

ULTRASTRUCTURE OF A PERIODIC PROTEIN LAYER IN THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

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ABSTRACT

Matrix protein (36,500 daltons), one of the major polypeptides of the *Escherichia coli* cell envelope, is arranged in a periodic monolayer which covers the outer surface of the peptidoglycan. Although its association with the peptidoglycan layer is probably tight, the periodic structure is maintained in the absence of peptidoglycan, and is therefore based on strong protein-protein interactions. A detailed analysis of the ultrastructure of the matrix protein array by electron microscopy and image processing of specimens prepared by negative staining or by freeze-drying and shadowing shows that the molecules are arranged according to three-fold symmetry on a hexagonal lattice whose repeat is 7.7 nm. The most pronounced feature of the unit cell, which probably contains three molecules of matrix protein, is a triplet of indentations, each approx. 2 nm in diameter, with a center-to-center spacing of 3 nm. They are readily penetrated by stain and may represent channels which span the protein monolayer.

The envelopes of Gram-negative bacteria have a multilaminar organization consisting of a cytoplasmic membrane, a rigid peptidoglycan layer, and an outer membrane. The latter is composed of phospholipids, lipopolysaccharides, and proteins. These include the matrix protein¹ (16) which, in *Escherichia coli*, has a mol wt of 36,500. The cellular complement of approx. 10^5 molecules forms a periodic array in close association with the underlying peptidoglycan layer. Numerically, the most abundant outer membrane protein is a lipoprotein (7,000 daltons) which has been extensively characterized (4). Each cell has approx. 6×10^5 copies, of which a third (11) are covalently linked to the peptide cross-bridges of the peptidoglycan layer (4). In addition, the outer membrane contains several other major (9) as well as a number of minor protein species, which have not yet

been so well characterized and will not be considered further here.

The structural organization of the outer membrane and the interactions between its components are not yet well understood. Likewise, the functions of its major polypeptides are still unclear. Motivated by the idea that information on the architecture of the periodic arrangement of matrix protein which effectively covers the peptidoglycan cell wall might afford insight into these questions, we have undertaken a study of it by electron microscopy combined with image processing. Our findings are reported in this communication.

MATERIALS AND METHODS

Preparation of Periodic Arrays of Matrix Protein

Two experimental approaches were followed. In the first, the matrix protein was prepared as a complex with

¹ This protein has been designated by various names (cf. reference 7).

the peptidoglycan-lipoprotein network, by a modification of the method described previously (16). Differential heat extraction in sodium dodecyl sulfate (SDS) was performed with unbroken cells, which had been washed previously with 1 mM EDTA. In the second, matrix protein was obtained free of peptidoglycan by a procedure in which the same differential heat extraction was applied to spheroplasts. *E. coli* B^P was grown as described previously (20), and spheroplasts were made according to the method of Kaback (12) or that of Osborn et al. (15). Pelleted spheroplasts were suspended in extraction buffer (10 mM Tris-HCl, pH 7.3, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol). The suspension was incubated for 30 min at 60°C, and the particulate fraction was collected by centrifugation at 40,000 g for 60 min. The pellet was washed repeatedly with water (1–4 cycles) and collected by centrifugation. The final pellet was resuspended in water and prepared for electron microscopy. Chemical analyses, polyacrylamide gel electrophoresis in dodecyl sulfate, and quantitation of peptidoglycan (using a diaminopimelic acid-requiring strain) were performed as described previously in detail (16).

Electron Microscopy

Negatively stained specimens were obtained from fresh preparations of extracted cells or of spheroplasts. They were fixed for 15 min with 1% glutaraldehyde, and adsorbed to 200-mesh copper grids covered with a thin collodion-carbon supporting film which had been glow discharged previously. The grids were floated on a drop of distilled water for 10 s and negatively stained with 2% sodium phosphotungstate at pH 7.2. Micrographs were recorded on a Philips 301 electron microscope, operating at 80 kV with a 30 μ m objective aperture. A liquid nitrogen anticontamination device was always used. Micrographs were recorded at a nominal magnification of 49,000 diam on 70-mm Kodalith LR 2672 film and developed in Kodak DK60 developer. The minimum beam exposure technique of Williams and Fisher (22) was used because we found the specimens to be highly sensitive to electron irradiation.

Specimens of freeze-dried and shadowed preparations were obtained as described elsewhere.² The shadowing material was tungsten, deposited at an elevation angle of 30°.

Image Processing

Optical diffraction and filtration were performed as described previously (1, 19). Lattice constants were measured using the meridional reflections of T4 phage tails, co-adsorbed to the grids, as internal magnification standard (2, 19). A spacing of 3.8 nm was assigned to these reflections (13). Once the lattice constant was determined (7.7 nm; cf. Results), it was used to calibrate the dimensions quoted for other ultrastructural features.

² Kistler, J. Submitted for publication.

Image processing by computer was carried out using the method for filtering hexagonal lattices, and computer programs developed by Dr. P. R. Smith. The method is described in detail elsewhere.³ The only point on which the filtrations presented here differ from that account was in the fine adjustment of orientational alignment and scaling of the digital pictures by bilinear interpolation. These operations were conducted according to two criteria which gave consistent results: (a) maximization of the total power in the Fourier orders of the indexed reciprocal lattice out to spatial frequencies with visible reflections on the corresponding optical diffractograms, and (b) maximization of power on specific outer Fourier orders. The photographic representations of the filtered digital pictures were recorded with an Optronics Photomat (Optronics International, Inc., Chelmsford, Mass.).

RESULTS

When the matrix protein was prepared in association with the lipoprotein-peptidoglycan network without cell disintegration, these complexes generally maintained their original rod shapes, as illustrated in Fig. 1. The quantitative chemical composition of these structures, which essentially consist of matrix protein, lipoprotein, and peptidoglycan, has been reported previously (16). Electron micrographs of negatively stained specimens consistently show them to be covered with a regular structure, evidently based on a hexagonal lattice, with the appearance of a honeycomb. In fact, the periodicity is not precisely maintained on these specimens, as we have found from examination of many such preparations that the regular structure is divided into subarrays by narrow cracks. On the individual subarrays, there is usually perceptible bending of lattice lines. Consequently, optical diffractograms (cf. Fig. 1) generally show only six hexagonally disposed reflections. Assuming that these reflections derive from the basic lattice repeat, and therefore represent the first order of a hexagonal reciprocal lattice, we have determined the lattice constant to be 7.7 ± 0.4 nm (12 measurements).

More regular structures were obtained in preparations from which the peptidoglycan network had been removed by lysozyme treatment before SDS extraction (Fig. 2). The mass fraction of peptidoglycan was reduced to less than 5% of its original level by this treatment (data not shown), which also removed the lipoprotein. Only trace amounts of the latter could be detected by slab gel electro-

³ Smith, P. R., and U. Aebi. The computer filtration of hexagonal lattices. Submitted for publication.

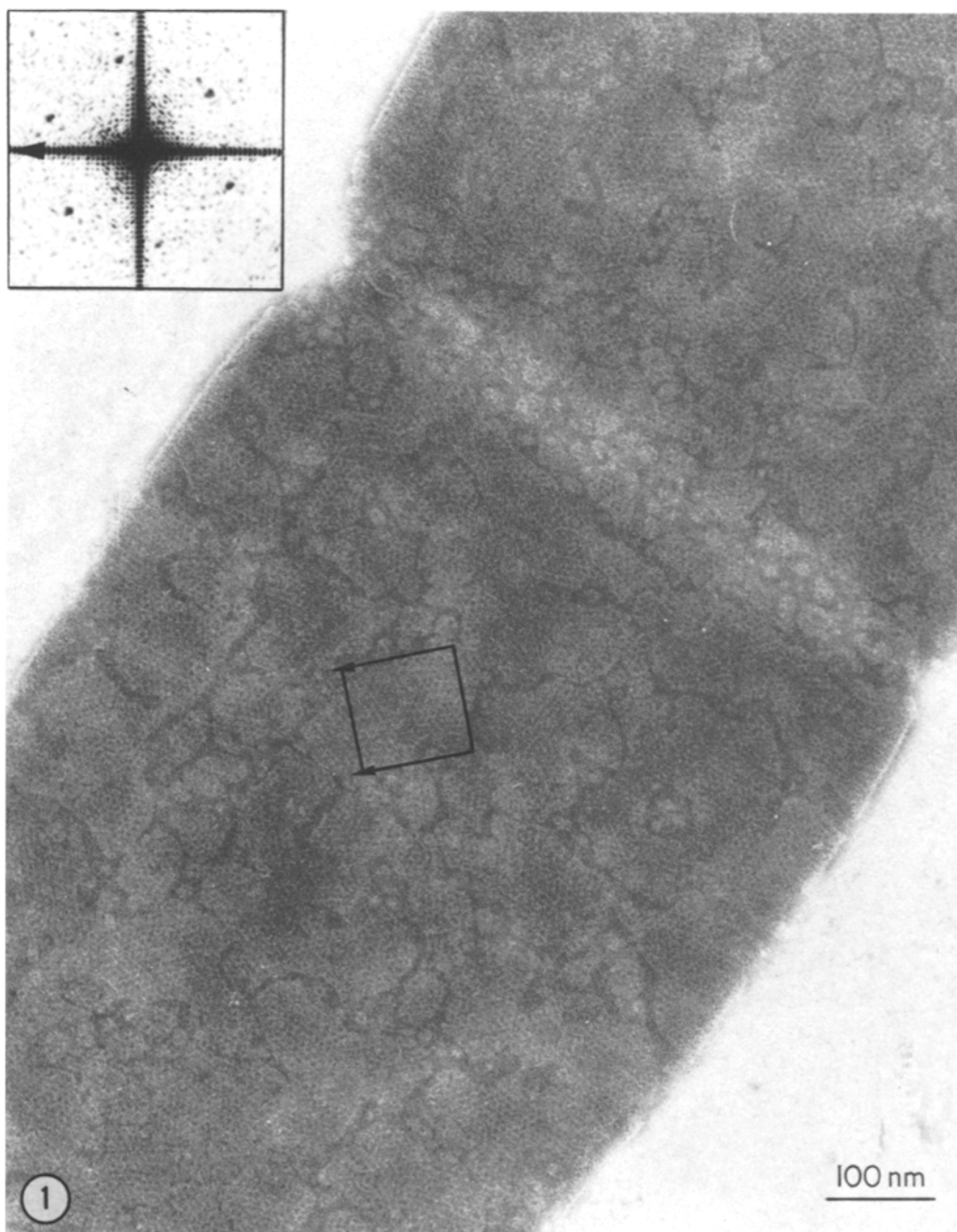


FIGURE 1 Electron micrograph of negatively stained (2% sodium phosphotungstate) peptidoglycan-matrix protein complex derived from *E. coli* by differential heat treatment in SDS (see Materials and Methods). The optical diffraction pattern of the marked area is *inset* with the arrowheads indicating the relative orientations of the specimen and the diffractogram.

phoresis in SDS with phosphate buffer, and only slight contaminations of lipopolysaccharide remained (16). Thus, the structures obtained by this

procedure are composed of essentially homogeneous matrix protein. They are predominantly in the form of what appear to be monolayer fragments

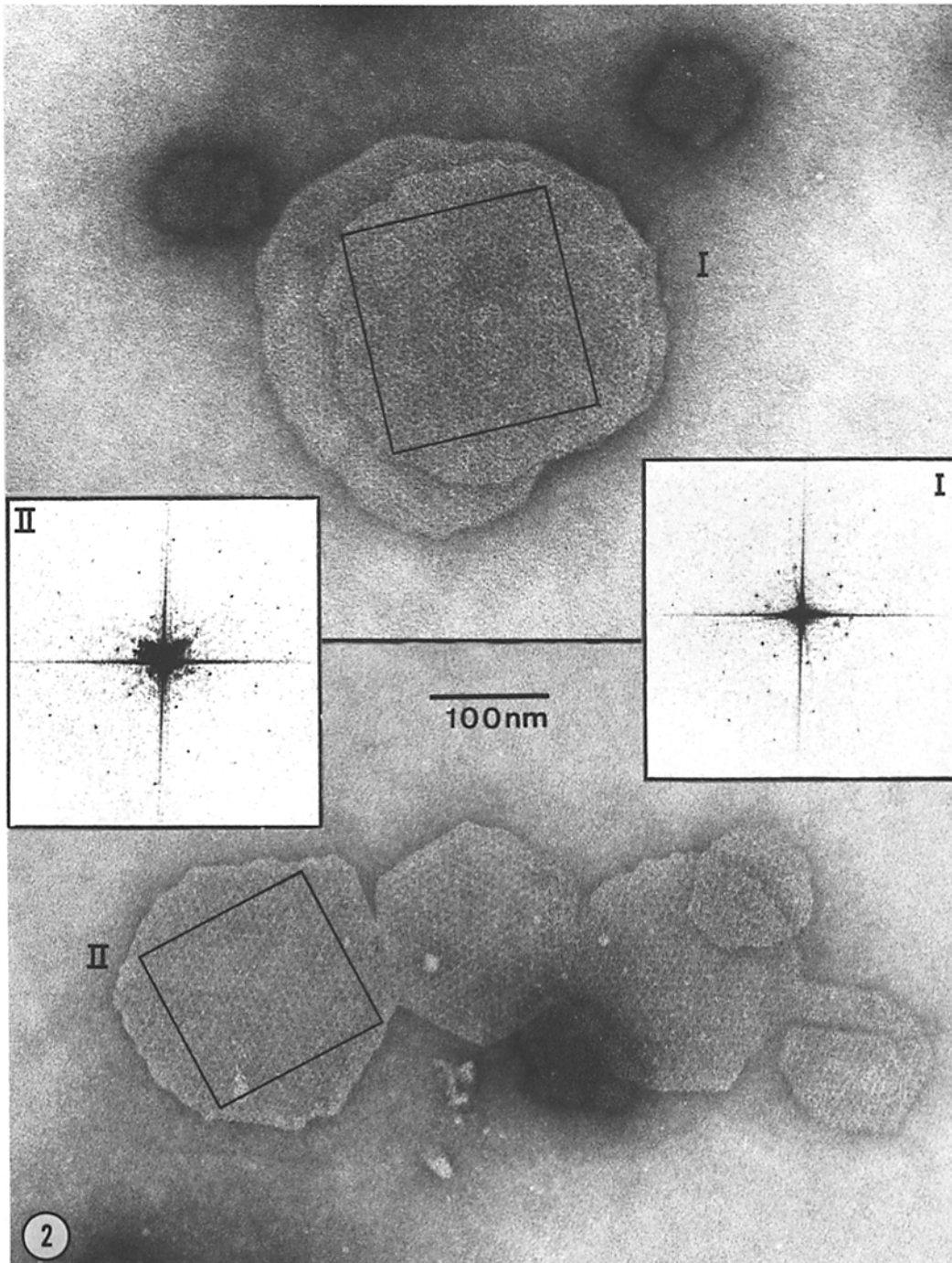


FIGURE 2 Electron micrograph of negatively stained (2% sodium phosphotungstate) periodic arrays of matrix protein derived from spheroplasted *E. coli* cells by differential heat treatment in SDS (see Materials and Methods). The optical diffractograms of the windowed areas (I and II) index on a hexagonal reciprocal lattice with visible reflections extending to the fifth radial order $(2.2 \text{ nm})^{-1}$. In area I, two such arrays are superimposed, of which one is less well preserved since it contributes only first order spots to the diffraction pattern.

exhibiting the same regular structure as seen on the intact complexes, together with relatively few closed vesicles. Therefore, the integrity of the periodic structure is based on protein-protein interactions and is not dependent on interaction of the matrix protein with the peptidoglycan layer.

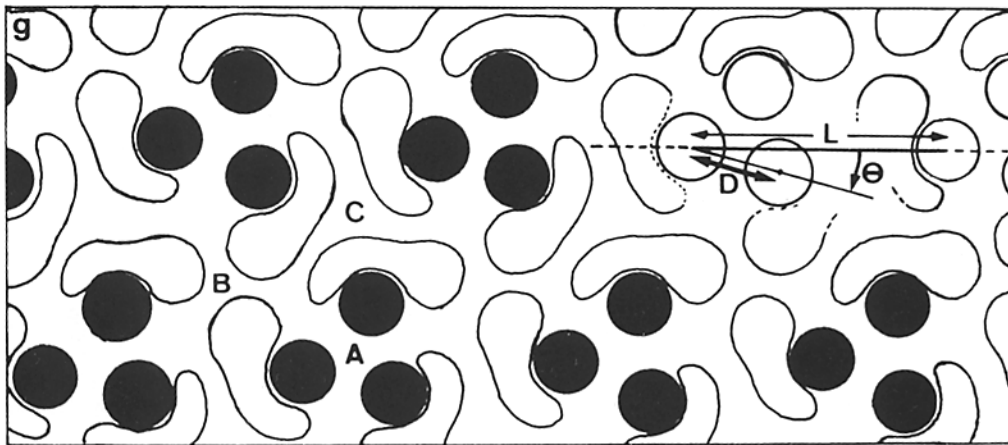
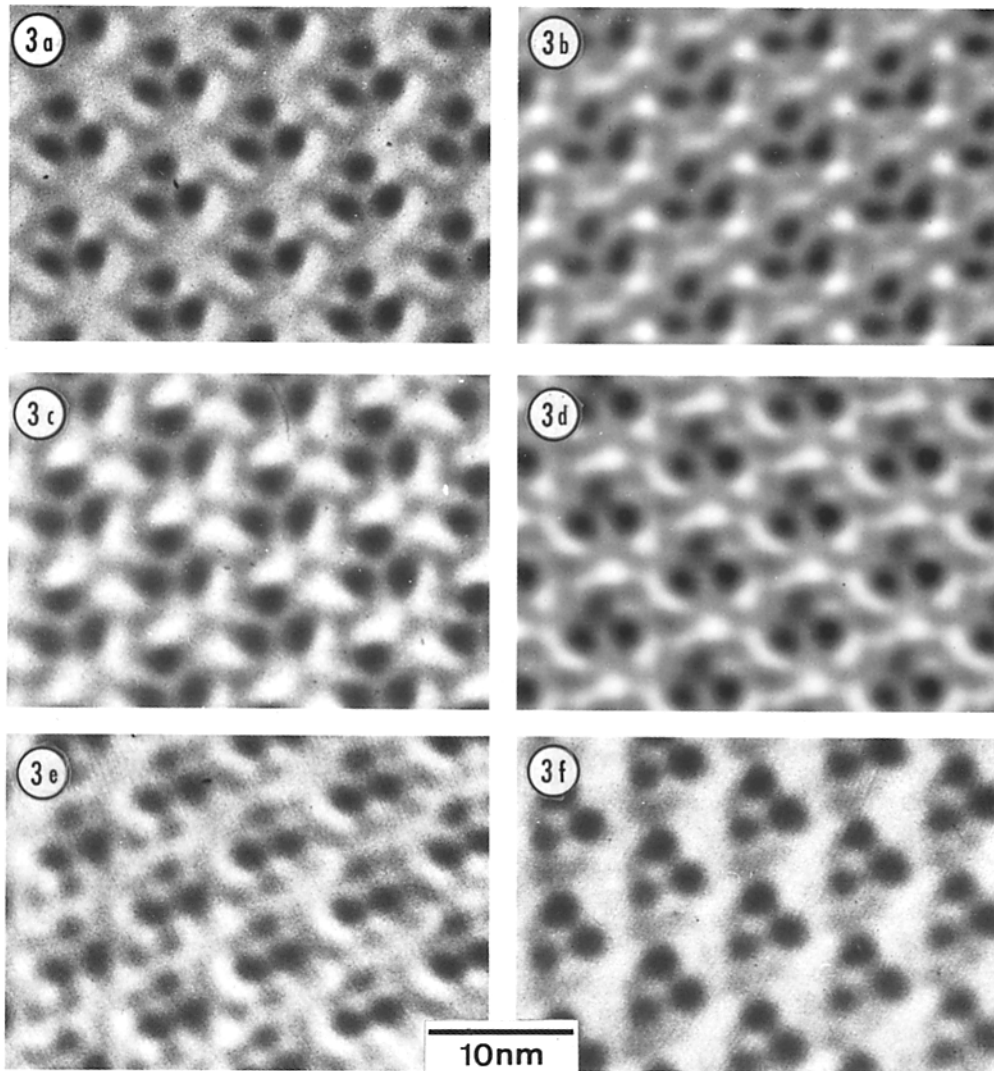
On optical diffraction, the best micrographs which we have obtained of such preparations show five radial orders (19) of the hexagonal reciprocal lattice, extending to spatial frequencies of $(2.2 \text{ nm})^{-1}$. Their lattice constant of 7.7 nm, taken together with the similarity between their general appearance and that of the regular arrays present on the intact complexes, confirms that they represent the same structure. These micrographs were treated by optical and computer image processing (Fig. 3). The most striking revelation of the filtered images is that the matrix protein is arranged according to local threefold ($p3$) symmetry on a hexagonal lattice. The most pronounced and reproducible feature is the resolution into triplet indentations of the dimples of the "honeycomb" which appear on the unprocessed micrographs as single sites of greater stain accumulation. These are positioned askew to the lattice lines at an angle of $19 \pm 3^\circ$, with a center-to-center spacing of $3.0 \pm 0.3 \text{ nm}$, as indicated in Fig. 3*g*. The boundaries of the evidently closely packed matrix protein monomers are difficult to infer from electron micrographs of negatively stained specimens since delineation of individual molecules is conditional upon substantial stain penetration into the areas of intermolecular contact (18). However, the filtered images show three stain-excluding "arms," grouped around lattice positions of local threefold symmetry which, taken together with the likely stoichiometry of three matrix protein monomers per unit cell (cf. reference 16 and Discussion),

suggest the interpretation given schematically in Fig. 3*g*.

Transmission electron microscopy of negatively stained specimens does not yield much information about these structures in the dimension perpendicular to the plane of the monolayer. For this purpose, the "one-sided" images obtained after shadowing freeze-dried specimens are more informative. Micrographs (cf. Fig. 4*a*) of the intact matrix protein-peptidoglycan complexes prepared in this way tally with those of negatively stained preparations. The complexes are covered by a regular structure, again interrupted by cracks, of a distinctly corrugated appearance. The periodicity of the hexagonal lattice measured by optical diffraction⁴ to be $7.5 \pm 0.3 \text{ nm}$ is also in agreement with that measured for negatively stained preparations. Freeze-dried and shadowed specimens of the peptidoglycan-free matrix protein arrays show two different aspects (Fig. 4*b-d*). The first of these is identical to that of the outer surface of the intact complexes. In the second case, a much smoother appearance is observed although some such specimens show weak diffraction spots corresponding to the same periodicity. From measurements of shadow lengths, we cannot distinguish a difference in thickness (approx. 4 nm for both) between these two types of surface image. Since they may be recognized respectively on adjacent particles, it is unlikely that this distinction is an artifact at the level of specimen preparation. Therefore, it is likely that the two aspects (corrugated and relatively smooth) represent fragments of the protein sheet deposited in differing orienta-

⁴ Average and standard deviation of 13 measurements. The magnification calibration used here was the manufacturer's specification.

FIGURE 3 Ultrastructure of the matrix protein layer by optical and computer filtration of electron micrographs. Protein is shown as white and negative stain as black. The optical filtrations (*a* and *c*) are of subareas of the specimens I and II, respectively, of Fig. 2. They are consistent with the corresponding computer filtrations, (*b* and *d*). For comparison, optical filtrations (*e* and *f*) of less well-preserved specimens are included. A schematic interpretation of these images is shown in (*g*). The symmetry of the structure is $p3$ with three different centers (*A*, *B*, and *C*) of local threefold symmetry. The lattice constant (*L*) is $7.7 \pm 0.4 \text{ nm}$. The "triplet indentations," penetrable by negative stain, have a center-to-center spacing (*D*) of $3.0 \pm 0.3 \text{ nm}$, and are arranged askew to a reference set of lattice lines at an angle (Θ) of $19 \pm 3^\circ$. The contours, inferred from the filtered images, enclose stain-excluding regions of protein, each probably contributed by a single molecule of matrix protein (cf. Discussion), not the outlines of entire molecules.



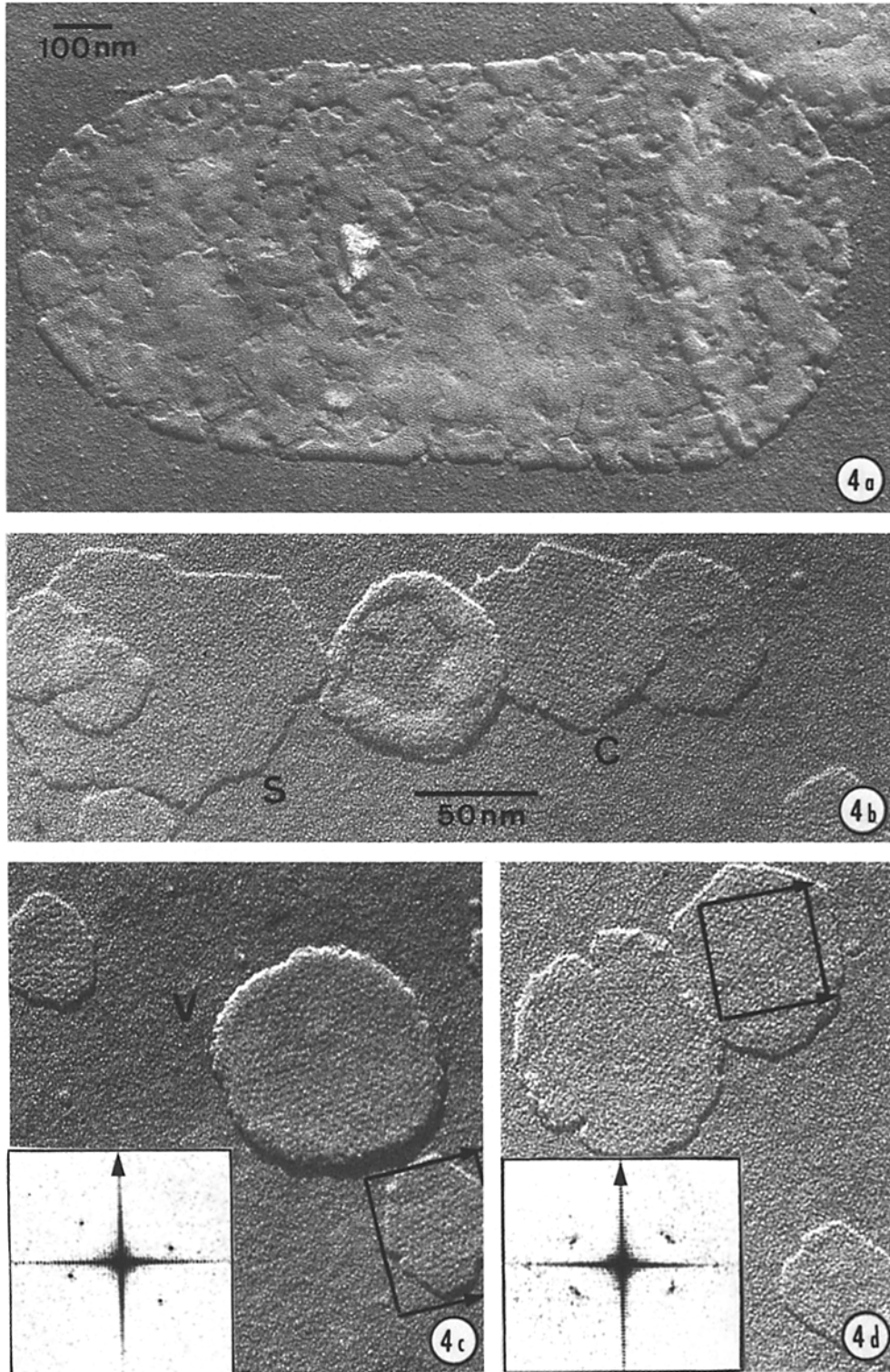


FIGURE 4 Electron micrographs of specimens tungsten-shadowed after freeze-drying. (a) An intact peptidoglycan-matrix protein complex retaining the rodlike shape of the cell. (b-d) Matrix protein arrays detached from the peptidoglycan. The one-sided images of monolayer fragments show either a corrugated (C) or a smooth (S) aspect. These preparations also contain vesicular forms (V). Optical diffraction patterns, which show the 7.7 nm periodicity, are not fully sixfold symmetric on account of the directional polarity introduced by the shadowing direction.

tions on the microscope grid, since negatively stained micrographs of the same preparations showed structures that were uniform in appearance apart from minor variations in staining and state of preservation of the periodic structure. In addition, there are what we judge to be flattened closed vesicles (Fig. 4c), on the grounds of their round shapes and the greater shadow length which they cast. The outer surface of these vesicles always has the corrugated appearance.

DISCUSSION

The periodic arrays of essentially homogeneous matrix protein, obtained after removal of peptidoglycan by lysozyme treatment and extraction in detergent, are very similar to those which cover the peptidoglycan-lipoprotein network of intact complexes. This observation,⁵ which applies both to their general appearance and to their lattice constants, allows the conclusion that the regular arrangement is maintained by strong protein-protein interactions between subunits of the matrix protein. Nevertheless, its association with the peptidoglycan-lipoprotein layer also appears to be tight, as is evident from the nature of the extraction procedure of the intact complexes from cell envelope preparations (16). Thus, the better ordering of the periodic structure in peptidoglycan-free preparations is probably due to release of this tight interaction. The latter might also be responsible for the cracks which we invariably observe to interrupt the array on specimens of the intact complex, prepared for electron microscopy either by negative staining or by freeze-drying and shadowing.

The superior ordering of the peptidoglycan-free specimens makes image processing of the electron micrographs worthwhile. Both optical and com-

puter filtrations consistently show that the molecular packing of matrix protein on the hexagonal lattice is based on threefold symmetry. Therefore, the number of monomers per unit cell must be a multiple of three. Assuming the minimal number (three molecules per unit cell), considerations of the lattice constant and the cellular complement of matrix protein (16) indicate that about 60% of the surface should be covered by the array, if the average cell surface area is taken to be $3 \mu\text{m}^2$ (8). Owing to experimental uncertainties, this figure of 60% does not contradict our observation that the cell surface is almost completely (>90%) covered with matrix protein. However, it does make improbable the alternatives of six or more molecules per unit cell.

The most pronounced features of the fine structure revealed in the filtered images are the triplets of stain-penetrable indentations. Since matrix protein covers most of the surface, the question arises as to how the lipoprotein is arranged in the outer membrane. This small protein is, in part, covalently linked to the peptidoglycan cross-bridges, but also appears to be exposed to antibody at the external surface of the outer membrane, at least in rough strains (4). Thus, if the peptidoglycan-linked molecules are not a population separate from those accessible to antibody, and if a similar arrangement of the lipoprotein is assumed in both rough and smooth strains, how is the lipoprotein arranged in the outer cell membrane relative to the layer of matrix protein? *A priori*, it is possible that the lipoprotein might be confined to specific domains, such as the cell poles or those areas which appear as cracks on electron micrographs. However, on account of the great quantity of lipoprotein and our observation that the matrix protein sheet extends over almost all of the cell wall (including the poles), we find this hypothesis implausible. Alternatively, if the lipoprotein is distributed over the entire cell, the observed indentations suggest themselves as the most likely location for its penetration through the matrix protein sheet. Do our results allow a conclusion with respect to the possibility that these indentations are channels? Although alterations of the protein structure during preparation for electron microscopy are to be expected, the argument that these indentations are real rather than artifactual is supported by their correlation with the corrugated surface in freeze-dried and shadowed specimens derived either from whole cells or from peptidoglycan-free preparations. The smooth fragments

⁵ In the literature, we have found only two references other than (16) which describe genuinely periodic layers in the *E. coli* cell envelope. The first (3) tallies with the structure analyzed here. We are unable to correlate our observations with those of Fischman and Weinbaum (6), who found a hexagonal array of apparently globular particles with a lattice constant of 14 nm, after heating *E. coli* cells to 90°C and treating them with proteolytic enzymes. Our attempts to prepare such specimens by following their experimental procedure have not been successful. The fracture face of closely packed particles of approx. 10 nm diam., described by Van Gool and Nanninga (21), does not constitute a periodic structure (N. Nanninga, personal communication, confirmed by ourselves).

seen in preparations of the latter type do not allow the drawing of a definitive conclusion. If, indeed, this aspect represents the inner face of the monolayer, the explanation which most easily accounts for these observations, then its relatively smooth relief might be due to local perturbations of the protein structure, or to a tapering of the "channels" from the outside in. The proposal that the lipoprotein, known to be predominantly α -helical (4), penetrates through the matrix protein layer is therefore reasonable. Also, the division into three indentations of the central depression (which appears as a single feature in unprocessed micrographs of negatively stained specimens) is likely to be significant and to represent the true local symmetry of the structure. Perturbations caused by preparative procedures would not be expected to generate a highly ordered structure or to systematically alter its symmetry. The diameter of the single indentations in the observed structures (approx. 2 nm and certainly less than 3 nm) signifies that they could not accommodate the hexameric assemblies of lipoprotein hypothesized by Inouye (10) to function as hydrophilic pores. However, they could contain double or triple coiled coils (5) of α -helices. This model could account for the peptidoglycan attachment of the lipoprotein and its accessibility to antibody in terms of a staggered oligomeric arrangement of that protein. The hypothesis that matrix protein from *E. coli* B may form channels is further suggested by the finding (14) that incorporation of this protein into reconstituted phospholipid-lipopolsaccharide vesicles enhances their permeability to sucrose. For this observation to have physiological significance, the protein would have to reach the external surface of the outer membrane. This has indeed been observed as the protein⁶ appears to be a phage receptor (17).

Our observations do not make it possible to distinguish between the alternatives given or to decide whether they are mutually exclusive. Nevertheless, they identify a limited range of models for the relative arrangements of matrix protein and lipoprotein which are accessible to experimental investigation. Experiments on the in vitro reas-

⁶ From packing density considerations, invoking the mass of the matrix protein and the unit cell complement of three molecules, as well as the lattice constant and the approximate thickness of the monolayer, it appears that only a small portion of the molecule can protrude through the lipid layer.

sociation of purified lipoprotein and matrix protein and on immunochemical studies are in progress in this laboratory. Hopefully, these experiments will allow us to distinguish between the alternative models formulated here and bring us closer to an understanding of the architecture and operation of the outer membrane of Gram-negative bacteria.

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