JOURNAL OF BACTERIOLOGY, Oct. 1973, p. 424-440
Vol. 116, No. 1

Printed in U.S.A.

# Localization of Alkaline Phosphatase in Three Gram-Negative Rumen Bacteria

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Received for publication 18 April 1973

Of the three species (*Bacteroides ruminicola*, *B. succinogenes*, and *Megasphaera elsdenii*) of anaerobic gram-negative rumen bacteria studied, only *B. ruminicola* produced significant amounts of alkaline phosphatase. This enzyme, which is constitutive, showed a greater affinity for *p*-nitrophenylphosphate than for sodium-β-glycerophosphate and was shown to be located exclusively in the periplasmic space of log-phase cells. Small amounts of this enzyme were released from these cells in stationary-phase cultures, but washing in 0.01 M $MgCl_2$ and the production of spheroplasts by using lysozyme in 0.01 M $MgCl_2$ did not release significant amounts of the enzyme. Exposure to 0.2 M $MgCl_2$ did not release significant amounts of the periplasmic alkaline phosphatase of the cell, and when these cells were spheroplasted with lysozyme in 0.2 M $MgCl_2$ only 25% of the enzyme was released. Spheroplasts were formed spontaneously in aging cultures of *B. ruminicola*, but even these cells retained most of their periplasmic alkaline phosphatase. It was concluded that the alkaline phosphatase of *B. ruminicola* is firmly bound to a structural component within the periplasmic area of the cell wall and that the enzyme is released in large amounts only when the cells break down. The behavior of alkaline phosphatase in this bacterium contrasts with that of conventional periplasmic enzymes of aerobic bacteria, which are released upon conversion into spheroplasts by lysozyme and ethylenediaminetetraacetic acid and by other types of cell wall damage. All three species of bacteria studied here, as well as bacteria found in mixed populations in the rumen, have thick, complex layers external to the double-track layer of their cell walls. In addition, *B. ruminicola* produces a loose extracellular material.

Alkaline phosphatase has been shown to be associated with cell walls of a number of gram-negative aerobic bacteria (16), by reaction product deposition (12, 37), by the use of ferritin-coupled antibody (25), and by biochemical means (2). In some cases cell wall-associated enzymes were seen to be confined to the periplasmic space (12, 22, 23), in others they were found at the cell surface (31), but in most cases they were present in both of these areas of the cell wall (14, 22, 25, 37). Cell wall-associated enzymes have been described in *Pseudomonas aeruginosa* (12-14), *Escherichia coli* (18, 24, 25, 31), *Salmonella typhimurium* (23, 24), and the marine pseudomonad B-16 (17, 25), but their existence in the anaerobic gram-negative bacteria has not been reported.

Cell wall-associated enzymes can be released from the cells of various aerobic gram-negative bacteria by a number of treatments that do not release cytoplasmic enzymes or unduly damage the cells. Ethylenediaminetetraacetate (EDTA)-osmotic shock procedures (18, 30) have been used to release these enzymes, as have spheroplast formation using EDTA and lysozyme (28), high $Mg^{2+}$ treatment (12-14), and cell wall removal (17). Cells with defective cell walls are known to release cell wall-associated enzymes during growth (23, 24, 36).

The presence of cell wall-associated enzymes is especially important in organisms that break down long, extracellular polymers (e.g., the wall-associated cellulase of *Cytophaga*) (35), and we must expect that rumen bacteria would use cell wall-associated degradative enzymes to break down organic compounds in their environment so that these compounds could be assimilated by the cells. Cheng, Hironaka, and Costerton (unpublished results) have found

that alkaline phosphatase is present in the bovine rumen in both cell-free and cell-associated states and that levels of the enzyme vary with the dietary regime of the cow. The present study examines three pure cultures of anaerobic, gram-negative, rumen bacteria, and spheroplasts derived from one of them (*Bacteroides ruminicola*) by aging and by lysozyme treatment, to determine the location of alkaline phosphatase and the effect of spheroplasting on the association of this enzyme with the cell wall.

## MATERIALS AND METHODS

**Organisms and culture conditions.** *Bacteroides ruminicola* subsp. *ruminicola* (strain 23), *B. succinogenes* (S85), and *Megasphaera elsdenii* (B159) were generously provided by M. P. Bryant, University of Illinois, Urbana (5, 8, 33).

The anaerobic technique used for culturing the bacteria was essentially that of Hungate (20) as modified by Bryant and Burkey (4). Rumen bacteria cultured anaerobically for 10 h at 38 C in prereduced rumen fluid medium (6) or synthetic medium (9) on a rotary shaker (75 rpm) were inoculated into 300 ml of fresh medium in 500-ml round-bottom flasks (5%, vol/vol) and incubated further with shaking. All experiments described herein used rumen fluid medium, unless otherwise stated. At given times of incubation, cultures were harvested by centrifugation in a Sorvall RC-2B refrigerated centrifuge for 10 min at 15,000 × *g*. The optical density of the culture was measured at 660 nm. The optical densities of cultures of *B. ruminicola*, *B. succinogenes*, and *M. elsdenii* were correlated with the dry weight of the cells by means of a calibration curve following drying to a constant weight at 90 C. From the curve it was determined that an optical density of 1.0 is equivalent to 0.409, 0.348, and 0.225 mg/ml for *B. ruminicola*, *B. succinogenes*, and *M. elsdenii*, respectively.

**Chemicals.** Tris(hydroxymethyl)aminomethane (Tris), *p*-nitrophenylphosphate (PNPP), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate, and lysozyme (EC 3.2.1.17) were purchased from the Sigma Chemical Co., St. Louis, Mo. Sodium-β-glycerophosphate was obtained from Fisher Scientific Co., Pittsburgh, Pa. Osmium tetroxide and Vestopal W were purchased from Polysciences Inc., Rydal, Pa. All other reagents and chemicals were the best grade obtainable from local commercial sources.

**Cell extracts and enzyme assays.** The cells harvested after given times of incubation were ultrasonically disrupted three times with a Bronson sonic oscillator at a tip energy of 100 W at 30-s intervals. The alkaline phosphatase was assayed with PNPP as the substrate as previously described (13).

The assay for glutamate dehydrogenase (EC 1.4.1.3) was similar to that described by Malamy and Horecker (27) except that the assay system contained 0.05 M 2-mercaptoethanol. NADH oxidase activity was followed in a 1-ml assay system consisting of $4 \times 10^{-4}$ M NADH, 0.05 M Tris buffer (pH 7.65), and 0.05 M 2-mercaptoethanol. The oxidation of NADH was followed at 25 C at 340 nm.

One unit of activity in all cases represents the conversion of 1 μmol of substrate to product per min at 25 C.

**Whole cell assay system for alkaline phosphatase localization studies.** The cells were harvested then washed three times with 0.01 M $Mg^{2+}$ in 0.01 M Tris (pH 8.4) to remove inorganic phosphate in the medium. The washed cells were incubated for 30 min in each of the following modified Gomori (12) mixtures: (i) sodium-β-glycerophosphate, 0.5%; sodium-barbital, 0.5%; $Ca(NO_3)_2$, 0.02 M; $MgCl_2$, 0.01 M; and Tris buffer, 0.05 M (pH 8.4); or (ii) PNPP, 0.0001%; $Ca(NO_3)_2$, 0.02 M; $MgCl_2$, 0.01 M and Tris buffer, 0.05 M (pH 8.4). Incubation mixtures lacking sodium-β-glycerophosphate and PNPP were also used with washed cells as controls.

**Preparation of spheroplasts for alkaline phosphatase localization studies.** Spheroplasts of *B. ruminicola* were prepared from 40 ml of 12-h log-phase cells (optical density 1.40; 660 nm, Gilford model 300-N spectrophotometer). Cells were centrifuged at 15,000 × g and were washed three times with 0.01 M $Mg^{2+}$ in 0.01 M Tris to remove inorganic phosphate in the medium. The washed cells were suspended in 20 ml of 0.2 M $Mg^{2+}$ in 0.01 M Tris (pH 8.4), or in 20 ml of 0.01 M $Mg^{2+}$ in 0.01 M Tris (pH 8.4), both containing 1 mg of lysozyme per ml. The cell suspensions were incubated at 37 C in a rotary shaker for 30 min. The cells were then centrifuged and suspended in 0.01 M $Mg^{2+}$ in 0.01 M Tris buffer (pH 8.4) for the formation of spheroplasts (10). In aging cultures of *B. ruminicola* (24 h), spheroplasts were formed spontaneously (10). Both lysozyme-induced spheroplasts and spontaneous spheroplasts were incubated with each of the above-mentioned modified Gomori mixtures to localize the alkaline phosphatase. During the processes used to convert *B. ruminicola* cells to spheroplasts, all supernatant fluids were collected and assayed (as in Table 1) for alkaline phosphatase, glutamate dehydrogenase, and NADH oxidase.

**Electron microscopy.** Fixation and embedding were as described by Kellenberger et al. (21) and modified by Margaretten et al. (29) except that phosphate buffer was replaced with Tris. The detailed procedure was described previously (12). The alkaline phosphatase activity in whole cells was localized by the deposition of electron-dense lead phosphate after incubation in a modified Gomori reaction mixture. Thin sections of reacted cells and controls were cut with a Sorvall Porter-Blum ultramicrotome, stained with 1% uranyl acetate and lead citrate (32), and examined with an AEI-EM-801 electron microscope having an acceleration voltage of 60 kV.

## RESULTS

**Alkaline phosphatase production by whole cells.** The rates of growth and of the production of both cell-free and cell-associated alkaline phosphatase were determined in cultures of the

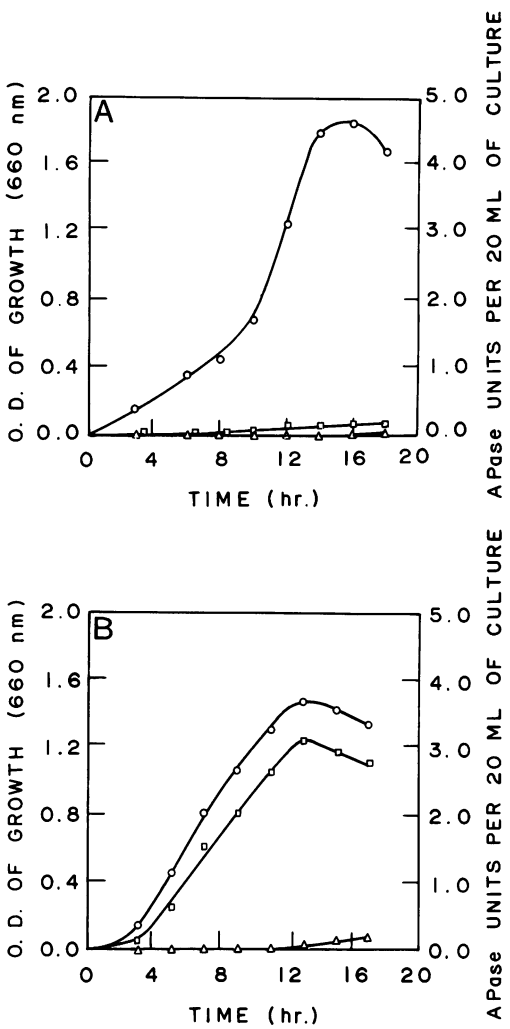



FIG. 1. *(A) Growth of Bacteroides succinogenes and productivity of alkaline phosphatase. B. succinogenes was grown in rumen fluid medium at 38 C. Growth was followed by observing the increase in optical density (OD) at 660 nm. Appropriate samples were removed from the 300 ml of culture, and the cells were recovered by centrifugation at 15,000 × g. The cells were resuspended into 0.01 M Tris (pH 8.4) containing 0.01 M $Mg^{2+}$ to determine the total cell-bound alkaline phosphatase. The cell-free culture filtrate was also assayed for alkaline phosphatase activity. Symbols:* (○) *OD at 660 nm,* (□) *units of cell-bound alkaline phosphatase per 20 ml of culture,* (△) *units of alkaline phosphatase per 20 ml of cell-free culture filtrate. (B) Growth of Bacteroides ruminicola and productivity of alkaline phosphatase. B. ruminicola was grown in rumen fluid medium at 38 C. Growth was followed by observing the increase in OD at 660 nm. Appropriate samples were removed from the 300 ml of culture, and the cells were recovered by centrifugation at 15,000 × g. The cells were resuspended into 0.01 M Tris (pH 8.4) containing 0.01 M $Mg^{2+}$ to determine the total cell-bound alkaline phosphatase. The cell-free culture filtrate was also assayed for alkaline phosphatase activity. Symbols:* (○) *OD at 660 nm,* (□) *units of cell-bound alkaline phosphatase per 20 ml of culture,* (△) *units of alkaline phosphatase per 20 ml of cell-free culture filtrate.*

three species used in this study. *M. elsdenii* grew well in rumen fluid medium but did not produce alkaline phosphatase. *B. succinogenes* produced small amounts of alkaline phosphatase, and all of the enzyme was cell-associated, until the stationary phase was reached when a small amount of cell-free enzyme appeared (Fig. 1A). *B. ruminicola* produced large amounts of alkaline phosphatase, and, as in the case of *B. succinogenes*, all of the enzyme was cell associated until a small amount of cell-free enzyme was released from the cells during the stationary phase (Fig. 1B). The enzyme is clearly constitutive in *B. ruminicola* and *B. succinogenes* because the level of inorganic phosphorus in the medium in which they were grown was very high (0.127 mg/ml).

**Alkaline phosphatase release during spheroplasting.** When log-phase cells (12 h) of *B. ruminicola* were exposed to lysozyme in 0.2 M $MgCl_2$, 25% of the alkaline phosphatase of the cells was released into the menstruum (Table 1). The formation of spheroplasts by resuspension in 0.01 M $MgCl_2$ caused only a negligible further release, and 75% of the enzyme remained associated with spheroplasts produced by this method. When spheroplasts were produced by the action of lysozyme in 0.01 M $MgCl_2$, only 5% of the alkaline phosphatase of the cells was released; the remaining 95% was associated with the spheroplasts (Table 1). Phase microscopy showed that the conversion of these cells to spheroplasts was complete in both cases (Fig. 2). Neither glutamate dehydrogenase nor NADH oxidase was released by the spheroplasting procedure at either of the $MgCl_2$ concentrations used.

**Ultrastructural localization of alkaline phosphatase.** The morphology of *B. ruminicola* was examined directly from the culture and after three washes in 0.01 M $MgCl_2$. The cells showed extensive accretions of extracellular material when prefixed directly in the medium (Fig. 3). Examination showed that washing removed this loose extracellular material (Fig. 4) but that the cell walls retained a thick (40 nm) particulate, electron-dense layer outside the double-track layer. Direct enzyme assay showed that none of the three 0.01 M $MgCl_2$

TABLE 1. *Enzyme release from cells and spheroplasts of Bacteroides ruminicola S-23*

| Treatment | Enzyme (U/200 ml of culture)[a] | | | | | |
|---|---|---|---|---|---|---|
| | 0.2 M $MgCl_2$[b] | | | 0.01 M $MgCl_2$[b] | | |
| | Alkaline phosphatase | NADH[c] oxidase | Glutamate dehydrogenase | Alkaline phosphatase | NADH[c] oxidase | Glutamate dehydrogenase |
| Supernatant fluid obtained from[d] cells treated with lysozyme in 0.2 M $MgCl_2$ or 0.01 M $MgCl_2$ | 6.4 | 0 | 0 | 1.2 | 0 | 0 |
| Supernatant fluid obtained from cells treated with lysozyme and $MgCl_2$ after resuspension into 0.01 M $MgCl_2$ (spheroplast formation) | 0.8 | 0 | 0 | 0.2 | 0 | 0 |
| Sonically treated spheroplasts | 22.0 | 6.8 | 39.0 | 25.4 | 6.8 | 39.0 |
| Cell-free extracts[e] | 26.4 | 7.0 | 38.0 | 26.4 | 7.0 | 38.0 |

[a] Dry weight of cells: 106 mg.

[b] 0.2 M $MgCl_2$ and 0.01 M $MgCl_2$ refer to the solution in which the cells were suspended.

[c] NADH refers to reduced nicotinamide adenine dinucleotide.

[d] Twenty milliliters of 12-h cells (10.6 mg [dry wt] of a culture grown to 1.4 optical density units) was centrifuged and resuspended in each of two solutions to which lysozyme (1 mg/ml) was added, and the suspension was incubated at 37 C on rotary shaker for 30 min. The suspension was centrifuged, the supernatant fluids were assayed for each enzyme, and the cell pellets were suspended in 0.01 M $MgCl_2$ and 0.01 M Tris (pH 8.4) for the formation of spheroplasts. The suspension was centrifuged, and the supernatant fractions were assayed for each enzyme. Spheroplast pellets were suspended in 0.01 M Tris buffer and sonically treated for enzyme assay.

[e] Twenty milliliters of 12-h cells were centrifuged and resuspended into 0.01 M Tris buffer and sonically treated for enzyme assay.

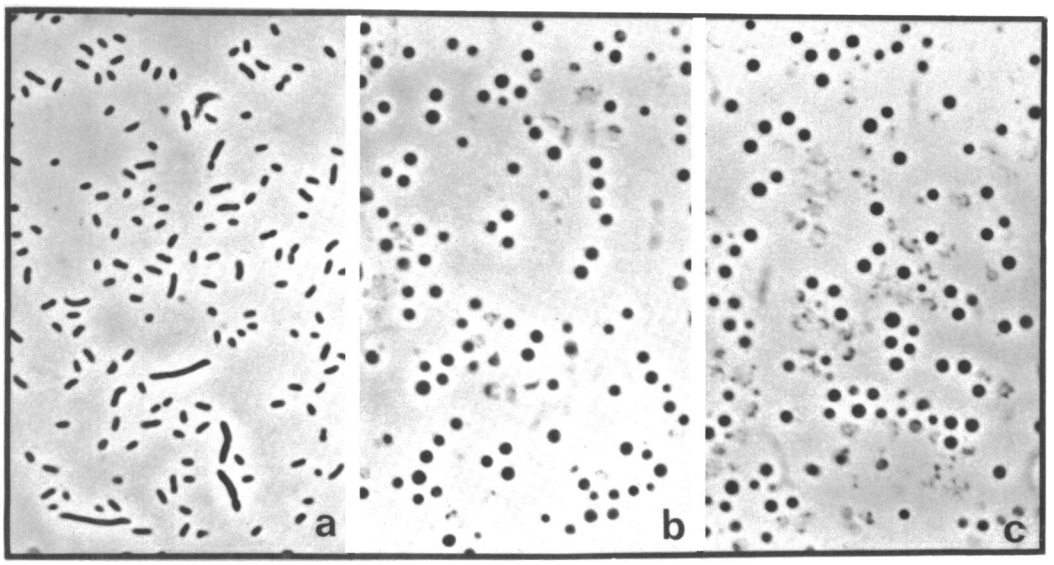



FIG. 2. *Phase contrast micrographs of (A) whole cells, (B) spheroplasts prepared in 0.01 M $Mg^{2+}$, and (C) spheroplasts prepared in 0.2 M $Mg^{2+}$.*

washes removed any alkaline phosphatase.

Washed cells were used to localize alkaline phosphatase to avoid interference by inorganic phosphate from the medium. The enzyme was localized exclusively in the periplasmic space of these log-phase cells (Fig. 5A and B), and only a

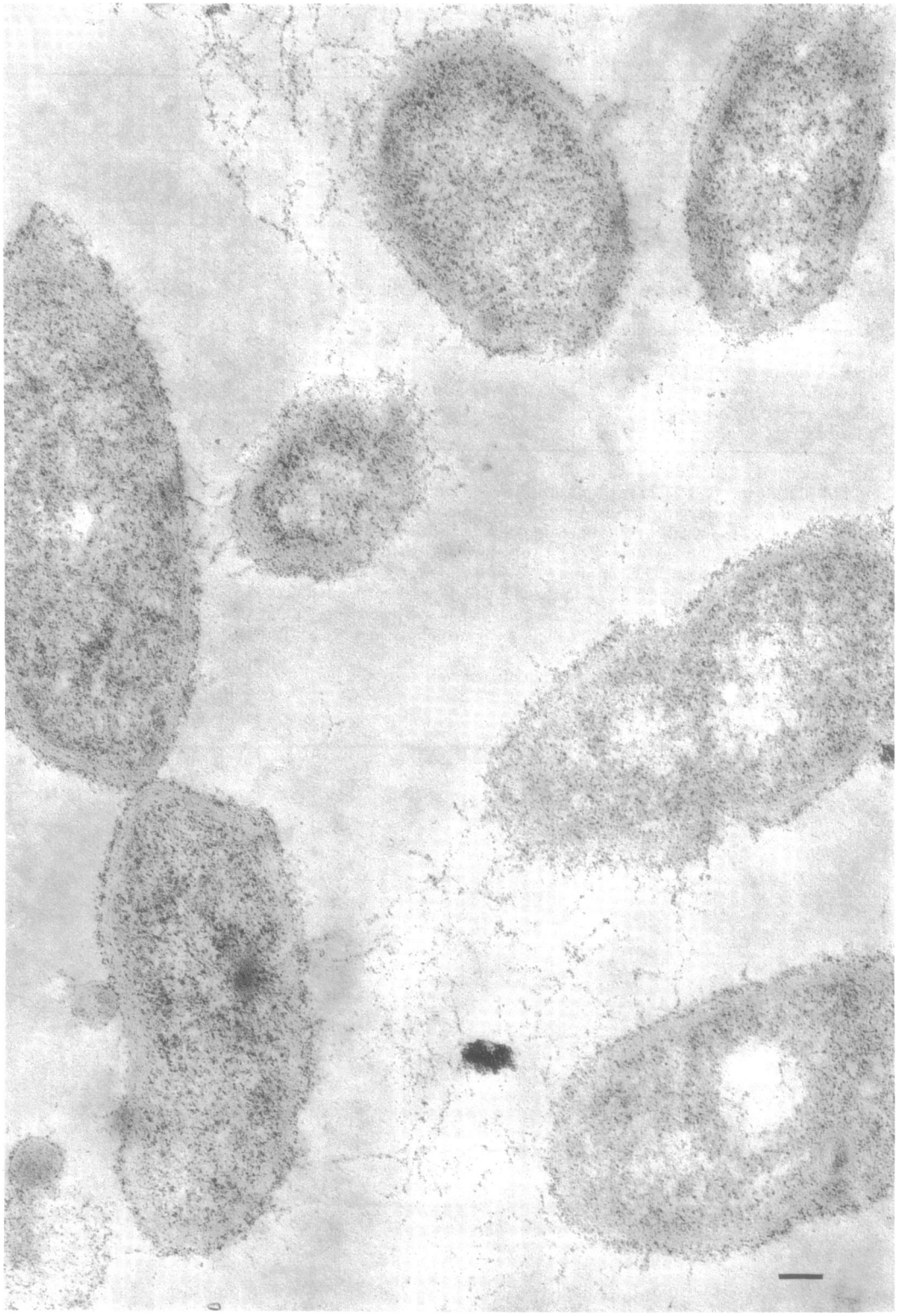


FIG. 3. *Electron micrograph of cells of Bacteroides ruminicola from a log-phase (12-h) culture. Note the acretion of extracellular material between these cells, which were prefixed in the medium. The bar in this and subsequent electron micrographs indicate 0.1 μm.*

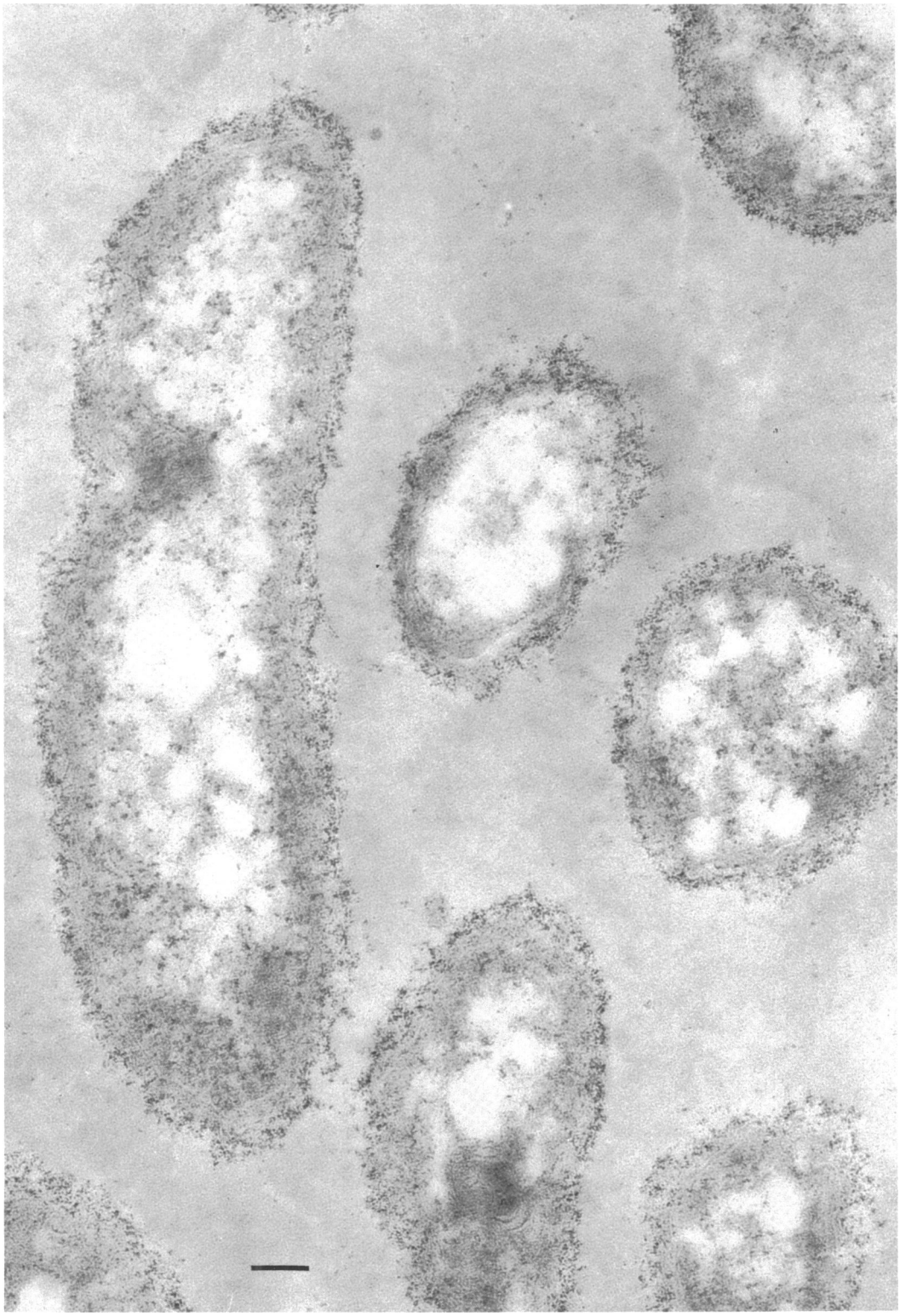


FIG. 4. *Electron micrograph of cells from a 12-h culture that had been washed three times in 0.01 M $MgCl_2$ before fixation. Note that washing has removed the extracellular material but left the thick and complex cell wall intact.*

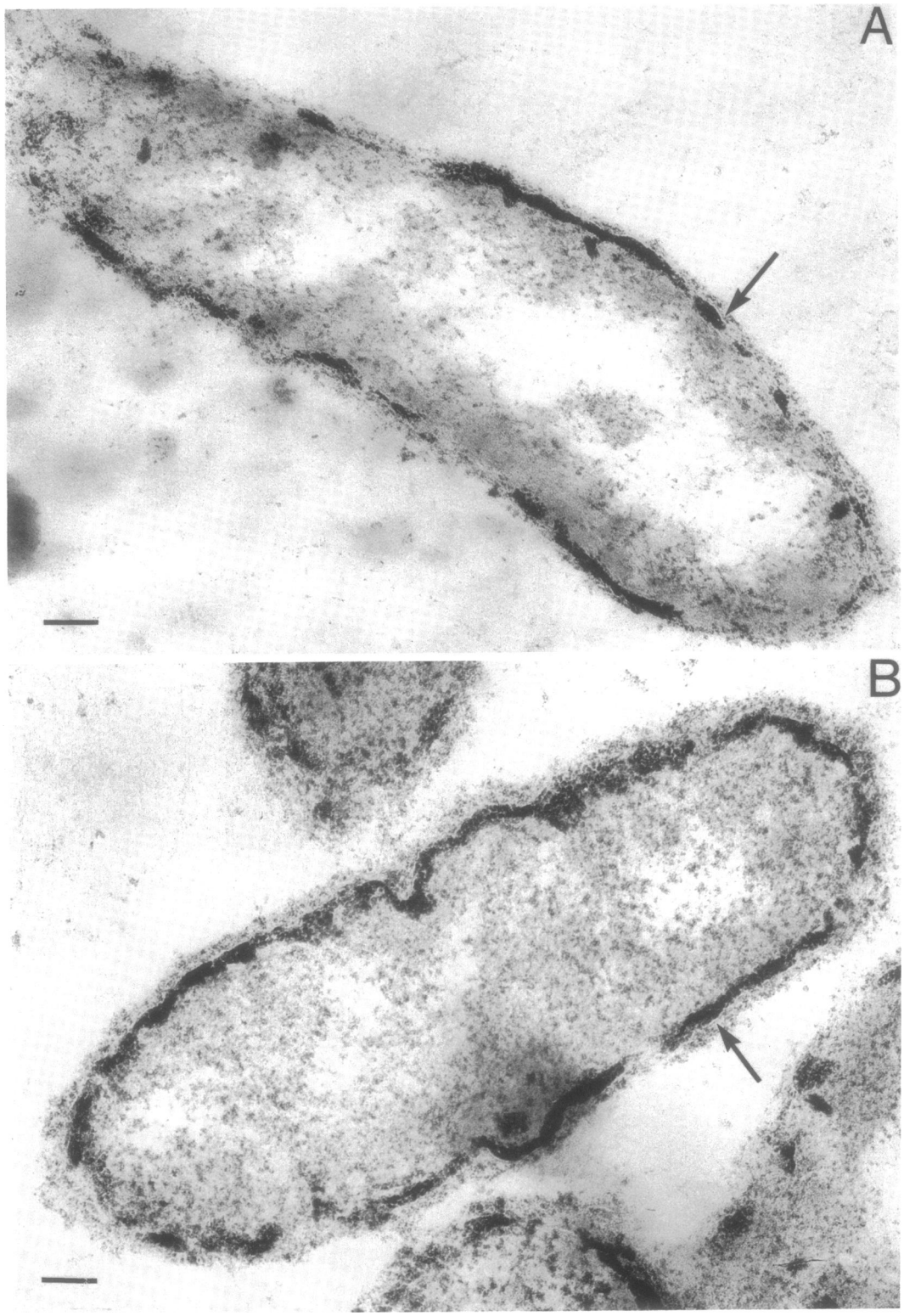



FIG. 5. *Electron micrograph of cells from a 12-h culture of Bacteroides ruminicola incubated for reaction product deposition by alkaline phosphatase using PNPP as the substrate. Note that the enzyme is entirely confined in the periplasmic space between the cytoplasmic membrane and the double-track layer of the cell wall, which can both be resolved in the areas indicated by arrows.*

small amount of reaction product was seen at the cell surface or in the menstruum. The particulate external layer of the cell wall is clearly seen in this preparation, and the general cell preservation is good. The alkaline phosphatase of *B. ruminicola* showed a distinct and specific affinity for PNPP and preparations in which sodium-$\beta$-glycerophosphate were used as the substrate showed only a very weak reaction (Fig. 6). No reaction product was found in control preparations in which the substrate had been omitted from the incubation mixture (Fig. 7). Preparations of log-phase cells at 8 and 10 h showed no significant differences from the 12-h cells.

Cultures of *B. ruminicola* were in stationary phase at 14 h, and reaction product deposition preparations showed that some of the cells were morphologically altered (Fig. 8, A) and that alakline phosphatase, which was present at the cell surface, had caused the formation of an intermittent crust of reaction product (Fig. 8, R) while the reaction product of cell-free enzyme had formed discrete crystals (Fig. 8, C).

Stationary-phase cells at 24 h were examined, and large numbers of irregularly shaped cells were seen (Fig. 9). Phase microscopy had shown 80% of these cells to be "spontaneous spheroplasts." In many of these cells the cytoplasmic elements were extensively damaged, cell envelopes sometimes lacked the outer particulate layer, and occasionally sections of the cell wall had been removed to produce a partial protoplast (Fig. 9, P). Reaction product deposition showed that a large proportion of the alkaline phosphatase had been retained in the periplasmic area of even severely damaged cells (Fig. 10, D), but surface crusts (Fig. 10, R) and discrete crystals (Fig. 10, C) formed by surface-associated and cell-free enzymes were seen also.

In log-phase cells (10 h) grown in a synthetic medium, some alkaline phosphatase activity was seen at the cell surface, while significant amounts of reaction product were seen also in the periplasmic area (Fig. 11). The total enzyme activity of these cells appeared to be reduced in comparison to cells grown in rumen fluid medium.

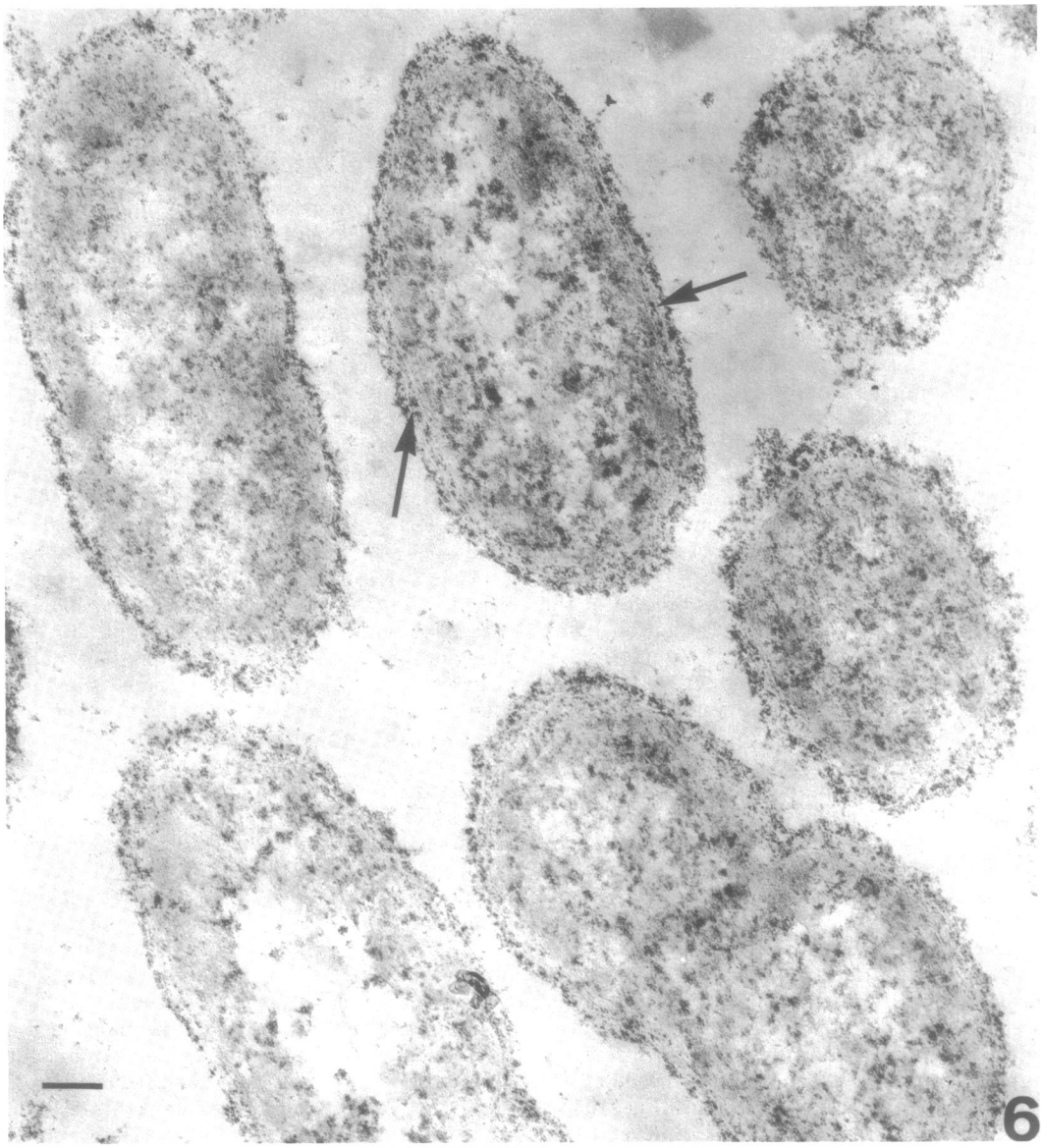
Spheroplasts prepared by the action of lysozyme in 0.2 M $MgCl_2$ had been shown to retain 75% of their alkaline phosphatase (Table 1). Reaction product deposition showed that the enzyme is retained in the periplasmic space of these cells (Fig. 12). Phase microscopy established that all of these cells were spherical, and reaction product deposition at the electron microscope level showed that all of the spheroplasts retained alkaline phosphatase. The spheroplasts prepared in 0.01 M $MgCl_2$ also retained alkaline phosphatase in their periplasmic areas.

*B. succinogenes* produced very small amounts of alkaline phosphatase (Fig. 1A) and log-phase (10 h) cells of this species showed very little reaction product (Fig. 13). Figure 13 shows clearly that washing three times in 0.01 M $MgCl_2$ has caused extensive damage to the cell walls of this organism. The large, spherical cells of *M. elsdenii* have a complex and interesting cell envelope structure, and reaction product deposition confirms the direct enzyme assay in that no reaction product is seen in these cells (Fig. 14).

## DISCUSSION

Cheng, Hironaka, and Costerton (unpublished results) have noted that the bovine rumen contains a significant amount of alkaline phosphatase, and reaction product deposition has shown that the enzyme is associated with the mixed bacterial population of this organ. Of the three anaerobic, gram-negative, rumen bacteria studied here, only *B. ruminicola* produces large amounts of this enzyme. Alkaline phosphatase is strictly cell associated in log-phase cells and, moreover, it is confined to the periplasmic zone of the cell wall, i.e., the area between the cytoplasmic membrane and the double-track layer of the cell wall. Only when cultures enter the stationary phase, with attendant cellular breakdown, is the enzyme released into the medium.

The strong binding of alkaline phosphatase by the cell wall is further evidenced by our observation that treatment with 0.2 M $MgCl_2$ and lysozyme, which removes all of this enzyme from cells of *P. aeruginosa* (14), removes only 25% of the enzyme from cells of this organism. We know that this proportion of the alkaline phosphatase is released because of cell wall damage since neither membrane-associated NADH oxidase nor cytoplasmic glutamate dehydrogenase enzymes are released by this treatment. The cell walls of these 0.2 M $MgCl_2$-washed cells are sufficiently disrupted to admit lysozyme, as are cells which are washed in 0.01 M $MgCl_2$, but alkaline phosphatase is retained in the periplasmic space of the resultant spheroplasts. The difference of enzyme release at different $Mg^{2+}$ concentrations may be due to a greater effect of high $Mg^{2+}$ (0.2 M) on the electrostatic association of the enzyme with a structural component of the cell wall (13). Morphological evidence shows that the cytoplasmic



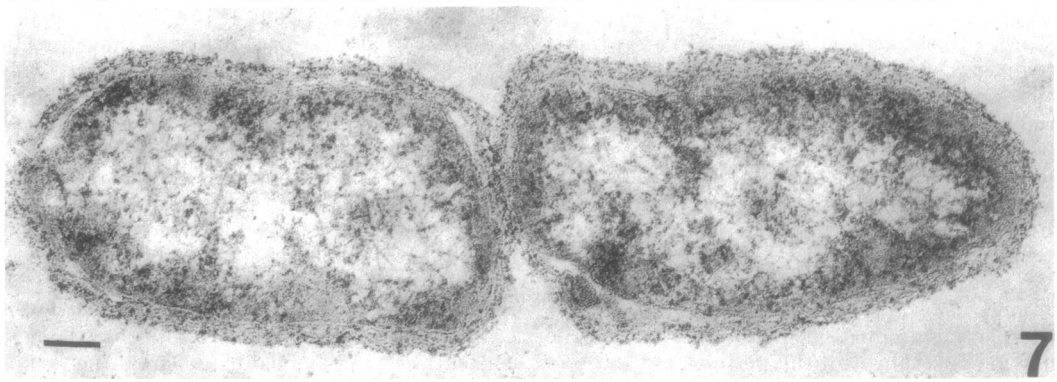




FIG. 6. *Electron micrograph of cells from a 12-h culture of Bacteroides ruminicola incubated for reaction product deposition by alkaline phosphatase using sodium-β-glycerophosphate as the substrate. Note the very small amounts of reaction product in the periplasmic space (arrows).*

FIG. 7. *The control preparation for the cells shown in Fig. 5 and 6 in which the substrate was omitted from the incubation mixture. Note the absence of reaction product in the cells of this preparation.*

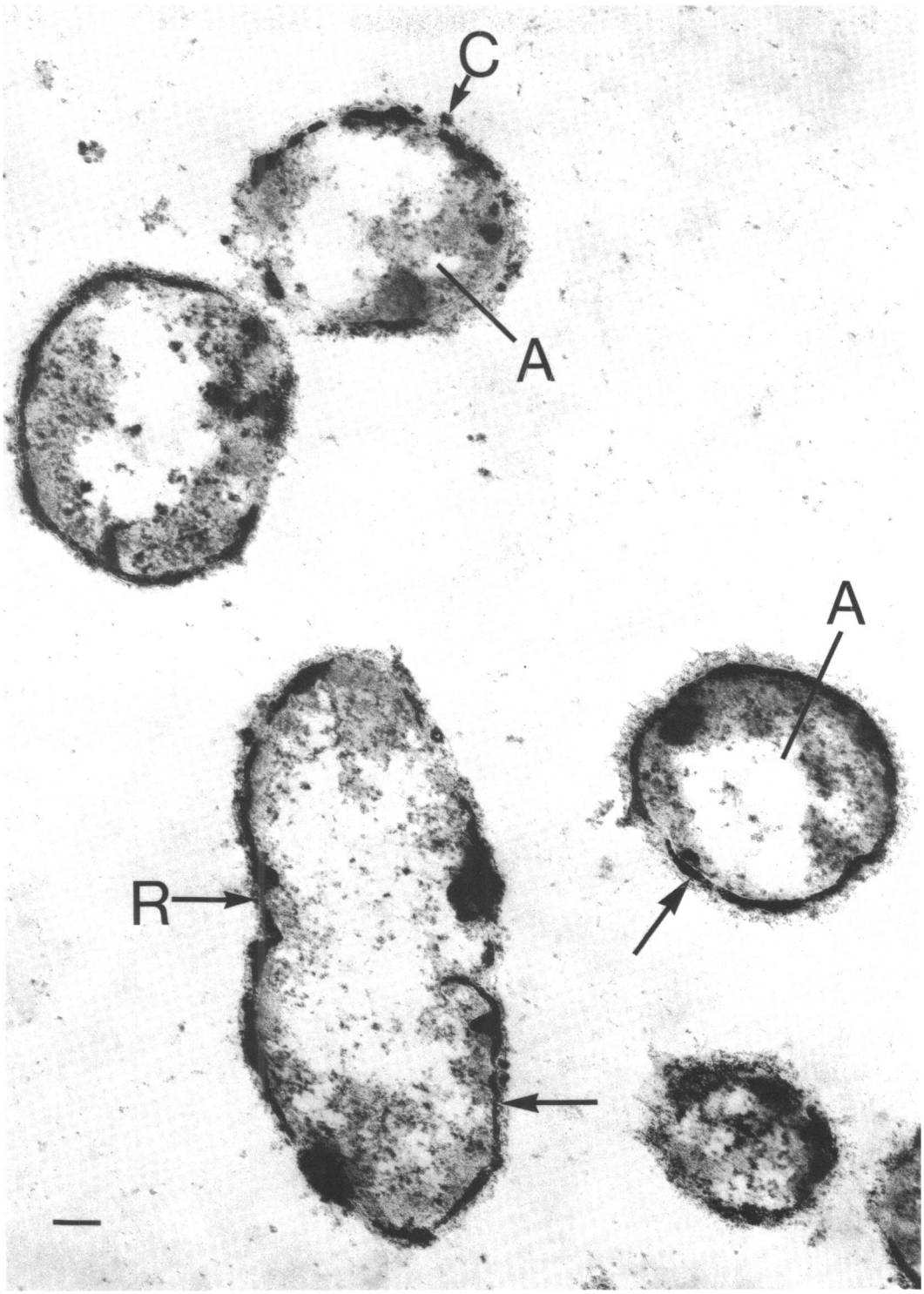



FIG. 8. *Electron micrograph of cells from a stationary phase (14 h) culture of Bacteroides ruminicola incubated for reaction product deposition by alkaline phosphatase using PNPP as the substrate. Note that some cells (A) show evidence of morphological alteration and that, while most of the enzyme activity is periplasmic (arrows), some reaction product has been deposited in a "crust" at the cell surface (R) and some has formed discrete crystals (C) that are attached to the cells.*

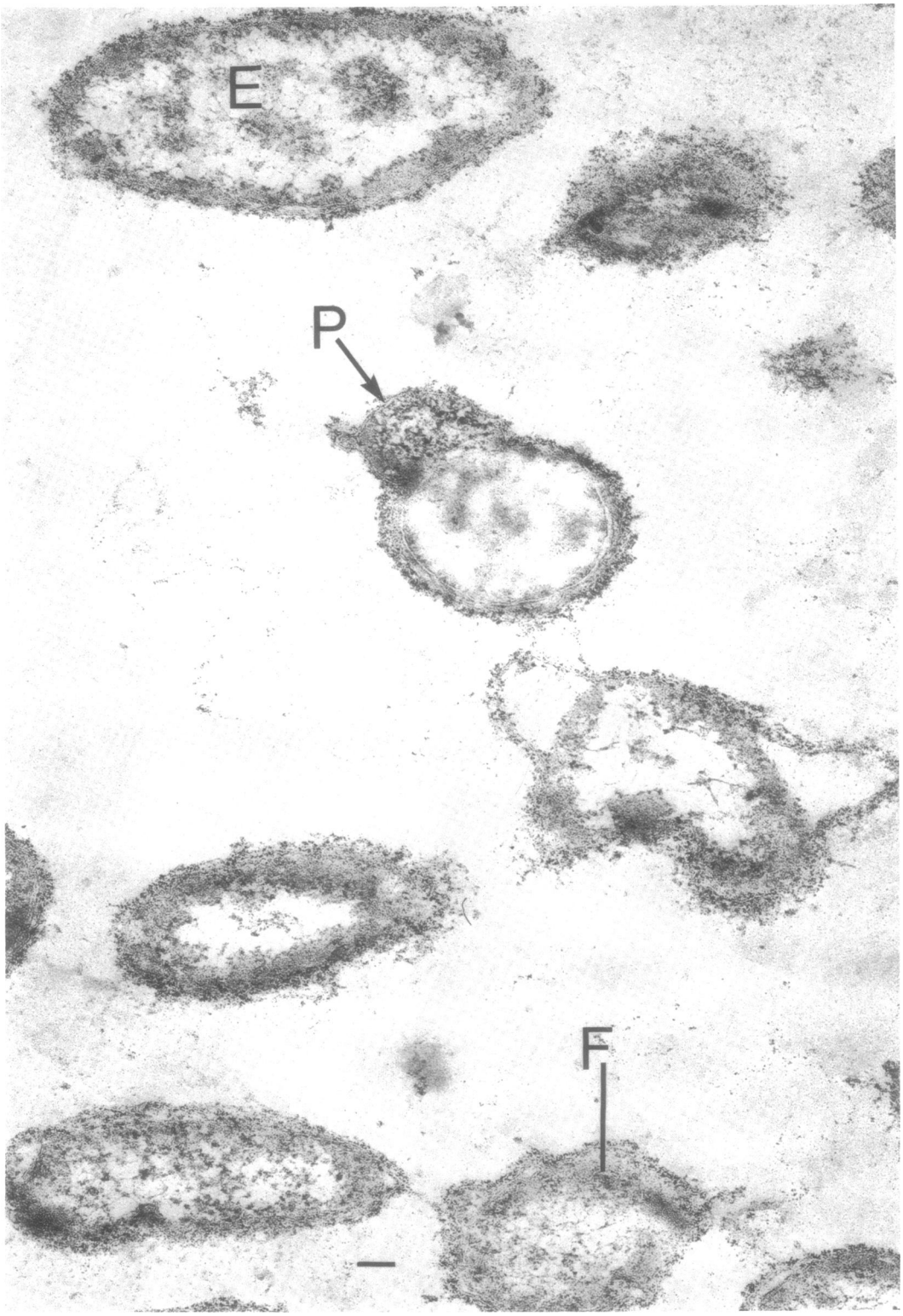



FIG. 9. *Electron micrograph of cells of a stationary-phase culture (24 h) of Bacteroides ruminicola. Note the morphological alterations of some of the cells that include enlargement of the electron-transparent chromatin zone (E), "rounding up" of cells (F), and changes in the cell envelope involving distortions and partial loss (P) of the double-track layer of the cell wall.*

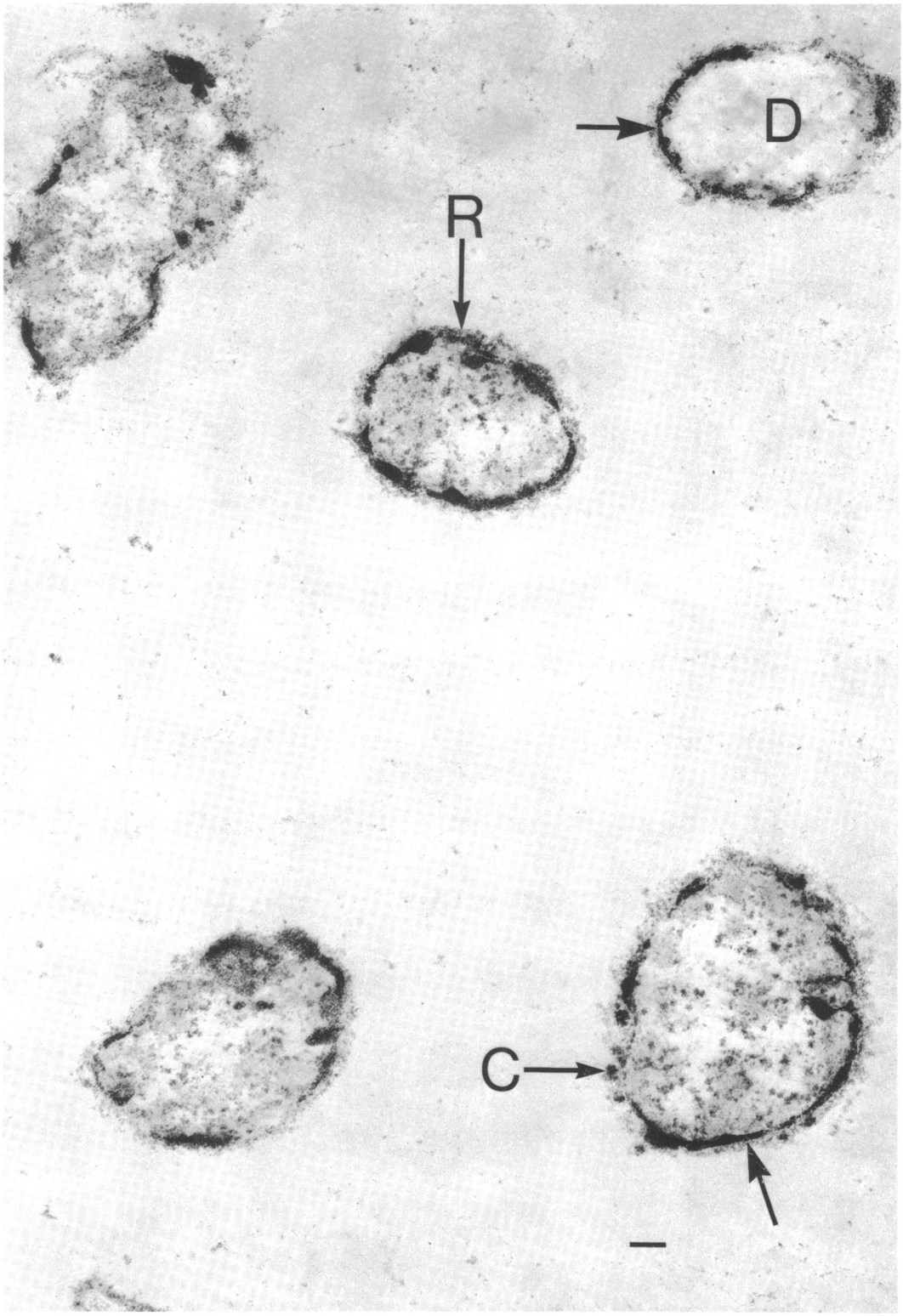
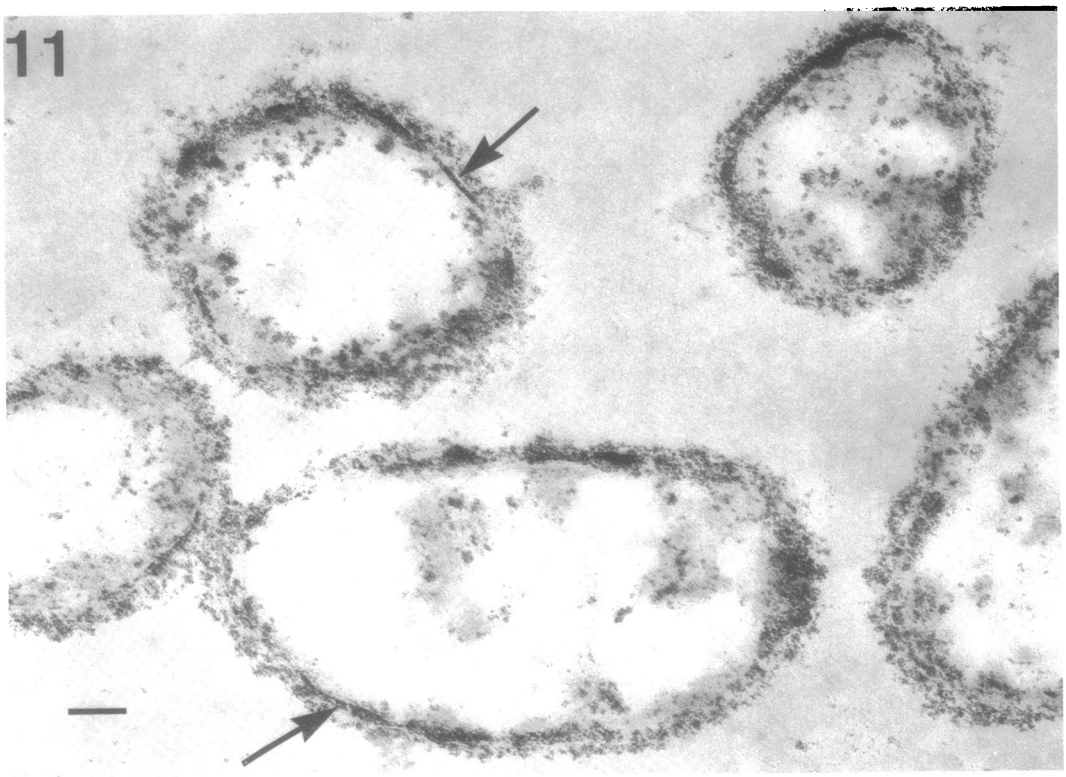



FIG. 10. *Electron micrograph of cells of a 24-h culture of Bacteroides ruminicola incubated for a reaction product deposition by alkaline phosphatase using PNPP as the substrate. Note that most of the enzyme activity is retained in the periplasmic space (arrows), even in severely damaged cells (D), while small amounts of reaction product form a "crust" at the surface of the cell (R) and small amounts of cell-free enzyme cause the production of discrete crystals (C) at the cell surface.*



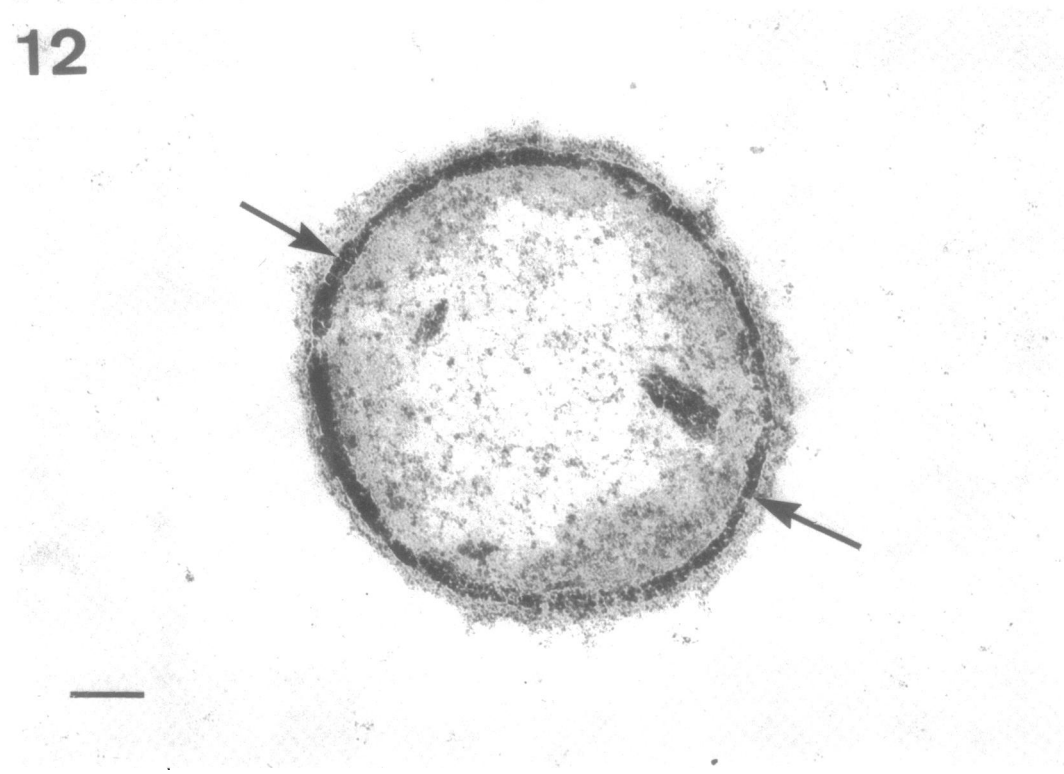
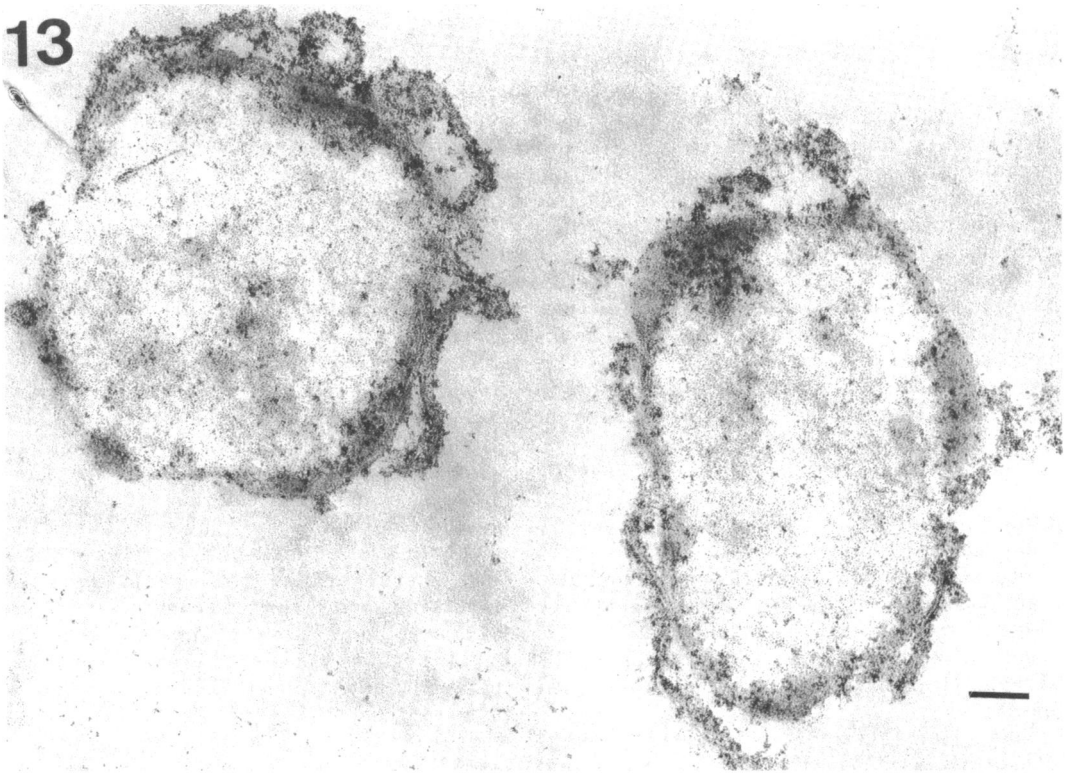




FIG. 11. *Electron micrograph of cells from a log-phase (10 h) culture of Bacteroides ruminicola grown in a synthetic medium. The cells had been incubated for reaction product deposition by alkaline phosphatase using PNPP as the substrate. Note that most of the enzyme activity is localized in the periplasmic space (arrows) but that small amounts of reaction product are associated with the outer layers of the cell wall.*

FIG. 12. *Electron micrograph of a spheroplast prepared from a 12-h cell of Bacteroides ruminicola by incubation with lysozyme in 0.2 M $MgCl_2$. These spheroplasts retain 75% of their alkaline phosphatase (Table 1), and this enzyme activity is exclusively localized in the periplasmic space (arrows) of cells incubated for reaction product deposition by alkaline phosphatase using PNPP as the substrate.*



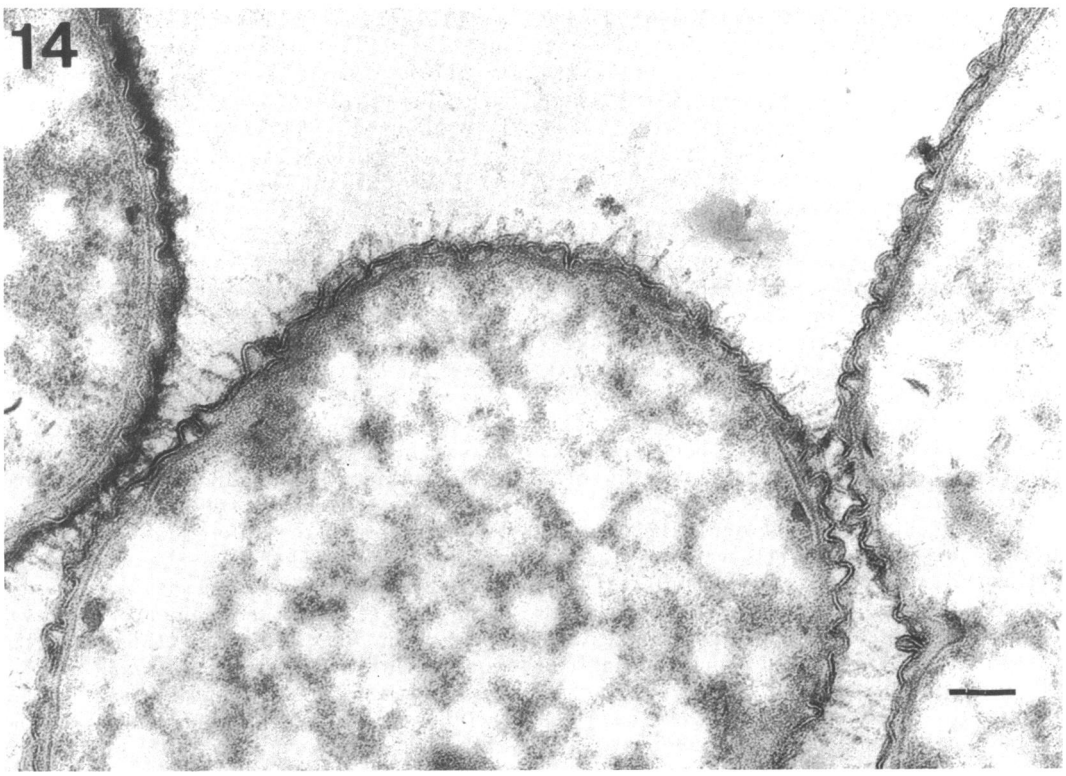




FIG. 13. *Electron micrograph of cells of a log-phase (10 h) culture of Bacteroides succinogenes incubated for reaction product deposition by alkaline phosphatase using PNPP as the substrate. Note that there is very little enzyme activity associated with these cells whose cell walls have been extensively damaged by washing in 0.01 M* $MgCl_2$.

FIG. 14. *Electron micrograph of cells of a log-phase (10 h) culture of Megasphaera elsdenii incubated for reaction product deposition by alkaline phosphatase using PNPP as the substrate. Note the virtual absence of reaction product in these cells.*

constitutents and cell walls of the "spontaneous spheroplasts" formed in old cultures of this organism are extensively damaged but that these cells retain large amounts of alkaline phosphatase in what remains of their periplasmic space.

The present data show that alkaline phosphatase is firmly associated with structural elements of the cell wall of this organism and is not released while the cells are alive. This enzyme was also found to be cell associated in the rumen fluid of cows fed a diet of hay or concentrate feed of coarse particle size (519 $\mu$m, geometric mean particle size). This suggests that the high levels of cell-free enzyme found in the rumen fluid of cattle fed fine particle size (344 $\mu$m, geometric mean particle size) concentrate feed (K.-J. Cheng and R. Hironaka, Can. J. Anim. Sci., in press) with high-energy content that induces "feedlot bloat" is the result of a massive breakage of bacterial cells (Cheng, Hironaka, and Costerton, unpublished data).

The alkaline phosphatase of *B. ruminicola* is constitutive, whereas that of *E. coli* (36) and that of *P. aeruginosa* (13) are inducible. Hence, the enzyme of *B. ruminicola* is present even though the concentration of inorganic phosphate in the rumen is maintained at a high level. This is advantageous to the organism because sugar phosphates can always be hydrolyzed to produce assimilable sugar molecules and phosphates.

Alkaline phosphatase has been shown to be associated with a structural component within the periplasmic space in *P. aeruginosa* (Cheng, Costerton, and Ingram, submitted for publication), and the tendency of this hydrophobic enzyme (D. F. Day and J. M. Ingram, Can. J. Microbiol., in press) to associate with lipopolysaccharide (LPS) in both this organism (J. M. Ingram, K.-J. Cheng, and J. W. Costerton, Can. J. Microbiol., in press) and in *S. typhimurium* (23), suggests that LPS is the structural component that binds the enzyme. LPS is present in the cell wall both in the periplasmic space and at the cell surface (34) in gram-negative aerobic bacteria and has also been shown to occur in gram-negative anaerobic bacteria (19). We cannot conclude that the alkaline phosphatase of *B. ruminicola* is specifically linked to LPS, and the tenacity of its binding suggests that other components or types of association may also be involved.

This binding of alkaline phosphatase by a structural component of the cell wall anchors the enzyme and provides it with a protected ionic environment because of the Donnan effect (16) exerted by the bound anions of the structural polymers. The periplasmic environment of the alkaline phosphatase of *P. aeruginosa* is so protective that the enzyme remains active in this space even when identical extracellular molecules have been inactivated by high $H^+$ concentration (pH 4.7) in culture (11).

The alkaline phosphatase of *B. ruminicola* is particularly well protected because it is exclusively periplasmic and is strongly bound to structural elements in this area and because the cell wall of the organism is unusually thick and elaborate. This thick external structure lying outside the double-track layer is found, to a greater or a lesser degree, in the cell walls of all three species studied here and in the mixed populations in rumen contents (Cheng, Hironaka, and Costerton, unpublished data), and its chemistry and structure will be reported in a subsequent paper. This thick, external layer of the cell wall would be expected to exclude certain ions and molecules and to attract others, thus conditioning the environment of cell wall-associated enzymes. In addition to this external layer, cells of *B. ruminicola* produced an extracellular material that adheres to cells in culture and is removed by washing with 0.01 M $MgCl_2$.

The taxonomy of rumen bacteria at species level is difficult due to the paucity of information available (3). The biochemical and morphological characteristics described here will help to bring order by the description of a wider variety of factors. For example, alkaline phosphatase is produced in large amounts by only one of the three organisms studied here and the enzyme of this species (*B. ruminicola*) shows a greater affinity for PNPP than for $\beta$-glycerophosphate, while organisms seen in mixed rumen populations readily use the latter as a substrate. The alkaline phosphatase level of *B. ruminicola* and *B. succinogenes* can be a useful criterion to differentiate these two species. The morphological characteristics of the cell wall of these two species are also very distinct, as is the ion requirement for cell wall integrity in *B. succinogenes* (Fig. 13). The requirement for $Na^+$ for *B. succinogenes* is of considerable interest, as only certain marine bacteria and a halophile, among bacteria so far studied, have shown a need for this ion in considerable amount (26). The amounts of $Na^+$ and $K^+$ necessary for best growth of strain S-85 are similar to those of marine bacteria (7, 26), and cell wall changes similar to those seen in

strain S-85 have been reported in the marine pseudomonad B-16 (15) when the ionic content of the menstruum is reduced. The large cytoplasmic glycogen granules seen in *M. elsdenii* are also a potentially useful taxonomic characteristic as are the granules of *Clostridium pasteurianum* (1). Detailed chemical studies of these granules will be reported in a subsequent paper.

There have been numerous reports that *B. ruminicola* and *B. succinogenes* are pleomorphic and that old cultures contain degeneration forms (1, 5, 8, 20), but we have observed a very constant morphology of the cells of both species except in aging cultures in which spontaneous spheroplasts are formed (10). In the present study, 80% of cells in a 24-h batch culture of *B. ruminicola* were seen to be spheroplasts. Electron microscopy has shown that these spontaneous spheroplasts are generally rounded and have enlarged chromatin areas and a somewhat disorganized cytoplasm, but that their cell walls generally retain the double-track layer so that they are true spheroplasts.

## ACKNOWLEDGMENTS

The capable technical assistance of Dale Cooper and C. L. J. Parfett is gratefully acknowledged.

This work was supported, in part, by grants from the National Research Council of Canada.

## LITERATURE CITED

1. Breed, R. S., E. G. D. Murray, and N. R. Smith (ed.). 1957. Bergey's Manual of Determinative Bacteriology, 7th ed. Williams and Wilkins, Baltimore.
2. Brockman, R. W., and L. A. Heppel. 1968. On the localization of alkaline phosphatase and cyclic phosphodiesterase in *Escherichia coli*. Biochemistry **7**:2554-2561.
3. Bryant, M. P. 1959. Bacterial species of the rumen. Bacteriol. Rev. **23**:125-153.
4. Bryant, M. P., and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205-217.
5. Bryant, M. P., and R. N. Doetsch. 1954. A study of actively cellulolytic rod-shaped bacteria of the bovine rumen. J. Dairy Sci. **37**:1176-1183.
6. Bryant, M. P., and I. M. Robinson. 1961. An improved non-selective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. **44**:1446-1456.
7. Bryant, M. P., I. M. Robinson, and H. Chu. 1959. Observations on the nutrition of *Bacteroides succinogenes*—a ruminal cellulolytic bacterium. J. Dairy Sci. **42**:1831-1847.
8. Bryant, M. P., N. Small, C. Bouma, and H. Chu. 1958. *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica* the new genus and species. Species of succinic acid-producing anaerobic bacteria of the bovine rumen. J. Bacteriol. **76**:15-23.
9. Caldwell, D. R., D. C. White, M. P. Bryant, and R. N. Doetsch. 1965. Specificity of the heme requirement for growth of *Bacteroides ruminicola*. J. Bacteriol. **90**:1645-1654.
10. Cheng, K.-J. 1973. Spheroplast formation by an anaerobic gram-negative bacterium *Bacteroides ruminicola*. Can. J. Microbiol. **19**:667-669.
11. Cheng, K.-J., D. F. Day, J. W. Costerton, and J. M. Ingram. 1972. Alkaline phosphatase subunits in the culture filtrate of *Pseudomonas aeruginosa*. Can. J. Biochem. **50**:268-276.
12. Cheng, K.-J., J. M. Ingram, and J. W. Costerton. 1970. Alkaline phosphatase localization and spheroplast formation of *Pseudomonas aeruginosa*. Can. J. Microbiol. **16**:1319-1324.
13. Cheng, K.-J., J. M. Ingram, and J. W. Costerton. 1970. Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and of pH. J. Bacteriol. **104**:748-753.
14. Cheng, K.-J., J. M. Ingram, and J. W. Costerton. 1971. Interactions of alkaline phosphatase and the cell wall of *Pseudomonas aeruginosa*. J. Bacteriol. **107**:325-336.
15. Costerton, J. W., C. W. Forsberg, T. I. Matula, F. L. A. Buckmire, and R. A. MacLeod. 1967. Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, spheroplasts and related forms from a gram-negative marine bacterium. J. Bacteriol. **94**:1764-1777.
16. Costerton, J. W. 1970. The structure and function of the cell envelope of gram-negative bacteria. Rev. Can. Biol. **29**:299-316.
17. Costerton, J. W. 1973. The relationship of a wall-associated enzyme with specific layers of the cell wall of a gram-negative bacterium. J. Bacteriol. **114**:1281-1293.
18. Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science **156**:1451-1455.
19. Hofstad, T., T. Kristoffersen, and K. A. Selvig. 1972. Electron microscopy of endotoxic lipopolysaccharide from *Bacteroides*, *Fusobacterium* and *Sphaerophorus*. Acta Pathol. Microbiol. Scand. section B **80**:413-419.
20. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1-49.
21. Kellenberger, E., J. Sechaud, and A. Ryter. 1959. Electron microscopical studies of phage multiplication. IV. The establishment of the DNA pool of vegetative phase and the maturation of phage particles. Virology **8**:478-498.
22. Kushnarev, V. M., and T. A. Smirnova. 1966. Electron microscopy of alkaline phosphatase of *Escherichia coli*. Can. J. Microbiol. **12**:605-608.
23. Lindsay, S. S., B. Wheeler, K. E. Sanderson, J. W. Costerton, and K.-J. Cheng. 1973. The release of alkaline phosphatase and of lipopolysaccharide during the growth of rough and smooth strains of *Salmonella typhimurium*. Can. J. Microbiol. **19**:335-343.
24. Lopes, J., S. Gottried, and L. Rothfield. 1972. Leakage of periplasmic enzymes by mutants of *Escherichia coli* and *Salmonella typhimurium*: isolation of "periplasmic leaky" mutants. J. Bacteriol. **109**:520-525.
25. MacAlister, T. J., J. W. Costerton, L. Thompson, J. Thompson, and J. M. Ingram. 1972. Distribution of alkaline phosphatase within the periplasmic space of gram-negative bacteria. J. Bacteriol. **111**:827-832.
26. MacLeod, R. A., and E. Onofrey. 1957. Nutrition and metabolism of marine bacteria. III. The relation of sodium and potassium to growth. J. Cell. Comp. Physiol. **50**:389-401.
27. Malamy, H. M., and B. L. Horecker. 1961. The localization of alkaline phosphatase in *E. coli* K12. Biochem. Biophys. Res. Commun. **5**:104-108.
28. Malamy, H. M., and B. L. Horecker. 1964. Release of alkaline phosphatase from cells of *E. coli* upon

lysozyme spheroplast formation. Biochemistry **3**:1889–1893.
29. Margaretten, W., C. Morgan, H. S. Rosenkranz, and H. M. Rose. 1966. Effect of hydroxy urea on virus development. I. Electron microscope study of the effect on the development of bacteriophage T4. J. Bacteriol. **91**:823–833.
30. Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685–3691.
31. Nisonson, I., M. Tannenbaum, and H. C. Neu. 1969. Surface localization of *Escherichia coli* 5′-nucleotidase by electron microscopy. J. Bacteriol. **100**:1083–1090.
32. Reynolds, E. S. 1963. The use of lead citrate at high pH as electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–242.
33. Rogosa, M. 1971. Transfer of *Peptostreptococcus elsdenii* Gutierrez et al. to a new genus, *Megasphaera* [*M. elsdenii* (Gutierrez *et al.*) comb, nov.]. Int. J. Syst. Bacteriol. **21**:187–189.
34. Shands, J. W. 1966. Localization of somatic antigen on gram-negative bacteria using ferritin antibody conjugates. Ann. N. Y. Acad. Sci. **133**:292–298.
35. Shilo, Miriam. 1970. Lysis of blue-green algae by myxobacter. J. Bacteriol. **104**:453–461.
36. Singh, A. P., K.-J. Cheng, J. W. Costerton, E. S. Idziak, and J. M. Ingram. 1972. Sensitivity of normal and mutant strains of *Escherichia coli* towards actinomycin-D. Can. J. Microbiol. **18**:909–915.
37. Wetzel, B. K., S. S. Spicer, H. F. Dvorak, and L. A. Heppel. 1970. Cyto-chemical localization of certain phosphatases in *Escherichia coli*. J. Bacteriol. **104**:529–542.