptide pattern of untreated material was, however, noted by paper chromatography. Lysozyme-resistant residues not only had lost all their ornithine, but also contained considerably more aspartic acid and threonine relative to glutamic acid, glycine, and alanine (still the dominant amino acids); valine and leucine became slightly more prominent, and cystine and arginine appeared.

Detailed analysis was carried out on the soluble lysozyme digest of crude walls of strain Sark, after removal of soluble protein and dialyzable substances (38). This fraction is designated as "soluble mucopeptide." The proportions of amino acids and amino sugars (Tables 3, 4) were remarkably similar to those found in the white band from strain R_1, except for smaller proportions of nonmucopeptide constituents in the soluble mucopeptide.

Effect of aqueous phenol on cell walls. Treatment of whole walls of *M. radiodurans* R_1 with aqueous phenol for 2 hr removed all the hexagonally packed structures. Structures resembling myelin figures were sometimes observed microscopically, lying beside the walls. These structures probably represent the dissolved lipoprotein which had not been washed away (5). Short treatments of only 2 min did not remove the hexagonal structures. When the material was shaken for 4 hr with phenol, all the diaminopimelic acid and most of the protein were removed. Extractable material was apparently partly lipoprotein in nature (Table 1); lipid, protein amino acids, and *meso-* and LL-diaminopimelic acid were major constituents, but there was no ornithine or muramic acid. The fatty acids were fairly similar to those of the lipoprotein pink layer (Table 2), except that palmitoleic acid was greatly decreased, C17:0 was increased, and some branched-chain acids were observed.

DISCUSSION

This investigation shows that the walls prepared from pink strains (R_1 and Sark) of *M. radiodurans* consist of at least three layers which differ morphologically and chemically. The morphological results agree with those of Murray (*personal communication*) and Thornley et al. (35), except for the absence in our wall preparations of a smooth membranous outer layer observed by Thornley in cell preparations disintegrated for a very short time. The outer structure in the walls we observed was the "network," and this structure was removed by trypsin. Therefore, the "network" may either contain protein or have peptide bonds binding it to the rest of the wall.

The Moiré patterns observed in the hexagonally packed regions are artifacts, shown here and elsewhere (15) to originate from displacement of two identical superimposed hexagonal patterns. These regions, containing peglike structures packed in hexagonal arrays, were soft and fragile and were partly detached during handling. They may be located in the wall either as two layers exterior to the holey layer, or, as suggested by Glauert (15), as a single exterior layer which is folded in places, or as one innermost layer, visible as a collapsed bag with its opposite sides pressed together (a situation analogous to that observed in collapsed phage polyhead tubes; 17). It is impossible to decide on any one of these alternatives. Figure 7a suggests that there is a double layer outside the "holey" layer; Fig. 7b could be interpreted as showing fragments emerging from within the wall, whereas Fig. 9 suggests that separated fragments may themselves form bags since single rows of pegs are visible all around the boundaries of many fragments.

The infrequency with which the hexagonal and holey layers were completely separated suggests that they are closely associated in normal preparations and are probably only visible after tearing of the fragile hexagonal layer. The holey layer, which contains the mucopeptide component, retains the shape of the cell, although its pattern appears to be deformed during preparation. When the original alignment of the undeformed holey and hexagonal patterns is maintained, the rarely observed composite pattern probably results. The axial position of these two layers in the wall cannot be revealed by thin sectioning, owing to the fact that the hexagonal layer cannot be identified in section, as it shows no differential staining.

Components characteristic of lipoproteins and polysaccharides were present in the soft hexagonal layer, and mucopeptide components were present in the more rigid holey layer. The presence of lipid, protein, and polysaccharide, in addition to mucopeptide, although exceptional in walls of gram-positive bacteria, is common-

place among gram-negative bacteria. Several such layers were described in *Escherichia coli* (20); they were not separated intact, but their structure and chemistry were deduced after stepwise removal of each component (20). Regular patterned layers in the outer structures of *Lampropedia hyalina* and *Spirillium serpens* showed hexagonal packing (8, 26) but the fine structures of these organisms differed from those of *M. radiodurans*. A second layer exhibiting a very regular distribution of holes was also found in *L. hyalina*, but it bore no relation to the holey layer of *M. radiodurans*, either in appearance or function, since it occurred in a delicate envelope surrounding groups of cells. It is probable that the lipid-containing layer of *M. radiodurans* walls does not contain the lipopolysaccharides characteristic of walls of gram-negative bacteria, as no heptoses could be found. Carotenoids, responsible for the pink color, are known to be present both in the cells (22) and in the walls of *M. radiodurans* R_1 (Work, *unpublished data*). Other pigmented gram-positive cocci possess carotenoids localized in the cytoplasmic membranes (31), the site of almost all the lipids of gram-positive bacteria.

The physical separation of the pink and white components of the walls of *M. radiodurans* R_1 apparently required two enzymatic steps, autolysis and digestion with trypsin. In the normal preparation of walls, the suspension was heated immediately after cell breakage to destroy autolytic enzymes. In a preparation (12) in which separation occurred, morphological investigations resulted in the broken cells being left at room temperature for an unrecorded period of time (not more than 2 hr). To repeat the separations, it was necessary to incubate the broken cells for as long as 5 hr at 37 C. The results of preparation 12 cannot be explained.

Autolysis was also observed in whole cells stored as pads at 2 C. In one preparation only, where whole cells were stored as pads for 5 days, a subsequent separation of wall components occurred after trypsin treatment. Glauert (15) reported that an unheated fragmented wall preparation of *M. radiodurans*, stored for 2.5 years at 5 C, lost its entire "holey" layer. Unexplained potentiation of autolysis by trypsin was observed in *Streptococcus* (6, 33). The site of attack on *M. radiodurans* by its autolytic system was different from the site of attack of lysozyme, since the dark dots in the middle of the pegs in the "hexagonal" layer were not affected by autolysis, whereas these dark dots disappeared during lysozyme treatment.

Lysozyme did not solubilize the entire holey mucopeptide layer; there remained a bag-shaped residue which had lost all its muramic acid and glucosamine and had a different amino acid pattern from the original mucopeptide. It is not possible to say whether this lysozyme-resistant component is another layer on which the holey structure is superimposed, or whether the lysozyme-resistant component is evenly dispersed in the holey fraction. It is noteworthy that the sheaves or ribbonlike inclusions were also unaffected by lysozyme (Fig. 11, 12a). No explanation of these inclusions can be made.

The chemical composition of the walls confirms that *M. radiodurans* is an atypical gram-positive bacterium, as suggested by Work (38). Separation of the lipoprotein-polysaccharide fraction resulted in confirmation of the mucopeptide composition previously deduced from examination of the soluble nondialyzable lysozyme digest (38). The diamino acid of the mucopeptide, L-ornithine, was identified in one other species (21); D-ornithine was present in the walls of several species of bacteria (12, 27, 37). Glycine was found in some staphylococcal and micrococcal mucopeptides, but most species contained aspartic acid and serine instead (4). Ribose, a common sugar in coccal walls, was not found in *M. radiodurans* walls. The lipoprotein-polysaccharide was unusual in that it contained significant amounts of odd-numbered fatty acids, which are not frequently found in bacteria. Rhamnose is known to be associated with bacterial lipid and polysaccharide fractions.

The constant occurrence of small amounts of *meso*- and LL-diaminopimelic acid in the mucopeptide fractions of walls of *M. radiodurans* is puzzling, since this amino acid is seldom found in conjunction with other diamino acids. A lipoprotein fraction extracted by aqueous phenol from walls of strain R_1 (but not from W_1), was relatively rich in this amino acid but contained no detectable ornithine. This suggests that diaminopimelate is not associated with mucopeptide in *M. radiodurans*.

Comparison of pink parent R_1 strain and the white W_1 mutant suggests that their mucopeptides are similar but that the lipoprotein component is largely absent from strain W_1. However, a small amount of a component exhibiting typical hexagonal packing was observed in walls from strain W_1 (Fig. 9). This component was once separated as a colorless lipid-free preparation, suggesting that the lipid component is not responsible for the hexagonally packed pegs and spokes of the wall of strain R_1. Strain W_1, although highly resistant to radiation, had an exponential survival curve to X-rays, instead of the sigmoidal curves shown by pink strain R_1 (22, 23). It is not known if the

loss of the carotenoid and lipid components are responsible for this difference.

This investigation of wall morphology and chemistry did not provide any direct clue to explain the high radiation resistance of *M. radiodurans*. The only features entirely specific to the walls of this organism are the holes, which completely traverse the mucopeptide layer and possibly align with other holes in the center of the pegs in the hexagonal layer.

ACKNOWLEDGMENTS

We would like to express our gratitude to B. Cole, who contributed much valuable work to this project. We also thank V. Okoro for preparing and purifying cell walls and for printing figures, V. Knivett for lipid analyses, and J. Shaw for amino acid analyses.

LITERATURE CITED

1. ALLEN, R. J. L. 1940. The estimation of phosphorus. Biochem. J. **34**:858–865.
2. ALLSOP, J., AND E. WORK. 1963. Cell walls of Propionibacterium species: fractionation and composition. Biochem. J. **87**:512–519.
3. ANDERSON, A. W., H. C. NORDAN, R. F. CAIN, G. PARRISH, AND D. E. DUGGAN. 1956. Studies on a radiation-resistant micrococcus. I. Isolation, morphology, cultural characteristics and resistance of γ-radiation. Food. Technol. **10**:575–577.
4. BAIRD-PARKER, A. C. 1965. The classification of Staphylococci and Micrococci from world-wide sources. J. Gen. Microbiol. **38**:363–387.
5. BIRDSELL, D. C., AND E. H. COTA-ROBLES. 1967. Production and ultrastructure of lysozyme and ethylenediaminetetraacetate-lysozyme spheroplasts of *Escherichia coli*. J. Bacteriol. **93**:427–437.
6. BLEIWEIS, A. S., AND R. M. KRAUSE. 1965. The cell walls of Group D streptococci. I. The immunochemistry of the type 1 carbohydrate. J. Exptl. Med. **122**:237–249.
7. BOLING, M. E., AND J. K. SETLOW. 1966. The resistance of *Micrococcus radiodurans* to ultraviolet radiation. III. A repair mechanism. Biochim. Biophys. Acta **123**:26–33.
8. CHAPMAN, J. A., R. G. E. MURRAY, AND M. R. J. SALTON. 1963. The surface anatomy of *Lampropedia hyalina*. Proc. Roy. Soc. (London) Ser. B **158**:498–513.
9. CHEN, P. S., T. Y. TORIBARA, AND H. WARNER. 1956. Microdetermination of phosphorus. Anal. Chem. **28**:1756–1758.
10. DISCHE, Z. 1962. Color reactions of heptoses and octoses. Methods in Carbohydrate Chemistry **1**:494–496.
11. DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
12. DUC-NGUYEN, H., AND L. L. WEED. 1964. D-Ornithine as a constituent of a bacterial cell wall. J. Biol. Chem. **239**:3372–3376.
13. DUGGAN, D. E., A. W. ANDERSON, P. R. ELLIKER, AND R. F. CAIN. 1959. Ultraviolet exposure studies on a gamma radiation resistant micrococcus isolated from food. Food. Res. **24**:376–382.
14. GLAUERT, A. M. 1962. The fine structure of bacteria. Brit. Med. Bull. **18**:245–250.
15. GLAUERT, A. M. 1966. Moiré patterns in electron micrographs of a bacterial membrane. J. Cell Sci. **1**:425–428.
16. HUGGET, A. ST. G., AND D. A. NIXON. 1957. The use of glucose oxidase, peroxidase and o-dianisidine in determination of blood and urine glucose. Lancet **2**:368–370.
17. KELLENBERGER, E., AND E. BOY DE LA TOUR. 1965. Studies on the morphopoiesis of the head of phage T-even. II. Observations on the fine structure of polyheads. J. Ultrastruct. Res. **13**:343–358.
18. KELLENBERGER, E., A. RYTER, AND J. SÉCHAUD. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671–678.
19. KNIVETT, V. A., J. CULLEN, AND M. JACKSON. 1965. Odd-numbered fatty acids in *Micrococcus radiodurans*. Biochem. J. **96**:2–3C.
20. MARTIN, H. H., AND H. FRANK. 1962. Quantitative Bausteinanalyse der Stützmembran in der Zellwand von *Escherichia coli* B. Z. Naturforsch. **17b**:190–196.
21. MILLER, I., R. PLAPP, AND O. KANDLER. 1966. The amino acid sequence of the serine containing murein of *Butyribacterium rettgeri*. Biochem. Biophys. Res. Commun. **25**:415–420.
22. MOSELEY, B. E. B. 1963. The variation in X-ray resistance of Micrococcus radiodurans and some of its less-pigmented mutants. Intern. J. Radiation Biol. **6**:489.
23. MOSELEY, B. E. B., AND H. LASER. 1965. Repair of X-ray damage in *Micrococcus radiodurans*. Proc. Roy. Soc. (London) Ser. B **162**:210–222.
24. MOSELEY, B. E. B., AND A. H. SCHEIN. 1964. Radiation resistance and deoxyribonucleic acid base composition of *Micrococcus radiodurans*. Nature **203**:1298–1299.
25. MURRAY, R. G. E. 1962. Fine structure and taxonomy of bacteria. Symp. Soc. Gen. Microbiol. **12**:119–144.
26. MURRAY, R. G. E. 1963. On the cell wall structure of *Spirillum serpens*. Can. J. Microbiol. **9**:381–392.
27. PERKINS, H. R., AND C. S. CUMMINS. 1964. Ornithine and 2,4-diaminobutyric acid as components of the cell walls of plant pathogenic Corynebacteria. Nature **201**:1105–1107.
28. RIBI, E., AND B. H. HOYER. 1960. Purification of Q-fever rickettsiae by density-gradient sedimentation. J. Immunol. **85**:314–318.
29. RHULAND, L. E., E. WORK, R. F. DENMAN, AND

D. S. HOARE. 1955. The behaviour of isomers of α,ϵ-diaminopimelic acid on paper chromatograms. J. Am. Chem. Soc. **77**:4844–4846.

30. ROTH, H., S. SEGAL, AND D. BERTOLI. 1965. The quantitative determination of galactose—an enzymic method using galactose oxidase, with applications to blood and other biological fluids. Anal. Biochem. **10**:32–52.
31. SALTON, M. R. J., AND A. F. M. EHTISHAM-UDDIN. 1965. The localisation of cytochromes and carotenoids in isolated bacterial membranes and envelopes. Austr. J. Exptl. Biol. Med. Sci. **43**:255–264.
32. SHOCKMAN, G. D., J. J. KOLB, AND G. TOENNIES. 1957. A high speed shaker for the disruption of cells at low temperatures. Biochim. Biophys. Acta **24**:203–204.
33. SHOCKMAN, G. D., J. S. THOMPSON, AND M. J. CONOVER. 1967. The autolytic enzyme system of *Streptococcus faecalis*. II. Partial characterisation of the autolysin and its substrate. Biochemistry **6**:1054–1065.
34. SPACKMAN, D. H., W. H. STEIN, AND S. MOORE. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **7**:1190–1206.
35. THORNLEY, M. J., R. W. HORNE, AND A. M. GLAUERT. 1965. The fine structure of *Micrococcus radiodurans*. Arch. Mikrobiol. **51**:267–289.
36. TREVELYAN, W. E., D. D. PROCTER, AND J. S. HARRISON. 1950. Detection of sugars on paper chromatograms. Nature **166**:444–445.
37. VEERKAMP, J. H., R. LAMBERT, AND Y. SAITO. 1965. The composition of the cell wall of *Lactobacillus bifidus* var *pennsylvanicus*. Arch. Biochem. Biophys. **112**:120–125.
38. WORK, E. 1964. Amino acids of walls of *Micrococcus radiodurans*. Nature **201**:1107–1109.
39. YOSHIDA, A., C.-G. HEDÉN, B. CEDERGREN, AND L. EDEBO. 1961. A method for the preparation of undigested bacterial cell walls. J. Biochem. Microbiol. Technol. Eng. **3**:151–159.