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Arrangement of Glycan Chains in the Sacculus of *Escherichia coli*

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A novel of *Escherichia coli* endopeptidase was used for a selective partial hydrolysis of the peptide bridges which interlink the glycan chains in *E. coli* sacculi. The loosening of the murein network revealed, in the electron microscope, a preferential orientation of the glycan chains, more or less perpendicular to the length axis of the cell. Control incubations with *E. coli* transglycosylase or egg-white lysozyme did not leave ordered structures behind.

In most gram-negative bacteria, cell shape is maintained by a sacculus, a single macromolecule enclosing the cell (18). The gross structure of the sacculus is known in detail (4); however basic information on its fine texture is still lacking. Chemical analysis had shown that the polymer murein (peptidoglycan) from which the sacculus is tailored is synthesized as a network; glycan chains are intermeshed by short peptide bridges. How the glycan chains are arranged relative to each other and in relation to the shape of the sacculus is still obscure. Our paper deals with this problem.

The spatial molecular structure of murein is not only an interesting problem per se. Knowledge of the arrangement of the glycan chains also might improve our understanding of the mode of enlargement and modification of the cell envelope in the growing and dividing cell and of the mechanisms of the topological control of the enzyme systems which synthesize and shape the sacculus.

The murein network in *Escherichia coli* is presumably present as a layer in which the components are tightly packed locally (1). It is generally assumed that the glycan chains are arranged in parallel (1-3, 8, 10). Different interpretations have been made on the basis of X-ray diffraction results of the detailed structure of the murein. Some workers prefer a tight chitin-like structure (1, 3, 8), whereas others arrive at a more loose conformation in which the glycan chains occur in the form of a fourfold right-handed helix (2). It was also inferred from X-ray analysis that the glycan chains run parallel to the surface of the sacculus (2). Such arrangement can be envisaged in many ways: highly ordered bundles of glycan chains can form microcrystalline patches (3) interlinked by less-ordered material; the chains would not show a long-range order. This also would be the case if the chains would not be ordered at all. A more or less parallel arrangement of chains in a loosely woven network, however, could result in a preferential, long-range orientation of the chains. A preferential orientation of stained material perpendicular to the long axis of the cell was observed with an electron microscope in sacculi of *Spirillum serpens* (11) and in isolated cell walls of *Bacillus subtilis* subsp. *niger* (16). In *B. subtilis* the striated appearance of the cell wall persisted after extraction of the teichoic acids (16). Though the above observations may give a clue to the arrangement of the glycan chains in the cell envelope, the approach is rather indirect.

In the electron microscope, spread sacculi of *E. coli* do not show a clearly discernible fine structure. We assumed that by digestion of sacculi with well-defined enzymes a loosening of the murein network could be attained which might reveal the arrangement of glycan chains in a straightforward way. Our results indicate that the glycan chains are preferentially oriented perpendicular to the long axis of the cell.

MATERIALS AND METHODS

Bacterial strain and growth condition. *E. coli* W7 (*dap lysA* [5]) was grown in minimal medium as described (5) and harvested at a density of 5×10^8 cells/ml.

Preparation of sacculi. Frozen cells of *E. coli* W7 were treated with a hot 4% sodium dodecyl sulfate solution. After centrifugation at 48,000 × *g* for 30 min, the pellet was washed five times with double distilled water and finally resuspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0). Digestion with trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio) was carried out overnight at 37°C. After centrifugation at 48,000 × *g* for 30 min, the pellet was resuspended in a

4% sodium dodecyl sulfate solution and kept at 100°C for 30 min. This preparation was washed three times with double distilled water and was finally resuspended in 0.01% sodium dodecyl sulfate in 0.05 M glycine–NaOH buffer (pH 9.0). Sodium azide was added to a final concentration of 0.1%.

Enzyme preparations. Transglycosylase was prepared from *E. coli* 3092 (6) as described before (7). The endopeptidase used is a novel enzyme derived from *E. coli* 3092. This enzyme could be completely freed from contaminating transglycosylase, a prerequisite for our experiments. Cells were opened by shaking with glass beads in a cell mill (7). The cell envelopes were removed by centrifugation, and the supernatant was used as enzyme source. Chromatography over CM-Sepharose CL-6B (Pharmacia) and hydroxylapatite (Bio-Gel HT; Bio-Rad) was followed by isoelectric-focusing in a flat bed of a granulated gel (LKB Ultrodex). This procedure results in a thousand-fold purification of an endopeptidase which accepts the isolated muropeptide dimer C3 (12) and purified sacculi as substrate. The enzyme collected within the isoelectric range of pH 6.5 to 6.8 has a pH optimum of 6 and is virtually free of transglycosylase activity. The enzyme is markedly different from the "classical" endopeptidase activities found in *E. coli* (4, 15) by its exceptional insensitivity to penicillins. The enzyme is fully active in the presence of 2×10^{-4} M penicillin G. Details of the purification and characterization of the enzyme will be published elsewhere (W. Keck and U. Schwarz, manuscript in preparation).

Measurement of enzyme activities. Sacculi used as a substrate were labeled with DL-[*meso*-^{3}H]diaminopimelic acid (specific activity, 19 Ci/mmol; Service des Molécules Marquées, France) and purified as described (5). Muropeptide C3, labeled with [^{3}H]diaminopimelic acid, was obtained from lysozyme digests of sacculi (5). Dimeric C3 is converted into a monomeric reaction product by endopeptidase (5).

Transglycosylase activity results in a liberation of small-molecular-weight reaction products from sacculi (7) which is the basis of our enzyme test. In a total volume of 100 μl of buffer (0.01 M Tris-maleate [pH 6.0], 0.01 M $MgSO_4$, 0.2% Triton X-100), enzyme was incubated at 37°C for 60 min with [^{3}H]diaminopimelic acid-labeled sacculi (5 μg of murein; 3×10^3 cpm/μg). Residual sacculi were precipitated by addition of 100 μl of 1% *N*-cetyl-*N,N,N*-trimethylammoniumbromide. After 15 min in an ice bath, the samples were centrifuged for 2 min at 12,000 × *g*; from the supernatant, 100 μl was counted in 2 ml of a toluene-Triton X-100 scintillation cocktail. Analysis of the reaction products by paper chromatography revealed the exclusive formation of products carrying 1,6-anhydrobond in the muramic acid moiety (cf. reference 7).

The test mixture for the assay of endopeptidase contained in total volume of 40 μl: 0.01 M Tris-maleate (pH 6); 0.01 M $MgSO_4$; 0.2% Triton X-100 and muropeptide C3 as substrate ([^{3}H]diaminopimelic acid-labeled; 3.5×10^4 cpm; 1 nmol). After incubation at 37°C for 60 min, samples were boiled for 2 min before separation of unreacted C3 and reaction products by paper chromatography (12). The compounds were identified and quantitated by measuring the radioactivity on dissected chromatograms in a scintillation counter.

Enzyme digestion of sacculi. From the transglycosylase and endopeptidase preparations samples were lyophylized and stored at −20°C. Shortly before a digestion experiment, 50 μl of double distilled water and 5 μl of 10-fold-concentrated Tris-maleate buffer (see above) were added to an enzyme sample. Lyophilized egg-white lysozyme (EC 3.2.1.17; grade I, Sigma, St. Louis, Mo.) was dissolved in 0.05 M Tris-hydrochloride buffer (pH 7.6) at a final concentration of 1 μg/ml. All digestion experiments were carried out at 37°C. For the spreading of sacculi, Formvar-coated grids were floated filmside down on a 50-μl droplet of sacculus suspension for 1 min. Excess fluid was removed with filter paper, and the grids were washed twice by floating on a droplet of 0.05 M glycine-NaOH buffer (pH 9.0). Excess fluid was removed with filter paper, and the samples were dried in the air. For the enzyme digestion, the grid with spread sacculi was applied to a droplet of enzyme solution for different times (17). Thereafter, the grid was floated on 4 drops of 0.05 M glycine-NaOH buffer (pH 9.0) during 5 min for each washing. Then, excess buffer was taken away with filter paper, and the sample was air dried.

Contrasting and electron microscopy. For positive staining the grids were floated for 1 min on an aqueous 1% uranyl acetate solution. The stain was removed with filter paper, and the specimen was dried in the air. Alternatively, sacculi were rotary-shadowed with Pt/Pd at an angle of 6°. In some cases, positively stained specimens were additionally shadowed to further improve contrast. Electron micrographs were taken with a Philips EM 300 or with a Siemens 102A Elmiskop apparatus.

RESULTS

We have followed the effects of three enzymes on purified sacculi with an electron microscope. Two of these enzymes (transglycosylase and lysozyme) split the β (1–4) bond linking *N*-acetylmuramic acid with *N*-acetyl-glucosamine within the glycan chain. Endopeptidase splits the peptide bond connecting the peptide side chains of two different glycan chains.

Control preparations. It proved essential to carry out the enzymatic treatment on sacculi already spread out on the grids. Attempts to make electron microscopic preparations of sacculi treated in suspension failed because of aggregation of the degraded material. Figure 1 shows an example of untreated sacculi. No clear-cut substructure is visible. Where the pole region has collapsed one often observes a fold. Since the digestion of sacculi was carried out for several hours, we have checked whether the appearance of the sacculi changes when they are treated with buffer without enzyme. No alterations were observed. Figure 2 shows as an example a preparation which was treated for 3.5 h with 0.05 M glycine-NaOH buffer (pH 9.0). Before carrying out an enzyme incubation, grids with well-spread sacculi were selected (Fig. 1).

Incubation with endopeptidase. The effect of this enzyme, which splits peptide bonds be-

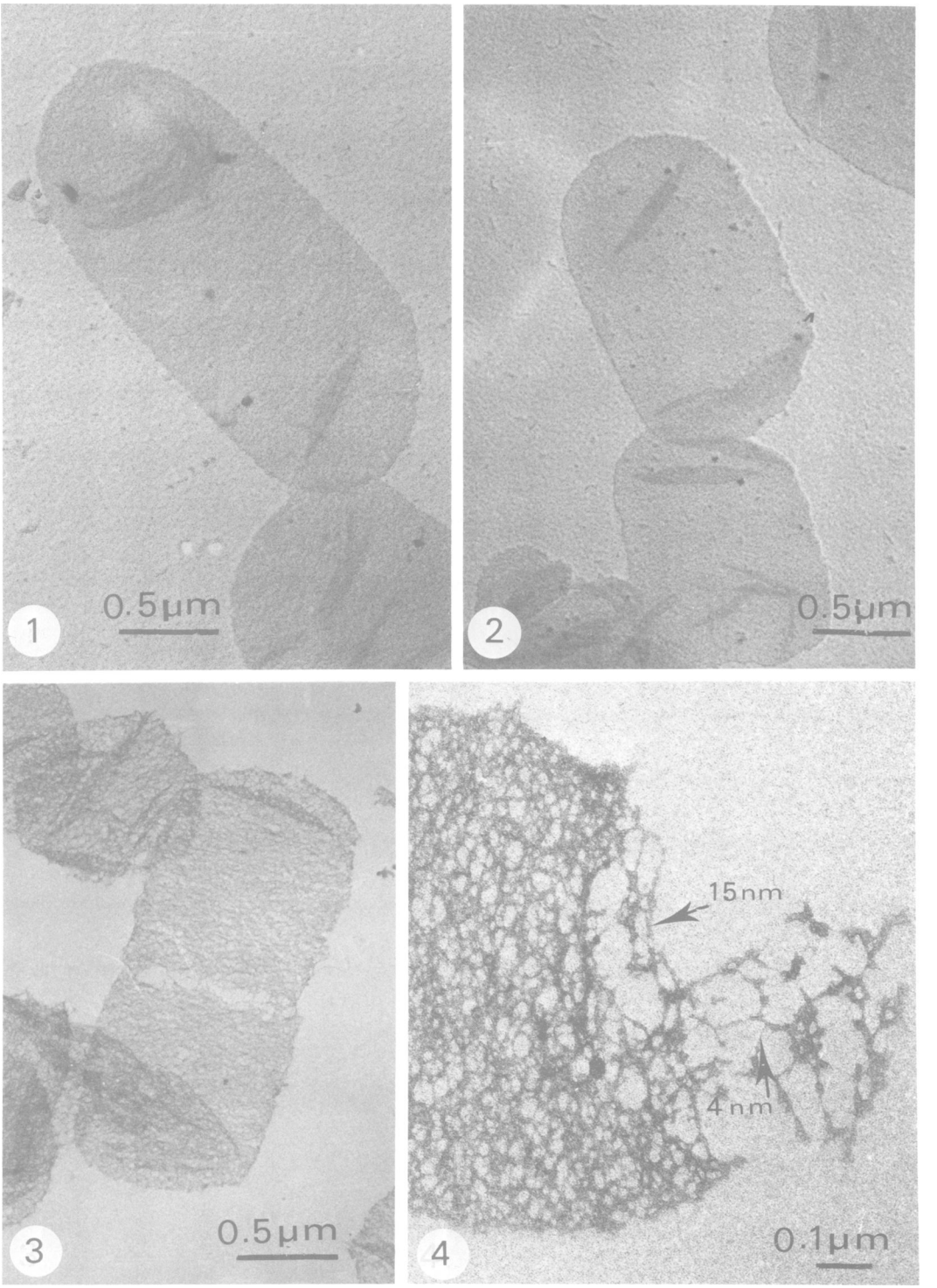
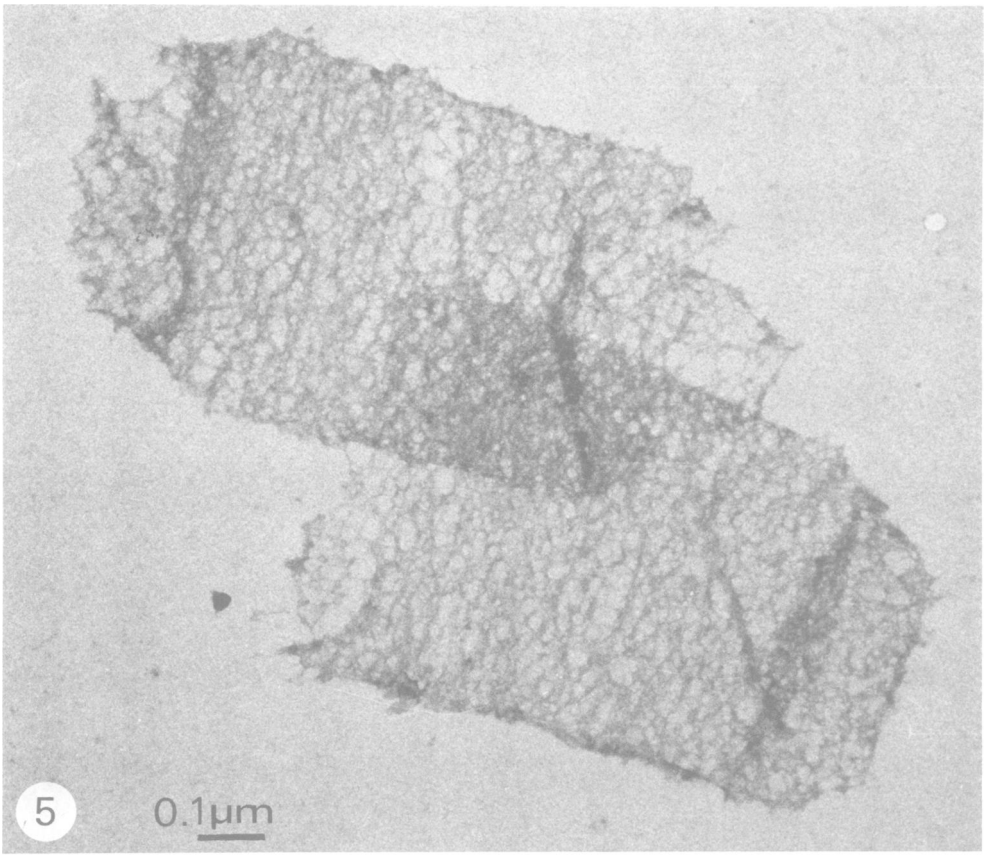


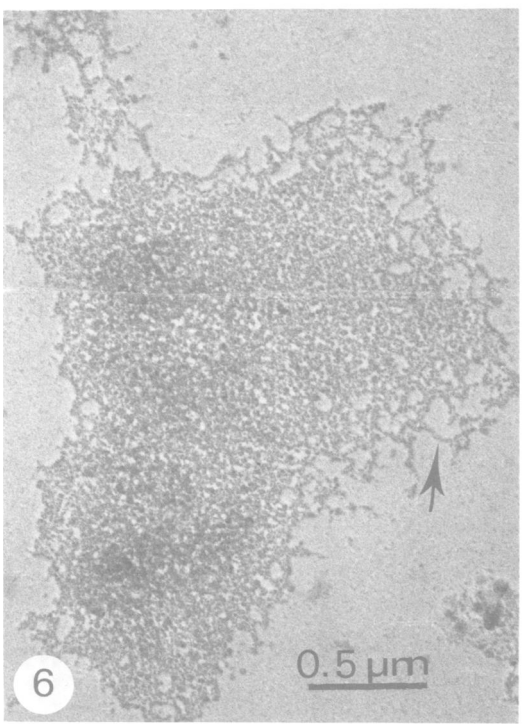
FIG. 1. *Untreated purified sacculi. The specimen was stained with uranyl acetate and then shadowed with Pt/Pd while rotating.*

FIG. 2. *Sacculi after floating for 3.5 h at 37°C on washing buffer (see Materials and Methods). Contrasting as in Fig. 1.*

FIG. 3. *Sacculi treated for 3 h with endopeptidase. Staining with uranyl acetate.*

FIG. 4. *Detail of a pole region after 6-h endopeptidase digestion. Staining with uranyl acetate.*





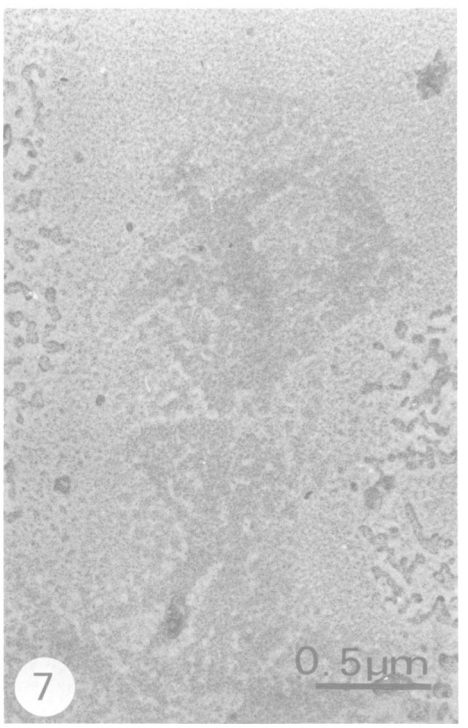


FIG. 5. *High magnification of endopeptidase-treated sacculi. Details as in Fig. 3.*

FIG. 6. *Sacculi treated for 15 min at 37°C with transglycosylase. Contrasting as in Fig. 1. The arrow points to a strand with a width of about 30 nm (evaporated metal included).*

FIG. 7. *Sacculi treated for 10 min at 37°C with lysozyme (2 µg/ml in 0.05 M Tris-hydrochloride, pH 7.6). Shadowing with Pt/Pd while rotating.*

tween neighboring glycan chains, can be seen in Fig. 3 to 5. Netlike structures appear, and the fibers in the net show a preferential orientation. The preference is in a direction perpendicular to the length axis of the cell. This can be seen in more detail at higher magnification as shown in Fig. 4 and 5 (arrows). The thickness of the strands is variable, and is on the order of 4 to 15 nm as indicated in Fig. 4. As deduced by Braun et al. (1), the maximal distance between two linked glycan chains with fully stretched peptide bridges is 4.2 nm. When the peptide side chains are maximally folded this distance becomes 1 nm. The strands are thus thought to represent single chains or small bundles of glycan filaments.

Incubation with transglycosylase. Treatment with this enzyme causes fragmentation of sacculi (Fig. 6). The size of the remaining substructure is about 30 nm, as indicated in Fig. 6, and it does not show a preferential orientation of its constituents.

Incubation with lysozyme. Images of lysozyme-treated sacculi (Fig. 7) resemble those of transglycosylase-treated ones. Holes arise, and the sacculus appears loosened up. Again no preferential orientation is observed.

DISCUSSION

Electron microscopy of purified sacculi after partial degradation of peptide bonds with endo-

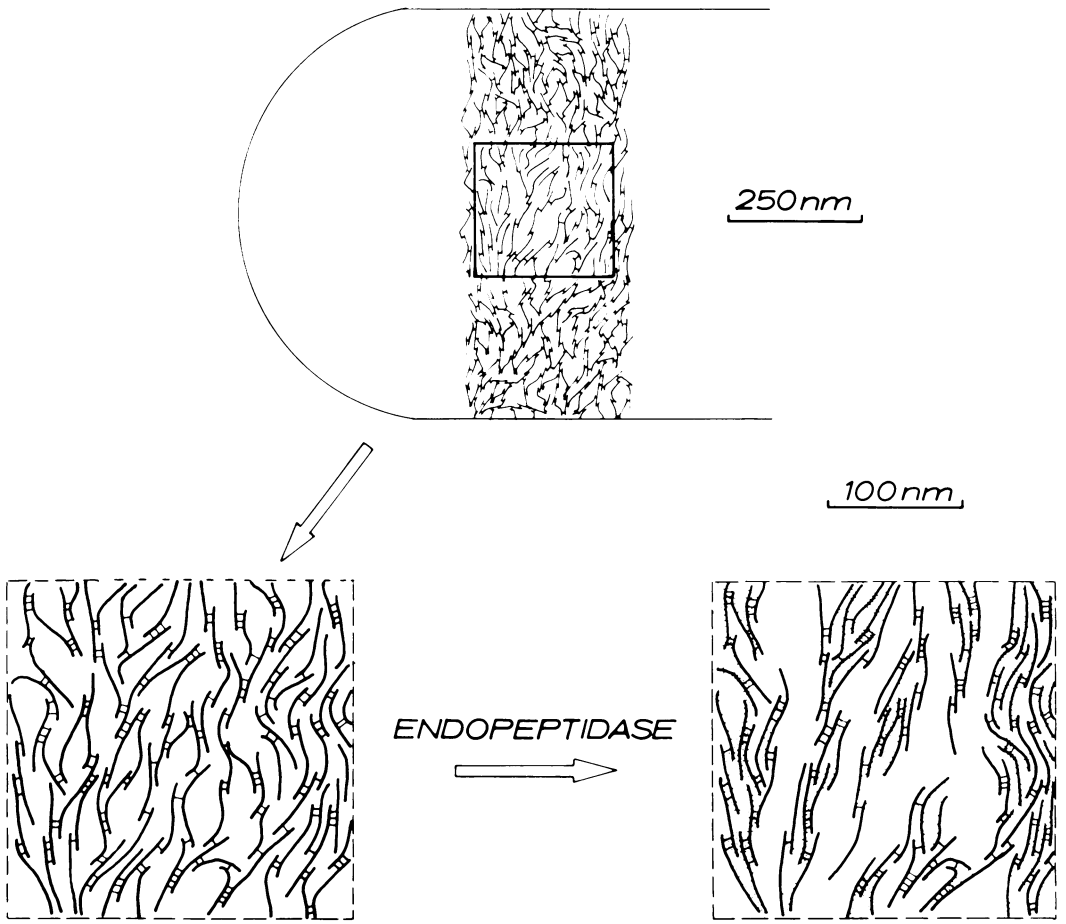


FIG. 8. *Schematic approximation of the arrangement of the glycan chains in the sacculus of E. coli W7. The upper part gives an impression of the size and proposed arrangement of the glycan chains over the bacterial cell surface. It should be noted that this structure is not necessarily completely flat. The lower part shows a detail of the network before and after endopeptidase digestion. For the sake of clarity, not all dimensions could be drawn as they really are. Approximately 30% of the glycan units are cross-linked (13), though it is not known whether the cross-links are grouped (as drawn) or occur more dispersedly. The glycan chains should be 35 to 50 nm long (13) and about 1 nm apart at the site of a cross-linkage (1).*

peptidase shows a loosely interwoven fibrillar network with a main direction perpendicular to the long axis of the cell. Such oriented fibers are not seen upon hydrolysis of glycosidic bonds either by *E. coli* transglycosylase or by egg-white lysozyme, as done in our controls. Unfortunately, we are not able to assess the extent of degradation on the grids in a quantitative way. The more amorphous sacculus appearance after incubation with lysozyme and transglycosylase is thought to be due to the removal of small fragments (because of glycan chain rupture).

The detailed packing of the glycan chains cannot be deduced from the electron micrographs. However, inspection of the pictures (Fig. 4 and 5) suggests a loose arrangement of the glycan chains in the murein layer which is reminiscent of the scheme of Burge et al. (Fig. 9a in reference 2). Although it is tempting to take our data as proof of their model, we are hesitant to do so; the wavy arrangement of material could be the result of a secondary reorientation during or after the digestion of sacculi on the grids. We only deduce from our data a long-range order of the glycan chains more or less perpendicular to the long axis of the cell. An arrangement of glycan chains perpendicular to the long axis of the sacculus had been inferred from the shrinkage pattern observed upon uranyl acetate treatment of *S. serpens* sacculi on moist grids (11). Possible artifactual striation in our experiments seems to us unlikely: the control sacculi, being immobilized on the grids before staining, do not show shrinkage and striation. Striated structures in shadow-cast preparations or dark-field images in which uranyl acetate had been avoided had already been observed in cell walls of *B. subtilis* (16).

There are other observations in the literature which agree with the glycan chain texture that we propose (Fig. 8). Sacculi, isolated from *E. coli* W173-25 treated with penicillin, showed a sharp cut in the cell center perpendicular to the length axis of the cell due to localized murein hydrolase action (14). Moreover, when sacculi break during the isolation procedure or when they are fragmented by sonic treatment, cuts occur preferentially in the same direction (unpublished observations). Taken together, the data again indicate that murein does not have an isotropic substructure.

During cell growth under balanced conditions the cell diameter is kept remarkably constant (9). This conservation is facilitated when the glycan chains show a circular long-range orientation more or less parallel to the short axis of the cell wall cylinder as supposed in our model. An insertion of new glycan chains into the growing sacculus would lead to an elongation of the cell wall, the cell diameter being kept constant. The orientation of the glycan chains also may ensure the strict topological control of murein hydrolase activity.

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