JOURNAL OF BACTERIOLOGY, Feb. 1973, p. 953-962

Vol. 113, No. 2
Printed in U.S.A.

Ultrastructure of the Cell Wall and Cytoplasmic Membrane of Gram-Negative Bacteria with Different Fixation Techniques

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Received for publication 26 October 1972

The ultrastructure of the cytoplasmic membrane and cell wall of two strains of *Escherichia coli*, *Proteus morganii*, *P. vulgaris*, *Acinetobacter anitratum*, *Moraxella lacunata*, *Erwinia amylovora*, *Acinetobacter* sp., and of a plant pathogen, unclassified gram-negative, fixed by the Ryter-Kellenberger procedure, was found to be significantly affected by the use or omission of the uranyl postfixation included in that procedure, and by the presence or absence of calcium in the OsO_4 fixative. The omission of the uranyl treatment results in a less clear profile of both the outer membrane of the cell wall and of the cytoplasmic membrane. The observation of these two membranes is further limited when both uranyl and calcium are omitted. The R-layer and the material covering the surface of the cell wall appear more distinct when the uranyl postfixation is not used. Evidence is given suggesting that the influence of uranyl and calcium ions on the appearance of the outer and cytoplasmic membranes would be primarily due to their action as fixatives, whereas the influence of uranyl on the appearance of the R-layer would be due to a direct action on the peptidoglycan component of this layer. When uranyl acetate is used as a section stain after the embedding in plastic, it improves the observation of the R-layer. In this case, a well contrasted R-layer is consistently observed in all strains studied, provided that the postfixation has been omitted. The frequent difficulty in clearly observing the R-layer in many published micrographs probably results from the common use of uranyl postfixation.

Previous studies (3, 18, 20, and M. T. Silva, Proc. 6th Int. Cong. Electron Microscopy, Kyoto, 1966, p. 275-276) using electron microscopy of thin sections have shown that the ultrastructure of the membranous components of several gram-positive bacteria is dependent on the conditions of chemical fixation. In the present paper, additional observations are described regarding the influence of calcium and uranyl ions, as used in the Ryter-Kellenberger (R-K) technique (15), on the ultrastructure of the cell wall and cytoplasmic membrane of several gram-negative microorganisms. Part of this work was previously presented in abstract (J. C. F. Sousa and M. T. Silva, Proc. 6th Annu. Meet. Portug. Soc. Electron Microscopy, Abstr. 13, 1971).

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MATERIALS AND METHODS

Microorganisms. The following strains were used: *Escherichia coli* (NCTC 8196); *E. coli* (enteropathogenic $O_{111}B_4$, clinical isolate); *Proteus morganii* (strain K from the collection of the Department of Bacteriology and Parasitology, Faculty of Medicine, University of Porto, Portugal); *P. vulgaris* (CCM 1956); *Acinetobacter anitratum* (NCTC 8102); *Moraxella lacunata* (NCTC 7784); *Erwinia amylovora* (strain 595, National Collection of Plant Pathogenic bacteria, Harpeden, Herts, England); *Acinetobacter* sp. isolated and kindly supplied by Dinah Abram. University of Pittsburgh, and previously considered as *Herellea vaginicola* (J. M. Klemencic and D. Abram, Bacterial Proc., p. 43, 1971); and an unclassified gram-negative isolated from the roots of a *Triticale*, (*Triticum vulgare* Linnaeus), currently under study and hereafter referred to as strain 2.

Culturing conditions. *E. amylovora* was grown in nutrient-yeast extract-glucose broth (23). *Acinetobacter* sp. was grown in Trypticase (BBL, 0.2%)-yeast extract (BBL, 0.04%), plus $Ca(NO_3)_2$ (0.013%);

pH 6.8. All other strains were grown in tryptone broth (1% tryptone [Difco], 0.5% NaCl; pH 7.2). All cultures were carried out at optimal temperatures with aeration by shaking, until the middle of the exponential phase was reached.

Production of ethylenediaminetetraacetic acid-lysozyme spheroplasts. *E. coli* NCTC 8196, grown as described above, was treated with lysozyme (muramidase, EC 3.2. 1.17; Sigma Chemical Co., egg white, 3× crystallized, specific activity 18,000 units/mg) by a modification of the method of Repaske (13): a pellet of centrifuged cells was washed once with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) and suspended in the same buffer (0.3 M) supplemented with sucrose (0.5 M), ethylenediaminetetraacetatic acid (EDTA) (1.6 mg/ml), and lysozyme (1 mg/ml). After 20 to 30 min at room temperature (24 C), the resulting spheroplasts were fixed as described below. Cells of the same culture treated as above, except that EDTA or lysozyme were omitted from the buffer, served as control.

Electron microscopy. Intact bacteria were fixed at room temperature (24 C) by the R-K procedure (15) usually without the prefixation step, as described elsewhere (18). In some experiments with *E. coli* 8196 and *A. anitratum*, calcium was omitted from the R-K OsO_4 fixative. After brief washing in R-K buffer, the fixed specimens were either directly dehydrated or postfixed with uranyl acetate (E. Merck AG, Darmstadt, Germany) (15, 20) using a 0.5% solution either in R-K buffer (final pH 5.1) or in water (final pH 3.9) for 30 to 120 min at room temperature (24 C). In experiments with *E. coli* 8196, the uranyl postfixation was also carried out for 24 hr. Samples of the same specimens were treated under the same conditions with 0.2 M aceto-acetate buffer (pH 3.9) to serve as a control for the standard uranyl treatment. To test the action of uranyl acetate after the dehydration step, the standard uranyl treatment was applied to samples of *E. coli* 8196 and strain 2, dehydrated to 100% ethanol, and then rehydrated back to water, as previously described (20).

For the fixation of the spheroplasts, glutaraldehyde (TAAB Laboratories, Reading, England; stock 25% solution) was added to the suspension of *E. coli* 8196 in sucrose-EDTA-Tris-lysozyme to a final concentration of 1.0%. After 1 hr at room temperature (24 C), the cells were collected by centrifugation (2,000 × *g*, 10 min) and the pellets were fixed in R-K OsO_4 fixative without any intermediate washing. The postfixation in uranyl acetate, when used, was carried out as described above.

The fixed specimens were processed for electron microscopy as described previously (20). The sections were contrasted with uranyl acetate (see legends to Fig.) and lead citrate (22) and, in some experiments, with thallium sulfate (10). For observation, an AEI EM 6 G electron microscope and a Siemens Elmiskop I A electron microscope were used at 80 kv, with double condenser, 50 μm objective apertures, and anticontamination devices.

Preliminary observations with *E. coli* 8196 showed that there is better view of the R-layer when the prefixation step of the R-K technique is omitted. All the micrographs presented in this work, regardless of the use or omission of the uranyl acetate postfixation or of the presence or absence of calcium in the OsO_4 fixative, are of bacteria prepared by the R-K procedure without prefixation.

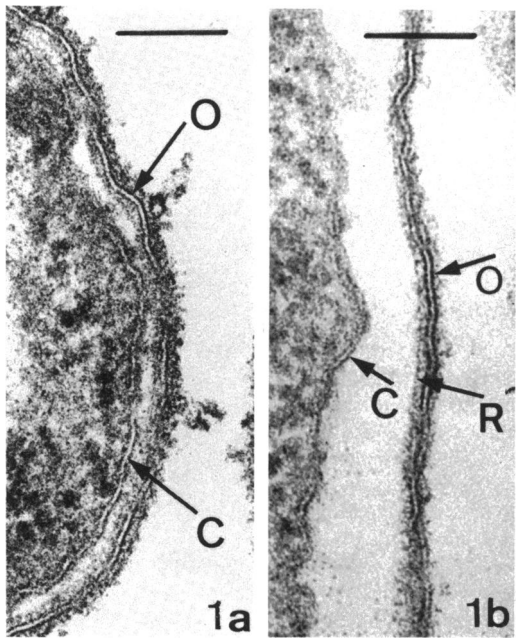
To determine the thickness of the envelope layers, a Bausch & Lomb Measuring Magnifier was used on prints enlarged to 200,000×. The electron microscopes were periodically calibrated with a grating replica grid (Ladd Research Industries, Inc.).

The terminology which has been used in the literature concerning the layers of the cell wall of gram-negative bacteria is not uniform. For the sake of clarity, in the present paper the following terms will be used: (i) outer membrane (indicated in the figures by an O), for the double-track component; (ii) R-layer (indicated in the figures by an R) for the single dense component located between the outer membrane of the cell wall and the cytoplasmic membrane.

RESULTS

The profile of the cytoplasmic membrane of all strains studied is clearest when both calcium and uranyl ions are used in the fixation procedure (Fig. 1a and b, 6, 9a and b, 11). When the postfixation with uranyl is omitted or substituted by the treatment with acidic buffer, bacteria fixed by the R-K OsO_4 with calcium usually exhibit a less defined profile of the cytoplasmic membrane (Fig. 2, 7, 10, 12). A poor definition of the membrane profile is also observed in cells fixed by the R-K OsO_4 without calcium followed by the uranyl postfixation (Fig. 3). When the uranyl postfixation is omitted in preparations fixed under conditions of calcium deficiency, the cytoplasmic membrane is usually unobservable in the strains studied (*E. coli* 8196 and *A. anitratum*, Fig. 4 and 8).

An outer cell wall membrane (Fig. 1a and b, 6, 9a and b, 11) with a very distinct profile is visible in all bacteria studied when the R-K OsO_4 fixative contains calcium and the postfixation with uranyl acetate is used. In all the strains studied, mainly in *E. coli* 8196, a slightly asymmetric profile (the outer layer being denser than the inner layer) is sometimes apparent (Fig. 1a). This is less frequent in samples postfixed with uranyl acetate for 24 hr. In the space between the outer membrane and the cytoplasmic membrane, a third layer is occasionally visible. This layer, due to its location in the cell wall (6) as well as its sensitivity to lysozyme in the presence of EDTA (10) (*see below*), corresponds to the R-layer. Frequently it has a loose structure and shows discontinuities (Fig. 1a and b, 6, 9a and b, 11). In many preparations of *E. coli* (both



All figures correspond to sections contrasted with uranyl acetate (aqueous saturated solution) for 30 min at room temperature (24 C), followed by lead citrate (5 min). In all figures line indicates 0.1 μm.

FIG. 1a and b. *E. coli 8196, fixed by the R-K technique followed by the standard postfixation in uranyl acetate. Notice the almost symmetric profile of the outer membrane, the loose and discontinuous aspect of the R-layer, and the symmetric cytoplasmic membrane.*

strains) and *Proteus* (both strains), the R-layer is completely absent or only traces of it can be discerned (as in Fig. 1a and b). The latter aspects are more frequent when uranyl acetate is used in water and for extended times (over 2 hr). The described appearance of the R-layer in cells fixed by the R-K procedure including the postfixation with uranyl acetate is observed in sections contrasted either with lead citrate or thallium sulfate.

When the postfixation is omitted, the cell wall of all the bacteria studied always has a structure composed of the outer membrane plus a distinct and continuous R-layer (Fig. 2, 7, 10, 12). This layer remains very distinct in sections contrasted with uranyl acetate for long periods of time (up to 24 hr) followed by lead citrate (5 min). The thickness of R-layer is somewhat different according to the strain (Table 1). In cells showing plasmolysis this layer remains in contact with the outer membrane (Fig. 1b, 2, 5b).

When the uranyl acetate treatment is applied to specimens (*E. coli* 8196 and strain 2) dehydrated to absolute ethanol and rehydrated back to water, the single dense layer does not appear well preserved. Instead, an aspect identical to that described above for samples postfixed with uranyl under the standard conditions is visible.

When the postfixation is omitted, the outer membrane of all strains studied exhibits a profile less clear than that observed when uranyl treatment is employed. In *E. coli* (both strains), *Proteus* (both strains), *Acinetobacter* sp., and strain 2, the inner layer of the outer membrane is more affected, resulting in an asymmetric profile (Fig. 2, 10).

The influence of calcium was studied in *E. coli* 8196 and *A. anitratum*. In *E. coli* 8196, omission of calcium from the R-K OsO_4 fixative results in an outer membrane almost as asymmetric as that described above (Fig. 3); omission of both calcium and uranyl ions results in an inner layer even less dense than when only uranyl was omitted, and sometimes this layer is almost invisible (Fig. 4). In *A. anitratum*, omission of both cations also results in a poor observation of the outer membrane, but the asymmetry is not so marked as in *E. coli* 8196 (Fig. 8).

Staining of sections even for long periods of time (up to 24 hr) with uranyl acetate does not result in any improvement in the observation of the outer membrane as compared with that obtained with the uranyl acetate treatment before dehydration (postfixation).

Treatment of specimens with aceto-acetate buffer (*p*H 3.9), in place of uranyl postfixation, does not result in the effects obtained with the uranyl postfixation.

Treatment with lysozyme in sucrose-EDTA-Tris buffer results in the disappearance of the R-layer in the strain studied (*E. coli* 8196) as shown in Fig. 5a. In control cells (lysozyme or EDTA omitted), this layer is clearly visible when uranyl postfixation is not used (Fig. 5b).

The surface of *E. coli* (both strains), *A. antitratum*, and *Acinetobacter* sp. appears covered with a material which is more abundant and compact when the postfixation is not used (Fig. 2, 7, 8, 12). This effect is particularly evident in the case of *E. coli* and *A. anitratum*.

DISCUSSION

In the present work it is demonstrated that variations in the R-K procedure, the most widely used technique in the study of bacterial ultrastructure, influence the final appearance of the cytoplasmic membrane and cell wall in thin sections of several gram-negative bacteria.

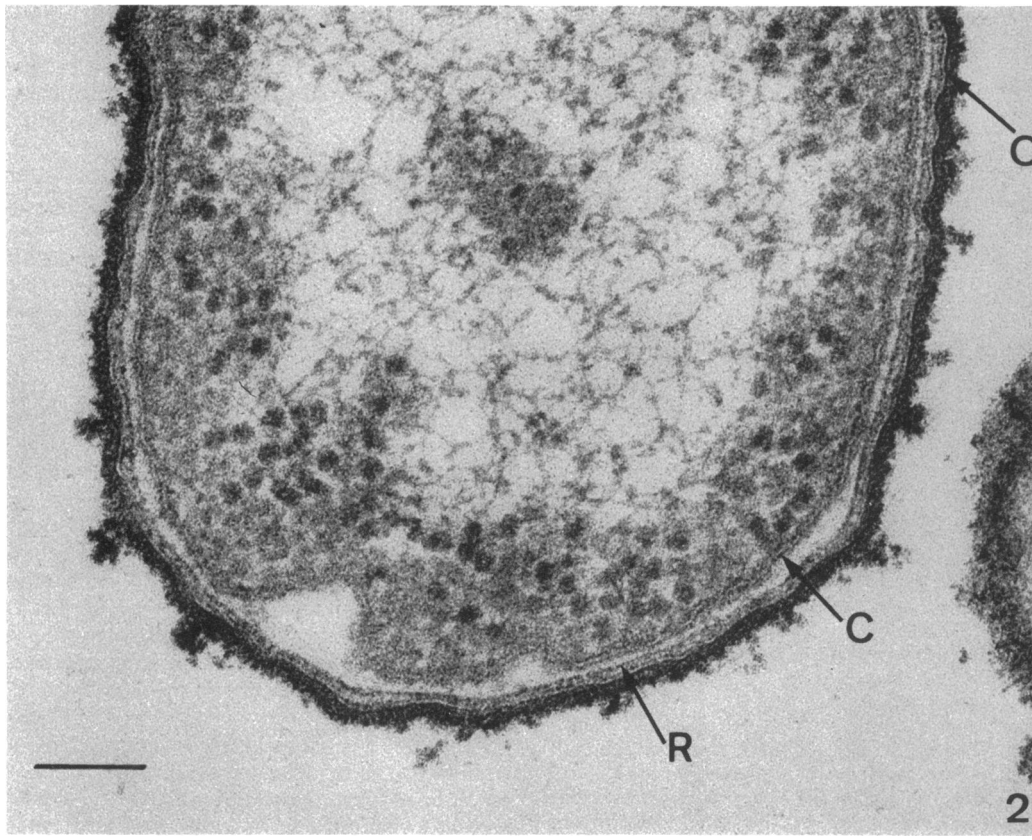


FIG. 2. *As in Fig. 1, but without postfixation in uranyl. The outer membrane has a very asymmetric profile. The R-layer is very clear. The cytoplasmic membrane is not clear. The material covering the cell wall is more abundant and compact than in Fig. 1.*

The presence of calcium in the OsO_4 fixative and the use of the postfixation with uranyl acetate are essential to obtain a well defined profile of the cytoplasmic membrane in the strains studied in the present work. The same was previously reported for gram-positives (18, 20, and M. T. Silva, Proc. 6th Int. Cong. Electron Microscopy, p. 275, 1966). When both cations are used, the cytoplasmic membrane of the gram-negatives studied appears continuous and exhibits a symmetric profile; i.e., both dense layers of the triple structure have similar densities. At present, it is not known whether the differences in the profile resulting from the variations in the fixative techniques under discussion are due to the contrasting effect of calcium and uranyl ions or whether they result from a fixative action, or from both. The fact that the omission of both calcium and uranyl results in an almost or completely invisible cytoplasmic membrane, even in sections intensely contrasted with uranyl acetate, suggests that these ions are acting primarily as fixatives, although a contrasting action is expected to coexist. A similar situation is known to occur when OsO_4 is used. This compound is employed primarily for its fixative action, but, due to the electron density of osmium, some contrast is conferred to the structures which interact with it. In gram-positive bacteria it was demonstrated (20), by the study of lipid loss during the dehydration step, that both calcium and uranyl do play a fixative role. A similar quantitative study (*unpublished results*) showed that the phospholipid loss during the preparation for electron microscopy of *E. coli* 8196 is greater when uranyl acetate is not used in the postfixation than when it is used; the amounts of lipid phosphorus lost when both calcium and uranyl ions are omitted are even greater. Since the outer membrane of gram negatives also contains phospholipids (*see below*), at least part of the lost lipid may originate from the cell wall.

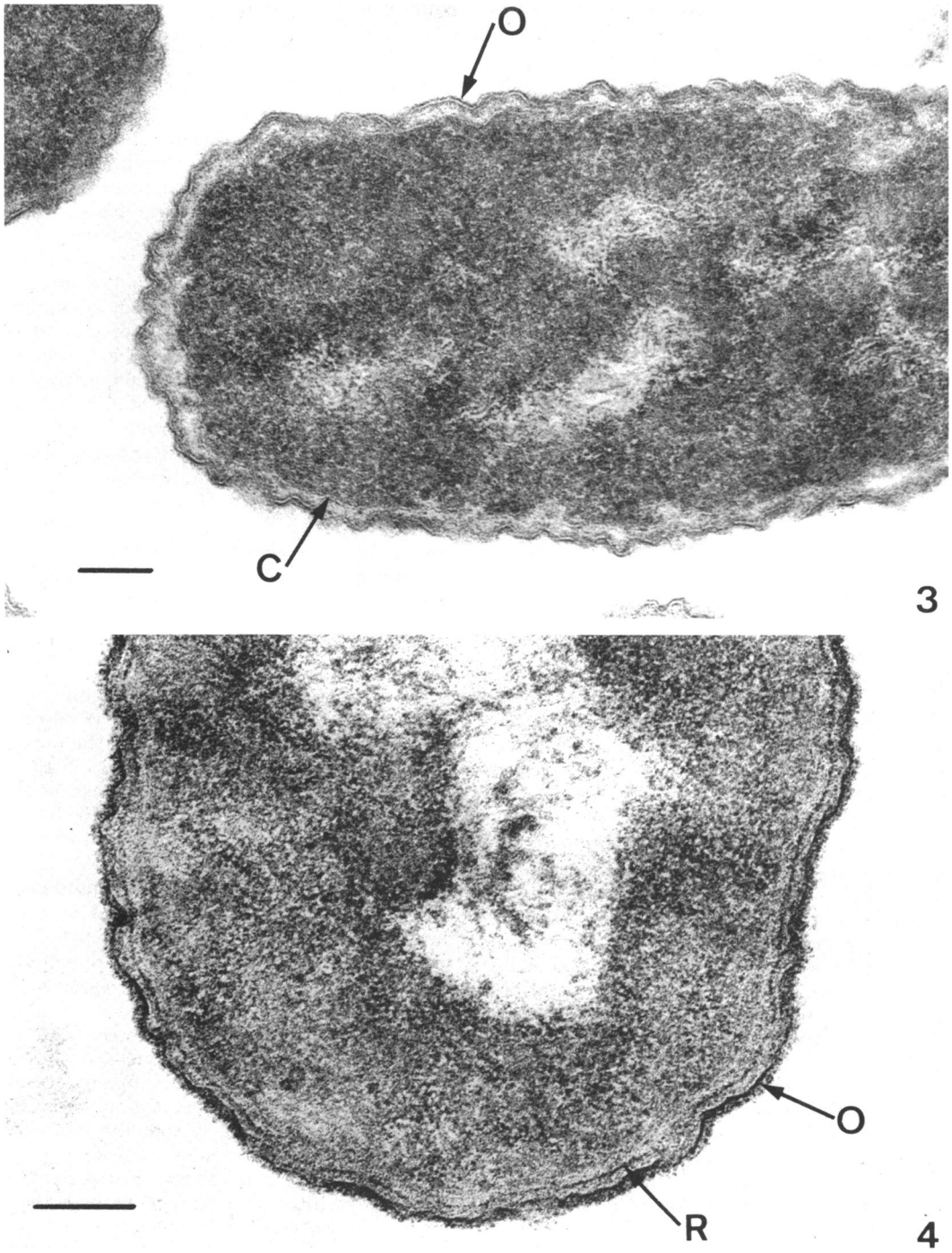


FIG. 3. *As in Fig. 1, but calcium omitted from the R-K OsO_4 fixative. The R-layer is almost invisible. The cytoplasmic membrane is not well defined. The outer membrane is asymmetric.*

FIG. 4. *As in Fig. 3, but postfixation in uranyl is omitted. The inner layer of the outer membrane and the cytoplasmic membrane are almost invisible. The R-layer is clearly seen.*

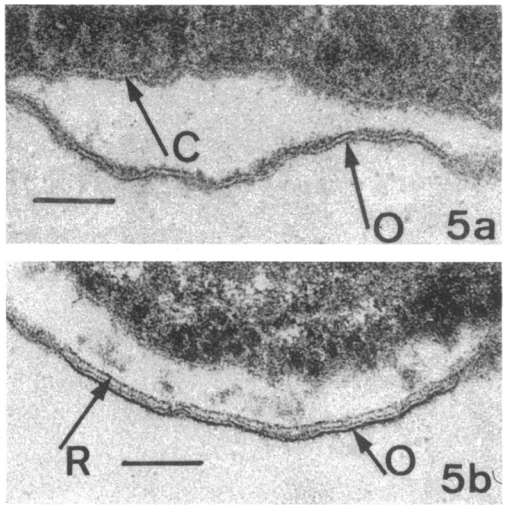


FIG. 5. *a, E. coli 8196 treated with lysozyme in sucrose-Tris-EDTA, prefixed with glutaraldehyde, and fixed with the R-K procedure without postfixation in uranyl. Notice the absence of the R-layer. b, As in Fig. 5a, but EDTA omitted from the buffer. The R-layer is clearly apparent.*

The observation that the treatment with acidic buffer does not result in any improvement in the preservation of the cytoplasmic membrane suggests that the effect of uranyl acetate postfixation is not due to the low *p*H of the solution. The same was previously reported for bacilli (20).

As to the ultrastructure of the cell wall, the present study shows that the fixation conditions have a significant influence on the visibility of the R-layer. As reported in the present paper, when the treatment with uranyl acetate is used, as is common practice since the work of Ryter and Kellenberger (15), the R-layer is not so clearly demonstrated as in the preparations obtained after fixative procedures omitting that postfixation. This observation may explain the fact, recently stressed by Forsberg et al. (4) that, in many published electron micrographs of the cell wall of gram-negative bacteria, the R-layer is frequently not visible. In most of these cases the postfixation with uranyl acetate was used. The loose material observed in place of the continuous dense R-layer in preparations obtained by using fixative procedures which include the uranyl acetate postfixation may represent the remnants of that layer. Whether the protein component described by Weidel et al. (25) is present in such loose material is not yet clear. Since the treatment with aceto-acetate buffer at the lowest *p*H value of the uranyl acetate solutions does not have the same effect as these solutions on the visibility of the R-layer, it seems likely that a direct action of UO_2^{2+} is involved. The main chemical component of the R-layer is the peptidoglycan macromolecule (25). Preusser (12) has shown, by electron microscopy of shadowed preparation of *Spirillum serpens*, that treatment of the isolated peptidoglycan "sacculus" with uranyl acetate results in a shrinkage or disruption of this polymer. The influence of the postfixation on the preservation of the R-layer described in the present paper may be due to this action of uranyl acetate. It must be considered, however, that in our case the peptidoglycan has been treated with OsO_4 and calcium prior to the postfixation with uranyl and then dehydrated and embedded. It is also possible that uranyl treatment renders the R-layer more sensitive to the subsequent treatments for ultramicrotomy. As described, when sections of *E. coli* 8196 and strain 2 fixed only with R-K OsO_4 are stained with uranyl acetate even for long periods (up to 24 hr) before lead staining, the R-layer appears very distinct. On the other hand, when the uranyl treatment was applied to specimens of those two strains after dehydration to absolute ethanol, damage of the R-layers still occurs. These observations indicate that only after embedding in plastic does the R-layer become resistant to the damaging action of the uranyl ions. However, it remains stainable by these ions. Consequently, to take advantage of the contrasting action of UO_2^{2+} towards the R-layer, the uranyl acetate should be used as a section stain.

The fine structure of the outer membrane of the cell wall of the bacteria studied also appears to be influenced by the use or omission of the uranyl postfixation. The visibility of the inner layer of that membrane is clearer when uranyl treatment is used than when it is omitted or substituted by treatment with an acidic buffer. The simultaneous omission of calcium and uranyl from the fixation procedure results in even poorer observation of the outer membrane. It seems likely that the improved observation of the outer membrane induced by the use of uranyl and calcium in the fixation procedure results from a fixative action of these ions, as in the case of the cytoplasmic membrane. As mentioned above in the discussion on lipid loss during the preparation of *E. coli* 8196 for ultramicrotomy, it is possible that part of the lost phospholipid is from the outer membrane. It is worth noting that the two components of the envelopes of gram-negative bacteria which exhibit a "unit membrane" (14)

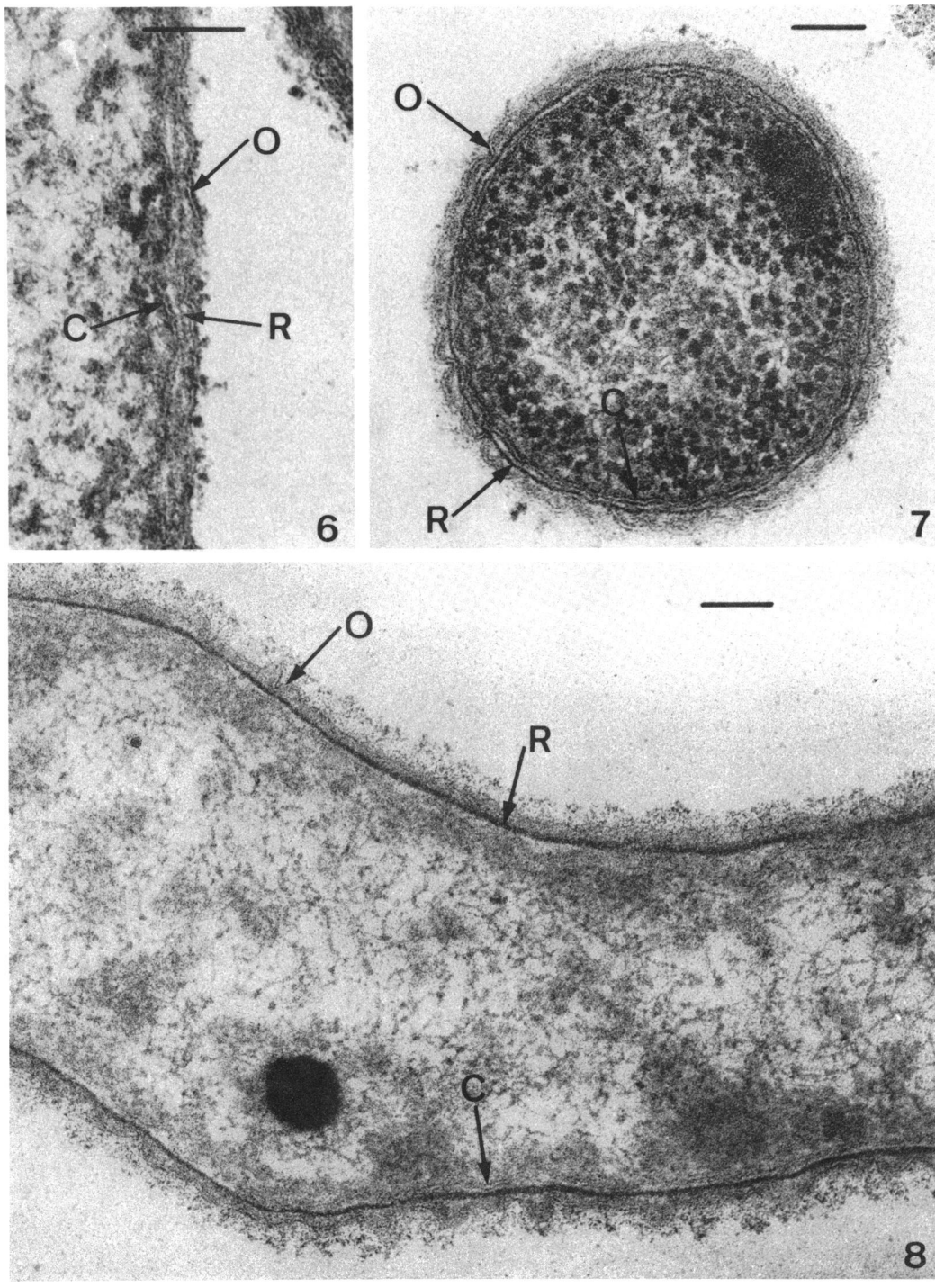


FIG. 6. *A. anitratum fixed by the R-K procedure, with postfixation in uranyl. The outer and cytoplasmic membranes have distinct and symmetric profiles. The R-layer is not well defined.*

FIG. 7. *As in Fig. 6, but without postfixation. The R-layer is better defined than in Fig. 6. The outer and cytoplasmic membranes exhibit less clear profiles.*

FIG. 8. *As in Fig. 7, but calcium omitted from the R-K OsO_4 fixative. The outer and cytoplasmic membranes are poorly defined. The R-layer is conspicuous.*

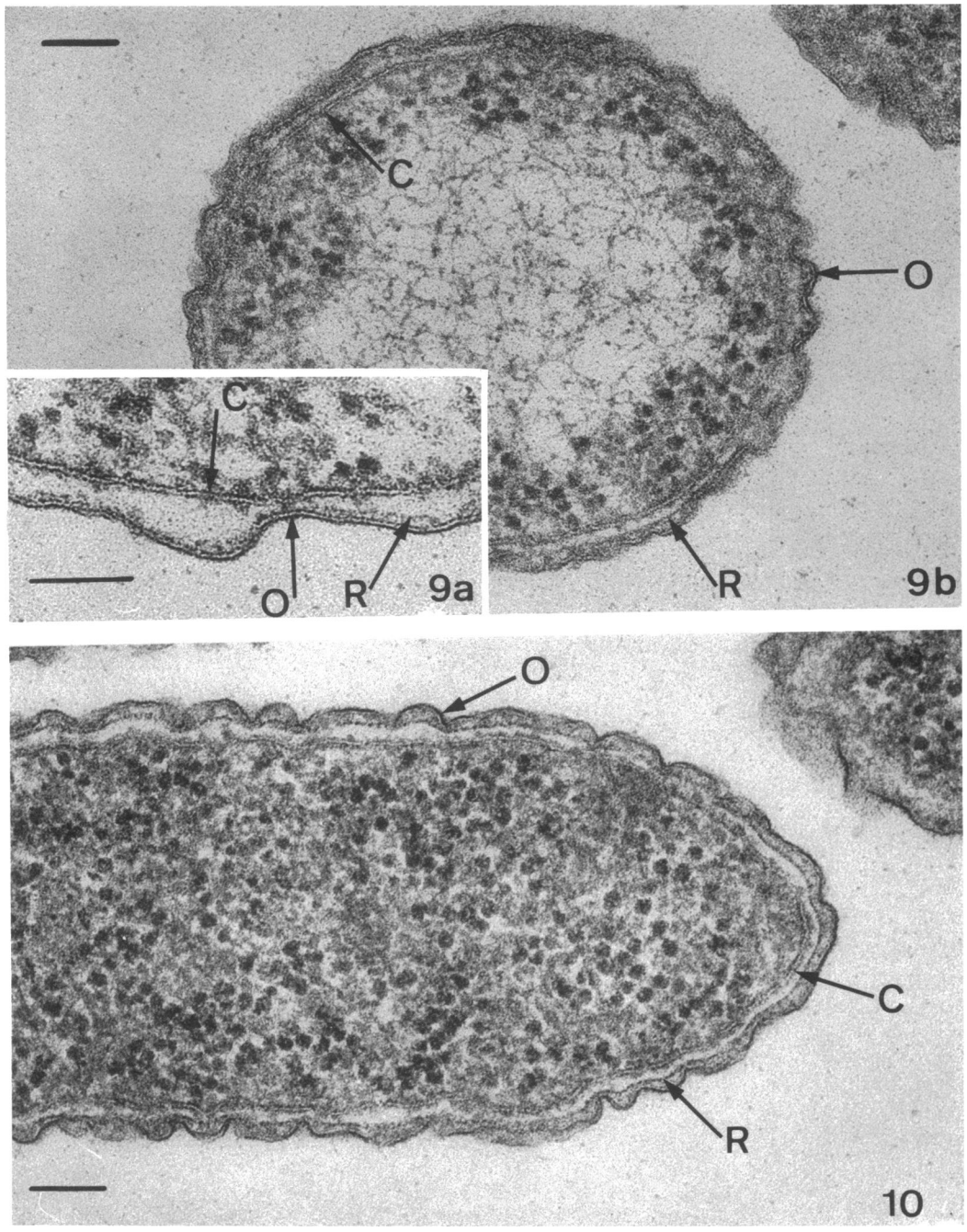


FIG. 9a and b. *Strain 2 fixed by the R-K procedure including the postfixation in uranyl. The outer and cytoplasmic membranes exhibit a symmetric and distinct profile. The R-layer is poorly defined.*

FIG. 10. *As in Fig. 9, but without postfixation. The R-layer is better defined, but the outer and cytoplasmic membranes are less distinct mainly in what concerns the inner layer of the outer membrane and the outer layer of the cytoplasmic membrane.*

profile, namely the cytoplasmic and the outer membranes, appear with an aspect which seems to correspond to a better preservation when calcium is present in the OsO_4 fixative and the postfixation with uranyl acetate is used. Under these conditions, these mem-

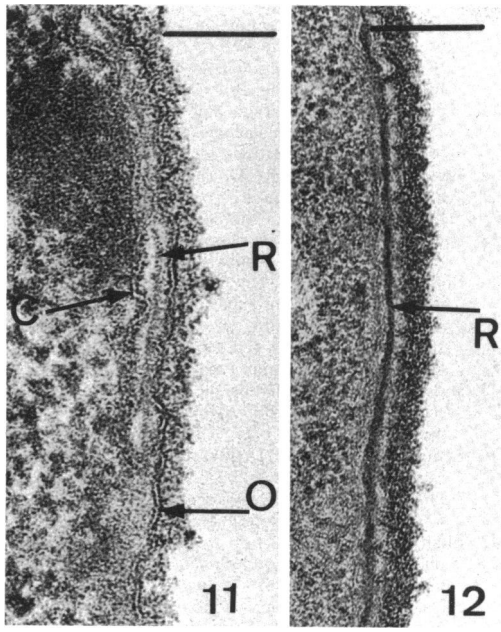


FIG. 11. *Acinetobacter sp. fixed by the R-K procedure with postfixation in uranyl. The outer membrane and the cytoplasmic membrane have clear and symmetric profiles. The R-layer exhibits a loose aspect.*

FIG. 12. *As in Fig. 11, but without postfixation. The R-layer is very conspicuous, and the profile of the outer and cytoplasmic membranes is less clear than in Fig. 11. The surface material is more abundant and compact than in Fig. 11.*

TABLE 1. *Thickness of the three components of the envelopes of five of the gram-negative organisms*[a]

Strain	Outer membrane	R-layer	Cytoplasmic membrane
E. coli 8196	7.6	3.4	7.5
E. coli $O_{111}B_4$	7.0	3.3	7.0
Acinetobacter sp.	7.2	5.3	7.3
A. anitratum	8.2	4.2	8.2
Strain 2	7.7	3.5	7.8

[a] The value (in nanometers) for each component is the mean of twenty independent determinations.

branes show, in general, a similar profile, in terms of thickness and geometry. The basic similarities between the outer and the cytoplasmic membranes were initially pointed out by Brown et al. (1). These authors showed that these two membranous components of the envelopes of gram-negative bacteria are not only structurally similar, but also function as permeability barriers. Another similarity between the outer and cytoplasmic membranes consists in the presence of phospholipids in both structures (see the review, reference 11). The improvement in the visualization of the two membranes under discussion induced by calcium and uranyl ions may be due to the strong affinity of these ions for the phosphate group of phospholipids (2, 5, 17). However, it should be taken into consideration that these ions also have affinity for proteins (9, 11*a*). In connection with this, it should be mentioned that Schnaitman (16) recently showed that the removal of two-thirds of the phospholipids from isolated *E. coli* cell walls does not significantly affect the ultranstructural appearance of the outer membrane.

The preservation of the surface material covering *E. coli*, *A. anitratum*, and *Acinetobacter* sp, also appears affected by the use of uranyl acetate in the fixative procedure. Such material is more abundant and compact when the postfixation is omitted, mainly in the two *E. coli* strains studied. In these strains it may correspond to the polysaccharide material described by Bayer (Bacteriol. Proc., p. 43, 1971). In *Acinetobacter* sp. it is composed, at least in great part, of proteins (J. M. Klemencic and D. Abram, Bacteriol. Proc., p. 43, 1971). In *A. anitratum* it looks similar to that described by Glauert and Thornley (7) in *Acinetobacter* strain 5. A similar effect of uranyl postfixation on capsular material was observed in *Bacillus anthracis* NCTC 7200 infecting mouse spleen (*unpublished results*). The mechanism by which the uranyl treatment affects the preservation of such materials with different chemical compositions is not clear.

Vye and Fischman (24) have shown that the glycogen in liver and heart tissues is removed by uranyl acetate postfixation, a treatment which is known to improve the preservation of other components of eukaryotic cells (19, 21, and M. A. Hayat, Proc. 27th Meet. Electron Microscop. Soc. Amer., p. 412, 1969). The observation that uranyl acetate postfixation in gram-negative bacteria improves the preservation of some components (outer membrane and cytoplasmic membrane) and reduces the preservation of others (R-layer and surface material) is another example of preferential fixation. Wood and Luft (26) have already stated that specific chemical effects are involved in fixations carried out under different conditions and that "at present the electron microscopist has less certainty about the concept of a single 'best' fixative but more flexibility in choice of fixative for specific purposes."

Finally, it should be stressed that not only the preservation of the membranous compo-

nents of gram-positive bacteria studied previously (3, 18, 20; M. T. Silva, Proc. 6th Int. Cong. Electron Microscopy, Kyoto, 1966, p. 275–276), but also that of the envelopes of several gram negatives, is significantly sensitive to the fixation conditions.

ACKNOWLEDGMENTS

The authors are grateful to Dinah Abram for stimulating discussions, to Audrey M. Glauert for reviewing the manuscript, and to M. Irene Barros for expert technical assistance.

This work was supported in part by Instituto de Alta Cultura and Fundação Calouste Gulbenkian, Lisboa, Portugal.

J.C.F.S. is a fellowship holder of the Fundação Calouste Gulbenkian, Lisboa, Portugal.

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