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Demonstration of Ribosomes in Mesosomes Associated with *Bacillus subtilis* Protoplasts

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The physiological differences between *Bacillus subtilis* (ATCC 6633) cells derived from a glucose-salts-yeast extract (GSY) medium and those of cells from tryptose broth permitted the identification of variables in protoplasting environments which noticeably affected the clarity of mesosomal ribosomes. They were the sucrose and magnesium ion concentrations and the type of buffer used. The environment suitable for conversion of GSY cells to the protoplast state was a 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.2, containing 0.6 M sucrose and 0.03 M $MgCl_2$. Branched mesosomal tubules and a unique organization of vesicles were detected in thin sections and in negative stains of the specimens. Ribosomes were demonstrable in the extruded structures associated with protoplasts that had been prepared according to four fixation schedules and embedded in either of two epoxy plastics. Adjustments in the fixation schedules improved the clarity of the large bodies of protoplast cytoplasm to a degree equivalent to that of their dangling appendages.

Electron-dense particles have been detected in mesosomal tubules and vesicles associated with protoplasts of *Bacillus subtilis* ATCC 6633 (7). They were assumed to be ribosomes on the basis of their size and staining properties. The reasons behind the unusual clarity of these specimens were not clear. The cells were derived from an enriched culture medium and were converted to protoplasts in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.2 M sucrose and 0.02 M magnesium chloride. In comparison to amounts used by others for protoplasting solutions, the sucrose concentration was low (3, 4, 9, 11-13), and the magesium ion level was very high (3, 11-13). In previous work with this species the organisms were grown in a semidefined medium (12, 13). A new fixation schedule and a new epoxy plastic were used in the recent study of *B. subtilis* protoplasts.

The objectives of this paper are to present the influence of these variables on the demonstration of mesosomal ribosomes and to report new features of complexity in protoplast appendages (branching and a unique organization of vesicles) that were detected in the course of these comparisons.

MATERIALS AND METHODS

B. subtilis ATCC 6633 was grown in tryptose broth (TB) (Difco, Detroit, Mich.) or in the glucose-salts medium of Roberts and Johnson (10) modified by the addition of 0.075% yeast extract (GSY broth). Cells or their derived protoplasts harvested from these media will be referred to in this paper as TB or GSY specimens. Twenty-milliliter amounts of media were inoculated with 4×10^3 spores. The cultures were incubated at 37 C on a gyratory shaker (model G25, New Brunswick Scientific Co., Inc.) set at 160 rpm. After 11 h in GSY broth and 12 h in TB, the cultures were diluted 1:20 in fresh media, and incubation was continued for an additional 1.75 h.

The buffers employed in solutions used for protoplast formation (PM) were 0.02 M sodium or potassium phosphate, pH 7.0, and 0.02 M tris(hydroxymethyl)-aminomethane (Tris)hydrochloride buffer, pH 7.2. A stock solution of 1.6 M sucrose and one of 0.2 M $MgCl_2$, dissolved in the desired buffer, were used to prepare solutions varying in sucrose and magnesium ion as indicated in the Results. To avoid repetition, the sucrose and magnesium ion concentrations and the kind of buffer used in the PM are sometimes given in parentheses. Cells were harvested by centrifugation at $9,200 \times g$ for 15 min. They were washed once with the desired media and were resuspended in them to a density of 10^9 to 2×10^9 colony-forming units/ml. Egg-white muramidase was added to the cell suspensions to a final concentration of 500 μg/ml. Conversion to protoplasts was complete within 30 min at 37 C for GSY cells in all the solutions tested, but with TB cells it was retarded in the presence of increased amounts of sucrose or magnesium chloride.

The details of the four fixation schedules used in this study are presented in Table 1. With the excep-

tion of the solutions prepared in Tris and Veronal acetate buffers (5), which were pH 7.2 and 6.1, respectively, the pH of all PM, washing buffers, and fixatives was 7.0. Primary fixation was accomplished by mixing the protoplasts 1:1 with chilled 12.5% glutaraldehyde containing sucrose equivalent to that present in the PM. The mixture was incubated in a refrigerator for 2 h. Fixed protoplasts were collected by centrifugation at 30,900 × *g* for 15 min. Specimens were embedded in agar directly after removing the primary fixative in schedules 1 and 4, and after osmium fixation in schedules 2 and 3. Washing of specimens between the primary and secondary fixations was carried out over a 16- to 18-h period with two changes of buffer in the first hour and a third the next morning. Since protoplasts are more like mammalian cells with respect to being wall-less, the 24-h osmium fixation period usually used with bacterial cells was reduced to 1 h in most of the schedules or 1, 2, or 4 h in schedule 4. Incorporation of specimens in agar was accomplished as follows. A test tube containing a pellet of protoplasts was placed in a water bath set at 45 C for a brief interval. A small amount (approximately 0.2 ml) of 2% buffered Noble agar (Difco) was added with a Pasteur pipette (with a 10-cm tip of not more than 1-mm inside diameter). The mixture was gently pipetted two times to obtain a fairly even suspension, and an amount not exceeding the tip length was permitted to gel therein. Slight positive pressure was sufficient to expel the agar column onto

TABLE 1. *Comparison of protoplasting environments and fixation schedules*

Stage of preparation	Schedule				
	1	2	3	4	Ryter-Jacob (12)
Induction of protoplasts	In suspension	Same as 1	Same as 1	Same as 1	In agar
Concn of Mg^{2+} in PM	≥0.02 M	0.02 M	0.02 M	0.02 M	5×10^{-4} M
Concn of stabilizer in PM	0.2–0.6 M sucrose	0.2 M sucrose	0.2 M sucrose	0.2 M sucrose	0.5 M succinate
Buffer salt in PM	Tris, Na^+ or K^+ phosphate	Na^+ phosphate	Tris	Tris	K^+ phosphate
Primary fixation	Mixed 1:1	Mixed 1:1	Mixed 1:1	Mixed 1:1	Agar cubes placed in fixative
Concn of glutaraldehyde added	12.5%	12.5%	12.5%	12.5%	6.25%
Buffer in fixative	0.1 M Na^+ or K^+ phosphate	0.2 M cacodylate HCl	0.1 M Na^+ phosphate	0.2 M cacodylate HCl	0.1 M cacodylate HCl
Sugar present	+	+	+	+	–
Duration of fixation	2 h	2 h	2 h	2 h	2 h
Washing Buffer	0.05 M Na^+ or K^+ phosphate	Veronal acetate	0.05 M Na^+ phosphate	Veronal acetate	Veronal acetate
Time	18 h	18 h	18 h	18 h	2 h
Secondary Fixation	In agar	In suspension	Same as 2	Same as 1	In agar
Concn of Osmium	2%	1%	1%	2%	1%
Buffer	0.05 M Na^+ or K^+ phosphate	Veronal acetate	Veronal acetate	Veronal acetate	
Fixation time	1 h	1 h	1 h	1, 2, or 4 h	24 h
Incorporation in agar	After primary fixation	After osmium fixation	After osmium fixation	After primary fixation	Cells were included in agar containing muramidase
Wash with dilute uranyl acetate[a]	–	–	–	±	+
Number of centrifugations required	1	4	4	1	0

[a] Symbols: –, no uranyl acetate wash was used; +, wash was used; ±, wash was used in some experiments and not in others.

a slide where it was then sliced into small pieces.

The agar-embedded specimens were dehydrated by passage through graded concentrations of ethanol, placed for 15 min each in two baths of propylene oxide, and then infiltrated with Luft's epon (LE) (6) or with a new Epon 812 medium (7) devised by one of us (Smith) (SE). The specimens were drained on absorbent paper, placed in capsules filled with degassed plastic, and left at 25 C until they had settled in the capsules. The blocks were cured at 60 C for 22 to 24 h. Sections (gray-silver) were stained for 30 min with saturated aqueous uranyl acetate, followed by 2 min with alkaline lead citrate. Electron micrographs were taken with an AEI model EM 6B electron microscope operating at 60 kV.

For examination of negatively stained specimens, protoplasts (TB, 0.2 M sucrose-0.02 or 0.03 M magnesium ion in sodium phosphate buffer) were mixed 1:1 with 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0, and 0.2 M sucrose. A 1:200 dilution was made with PM 1.5 h later. Using the virus rotor (type SU) made by Ivan Sorvall Inc. (Norwalk, Conn.), the protoplasts were gently deposited by centrifugation at 77 $\times$ *g*, for 5 min, on carbon-collodion-coated grids that had been mounted in the Lucite cells. The grids were removed, drained of excess fluid with filter paper, stained with 1% phosphotungstic acid (adjusted to pH 7.0 with sodium hydroxide) (PTA) in 6.3 $\times$ 10^{-3} M sucrose, dried briefly, and placed in the electron microscope for examination.

RESULTS

That physiological differences of GSY and TB cells dictate the conditions necessary to obtain equivalent specimen clarity was demonstrated in preliminary work with cells of this less enriched growth history. The PM used with the TB organisms (0.2 M sucrose-0.02 M magnesium ion in sodium phosphate buffer) was grossly inadequate for GSY cells. A few of them lysed spontaneously in this environment. The contrast of the protoplasts that formed either spontaneously or through muramidase action was inferior to that of the TB specimens. The most likely factor involved is the low level of sucrose, but the monovalent cation, sodium, or the orthophosphate present could also play roles. To isolate and assess the influence of the buffer salt on specimen clarity in the absence of the other variables, TB and GSY protoplasts were formed in low sucrose, sodium, potassium and Tris-buffered solutions (0.2 M sucrose-0.02 M magnesium ion, schedule 1). The GSY protoplasts, formed in the presence of potassium (Fig. 1), did not appear very different from those formed in the sodium PM (not shown). The tubules and vesicles were not quite as numerous, and those remaining appeared to be in advanced stages of degeneration. The overall contrast was very poor, necessitating correction in the photographic reproduction. The cytoplasmic ribosomes were not distinct. The contrast of GSY protoplasts formed in the Tris PM (Fig. 2) was good in spite of the fact that the level of sucrose and magnesium present was minimal. The membrane surfaces were smooth, and the cytoplasm and mesosomal content (arrows) were more defined than in Fig. 1. As expected, the clarity of GSY Tris protoplasts improved as the environmental sucrose and magnesium ion used for induction were increased. Ribosomes, 170 to 190 nm in size, were visible in the extruded structures as well as in the cytoplasm of protoplasts induced in the presence of 0.6 M sucrose-0.03 M magnesium chloride (Fig. 3). The integrity of the mesosomal appendages of TB protoplasts and their overall contrast was equivalent to that reported (7), regardless of the PM used. Those prepared in low sucrose Tris PM (0.2 M sucrose, 0.02 M divalent ion) are illustrated in Fig. 4 for comparison with the GSY protoplasts (Fig. 1-3).

TB protoplasts (sodium phosphate buffer containing 0.2, 0.3, 0.4 M sucrose and various amounts of magnesium) were fixed (schedule 1), dehydrated, and embedded in either LE or SE to compare the staining properties of these plastics. Regardless of the kind of environment employed in preparation, the ribosome contrast in the paired specimens was similar. Protoplasts prepared in the presence of 0.2 M sucrose-0.04 M magnesium ion (LE, Fig. 5a, and SE, Fig. 5b) and in 0.4 M sucrose-0.02 M magnesium chloride PM (Fig. 7) are shown to illustrate the effect of increased levels of divalent cation and sucrose on the conversion of enriched cells to the protoplast state.

The vesicles in Fig. 5a and 5b, indicated with large arrows, contain ribosomes. Unlike the GSY protoplasts, these specimens were surrounded by considerable amounts of wall material. The tubules are branched. The inner membrane surfaces forming the core can be traced into each new limb of the tubule in Fig. 5a. Structures in different planes of the section show increased density in that area. A ribosome is discernible in the fork of the tubule in Fig. 5b.

An unusual organelle, encountered in the examination of the TB protoplasts prepared in 0.4 M sucrose-0.02 M magnesium ion in sodium phosphate PM is illustrated in Fig. 7. Inhibition of protoplast formation was somewhat less in this environment than in one of high magnesium content, since only a few of the specimens were surrounded by residual wall. The large vesicle (1,800 nm) is reminiscent of one that was detected in the mesosome associated with new cross-wall formation in *B. megaterium* (2). It is surrounded by at least six 100-nm tubules (or vesicles) and by a body of cytoplasm whose

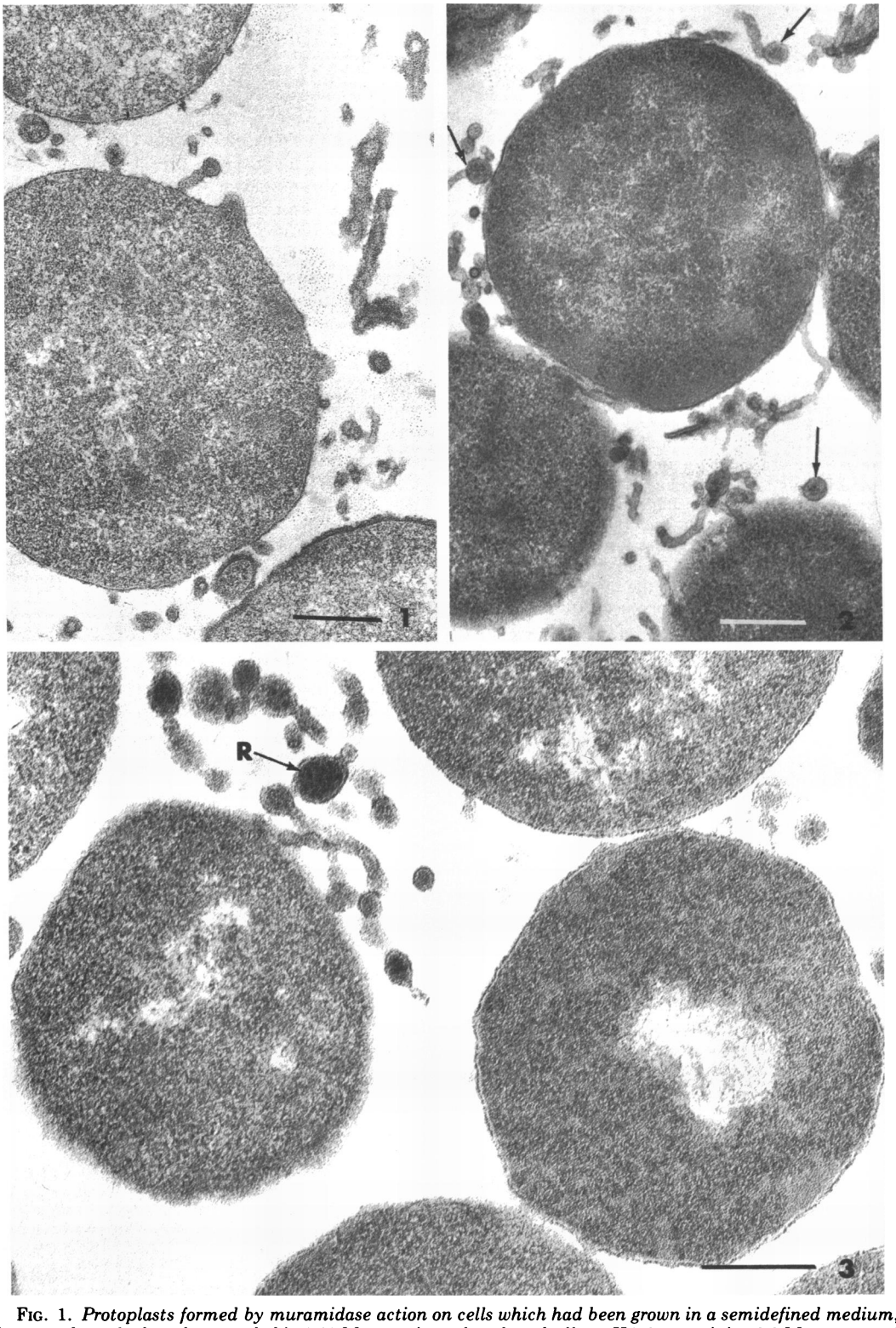

FIG. 1. *Protoplasts formed by muramidase action on cells which had been grown in a semidefined medium, harvested, washed, and suspended in 0.02 M potassium phosphate buffer, pH 7.0, containing 0.2 M sucrose and 0.02 M Mg^{2+}. The contrast was inferior to that of TB and GSY (Tris) protoplasts. Schedule 1 fixation. SE infiltration. Scale markers on this and subsequent figures indicate 0.25 μm.*

FIG. 2. *GSY protoplasts formed in 0.2 M sucrose and 0.02 M Mg^{2+} in 0.02 M Tris Buffer, pH 7.2. Schedule 1 fixation. SE infiltration.*

FIG. 3. *Ribosomes (R) are visible in vesicles associated with GSY protoplasts formed in 0.6 M sucrose and 0.03 M Mg^{2+} in Tris buffer. Schedule 1 fixation. SE infiltration.*

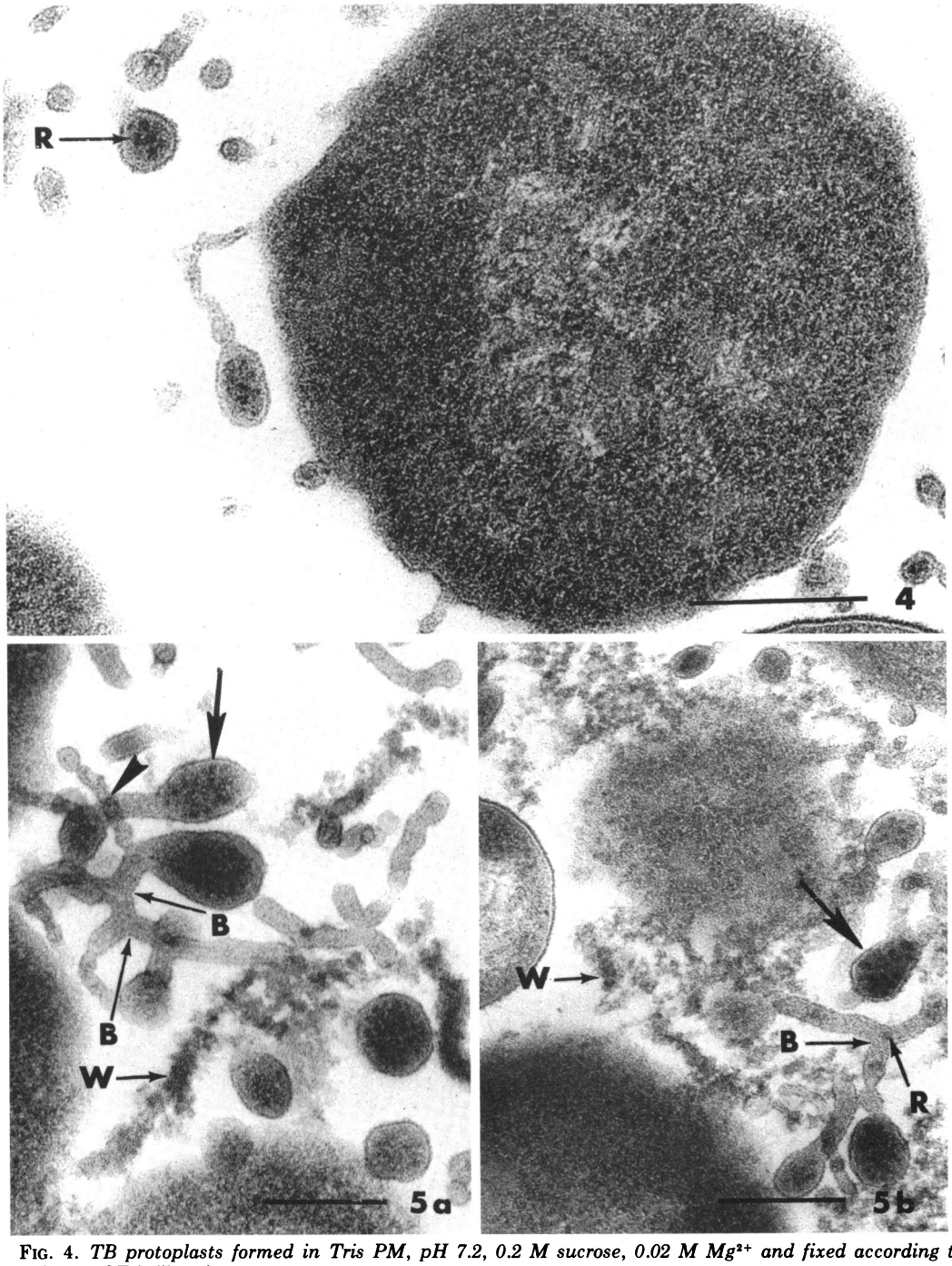

FIG. 4. *TB protoplasts formed in Tris PM, pH 7.2, 0.2 M sucrose, 0.02 M Mg^{2+} and fixed according to schedule 1. SE infiltration.*

FIG. 5. *a, TB protoplasts embedded in Luft (6) Epon. The mesosomal structures are enmeshed in wall (W) residue. Two branch points (B) are present. Tubules located in different planes of the section are indicated by a marker (➤). Schedule 1. b, The same protoplasts as those in Fig. 5a except embedded in the new plastic (SE). The tubule is branched, and a ribosome (R) is situated in the fork. Schedule 1.*

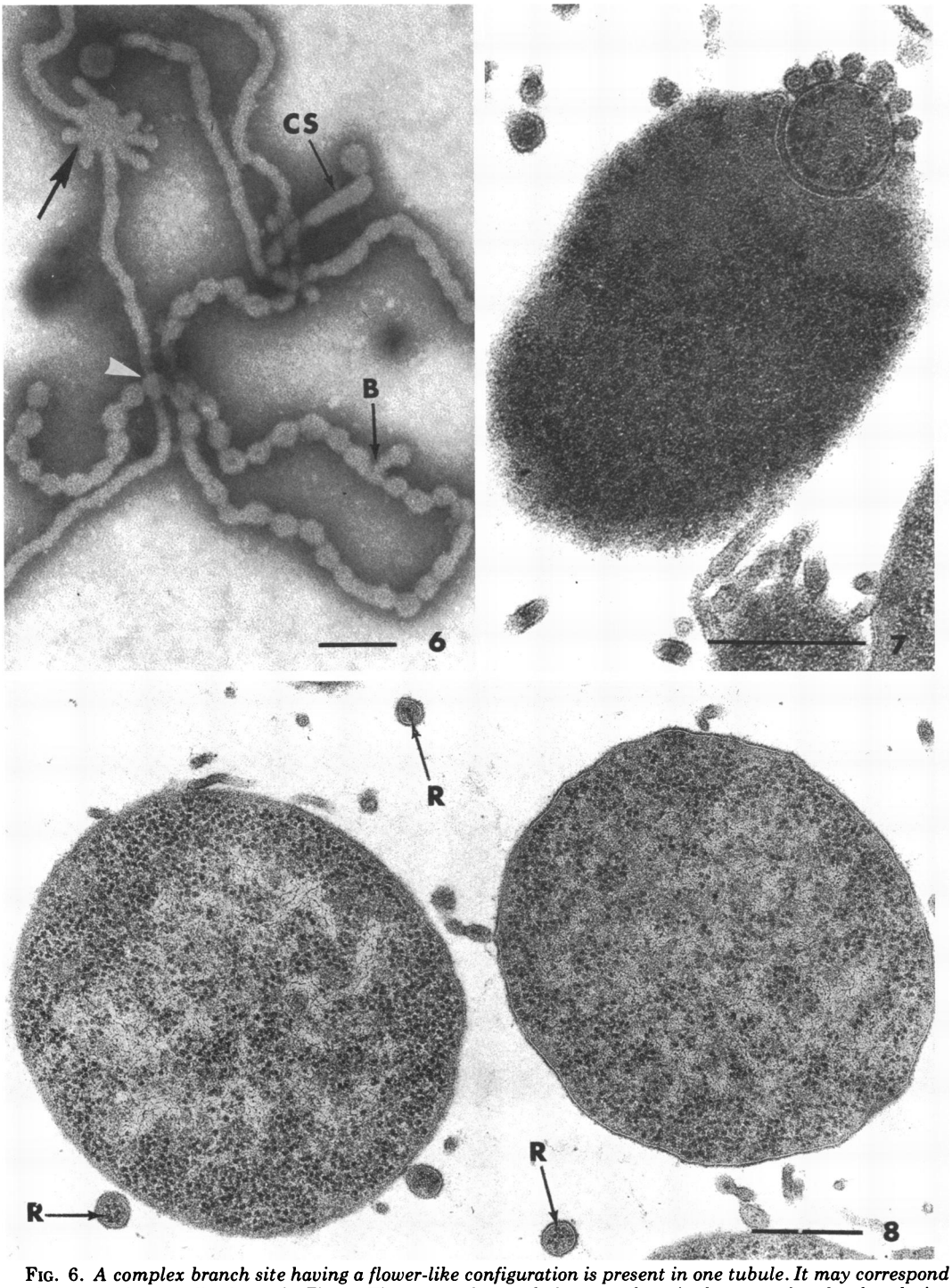

FIG. 6. *A complex branch site having a flower-like configuration is present in one tubule. It may correspond to the unusual organelle shown in Fig. 7. The areas where tubules were deposited on top of each other during centrifugation are indicated (➤). CS, Cylindrical segments.*

FIG. 7. *A large spherical vesicle, 1,800 nm in diameter, containing ribosomes, is surrounded on its exposed half by at least six 100-nm tubules. The protoplast cytoplasm in the immediate vicinity of this organelle is free of ribosomes. The PM for this TB specimen was 0.4 M sucrose and 0.02 M $MgCl_2$ in Na^+ phosphate buffer. Schedule 1. SE infiltration.*

FIG. 8. *With the exception of 1 h instead of 24 h for osmium fixation, the schedule of Ryter and Jacob (12) was used with these protoplasts (TB, 0.2 M sucrose, 0.02 M Mg^{2+}, Na^+ PM, schedule 2). SE infiltration.*

membrane is visible only near the point of contact. A tengential cut through the protoplast would account for the invisibleness of the remaining portion of plasma membrane. The cytoplasm in the immediate vicinity of the 1,800-nm sac is free of ribosomes.

Protoplasts (TB, 0.2 M sucrose, 0.02 or 0.03 M magnesium chloride in sodium phosphate) were stained with PTA for examination of the entire appendage structures since thin sections are limited in being two-dimensional slices of them. The appendages released in 0.02 M magnesium PM were much less stable than those associated with the 0.03 M protoplasts (Fig. 6). This damage was caused by PTA and was related to the low glutaraldehyde concentration (1%) used as the fixative. Portions of the tubules were beaded, and cylindrical segments like those mentioned by Ryter et al. (11) as part of *B. subtilis* mesosomal structures were also evident. Simple branch sites, an example of which is indicated in Fig. 6, were characteristic features of most tubules. Sites of multiple branching (large arrow, this figure) were not as common. Since the morphology is similar to that reported by others (8, 12, 13) and since the greater complexity can be attributed to the environmental divalent cation being 40-fold higher (Table 1) than that employed in the previous work on this species, the protoplast appendages observed in these experiments are considered to be mesosomal structures.

The interior of protoplasts prepared according to schedule 1 was often poorly defined, even when Tris buffer was used in the PM (Fig. 3 and 4), yet the mesosomal appendages seemed to have been adequately preserved. Three additional schedules were used to fix TB protoplasts to improve the clarity of the cytoplasm. Procedures of schedules 2 and 3 differ from schedules 1 and 4 in that the protoplasts were fixed with osmium as a suspension instead of through an agar matrix. In one case (schedule 2) the PM contained sodium phosphate while the other was a Tris PM (procedure of schedule 3). In schedules 2 and 4, sodium cacodylate, which is toxic and an enzyme inhibitor, was used to buffer the glutaraldehyde. Sodium phosphate was used as the buffer for the primary fixative in schedules 1 and 3. The interval for osmium fixation was varied 1, 2, and 4 h in schedule 4. The overall clarity of the products obtained with procedures 2 and 4 (Fig. 8 and 10) was good, but the interior of the Schedule 3 specimen (Fig. 9) was no better than that of the protoplasts in Fig. 4. Increasing the osmium fixation time (schedule 4) to 2 h caused a noticeable improvement in the quality of the interior, but post osmium treatment with aqueous uranyl acetate did not affect it.

DISCUSSION

The buffer salt and the sucrose and magnesium ion concentrations in protoplasting environments are the most important factors in the demonstration of ribosomes in *B. subtilis* mesosomes. The staining characteristics of the plastic embedment were not significant. Mesosomal ribosomes were demonstrable with specimens fixed by any of four schedules, but fixation variables could be important with this organism in significantly different physiological states or with other organisms. This belief is based on the finding that it was possible to improve the clarity of the larger bodies of protoplast cytoplasm by making adjustments in the fixation schedule.

An ideal environment for protoplast formation should (i) permit ready conversion of cells, (ii) preserve as nearly as possible the native degree of order, (iii) prevent degeneration of the membranes, cytoplasm, and delicate mesosomes, and (iv) yield a product with excellent contrast in all structures. There was considerable variation with respect to these requirements with *B. subtilis* in different physiological states. High concentrations of sucrose or divalent cation, or both were unnecessary for achieving excellent specimen detail with TB cells and had the undesirable effect of inhibiting protoplast conversion. Both variables were required to prevent the degeneration of the protoplast structures and to obtain an equivalent degree of order and contrast with GSY cells. The contribution of Tris in the environment toward increased contrast of GSY protoplasts is less clear. It prevented spontaneous protoplast conversion. The absence of orthophosphate or the Tris may have prevented certain biochemical activities leading to degenerative changes in a non-nutritive environment.

The critical variable in achieving a degree of clarity in the cytoplasm of *B. subtilis* protoplasts equivalent to that of their mesosome structures was the use of sodium cacodylate as buffer for the primary fixative. The cytoplasm of schedule 3 protoplasts, even though they had been induced in a Tris PM and fixed with osmium as a suspension, was not as clearly defined as that of schedule 2 and 4 specimens. A comparison of the latter procedures with that reported by Ryter and Jacob (12) (Table 1) indicates four differences: (i) the concentration of magnesium in the protoplasting environment is high, (ii) the protoplasts are fixed by adding

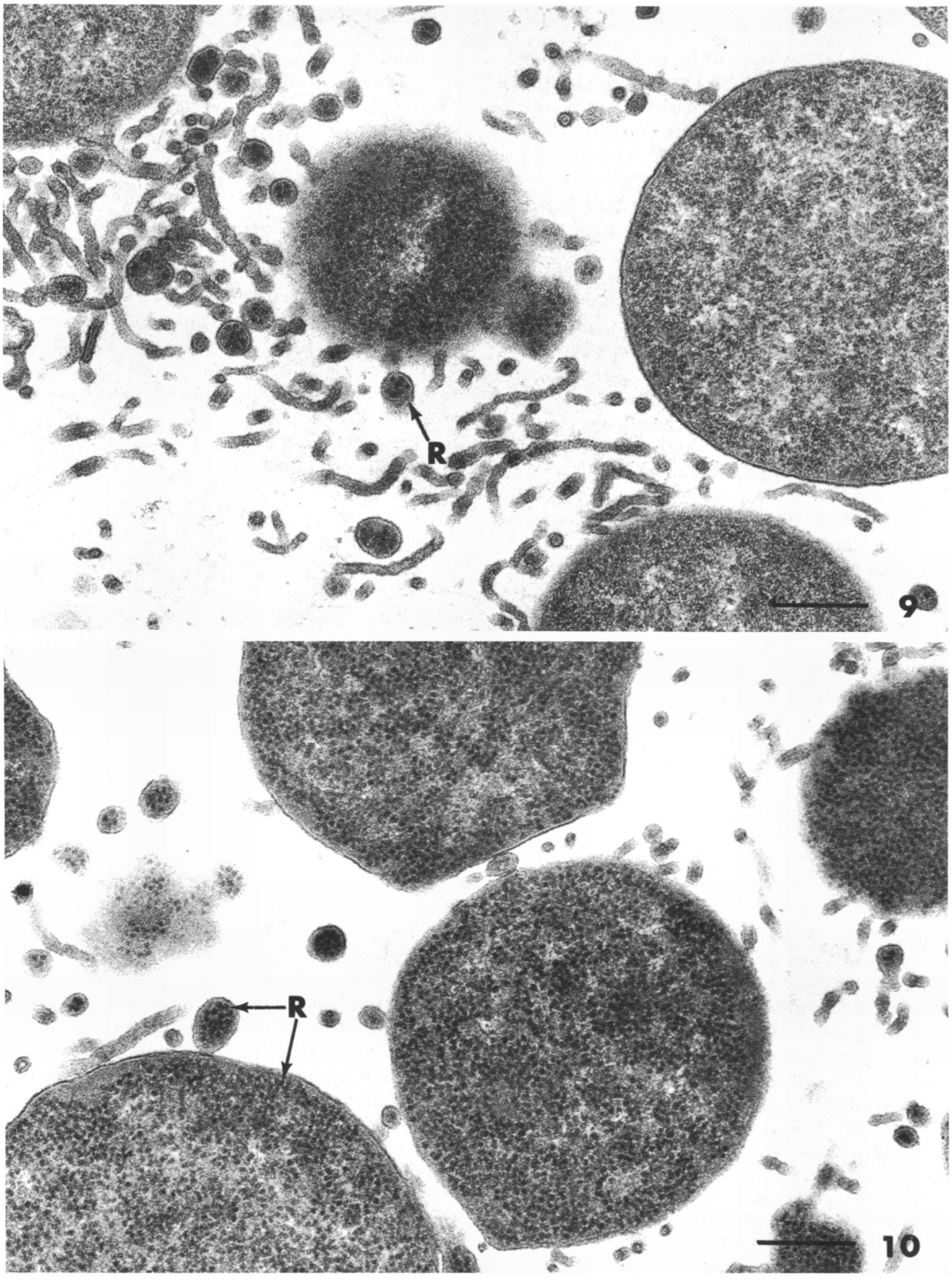

FIG. 9. *TB protoplasts prepared in Tris PM and fixed with glutaraldehyde buffered with sodium phosphate. The fixed protoplasts were collected by centrifugation and washed with three changes of Veronal acetate buffer (5) in a 16- to 18-h period. They were fixed for 1 h with 1% osmium as a suspension, collected by centrifugation, and incorporated in agar (schedule 3). SE infiltration.*

FIG. 10. *TB protoplasts prepared in Tris PM and fixed with glutaraldehyde in sodium cacodylate, incorporated in 2% agar, washed overnight with Veronal acetate (5), fixed again with 2% osmium in Veronal acetate for 2 h (schedule 4). SE infiltration.*

an equal volume of 12.5% glutaraldehyde instead of through an agar matrix, (iii) the washing time is increased between glutaraldehyde and osmium fixations, and (iv) the osmium fixation time is reduced.

Burdett and Rogers (1) reported the occurrence of branched mesosomal tubules associated with *B. licheniformis* protoplasts prepared by the usual droplet type, negative staining procedure. They minimized damage by mixing specimen with stain. Thus, the dangling appendages were subjected only once to shear forces while draining off excess fluid with filter paper. In the present technique the specimens were first gently deposited on the film-coated grid by using a very mild centrifugal force before removing excess fluid. The detection of simple and multiple branch sites in thin section (Fig. 5a and 5b) argues against an explanation that they are artifacts inherent in the negative staining procedure. The significance of the organelle illustrated in Fig. 7 is unknown. Its dimensions and the structures in the lower right portion of this micrograph and in the upper left corner of Fig. 3 of reference 7, suggest that it may be related to the sites of multiple branching observed in the negative stains.

The present findings are not consistent with an argument that the presence of ribosomes in protoplast mesosomes arises as the appendage everts during conversion or when a poor stabilizing environment permits swelling of the protoplast and gross eversion of cytoplasm through the disintegrating wall. They were visible in the appendages associated with protoplast cells that were induced in environments which retarded and even inhibited conversion. They have been seen in one or two small vesicles among mesosomal tubules still confined by wall material when cells were fixed as early as 3 min after the addition of muramidase (unpublished observations) and in mesosomes associated with septa of control cells of these experiments. It is possible that, during and after treatment with muramidase, there is a stimulus for cytoplasmic ribosomes to move into the appendages to join those already present. Small groups of electron-dense particles are often seen at the appendage attachment area. Such protoplasts are always free of any wall material.

ACKNOWLEDGMENT

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