0021-9193/78/0133-0372$02.00/0
JOURNAL OF BACTERIOLOGY, Jan. 1978, p. 372-386

Vol. 133, No. 1
Printed in U.S.A.

Structural Arrangement of Polymers Within the Wall of *Streptococcus faecalis*

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Received for publication 28 June 1977

The structure of the cell wall of *Streptococcus faecalis* was studied in thin sections and freeze fractures of whole cells and partially purified wall fractions. Also, the structures of wall preparations treated with hot trichloroacetic acid to remove non-peptidoglycan wall polymers were compared with wall preparations that possess a full complement of accessory polymers. The appearance of the wall varied with the degree of hydration of preparations and physical removal of the cell membrane from the wall before study. Seen in freeze fractures of whole cells, the fully hydrated wall seemed to be a thick, largely amorphic layer. Breaking cells with beads caused the cell membrane to separate from the wall and transformed the wall from a predominantly amorphic layer to a structure seemingly made up of two rows of "cobblestones" enclosing a central channel of lower density. Dehydration of walls seemingly caused the cobblestones to be transformed into two bands which continued to be separated by a channel. This channel was also observed in isolated wall preparations treated with hot trichloroacetic acid to remove non-peptidoglycan polymers. These observations are consistent with the interpretation that both peptidoglycan and non-peptidoglycan polymers are concentrated at the outer and inner surfaces of cell walls. These observations are discussed in relation to possible models of wall structure and assembly.

Walls of gram-positive bacteria are composed of peptidoglycan to which is covalently linked one or more non-peptidoglycan polymers, such as anionic, teichoic, or teichuronic acids, and neutral polysaccharides. Currently, little is known about the exact topological localization of the various wall polymers. The question of exclusive or enriched domains within walls remains open.

Several kinds of evidence have been used to promote the idea that various polymers in walls are enriched in specific wall areas. First is the appearance of walls of a variety of gram-positive species in thin sections. Frequently, two electron-dense bands enclosing a less dense central zone are seen (16). This appearance has led to the idea that the outer and inner dense bands might be enriched in anionic polymers which would bind larger amounts of the heavy metal stains used to improve the contrast of these preparations. Although phosphate groups in the teichoic acid of *Streptococcus faecalis* have a high affinity for uranyl acetate, this affinity was greatly reduced after exposure of walls to osmium tetroxide (14). Since fixation with osmium almost invariably precedes uranyl acetate staining of thin sections, it seems unlikely that the tribanded appearance is due solely to selective binding of uranyl ions by phosphate groups. However, in support of the idea that anions are concentrated at wall surfaces are studies of sections of *Bacillus subtilis* in which tribanded walls were visualized in sections that had never been exposed to either heavy metal stains or osmium tetroxide (29). The tribanded image observed was interpreted as indicating an accumulation of phosphate groups at wall surfaces. The idea of a surface concentration of anions is also supported by a group of observations which indicate that the typical tribanded wall profile is lost or greatly diminished in preparations where non-peptidoglycan polymers were extracted (1, 23, 25) or were reduced by a mutational event (9, 13). Although extraction of non-peptidoglycan polymers from walls of several gram-positive species resulted in thinner walls (14, 23, 25), reduction in width (and volume) was not found to follow a pattern consistent with a layered structure (23). Studies using lectins (2, 3, 5) or specific antibodies (7) to interact with specific wall polymers also suggest a location of secondary polymers at wall surfaces. For example, the interaction of concanavalin A with α-D-glucose units of the wall teichoic acid of *B.*

subtilis was seen on the outer but not the inner wall surface (5). While this would be consistent with an outer electron-dense layer seen in thin sections of these walls, it would not explain the opacity of the inner wall layer that is also observed. Furthermore, some studies with lectins and antibodies suggest an enrichment rather than an exclusive location, since, at least in some cases (7, 11), partial or complete dissolution of walls with lysozyme increased the number of binding sites.

In an attempt to obtain further information on the localization of polymers within walls, we have now examined the ultrastructure of walls in intact cells and in isolated wall fractions by both freeze-fracture and thin-section techniques. Freeze-fracture preparations have the advantage of permitting visualization of structures in their hydrated state. Although we have successfully observed a type of tribanded wall structure in freeze-fractured preparations of isolated walls consistent with that observed in thin sections, we have not been able to correlate this structure with an enrichment of specific wall polymers.

MATERIALS AND METHODS

Cell growth. Exponential phase cultures of *S. faecalis* ATCC 9790 were grown for at least six doublings at generation times of 31 to 33 min at 37°C in a chemically defined medium (27) to a culture density equivalent to 0.32 mg of cellular dry weight per ml. Cultures were chilled to 0°C, and the cells were removed by centrifugation (1,500 × *g*, 15 min, 3°C) and immediately treated as described below.

Wall isolation. Cells were suspended in ice-cold double-distilled water, mixed with an equal volume of styrene divinylbenzine copolymer beads (18 to 50 mesh), and shaken for 8 min at 4,000 rpm in a Braun MSK cell homogenizer, maintaining the temperature below 5°C using liquid carbon dioxide. The beads were removed by filtration through a scintered-glass filter. This filtrate was then centrifuged (1,000 × *g*, 20 min, 3°C) to remove unbroken cells, and the walls were then sedimented (12,000 × *g*, 15 min, 3°C). Crude wall pellets were suspended in 2% sodium decyl sulfate (SDS) and incubated at 37°C for 30 min. The SDS-treated walls (SDS-walls) were sedimented and washed four times in double-distilled water. Trichloroacetic acid-extracted walls (TCA-walls) were prepared from SDS-walls by suspension in 10% TCA and incubation at 90°C for 10 min. After treatment, the walls were washed four times with double-distilled water.

Labeling with cationized ferritin. Intact cells (100 mg), SDS-walls (25 mg), and TCA-walls (10 mg) were suspended in 1 ml of 10 mM phosphate, pH 7.2, to which 0.3 ml of cationized ferritin (12 mg/ml; Miles, Yeda, Ltd.) was added. Mixtures were incubated for 30 min at 25°C and then washed three times with 10 ml of 10 mM phosphate, pH 7.2.

Chemical determinations. Phosphorus content of the TCA- and SDS-walls was determined by the method of Lowry et al. (21), using sodium phosphate as a standard. Hexosamine was determined by a modification of the Morgan-Elson procedure (15) after hydrolysis in 3 N HCl for 4 h at 95°C and reacetylation with acetic anhydride, using *N*-acetylglucosamine-HCl as a standard. The method of Dische and Shettles (10) was used for the determination of rhamnose.

Electron microscopy. Cells were fixed for thin sectioning by adding glutaraldehyde directly to the growth medium to a final concentration of 2% (vol/vol). Walls to be sectioned were similarly initially fixed by adding glutaraldehyde (final concentration, 2%) to 10 to 20 mg of walls in 20 ml of 10 mM phosphate, pH 7.2. After 2 h of glutaraldehyde fixation at 25°C, both walls and cells were postfixed in osmium tetroxide, embedded in Epon 812, and stained with uranyl acetate-lead citrate as previously described (17, 19).

For freeze fracture, unless otherwise specified, intact cells and walls were suspended in 30% glycerol in 10 mM phosphate, pH 7.2. The incubation time in 30% of glycerol was 20 min at ice bath temperatures.

After incubation, cells and walls were concentrated by centrifugation and then frozen in liquid Freon 22. All subsequent procedures were as described in the instruction manual of the Balzer Freeze microtome (BA 360 M) used in this study. Unless otherwise stated, all freeze fractures were etched 1 min before being replicated. A Siemens Elmiskop 1A electron microscope was used for all observations. Measurements of wall thickness were taken from whole cells and wall fragments which had the bisymmetrical shape of a longitudinally sectioned whole cell and showed a maximum diameter of about 1 μm. In the case of thin-sectioned cells and walls, only portions of the wall that had a sharp tribanded wall profile were measured. The average thickness of the walls in each sample was determined by taking at least 100 measurements of the walls observed on a minimum of 50 cell walls.

RESULTS

Chemical composition of wall preparations. TCA extraction efficiently removed over 95% of both rhamnose and phosphorus from SDS-walls (Table 1). TCA extraction also removed about one-third of the total hexosamine from SDS-walls. Loss of a portion of the hexosamine from SDS-walls by TCA extraction was expected, since hexosamine has been reported as a component of the wall polysaccharide of *S. faecalis* (6) as well as of the peptidoglycan. Treatment of SDS-walls with hot TCA was accompanied by a 60 to 65% decrease in dry weight of the wall suspensions. The extent of removal of indicators of non-peptidoglycan polymers present in SDS-walls of this species by TCA extraction is consistent with the nearly complete absence of such polymers in the TCA-walls.

Ultrastructural studies of thin-section walls on intact cells and in isolated wall

preparations before and after TCA extraction. The structure of the cell walls of *S. faecalis* observed in thin sections has been reported (18, 19). Typically for gram-positive bacteria in axial section, walls in sectioned cells appear as a "sandwich" made up of two electron-dense layers enclosing a central, less dense region (Fig. 1A). Total thickness averaged about 28 ± 5 nm (Table 2).

A similar type of sandwich structure was also observed in sections of isolated untreated walls (Fig. 1B) and SDS-wall preparations (Fig. 1C and 2A). However, as shown in Fig. 1, the outer and inner margins of the isolated walls often seemed to take up somewhat less heavy metal stain than did the walls observed in whole cells. However, these staining reactions were not reflected in measurements of wall thickness (Table 2), which indicated that the thickness of the cell wall of *S. faecalis* was not significantly altered by cell breakage or SDS treatment. While the wall is seemingly not significantly thinned during isolation, Table 2 does indicate that walls undergo about a 30% decrease in thickness when their non-peptidoglycan polymers are removed by hot TCA. This decrease in thickness is also accompanied by a further decrease in the prominence of the tribanded profile (Fig. 1D and 2B).

These results are similar to those of studies of other organisms (but not in all [1]) in which

TABLE 2. *Thicknesses of walls as measured in freeze fractures and thin sections after various treatments*

Types of prepn	Freeze-fracture prepn		Thin sections		Thin section/freeze fracture (nm)
	nm	% of SDS-wall	nm	% of SDS-wall	
Intact cells	42 ± 2.6[a]		28 ± 5.0[a]		0.67
SDS-treated cells	42 ± 3.1		28 ± 3.3		0.67
Unfractionated disrupted cells	44 ± 3.5				
SDS-walls	44 ± 3.0		28 ± 2.9		0.64
TCA-walls	29 ± 2.9	65	20 ± 2.4	71	0.69
Cationized ferritin-labeled:					
SDS-walls			43 ± 3.0		
TCA-walls			28 ± 2.3		

[a] Standard deviation of 50 to 100 measurements.

TABLE 1. *Chemical composition of SDS- and TCA-walls of S. faecalis*

S. faecalis cell walls	Dry wt (% of SDS-walls)	Total hexosamines		Rhamnose		Phosphorus	
		nmol/mg	% of SDS-walls	nmol/mg	% of SDS-walls	nmol/mg	% of SDS-walls
SDS-walls		840 ± 90[a]		915 ± 30		410 ± 30	
TCA-walls	35	505 ± 80	60	8 ± 0.7	1	20 ± 2	5
TCA-soluble fraction		280 ± 20	33	860 ± 40	94	450 ± 50	110

[a] Standard deviation of determinations.

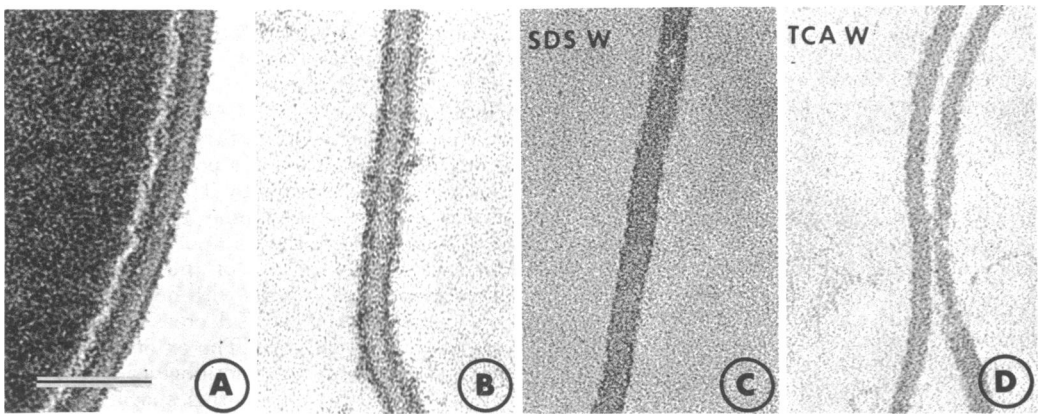

FIG. 1. *Thin sections showing the morphology of the cell wall of S. faecalis as seen in (A) whole cells, (B) cells after being broken by beads, (C) SDS-walls, and (D) TCA-walls. See Materials and Methods for a description of wall purification and electron microscope procedures. Bar in (A) applies to the entire plate and equals 0.1 μm.*

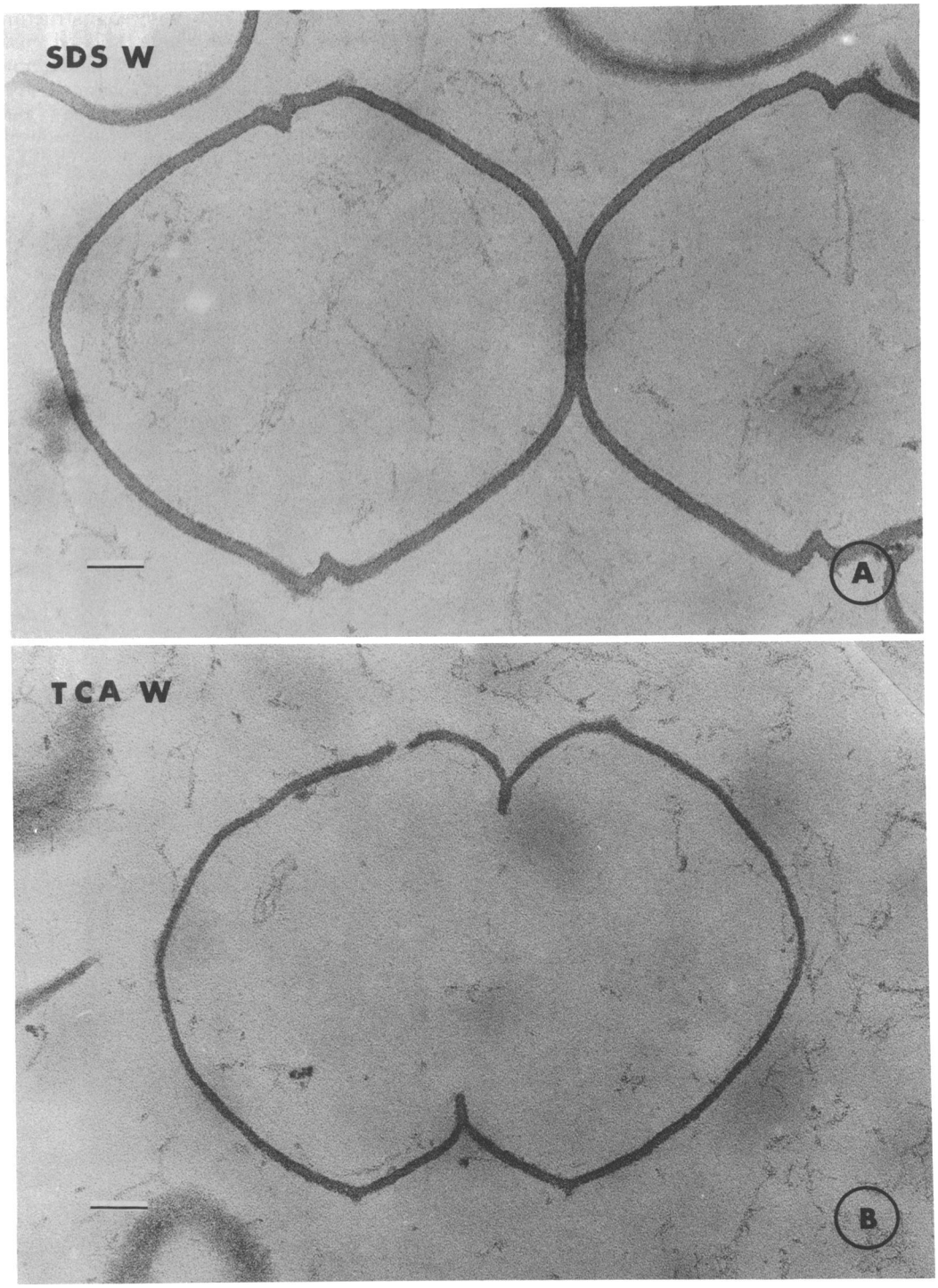

FIG. 2. *Thin sections of (A) SDS- and (B) TCA-wall fractions showing the relative thickness and morphology of cell-shaped wall fragments. Bars = 0.1 μm.*

decreased thickness and strain affinity accompanied reduced content of non-peptidoglycan polymers (14, 23, 25).

Freeze-fracture studies. Cross fractures of intact cells showed the wall to be a largely amorphic layer (Fig. 3). However, cross fractures of isolated SDS-walls no longer appeared amorphic and consistently showed a structural arrangement (Fig. 4A). These isolated SDS-walls seemed to be made up of irregular rows of "globules" separated by a "channel" (Fig. 4A). This type of globular morphology could not be discerned in surface views of etched walls (Fig. 5) and was only observed in cross-fractured

FIG. 3. *Freeze fracture of a whole cell of S. faecalis illustrating the morphology of the cell wall in a cross-fractured perspective. Arrow in the left bottom corner of this and all other freeze fractures gives the approximate direction of the shadowing. Bar = 0.1 μm.*

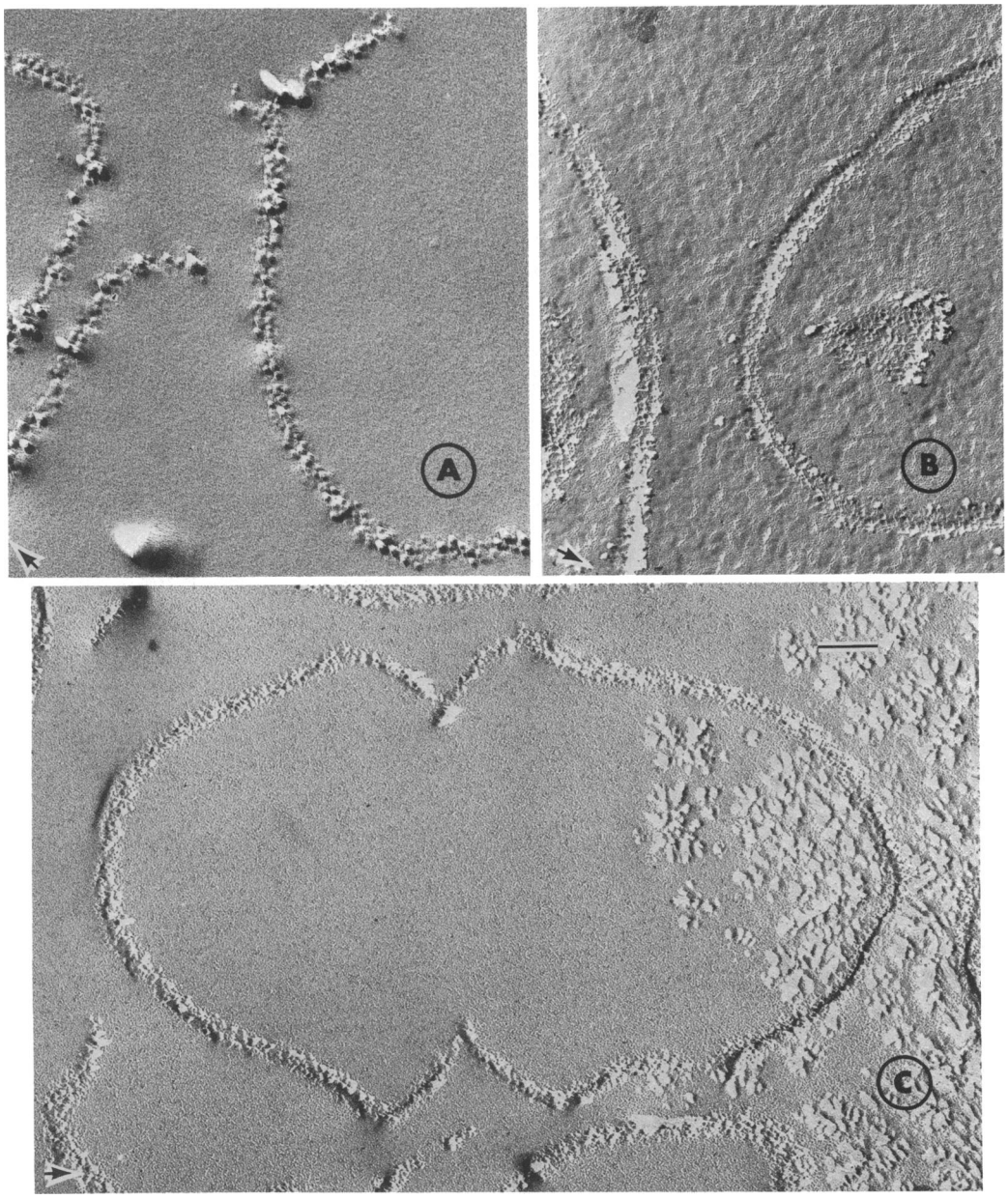

FIG. 4. *Morphology of SDS-wall fractions as affected by the rate of initial freezing. In portions of replica where no ice crystals are observable, the walls appear globular (A). When such fractures are etched for 15 min, the walls appear compactly tribanded (B). This tribanded morphology can also be seen in portions of replicas where the initial freezing rate was slower. Bar in (C) applies to all micrographs and equals 0.1 μm.*

preparations where the initial rate of freezing was fast enough to prevent separation of water and glycerol in the freezing medium. At slower rates of freezing (i.e., in those regions where the background appears rough or matted; see Fig. 6 and 7 and [28]), the globular nature of the walls was much less prominent, and these walls seemed morphologically to look much more like the tribanded structures seen in thin sections (Fig. 1). In fact, it was possible to find regions of replicas in which a transition in freezing rate apparently occurred (Fig. 4C). In those regions where one begins to notice ice-crystal growth (e.g., the right side of Fig. 4C), the wall begins

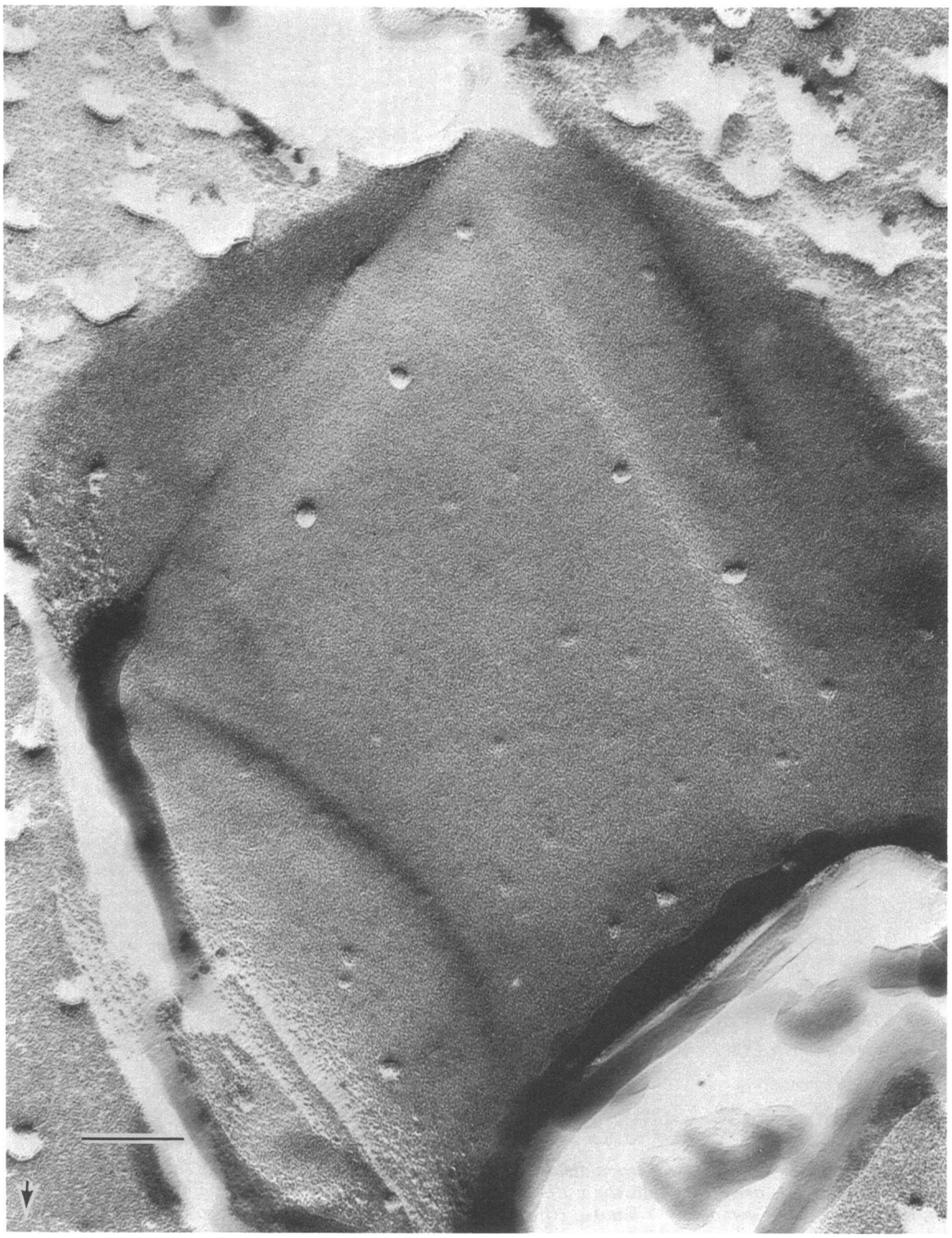

FIG. 5. *Surface view of an SDS-wall fragment frozen in water and seen after 1 min of etching. Bar = 0.1 μm.*

to take on a much more prominent "dual track" morphology.

Although a channel can be faintly seen in some whole cell preparations, the channel structure seems to become prominent in rapidly frozen samples only after cell breakage (Fig. 8). The visualization of a prominent channel seemingly requires neither SDS treatment nor glycerol infiltration (Fig. 8). One implication of these observations is that the channeled type of profile may be a result of the dissociation of the close apposition of wall and membrane observed in

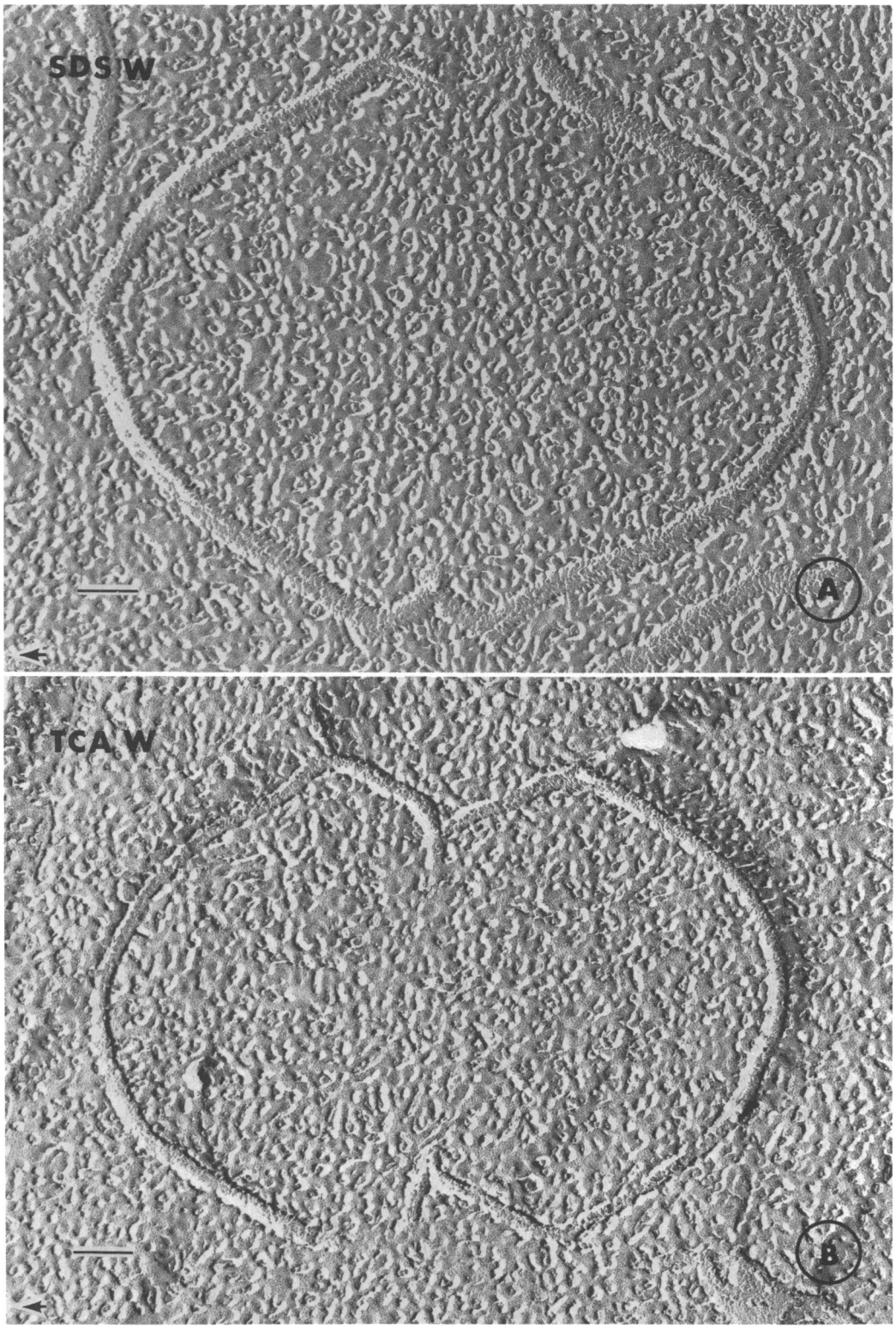

FIG. 6. *Channeled appearance of (A) SDS- and (B) TCA-walls in freeze fractures. Bars = 0.1 μm.*

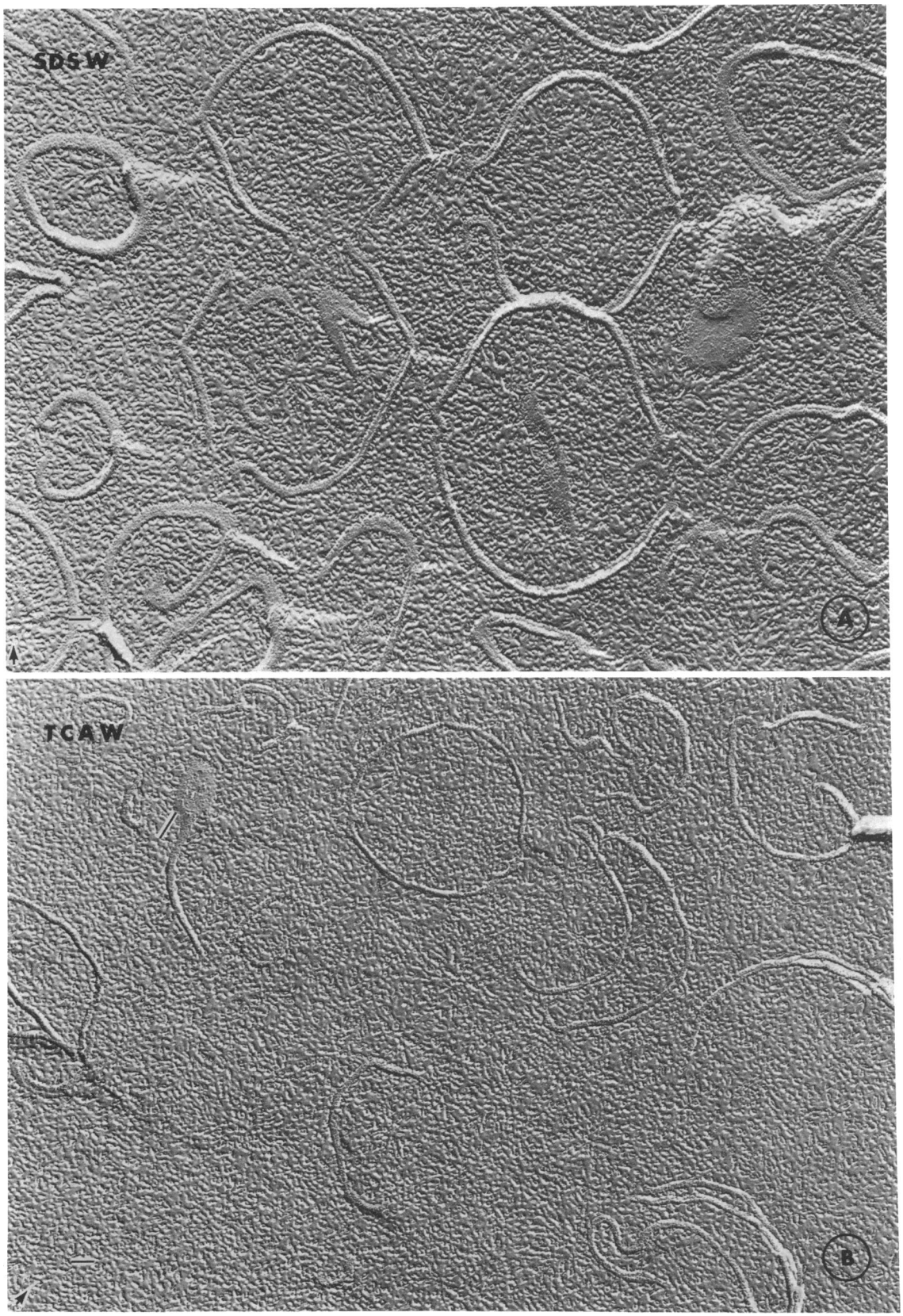

FIG. 7. *Lower magnification views of channeled (A) SDS- and (B) TCA-wall fractions in freeze fractures. Bars = 0.1 μm.*

FIG. 8. *Freeze fracture of a cell wall fragment of S. faecalis frozen in water just after a cell suspension had been shaken with beads. Specimen was fractured at −105°C and replicated immediately. Solid black arrow points to the center of channel observed in cross fractures of such walls. Bar = 0.1 μm.*

intact cells (Fig. 3). A direct way of testing this hypothesis would be to see if the channeled type of organization is observed upon plasmolysis of cells. Unfortunately, attempts to plasmolyze *S. faecalis* with sucrose or saline (1 M sucrose or 5% NaCl) were unsuccessful.

The interpretations and possible implications of the apparent globular-like wall structure will be treated in the Discussion. However, for purposes of comparing the thickness of various wall preparations, we decided to use glycerol-infiltrated walls which showed the compact tri-banded type of morphology, such as that shown in Fig. 6A and B. This was done simply because walls showing the globular configuration had irregular margins which prevented easily reproducible thickness measurements. Regions of replicas in which the background granularity was similar to that shown in Fig. 6 were selected for photography and measurement. The small variations in thickness measurements (±2 nm) obtained from replicas taken from identical samples indicated that this approach produced reproducible and consistent results.

Hydrated walls of both freeze-fractured preparations of intact cells and isolated SDS-walls were 42 ± 3 nm thick (Table 2), about 33% thicker than walls seen in thin sections. These observations are consistent with previous studies in other organisms in which the thickness of hydrated walls estimated by physical methods appeared to be about 2 to 5 times greater than those measurements taken from thin sections of cells (8, 32).

After TCA extraction of non-peptidoglycan wall polymers, freeze-fractured walls measured 29 ± 3 nm thick (Table 2), thus yielding a comparative picture very similar to that obtained by the thin-section technique, namely that removal of accessory polymers is accompanied by a marked decrease in wall thickness. However, other than thickness, freeze-fracture preparations of TCA-walls showed the same basic channeled structure seen in SDS-walls (Fig. 6 and 7).

Cationized ferritin. In 10 mM sodium phosphate, pH 7.2, both SDS- and TCA-walls bound polycationic ferritin. Etched preparations of either type of wall preparation frozen in water showed surface arrays of bound particles (Fig. 9). However, in sections, more particles seemed to be bound to the surfaces of the SDS- than to the TCA-walls (Fig. 10). Also, the sections showed that, in general, ferritin particles were located much closer to the center of TCA-walls. Center-to-center distances between particles on one side of the wall to particles on the other side averaged 43 ± 3 and 28 ± 2.3 nm for the SDS- and TCA-walls, respectively (Table 2). Comparison of these thicknesses with those obtained from freeze fractures of SDS- and TCA-wall preparations not exposed to ferritin (e.g., 44 and 29 nm, respectively; Table 2), suggest that binding of cationized ferritin to walls before dehydration partially protected them from the collapse that apparently occurs during the usual processing for thin sectioning. This effect can be seen by comparing the thicknesses of the walls in Fig. 2A and B with those in Fig. 10A and B.

DISCUSSION

Most of the current information concerning the ultrastructure of walls of gram-positive bacteria is derived primarily from studies of thin sections of cells and various wall fractions. However, the thin-section technique has the disadvantage that the image produced is not actually that of the wall, but rather the image that the wall produces after it has been fixed, bound to metals (4), dehydrated, and infiltrated with other polymers. In this regard, the freeze-fracture technique seems to present the marked advantage in that walls can be studied without fixation and presumably at close to normal levels of hydration. In the present study, when the thin sections and fracture images of *S. faecalis* walls were compared in whole cells, isolated walls, and walls extracted of their accessory polymers (TCA-walls), the effects of dehydration were clear. In each case, the thin-sectioned walls were about 31 to 35% thinner than their freeze-fractured counterparts. Moreover, the freeze-fractured walls indicated structured details which have not been previously observed in thin sections. For example, there seems to be a large change in the appearance of the wall when cells are broken and the apposition of the underlying cell membrane is dissociated from the wall's surface (Fig. 8). Thus, when the cells are broken, a central channel becomes a prominent feature of the wall's morphology. This channel is interpreted as being a zone of lower water-binding capacity and/or lower density. On the other hand, interpretation of the two rows of "cobblestones" separated by the channel, which are seen in hydrated preparations, is more difficult. For example, since the globules which make up the cobblestone rows could not be seen in etched-surface views of wells (Fig. 5), it is not known if they are actually globules or rather cross fractures of some other regular or irregular fracture product. (For example, it is possible that they could be cross fractures of fibrillar bundles such as those seen during the reversion of L-forms of *Bacillus licheniformis* [12] or even a plastic deformation of some other structure.) However, even if the observed cobblestone mor-

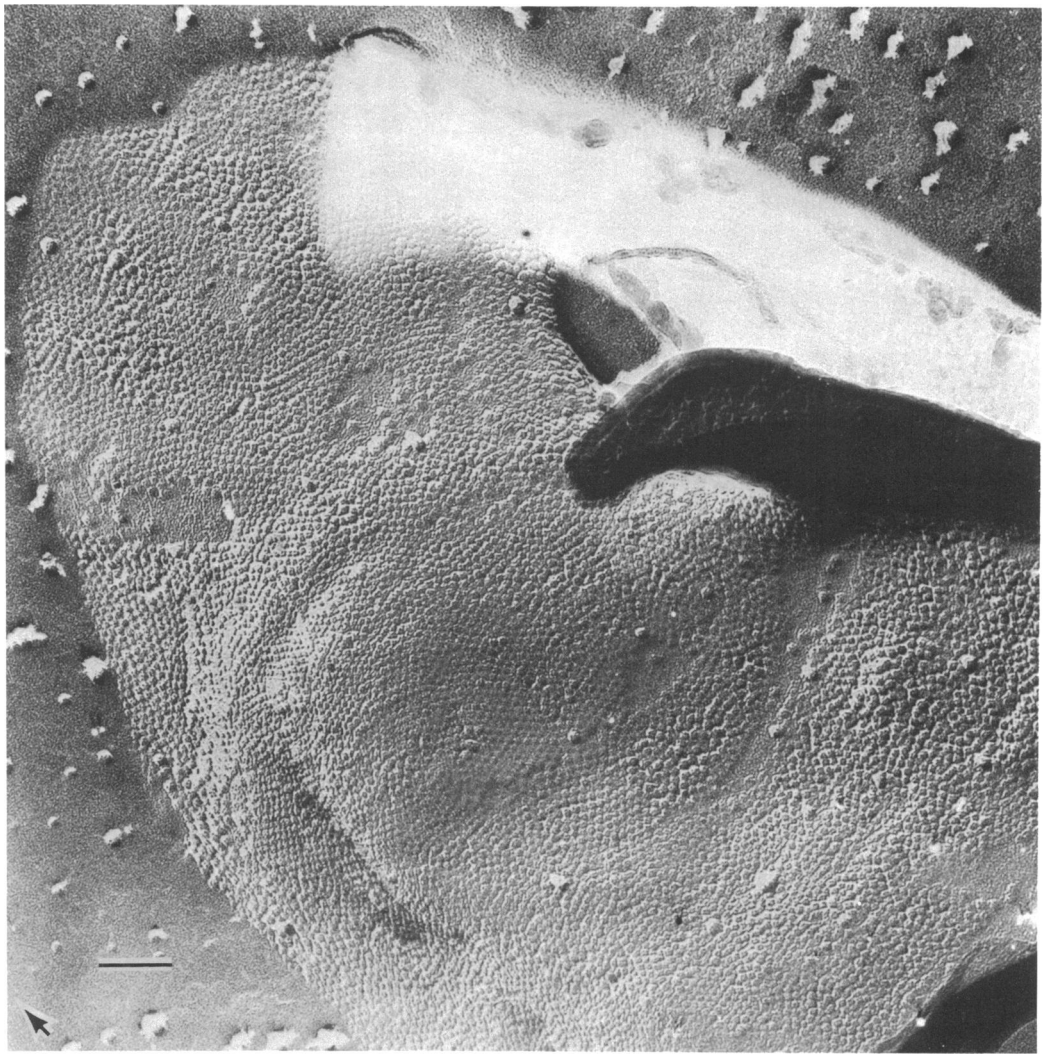

FIG. 9. *Freeze fracture of an SDS-wall fragment frozen in water, showing a crystalline array of cationized ferritin particles bound to its surface. Bar = 0.1 μm.*

phology was a product of (i) the manipulation required for wall isolation and purification, or (ii) some event that occurs during freeze fracture, such as a contamination process occurring at points along the fractured wall, it still seems that all of the evidence at hand suggests a nonhomogenous arrangement of polymers within isolated walls.

Dehydration of the wall by a variety of means (i.e., sublimation, Fig. 4B; phase separation of glycerol and water molecules, Fig. 6A; or dehydration for thin sectioning, Fig. 1) all seem to transform the two rows of cobblestones observed in freeze fractures into two denser bands of polymers enclosing a less dense central region (again compare Fig. 4A with Fig. 4B and Fig. 1). It seems that on dehydration, the globules may collapse to form the much more regular tribanded structure commonly observed in thin sections. That the wall remains tribanded even after non-peptidoglycan polymers are extracted with hot TCA (Fig. 6B) further supports the idea (23) that both peptidoglycan and accessory polymers are found at higher concentrations on either side of a central zone of lower density. However, we were not able to obtain evidence indicating that either accessory or peptidoglycan polymers might be enriched within any location within the tribanded wall. Our cationized ferritin-binding studies suggested that more anionic sites were located on the surfaces of SDS-treated walls than on the same walls after TCA extrac-

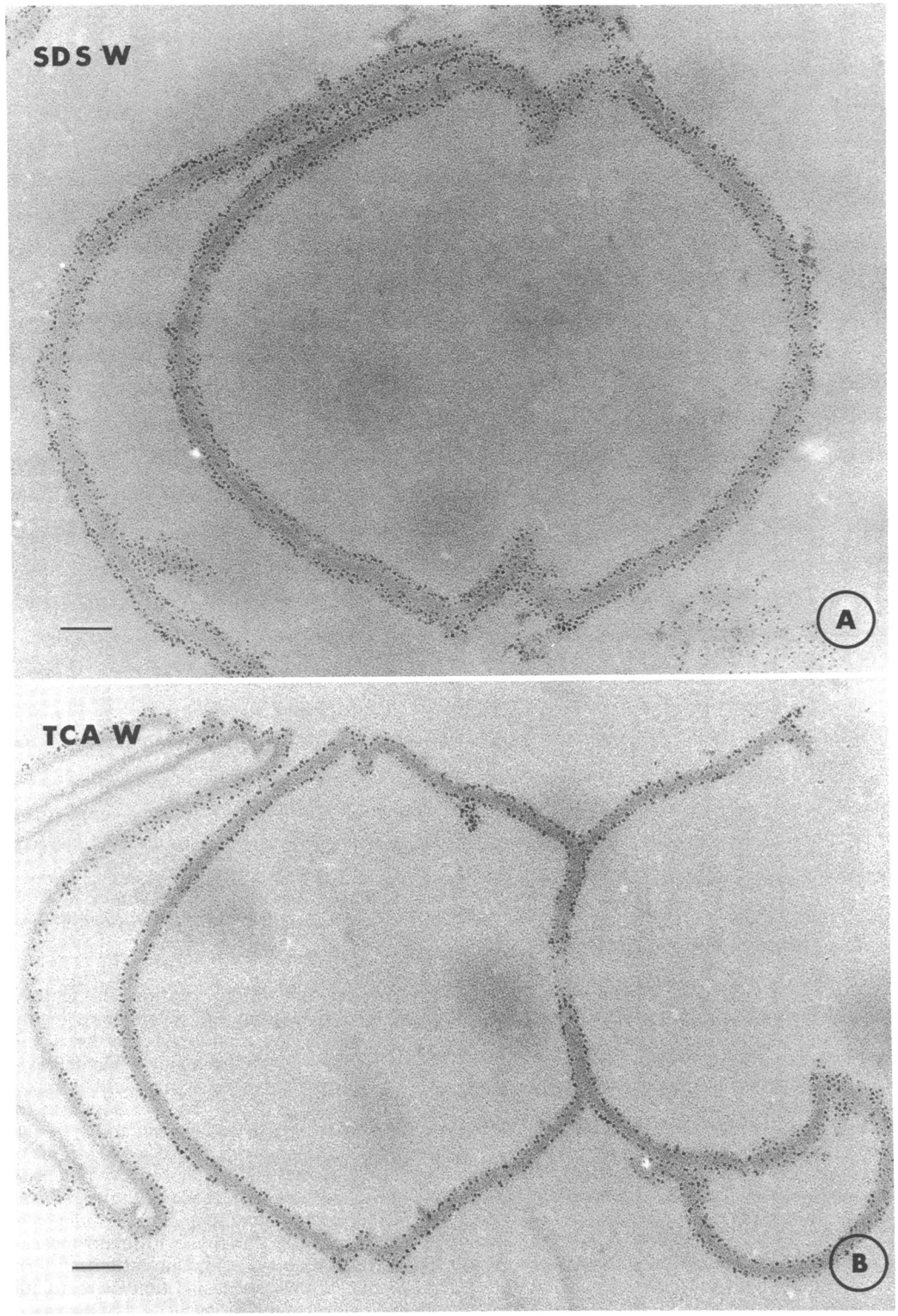

FIG. 10. *Thin sections of (A) SDS- and (B) TCA-walls which, prior to fixation and processing, were exposed to cationized ferritin. Bars = 0.1 μm.*

tion. However, this does not indicate that these superficial binding sites represent an enrichment of secondary or accessory wall polymers on wall surfaces.

While caution is obviously needed in interpreting our observations, it is tempting to consider that the rows of globules seen in fully hydrated walls might be subassemblies of wall units, or the "bricks" from which the wall is made. Such a subassembly would be consistent with current views of wall biosynthesis and assembly (22, 24, 30) and would also provide a mechanism for the remodeling of the assembled wall during surface growth and division (26). For example, one could visualize the hydrolysis of a few bonds between the bricks, permitting their topological rearrangement without loss from the wall structure and minimal decreases in osmotic protection. Subsequent formation of bonds between rearranged subunits via transpeptidation, transglycosylation, or transamidation would serve to stabilize a new conformation.

It is also tempting to speculate that the globules and channels observed in cross fractures of the isolated wall would not be a prominent feature of the morphology of the wall observed in whole cells (compare Fig. 3 with Fig. 4A) due to the regions between the globules being normally "filled in" with nonwall polymers. Proteins involved in wall assembly or modification or lipoteichoic acids might be good candidates for such "filler polymers." Lipoteichoic acids are membrane-associated polymers which, at least in several gram-positive species, are known to extend into and through the wall structure (31) and be excreted into the growth medium (20). Supposedly, during cell breakage, enough of these polymers would be extracted from the wall to make the globules and central channel visible. But, once again, caution is stressed, for these observations could also be explained equally well on the basis of a rearrangement of wall polymers (possibly aided by hydrolytic activity) which could occur as cells are broken and walls are purified.

It is apparent that to differentiate between various interpretations, much more work will be required. However, it seems from the results at hand that the structure of the gram-positive wall may be much more complex than our present knowledge of its biosynthesis and assembly might suggest.

ACKNOWLEDGMENTS

We thank U. Sleytr for preparing the replica for Fig. 8 and for his many invaluable comments during the preparation of this manuscript, M. P. O'Connor for excellent technical assistance in electron microscopy, and P. Tocci for her assistance in cell growth and chemical determinations.

This work was supported by research grant GB 31920 from the National Science Foundation and Public Health Service Grant AI 10971 from the National Institute of Allergy and Infectious Diseases. During the period of this work, M. L. H. was the recipient of Public Health Service Career Development Award KO4 GM 70251 from the National Institute of General Medical Sciences.

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