

# Ultrastructural Alterations Induced by Moist Heat in *Bacillus cereus*<sup>1</sup>

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The ultrastructural alterations induced in vegetative, exponentially growing *Bacillus cereus* cells suspended in broth, by heating at 65 C for 2 and 15 min and 100 C for 5 min, were studied by electron microscopy of thin sections. The following alterations were observed: (i) change in the triple-layered profile of the membranes from the normal asymmetric to a symmetric geometry, appearance of fractures in the membranes, occurrence of prominent myelin-like systems of concentric membranes; (ii) disappearance of the ribosomes in most cells heated at 65 C and in all cells heated at 100 C; (iii) cytoplasmic precipitation resulting in the appearance of dense blocks of Pronase-sensitive material. The cell wall appears unaffected in most preparations. No signs of deoxyribonucleic acid damage are observed. These ultrastructural data are discussed in relation to the alterations in the chemistry and physiology of heated bacteria described in the literature.

Thermal injury had been studied in several laboratories to try to understand the mechanism(s) by which bacteria are killed by heat. Most of the published works deal with the study of biochemical alterations, and only very few describe the ultrastructural modifications in gram-positive mesophilic nonsporulated bacterial cells (see references 6 and 9). In this paper some alterations in the fine structure of the vegetative cells of *Bacillus cereus* induced by moist heat, studied by electron microscopy of ultrathin sections, are described.

## MATERIALS AND METHODS

*B. cereus* strain NCTC 7587 was grown in tryptone broth (1% tryptone [Difco], NaCl 0.5%, pH 7.0) at 35 C, with aeration by shaking for 6 to 7 hr. For inoculum, a suspension of spores was used. The cells in the growth medium were placed in narrow test tubes prewarmed to the desired temperatures in thermostatically controlled water baths. Three different heat conditions were studied: 65 C for 2 and 15 min and 100 C for 5 min. The desired temperatures were attained in the bacterial suspensions in less than 30 sec.

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Control and heated cells, after quick cooling to room temperature (24 C), were fixed by the Ryter-Kellenberger (R-K) procedure (27), with or without the prefixation step. The postfixation with uranyl acetate (27, 33) was always used. The fixed specimens were embedded in Epon (18) after dehydration in ethanol, without infiltration in propylene oxide. Ultrathin sections were cut with an LKB Ultratome III with a diamond knife. The sections were mounted on copper 400-mesh grids coated with a Zaponlak (Riedel) film, and contrasted with lead-citrate (36) for 5 min. Micrographs were taken on Agfa-Gevaert Scientia 23 D 56 film in a Siemens Elmiskop I A or in an AEI EM 6G. Both microscopes were used at 80 kv, with a double condenser, 50- $\mu$ m objective aperture and anticontamination device.

Pronase (Taab Laboratories, Reading, England) dissolved in freshly distilled and deionized water to a final concentration of 1% was used to treat, for 1 hr at 37 C, sections previously oxidized with 3% (w/v) H<sub>2</sub>O<sub>2</sub> (Perhidrol Merck) for 10 min at room temperature (7, 22, 23, 30). After this treatment, the sections were stained with lead, as described above. For control, sections of similar thickness were treated under the same conditions, with the Pronase solution previously heated at 100 C for 4 min.

## RESULTS

Control cells fixed by the R-K procedure, including the prefixation step, are shown in Fig. 1 and 7. Figure 2 represents a control cell fixed by the same method without the prefixation step. The configuration of the membrane systems, i.e., mesosomes (13) is different ac-

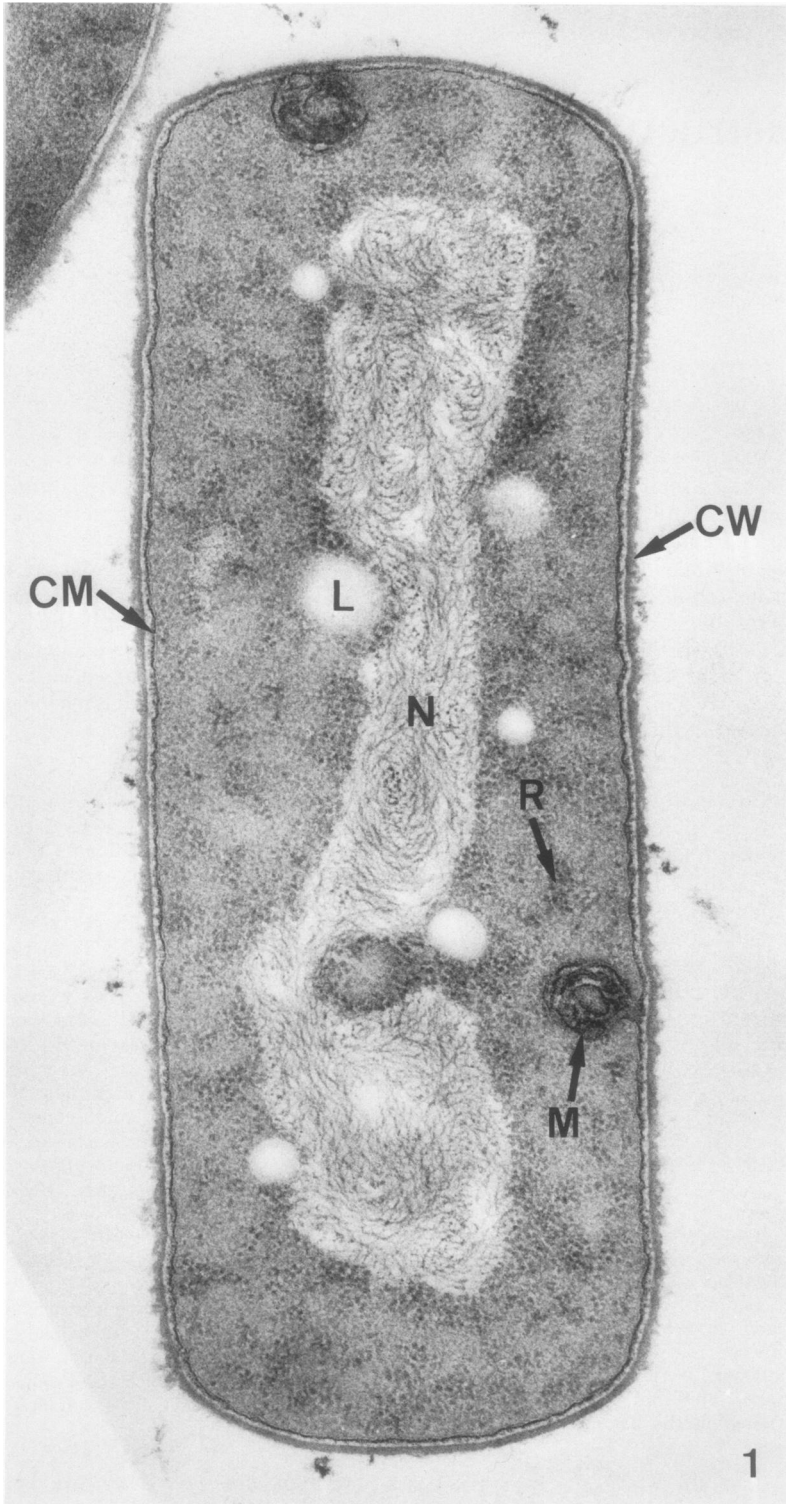


FIG. 1. Control *B. cereus* cell fixed by the R-K procedure including the prefixation. Notice the fibrillar nucleoid (N), the cytoplasm with clusters of ribosomes (R), empty lipid droplets (L), and mesosomes (M). The cytoplasmic membrane (CM) has an asymmetric profile. The periplasmic space between the cytoplasmic membrane and the cell wall (CW) is restricted. This and all subsequent sections were contrasted with lead for 5 min.  $\times 64,000$ .

ording to the fixation technique. More pictures of this *B. cereus* strain prepared under the same conditions can be seen in previous papers (32, 33).

Several ultrastructural alterations are apparent in heated bacteria. The distribution of the alterations among the cells of the same preparation is not regular, bacteria with different ultrastructural patterns being visible side by side.

**Cell wall.** The majority of the preparations show no significant modifications in the cell wall fine structure (Fig. 3, 4, 12-14). However, in a few samples, the cell wall appears with a disrupted surface, a continuous layer remaining in its inner portion (Fig. 6 and 10). In such cells the septa exhibit a loose layer sandwiched between two continuous layers (Fig. 10).

**Membranes.** In cells heated at 65 C for 15 min or at 100 C for 5 min, the cytoplasmic membrane always appears with a symmetric profile, that is, the triple-layered structure exhibits two dense layers with similar densities (Fig. 3-6, 8-12). Fractures are frequently seen in these symmetric membranes (Fig. 3 and 8).

Prominent systems of concentric membranes, resembling hydrated "myelin-like figures," occur in these cells, both after the complete R-K procedure and after this method with prefixation omitted (Fig. 3-6). In some sections these systems are seen in continuity with the cytoplasmic membrane (Fig. 4). The membranes composing these systems are always symmetric and frequently appear broken (Fig. 3 and 4). Parallel arrays of symmetric membranes are occasionally seen at the cell periphery (Fig. 10). No vesicular intracytoplasmic systems are seen in these cells, only a few dispersed vesicles being present in some sections (Fig. 3-5). The periplasmic space is usually enlarged (Fig. 3-5).

The alterations described above are visible in some cells heated at 65 C for 2 min. However, in other cells the membranous alterations are much less important. For instance, the occurrence of fractures is observed in very few cells. The cytoplasmic membrane appears sometimes with an asymmetric profile similar to that of control cells (Fig. 13 and 14). Vesicular membrane systems (Fig. 12-14), identical to those observed in control cells when the pre-

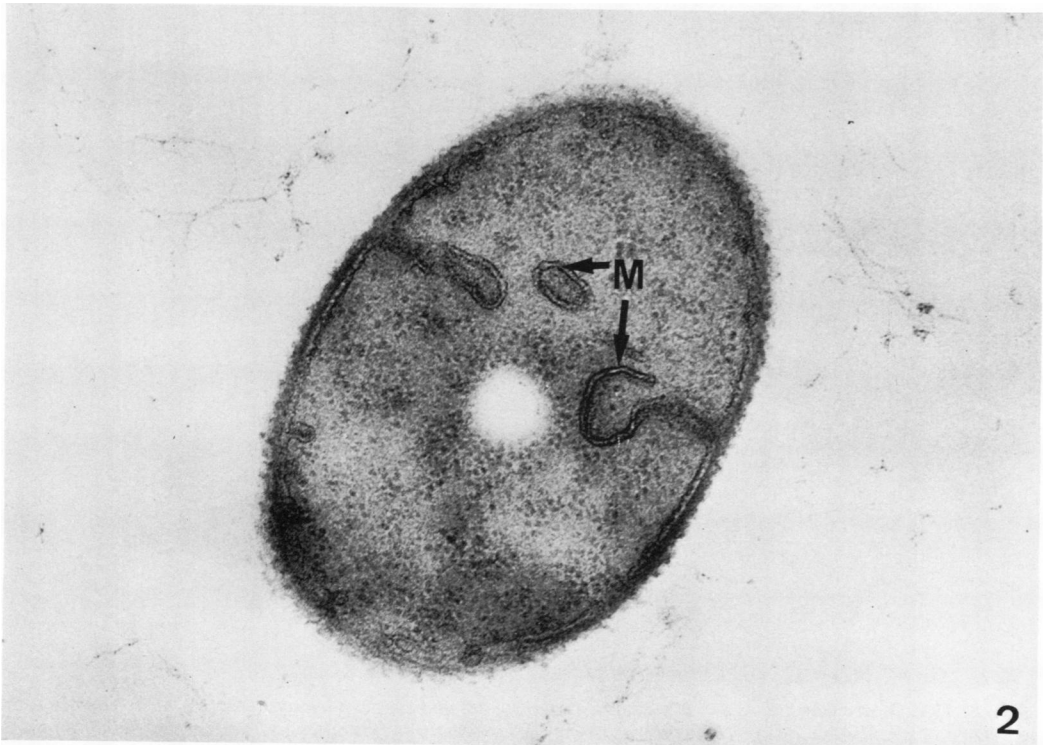


FIG. 2. As in Fig. 1, except that the prefixation step was omitted. Mesosomes (M) are very simple and small.  $\times 68,200$ .

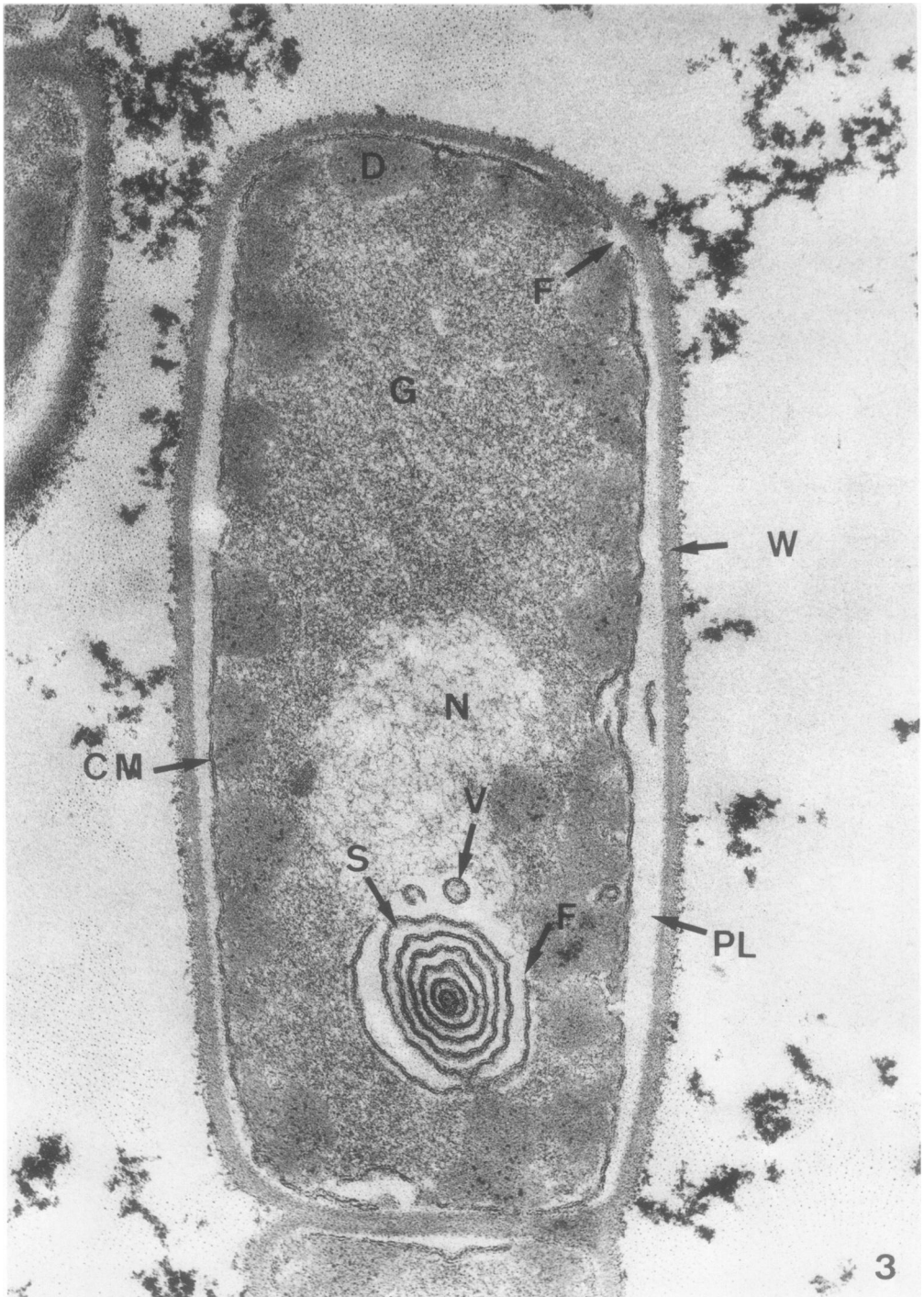


FIG. 3. Cells heated at 100 C for 5 min and fixed by the R-K procedure without pre-fixation. Notice the occurrence of prominent intracytoplasmic systems of concentric membranes (S). A few small membranous vesicles (V) are present. The cytoplasmic membrane (CM) as well as the membranes of the intracytoplasmic systems show symmetric profiles. Fractures (F) are visible in both types of membranes. No ribosomes are visible in the cytoplasm which exhibits a granular (G) background and numerous dense blocks (D). The cell wall (CW) and the nucleoid (N) appear comparatively unaffected.  $\times 68,000$ .

fixation step is used (Fig. 1), are present in some sections regardless of the use or omission of the prefixation. These mesosomes (13) exhibit the typical connection with the periplasmic space (Fig. 12-14) and contacts with the nucleoids (Fig. 12). In a few cells prepared by the R-K procedure without prefixation, the mesosomes appear small and simple as in control cells prepared by the same technique. In the bacteria exhibiting these small and simple mesosomes, no ultrastructural alterations concerning other cell components are observed. Plating of suspensions heated at 65 C for 2 min revealed that many bacteria remain viable. It is possible that the cells exhibiting the reduced alterations just described correspond to those bacteria able to resume growth.

**Cytoplasm.** Two main features are characteristic of cells heated at 65 C for 15 min or at 100 C for 5 min. No ribosomes are visible, except in a few cells corresponding to bacteria heated at the lower temperature (Fig. 6 and 11). Most sections show a granular cytoplasm arranged in compact and dense blocks (Fig. 3-6, 10) dispersed among areas of a much looser texture (Fig. 3, 4, 6, 10). The dense blocks show some tendency to be located at the cell periphery (Fig. 3-5). The density of these blocks is reduced by the treatment with

Pronase as shown in Fig. 11. The lipid droplets present in control cells (Fig. 1) are usually absent in these heated bacteria. Some cells heated at both temperatures studied exhibit an empty cytoplasm (Fig. 5) suggesting a considerable loss of cytoplasmic material.

Heating at 65 C for 2 min occasionally results in the appearance of the above-described cytoplasmic alterations in the same cell. The first alteration to appear seems to be the production of dense blocks which can be seen in bacteria without any other sign of damage (Fig. 13 and 14). Cells with ribosomes are much more frequent than after heating at 65 C for 15 min. Lipid droplets are frequently seen in cells subjected to this less intense heating (Fig. 12).

**Nucleoid.** Areas with the structural characteristics of deoxyribonucleic acid (DNA) material are visible in favorable sections. These areas show fibrils cut in several plans. The resulting image (Fig. 10 and 11) is identical to that of the nucleoid of control cells (Fig. 1), except in cells with signs of increased hydration. The periphery of the nucleoid is sometimes separated from the surrounding cytoplasm by a clear space (Fig. 10). It seems that such separation, not seen in the control cells (Fig. 1), is due to a shrinkage of the cytoplasm.

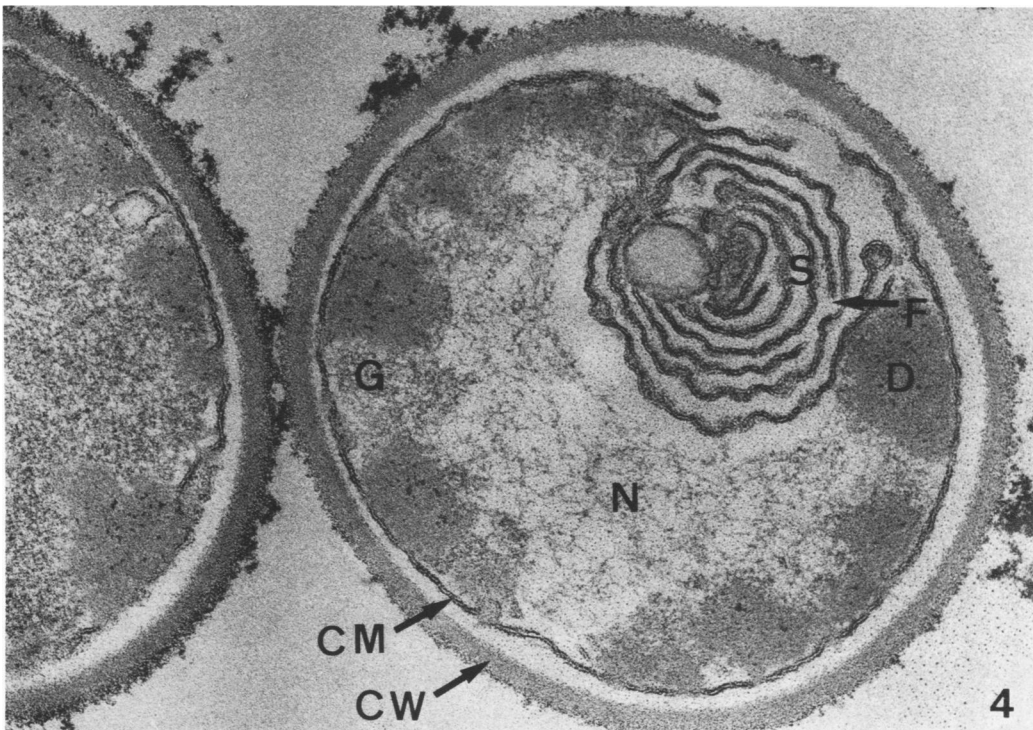


FIG. 4. Same as Fig. 3, except  $\times 78,000$ .



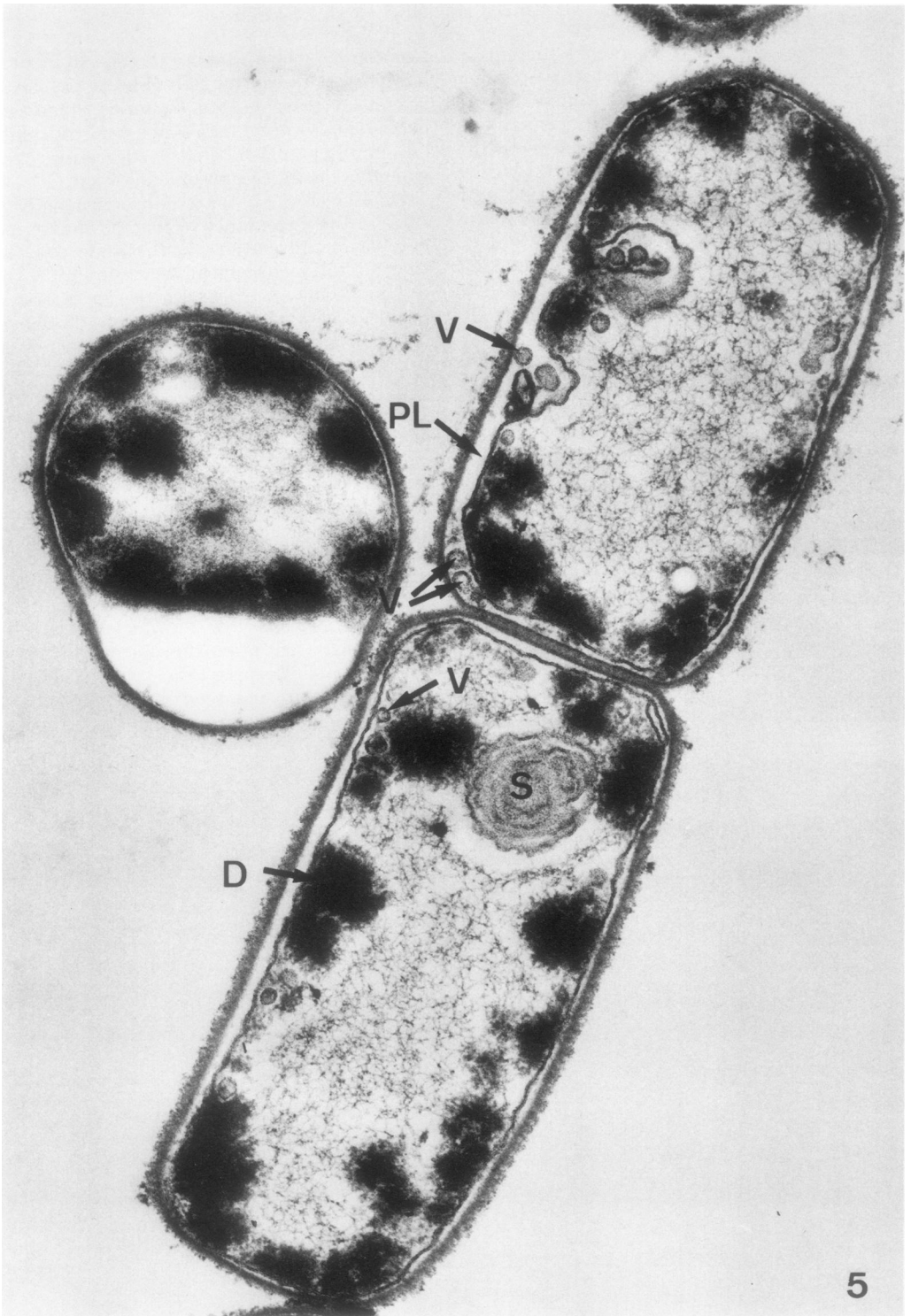


FIG. 5. Cells heated at 65 C for 15 min and fixed by the R-K procedure with prefixation. Note the empty aspect of the cytoplasm in which only the dense blocks (D), DNA-like fibrils, and membranous vesicles (V) are visible. A tangential section of a concentric membrane system (S) is apparent. In the upper cell two intracytoplasmic membrane systems are visible in connection with the enlarged periplasmic space (PL). Some membranous vesicles are present in this space.  $\times 53,000$ .

## DISCUSSION

The ultrastructural alterations we observed in *B. cereus*, heated while suspended in the culture medium, agree with the data described in the literature concerning the biochemical changes induced by moist heat in bacterial cells. In fact, several papers have reported on the damage of membrane permeability (1-4, 11, 12, 15, 17, 19, 20, 24, 26, 37), assessed either by the efflux of intracellular constituents or the influx of ANS (8-anilino-1-naphthalene-sulfonic acid). Ray and Brock (25) reported on the lysis, due to the membrane damage, of protoplasts from *Sarcina lutea* and *Streptococcus faecalis* heated at temperatures of 60 C and above. The observation of fractures in the cytoplasmic membrane of heated *B. cereus* is a sufficient explanation for the referred damage of membrane permeability. It is possible, however, that before the production of these fractures, other less dramatic structural alterations may occur in the membranes as a consequence of the action of moist heat. The study of sections of *B. cereus* cells heated at 65 C for 2 min shows

that the change in the geometry of the cytoplasmic membrane from asymmetric to symmetric seems to occur before the production of fractures. It is possible that such a change reflects structural alterations which in turn may be related to the described disturbance in membrane permeability. Similar change in the profile of the membranes has been described in several bacteria under different conditions leading to lysis (13, 14, 29, 31). It is not proved, however, whether such change in the profile of the membranes is related to a real alteration of the membrane structure. It may result from changes in the environmental conditions including ionic composition and strength which are certainly induced by the heat treatment. It is known (10, 32; M. T. Silva, Proc. 6th Int. Cong. E. M., p. 275-276, 1966) that the conditions prevailing during the fixation of bacterial cells markedly affect the final electron microscopic appearance of their membranes.

Degradation and leakage of ribonucleic acid (RNA) has also been widely described by several authors (4, 10, 19, 34, 35) as a constant result of thermal injury. The coarse granular

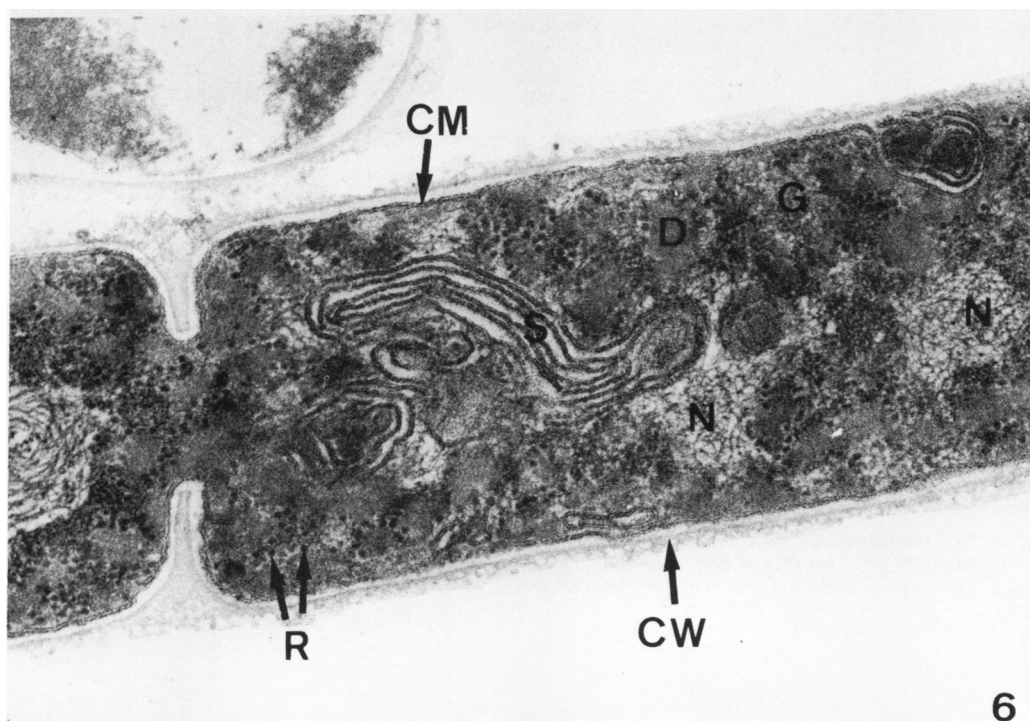


FIG. 6. In this figure, which corresponds to a section oxidized with  $H_2O_2$  before lead staining, several ribosomes (R) are present. On the right side of the section, a system of concentric membranes (S) is seen in continuity with the cytoplasmic membrane. The outer part of the cell wall (CW) is somewhat damaged. The nucleoids appear unaffected. Cells treated as in Fig. 5. For abbreviations, see legend to Fig. 3.  $\times 74,000$ .

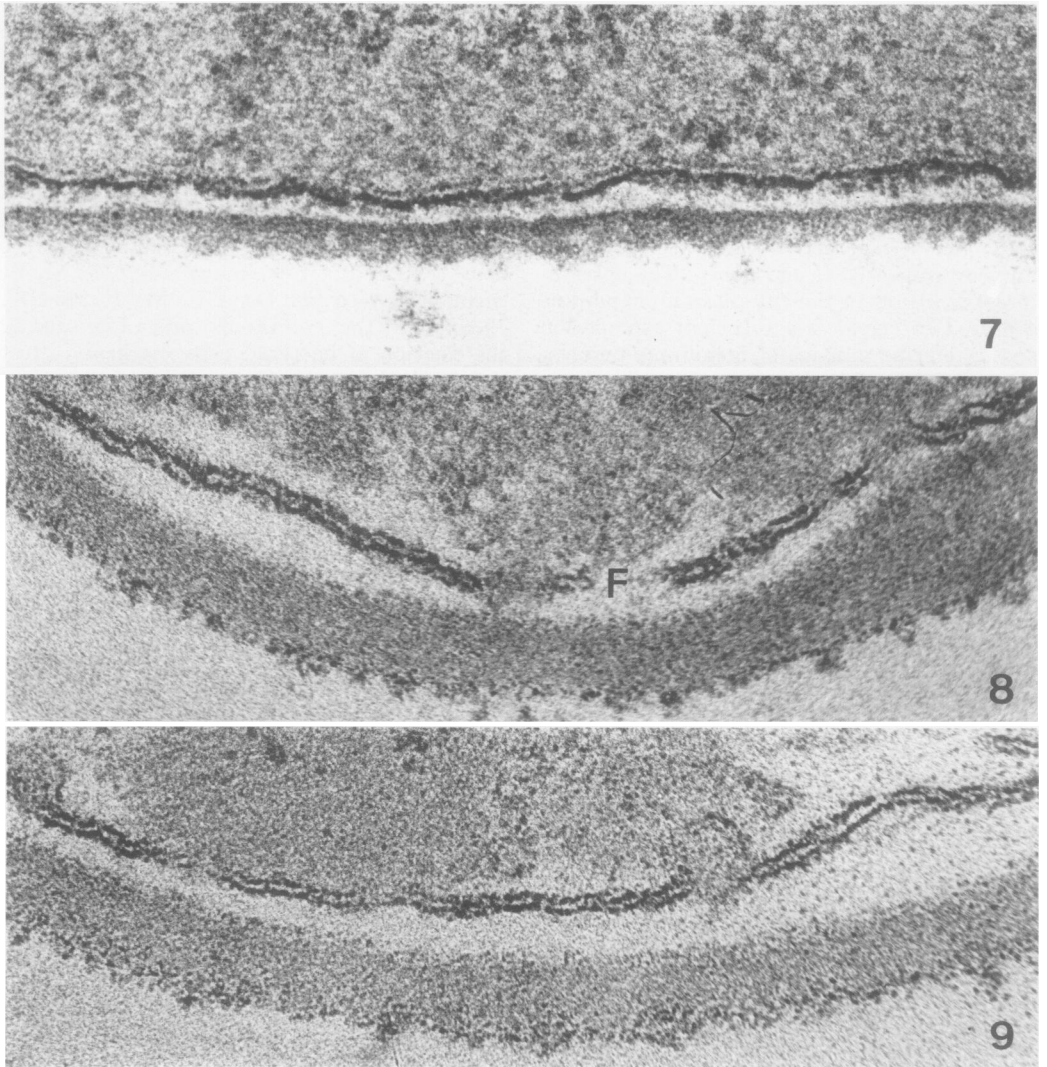


FIG. 7-9. Higher magnifications showing the profile of the cytoplasmic membrane in control (Fig. 7) and in heated (100 C, 5 min) (Fig. 8 and 9) cells. Notice the asymmetric and continuous profile in the control cell and the symmetric and fractured structure in heated cells.  $\times 244,000$ .



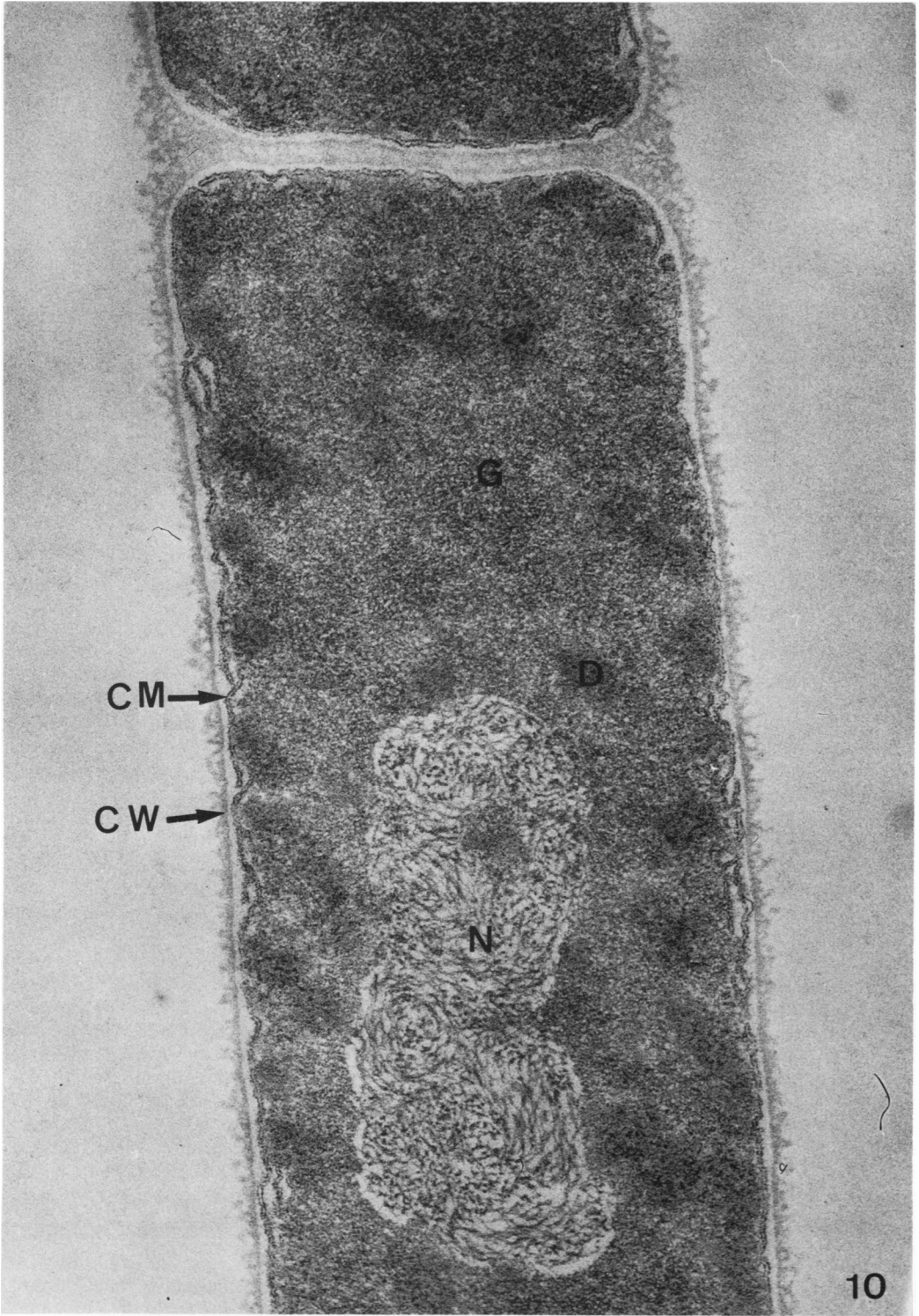


FIG. 10. As in Fig. 3 and 4. Notice the fibrillar nucleoid (N), the dense blocks (D) and the granular background of the cytoplasm, the symmetric cytoplasmic membrane (CM), and the cell wall (CW) with a damaged outer portion. The nucleoid is separated from the cytoplasm.  $\times 87,500$ .

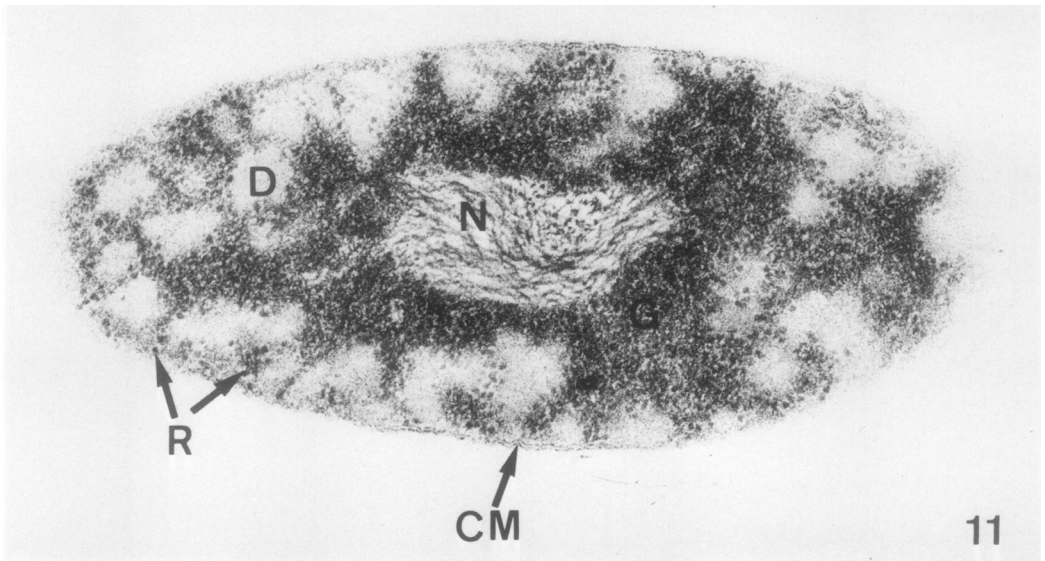


FIG. 11. As in Fig. 5 and 6, but section oxidized with  $H_2O_2$  and then treated with Pronase. Notice the reduction in the density of the cytoplasmic dense blocks (D).  $\times 74,000$ .

areas observed in the cytoplasm of heated *B. cereus* may contain the products of degradation of ribosomes. In fact, these areas appear in cells completely depleted of ribosomes or in cells with only a few ribosomes. In the latter case, the remaining ribosomes appear in the areas under discussion.

The dense blocks, containing Pronase-sensitive material very likely correspond to coagulated proteins. Protein coagulation was previously reported by Heden and Wyckoff (16), in an electron microscopic study of heated bacteria. Allwood and Russell (4) indirectly concluded protein coagulation that occurred in *Staphylococcus aureus* subjected to treatment with moist heat, but these authors could not distinguish between intra- or intercellular coagulation. In a more recent paper including an electron microscopy study on that bacterium (5), these authors found it difficult to correlate the alterations in the ultrastructural image of the cytoplasm with protein coagulation. Our results indicate that intracellular protein coagulation indeed occurs. Moreover, the study of sections of *B. cereus* heated at 65 C for 2 min suggests that, among the several alterations induced by the heating at this temperature, protein coagulation is the first one to be ultrastructurally detectable. It is interesting to mention, in connection with these observations, that protein coagulation is unlikely to be the prime lethal event (9). The above mentioned alteration in the permeabil-

ity of the membranes explains the important leakage of intracellular components, as amino acids, peptides,  $K^+$ , RNA or the products of its degradation, or both. This leakage explains, in turn, the empty aspect observed in many sections of heated *B. cereus*, as described above.

The observation that no significant alterations are exhibited by the nucleoids in our study agrees with the findings of Strange and Shon (35) and Allwood and Russell (4), who found that the bacterial DNA is not degraded or leaked after heating at temperatures not exceeding 100 C. Also the comparatively minor alteration in the ultrastructure of the cell wall observed in the present study is in accordance with the results of Salton (28) who showed that the cell wall of *S. aureus* is not affected by heating at temperatures up to 100 C.

As discussed by Allwood and Russell (6), it is expected to have important effects of heating on the lipid components of bacterial membranes. Phospholipids are expected to be lost, at least in part, either by melting or by hydrolysis. It was observed (J. C. F. Sousa, M. T. Silva, J. M. Santos Mota, and M. Luisa Abreu, Proc. 1st Latin-American Cong. for E.M., p. 36, 1972) that heated bacteria have a much lower lipid P content due to the loss of some of the different phospholipids of the membranes. Hydrolysis of phospholipids seems to be involved in this process. The occurrence of prominent membranous systems in heated

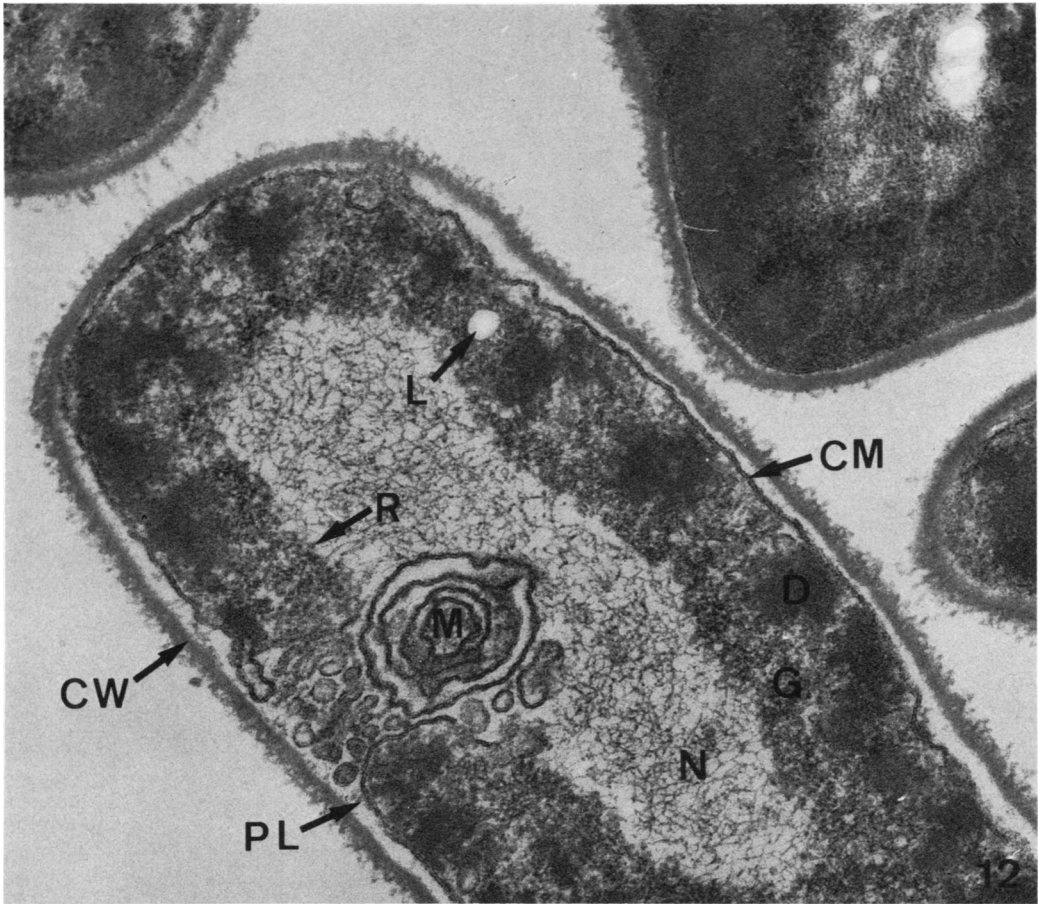


FIG. 12. Cell heated at 65 C for 2 min and fixed by the R-K procedure without prefixation. The cytoplasmic membrane (CM), as well as the other membranes, have symmetric profiles. Notice the presence of a partly vesicular mesosome (M) in connection with the periplasmic space (PL). Both the cell wall (CW) and the nucleoid (N) appear unaffected. In the cytoplasm of the central cell, several granules which look like ribosomes (R) under the process of degradation are visible. These granules are located in the coarsely granular areas (G); compare these granules with the normal appearance of the ribosomes in the cell at the upper left corner of the picture. Dense blocks (D) are visible in the cytoplasm.  $\times 77,000$ .

cells should be commented on. These prominent membranous systems are observed regardless of the use or omission of the prefixation step of the R-K technique. On the contrary, in intact *B. cereus* prominent membranous systems are observed only when the prefixation is used, as previously reported (33). In connection with this, it has to be considered that, when heated bacteria are fixed, either by procedures including or omitting the prefixation, the permeability of their membranes has already been severely damaged by heating, as demonstrated by several authors (see above) and confirmed in our laboratories for *B. cereus* (unpublished results). As a consequence of this permeability damage, which

allows an efflux of several constituents of the bacterial cell, as mentioned above, the normal high internal osmotic pressure characteristic of gram-positive bacterial cells (21) is certainly very much reduced at the time of fixation. As will be reported in another paper, the value of the internal osmotic pressure in bacterial cells subjected to the fixation with  $\text{OsO}_4$ , may influence the final ultrastructural image, mainly in what concerns the membranes.

In a study of the ultrastructure of heated *S. aureus*, using the ultrathin section method, Allwood and Russell (5) reported only on the loss of homogeneity of the cytoplasm, with vacuolation. However, their published electron micrographs also show the occurrence of

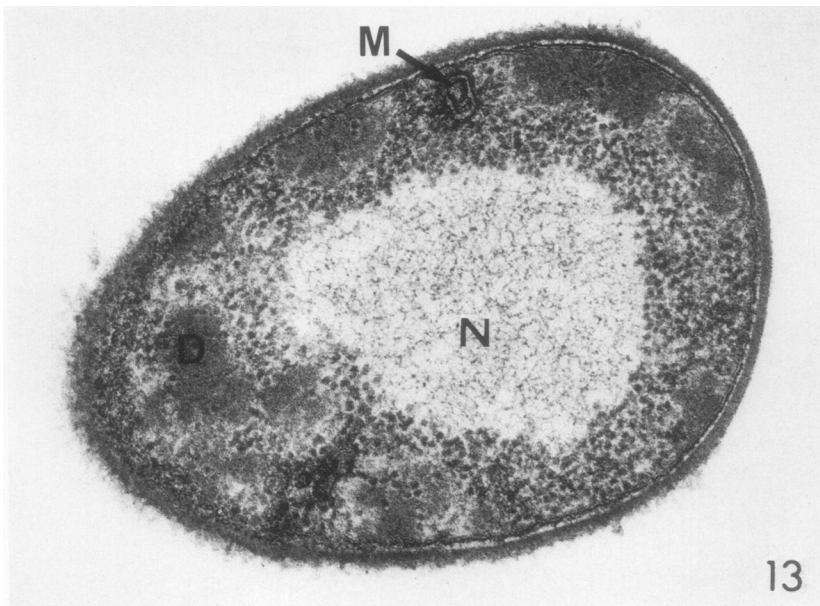


FIG. 13. As in Fig. 12. The only detectable ultrastructural alteration is the presence of dense blocks (D) in the cytoplasm. Notice that the cytoplasmic membrane (CM) has an asymmetric profile. A small and simple mesosome (M) is visible.  $\times 94,600$ .

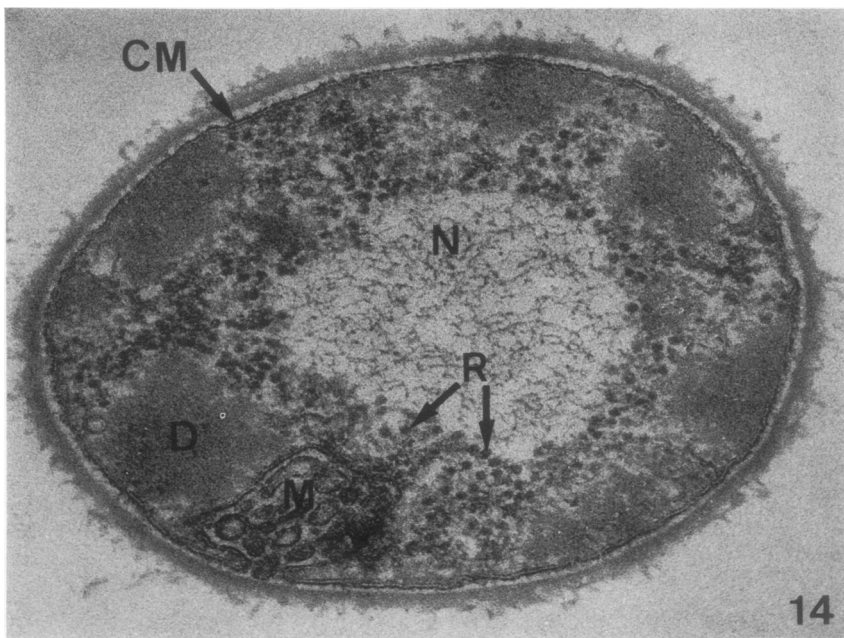


FIG. 14. As in Fig. 12. Notice the presence of a prominent vesicular mesosome (M). The cytoplasmic membrane (CM) is asymmetric, and the cytoplasm has numerous ribosomes (R).  $\times 123,000$ .

"myelin-like figures" similar to those we described in *B. cereus*.

To get a dynamic view of the occurrence of the ultrastructural alterations induced by moist heat in *B. cereus*, a time-lapse study is necessary. It is not to be expected, however, that a pure ultrastructural study would provide important information useful to solve the interesting problem of whether there is a prime cause of death by moist heat (and what it is) or whether several different sites are involved (and what they are). In fact, alterations in biochemical or physiological parameters not having an ultrastructural counterpart may be responsible for the death of bacteria induced by moist heat.

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