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Sequence of Events in the Digestion of Fresh Legume Leaves by Rumen Bacteria

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When fresh whole leaves of six different species of forage legumes were suspended in an artificial rumen medium and inoculated with rumen bacteria, bacterial adhesion and proliferation were noted at the stomata, and penetration of the stomata by these bacteria was documented by electron microscopy. The invading bacteria adhered to surfaces within the intercellular space of the leaf and produced very extensive exopolysaccharide-enclosed microcolonies. After some of the legume leaf cell walls were disorganized and ruptured by bacterial digestion, these cells (notably, parenchyma and epidermal cells) were invaded by bacteria, with subsequent formation of intracellular microcolonies. However, other cells were neither ruptured nor colonized (notably, stomata guard cells and vascular tissue). At all stages of the digestion of intact legume leaves, the rumen bacteria grew in microcolonies composed of cells of single or mixed morphological types, and a particular ecological niche was often completely and consistently occupied by a very large microcolony of cells of single or mixed morphological types.

The bacterial digestion of cut grass leaves and of cell walls prepared from various grasses has been documented by several groups (1, 2, 9, 13, 14). Brazle and Harbers (3) studied the digestion of air-dried alfalfa hay by scanning electron microscopy, but no detailed studies of the sequence of events in the bacterial digestion of fresh whole legume leaves have been reported.

Various bacteria have been shown to adhere to plant cell walls to produce "pits" by their cellulolytic activity (1, 2, 5). The cell walls of different plant tissues have shown sharp differences in the extent to which they are colonized and digested by bacteria (1, 2, 5), and workers in the United Kingdom (9, 13, 14) and in the southern United States (1, 2) have found that morphologically different cellulolytic bacteria predominate in the digestion of plant materials in their particular geographic regions. Studies of the adhesion of groups of rumen bacteria to their polymeric substrates have shown that amylase producers adhere to starch (11) but not to cellulose and that cellulose decomposers adhere to cellulose (17, 18) but not to starch. Thus, we expect that plant material will be heavily colonized by various types of bacteria soon after it is introduced into the rumen; therefore, it is not surprising that Forsberg and Lam (10) found 75% of the adenosine triphosphate of the bacteria in the rumen contents to be associated with food particles.

Because transmission electron microscopy of ruthenium red-stained sections allows both bacteria and their exopolysaccharide products to be seen throughout leaf tissues during digestion, we incubated intact legume leaves with rumen bacteria and examined them at intervals using this method. We have shown (7) that the rumen bacterial population is made up of three distinct subpopulations—the rumen fluid bacteria, the food particle-associated bacteria, and the bacteria adherent to the rumen epithelium. To obtain a good representation of plant cell-digesting bacteria in these studies, we used both rumen fluid bacteria and food particle-associated bacteria as a combined inoculum (J. P. Fay, K.-J. Cheng, M. R. Hanna, R. E. Howarth, and J. W. Costerton, J. Dairy Sci., in press) in this study of the in vitro bacterial digestion of the intact leaves of six different species of forage legumes.

MATERIALS AND METHODS

Plant material. Trek alfalfa, Lasalle red clover, Merit white clover, Oxley cicer milkvetch, Melrose sainfoin, and Leo birdsfoot trefoil were grown in a greenhouse. Leaves were manually picked from vigorous plants in the prebud or bud stages of growth and 5 g (fresh weight) was placed into each digestion flask.

Microbial inocula. Rumen fluid and solid contents were obtained from a fistulated Holstein cow self-fed on cubed alfalfa hay. Solid contents were removed from the upper third of the ingesta. A combined inoculum (Fay et al., in press) was made from both solid and liquid ruminal contents: 300 g of solid ruminal contents and 300 ml of rumen fluid were homogenized

in a Waring blender at full speed for two 30-s periods, squeezed through two layers of cheesecloth into a graduated cylinder, and transferred directly to the incubation flasks. A CO_2 atmosphere was maintained in all vessels used for the collection and preparation of inoculum. Inoculum (60 ml) was transferred into 500-ml digestion flasks containing 315 ml of Dehority basal medium (20) and plant material or medium alone.

Medium and incubation conditions. An artificial medium (20) without carbohydrate addition was used for all experiments. Incubation was carried out in anaerobiosis (under an initial CO_2 atmosphere) in 500-ml digestion flasks placed in a water bath at 38°C. Control flasks without microbial inoculum were set up in all cases. The anaerobic technique used to culture mixed rumen bacteria throughout this investigation was essentially that of Hungate (12) as modified by Bryant and Burkey (4). In this technique, anaerobic conditions are obtained by displacement of all air in flasks with CO_2 made oxygen-free by passage over heated copper. Cysteine hydrochloride (0.1%) is added to media to produce in them a low oxidation-reduction potential, and resazurin (0.0001%) is added as an indicator of anaerobiosis.

Ruthenium red staining of whole leaves. Leaves were removed from a parallel set of digestion flasks at 10 min and 2, 8, 16, and 22 h and stained in 10 ml of a solution containing 0.15% (wt/vol) ruthenium red and 0.5% (vol/vol) glutaraldehyde in 100 mM cacodylate buffer at pH 7.2 (19). After 1 h at room temperature, this solution was decanted and replaced by one containing 0.05% (wt/vol) ruthenium red and 5.0% (vol/vol) glutaraldehyde in 100 mM cacodylate buffer (pH 7.2). After 2 h at room temperature, this solution was decanted, and the leaves were washed five times (10 min per wash) in cacodylate buffer containing 0.05% ruthenium red. The whole leaves were prepared as wet mounts and photographed with Kodachrome film.

Electron microscopy. Leaves were fixed in glutaraldehyde in the presence of ruthenium red and washed in cacodylate buffer containing ruthenium red as described for ruthenium red staining of whole leaves (above). After these treatments, leaves to be embedded for transmission electron microscopy were postfixed in 2% OsO_4 in cacodylate buffer with 0.05% (wt/vol) ruthenium red and washed five times in this buffer before dehydration in acetone and propylene oxide and embedding in Vestopal. The dehydration solutions were made up with the buffer with 0.05% ruthenium red up to the 50% acetone stage. Above this level, buffer alone was substituted because the stain was not sufficiently soluble. Sections were cut, stained with uranyl acetate and lead citrate, stabilized by carbon evaporation, and examined using an A.E.I. EM801 electron microscope at an accelerating voltage of 60 kV.

Samples for scanning electron microscopy were fixed as for transmission electron microscopy, treated with thiocarbohydrazide and OsO_4 by the method of Malick and Wilson (16) to induce conductivity, dehydrated in ethanol, dried by the critical-point method using Freon 13, gold coated, and examined with a Hitachi 450 scanning electron microscope.

RESULTS

Ruthenium red is an electron microscopy stain that is specific for anionic polymers (15), most of which are carbohydrates. When intact leaves of forage plants are incubated with rumen bacteria, this stain is particularly useful because it binds to carbohydrates produced or released by bacterial activity and allows the identification of foci of bacterial digestion by the unaided eye (Fig. 1B). Figure 1 shows leaves of a representative species of forage plants that had been inoculated with rumen bacteria and incubated for 16 h (Fig. 1B) and 22 h (Fig. 1C and D), and the dark red foci of digestion are easily discerned.

Examination of these dark red-stained digested areas by light microscopy shows that the leaf cells are extensively degraded so that only the red-staining cell walls remain and that the intracellular spaces and even the surface of these digested leaves contain large masses of bacteria. This technique is useful because, in addition to providing a qualitative estimate of bacterial activity, it allowed us to identify foci of digestion for excision and subsequent examination by electron microscopy so that comparable affected areas in the forage leaves were examined.

Control preparations, in which paired leaves were incubated under the same conditions but without the addition of rumen bacteria, showed very little ruthenium red-positive reaction (Fig. 1A). Examination of these control preparations by electron microscopy showed that the plasmalemma of the leaf cells was disrupted by suspension in the incubation medium. Small numbers of bacteria could be seen adhering to the cuticle of the epidermal surface of these uninoculated control leaves at the beginning of the incubation. No bacterial invasion of intercellular spaces was seen in the uninoculated control leaves incubated for 22 h, but some surface-associated bacteria had proliferated to form microcolonies during this incubation.

When intact leaves were recovered 10 min after incubation with rumen bacteria and compared with the control preparations (not shown), the same extensive cytoplasmic damage was evident in the plant cells (Fig. 2). The plasmalemma could no longer be resolved, nor could the cytoplasmic elements except the chloroplasts and nuclei be resolved. No bacteria were seen in the intercellular spaces of the plant tissue.

As incubation proceeded, the bacterial microcolonies adjacent to stomata proliferated more than did those on the general surfaces (Fig. 3a and b), and individual bacterial cells were seen

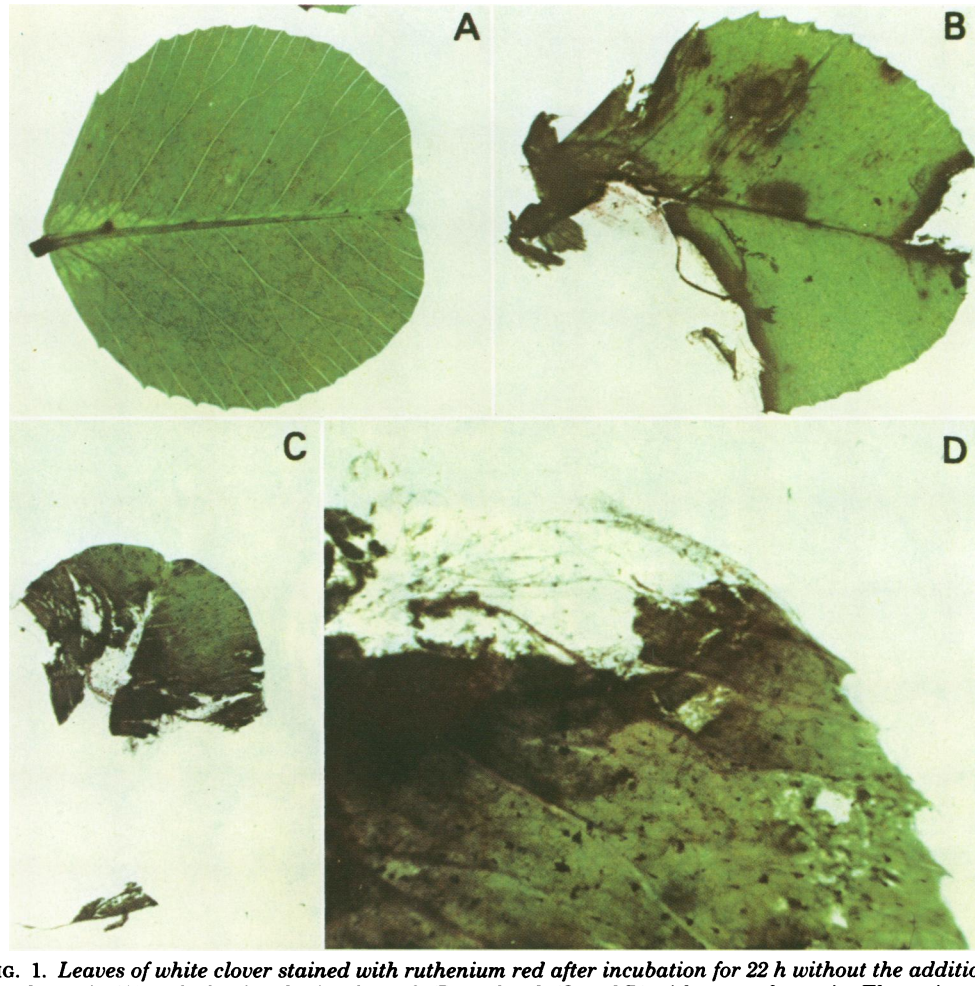

FIG. 1. *Leaves of white clover stained with ruthenium red after incubation for 22 h without the addition of rumen bacteria (A) and after incubation for 16 h (B) and 22 h (C and D) with rumen bacteria. The stain reacts with extensive foci of digestion in the digested leaves (B and C), and higher magnifications show that leaf tissue is extensively destroyed in some of these foci (D).*

to penetrate these openings (Fig. 3c). In these leaves, the cuticle covers the epidermis (Fig. 3a) and lines the stomata (Fig. 3c), and it is through the opening of these cuticle-lined structures that bacteria penetrated (Fig. 3c). Small numbers of bacteria appeared in the intercellular spaces of the parenchyma tissue after 2 h of incubation, and many such bacteria were seen after 8 h (Fig. 4a). The plant cell contents showed further degenerative changes; nuclei disappeared, and chloroplasts were reduced to fragments. The bacterial population of these intercellular spaces proliferated rapidly, but the bacteria were definitely confined to the intercellular spaces (Fig. 4a and b), and cellular compartments (identifiable by their content of cytoplasmic debris) were not perceptibly invaded during the early stages of digestion.

The bacteria in these intercellular spaces formed microcolonies (Fig. 5) that proliferated until they became enormous masses of morphologically similar cells and eventually filled most of the intercellular spaces of the parenchyma. Although bacterial microcolonies developed to fill the intercellular spaces, no bacterial penetration of intracellular spaces was seen in either parenchyma or vascular tissue (Fig. 5).

During the colonization of the intercellular spaces of the legume leaves, certain bacteria, presumed to be *Ruminococcus* sp. (Fig. 6a) and *Bacteroides* sp. (Fig. 6b) by their unique morphological structures, were seen to adhere to the plant cell walls by means of their ruthenium red-positive glycocalyx fibers (Fig. 6). Bacterial adhesion to the cellulose substrate of the plant cell wall stabilized the structure of these glyco-

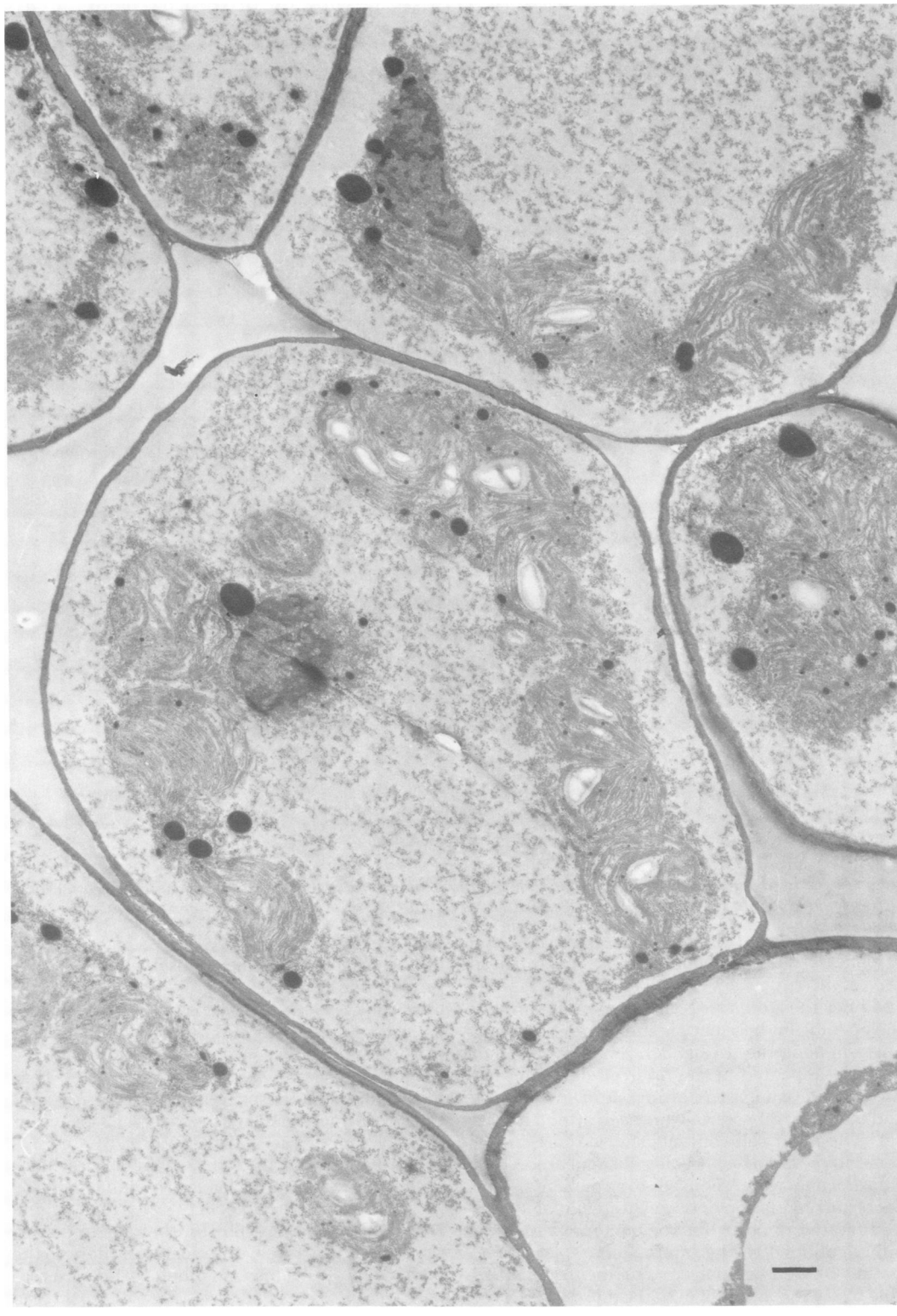

FIG. 2. *Parenchyma tissue of a legume (cicer milkvetch) at 10 min after incubation in the artificial rumen medium. Note the absence of bacteria and the extensive damage to the plasmalemma and all cytoplasmic organelles except the nuclei and chloroplasts. The bar in this and all subsequent electron micrographs indicates 1 μm.*

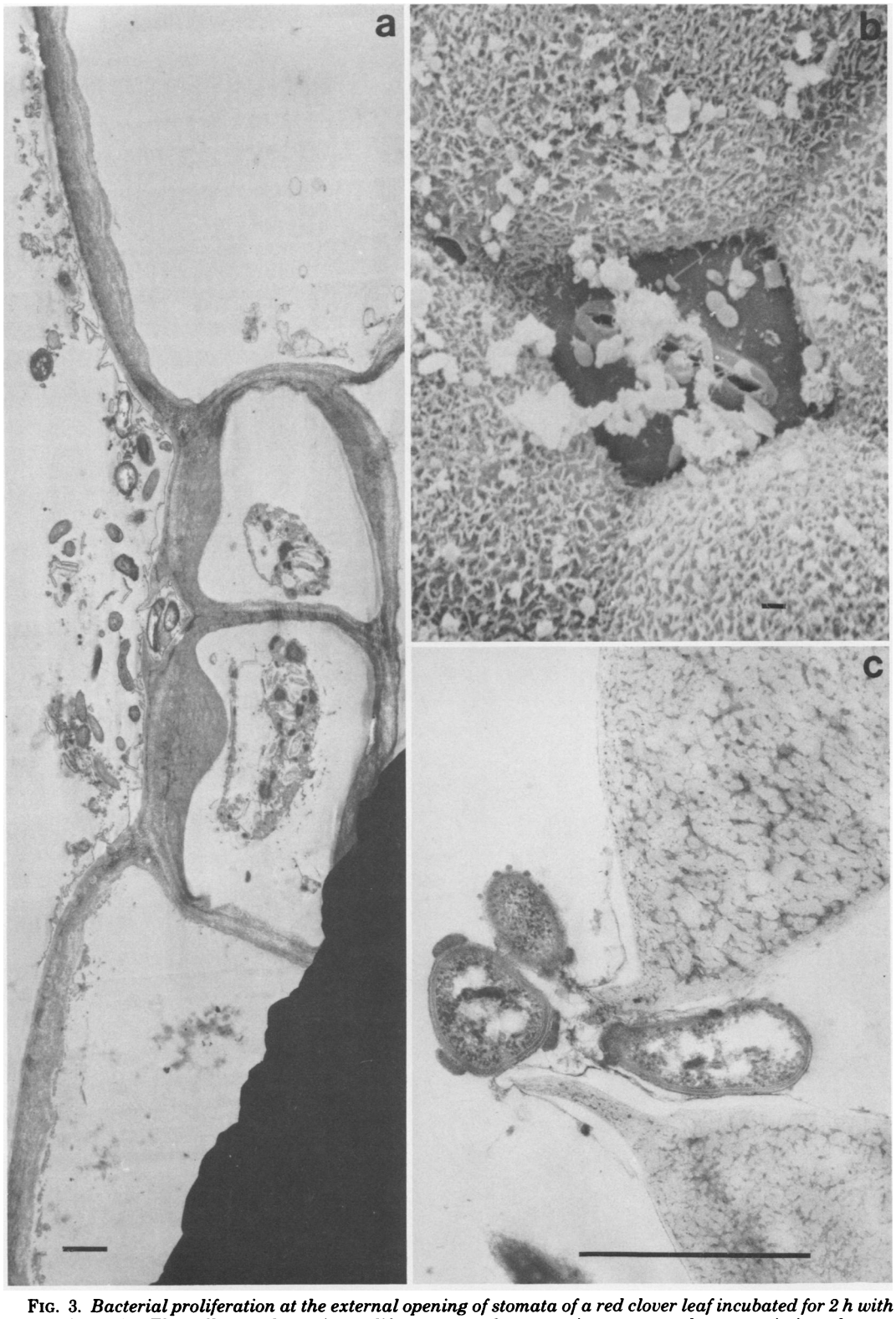

FIG. 3. *Bacterial proliferation at the external opening of stomata of a red clover leaf incubated for 2 h with rumen bacteria. The adherent bacteria proliferate near these openings as seen by transmission electron microscopy (a) and scanning electron microscopy (b); bacteria can be seen within the external opening of individual stoma (c).*

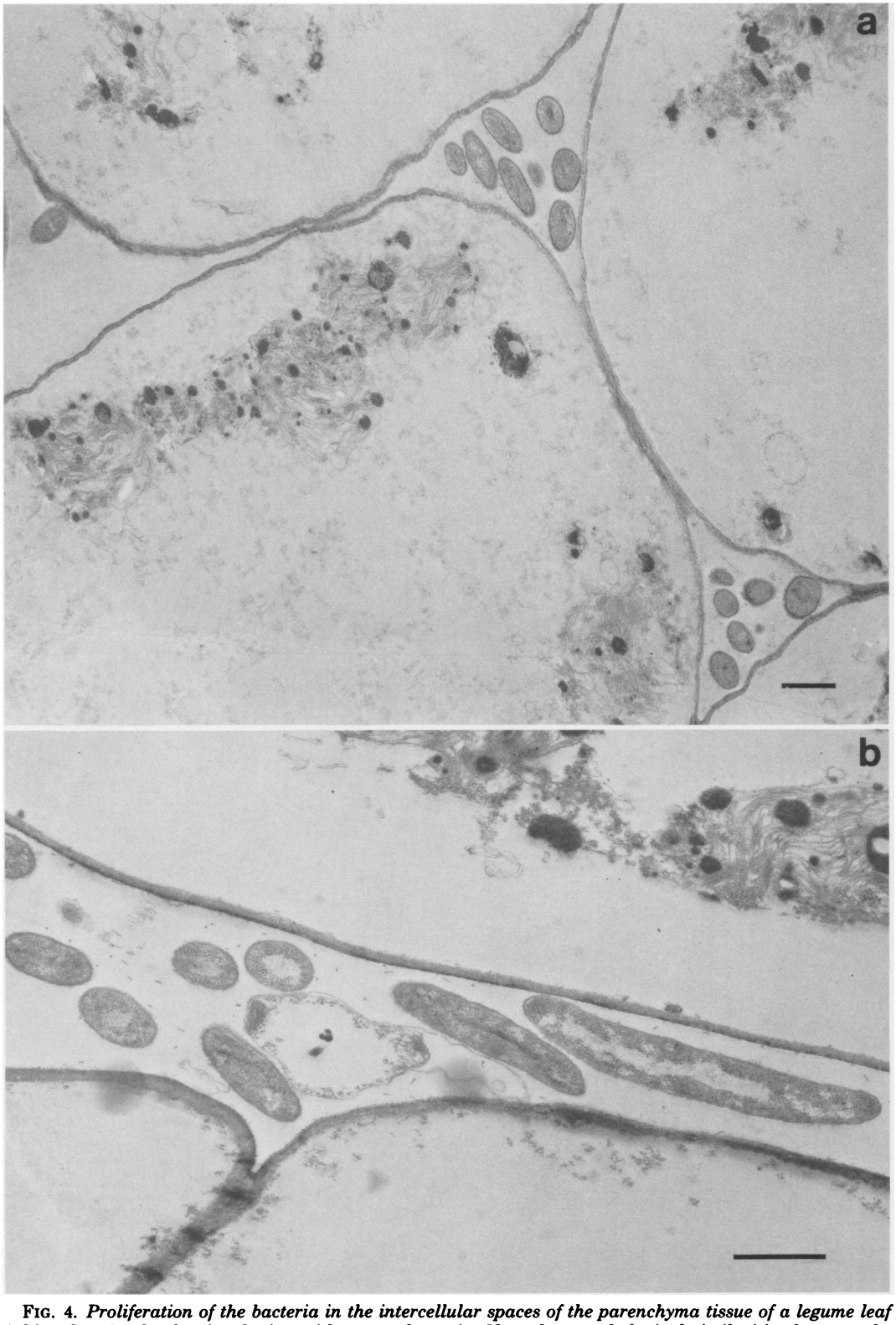

FIG. 4. *Proliferation of the bacteria in the intercellular spaces of the parenchyma tissue of a legume leaf (white clover) 8 h after incubation with rumen bacteria. Note the morphological similarities between the individual bacterial cells in these discrete microcolonies (a) and the presence of both intact and dead cells (b).*

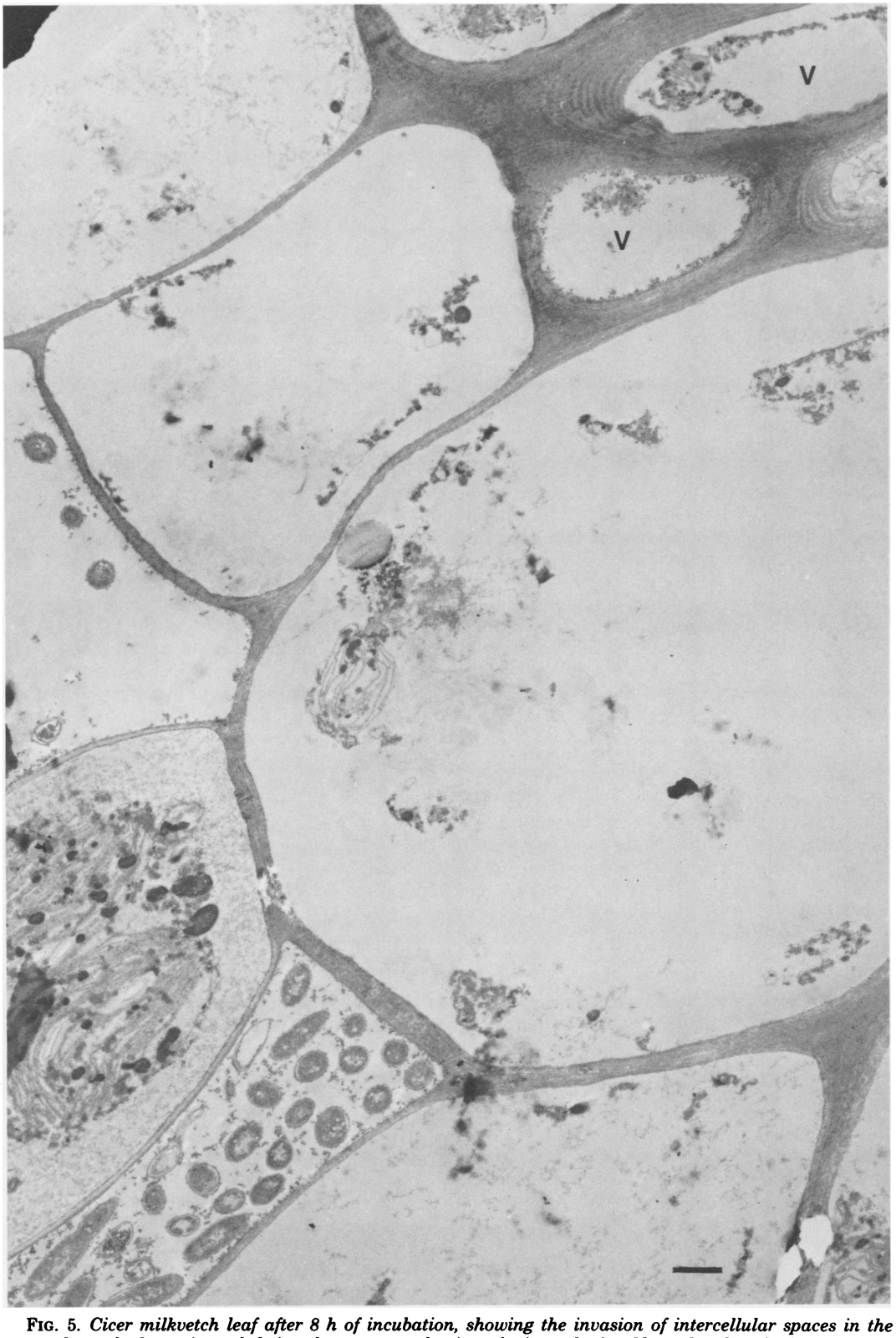

FIG. 5. *Cicer milkvetch leaf after 8 h of incubation, showing the invasion of intercellular spaces in the parenchyma by bacteria and their subsequent production of microcolonies. Note also that the intracellular spaces of the parenchyma and the vascular tissue (V) have not been penetrated by bacteria.*

calyx fibers during fixation and embedding so that they were in an extended configuration where they were attached to both surfaces (Fig. 6, A) and were collapsed back onto the bacterial cell in areas where they were not directly involved in adhesion (Fig. 6, arrows).

Although these bacteria adhered to the cell walls in legumes, they did not produce pits in these structures by cellulolytic activity (1, 2), but their presence appeared instead to induce a disorganization of the plant cell walls in that the electron-dense layer at the plant cell wall surface was partially removed. After bacterial digestion ruptured the plant cell walls, bacteria invaded the intracellular spaces of the plant tissue (Fig. 7a), and chloroplast fragments were released into the menstruum. At this advanced stage of breakdown, phenomenal masses of bacteria were visible within the plant tissue (Fig. 7b) and very distinct microcolonies were distinguishable within this slime-enclosed mass on the basis of the morphological similarities of their component cells or of the discrete borders of their enveloping slime matrix.

At the leaf surface, the microcolonies of bacteria developed to produce a very thick adherent layer (Fig. 8), and the epithelial cells of the leaf were invaded and completely filled by a tightly packed mass of bacteria (Fig. 8, E; Fig. 9, inset). However, the guard cells of the stomata (Fig. 9) were not invaded by these rumen bacteria.

DISCUSSION

Live leaves are externally colonized by a natural population of aerobic and facultative bacteria when they are introduced into the anaerobic rumen fluid medium. These adherent aerobic bacteria would not be expected to proliferate in this environment, and the uninoculated control at 22 h showed only moderate growth of some adherent facultative organisms. No foci of bacterial digestion of these control leaves were observed. Clearly, contact for 10 min with fatty acids, the anaerobic environment, other factors in rumen fluid, or a combination of all of these are sufficient to destroy the plasmalemma and much of the cytoplasmic structure of the plant cells, thus making plant cell constituents available in the intercellular space by diffusion. Soon after incubation began, bacteria were seen in large numbers around the stomata, from which the leachates may be presumed to be mainly escaping, and bacteria penetrated the openings of the cuticle-lined stomata. Chet et al. (8) found that leaf leachates exerted a positive chemotactic effect on *Pseudomonas lachrymans*, and we suggest here that chemotaxis may also be involved in the process of attracting rumen bacteria to the stomatal openings.

We have examined the invasion of legume leaves by rumen bacteria by treating digested leaves with ruthenium red, which reacts with polyanions (15) at foci of digestion to yield qualitative data on the comparative rates of plant cell breakdown in different legumes. Electron microscopy has shown that once they had penetrated the intercellular spaces of the leaf, some of the invading bacteria attached themselves to cell walls and proliferated in this nutrient-rich niche to produce coherent microcolonies of very considerable dimensions. This bacterial proliferation may increase the intercellular spaces by separating the plant cells and thus provide access to more adhesion sites and more nutrients. Thus, a digestion pattern develops which is similar to that caused in legume tissues by the pectin-digesting activity of pure cultures of *Lachnospira multiparus* (6).

Because the cellular compartments of the leaf were not breached (i.e., bacteria were absent) at this time, which varied in different plant species and in different locations in the leaf, we can conclude that this bacterial proliferation is dependent on nutrients that are normally present in the intercellular spaces (i.e., pectins) or are leached into them from the cellular compartments. At a particular time, which varied with the location within the leaf, the walls of some plant cells were disorganized and ruptured by bacterial activity, and bacteria appeared in the intracellular spaces where they could have direct contact with residual cytoplasmic structures such as chloroplast fragments.

The bacterial penetration of the cell walls of legume leaf tissue proceeded by means of general disorganization rather than by the specific pit formation elegantly shown in grass forage plants by Akin et al. (1, 2). Legume cells differed sharply in the extent to which they were penetrated, just as the various tissues of grass leaves are colonized to different extents by rumen bacteria (13, 14). For example, vascular tissues and the guard cells of stomata were not penetrated even when the majority of parenchyma and epidermal cells contained large amounts of bacteria. After their cell walls were ruptured, individual plant cells were invaded by bacteria that proliferated until they filled the cellular compartment with a tightly packed microcolony of cells. This demonstrates the importance of microcolony formation in the digestion of solid substrates.

It must be noted that the invasion stages described above did not take place simultaneously in all regions of the leaf, as is clear from the ruthenium red-stained whole mounts. At a certain time, in one zone bacteria may have breached the cellular compartments, while in

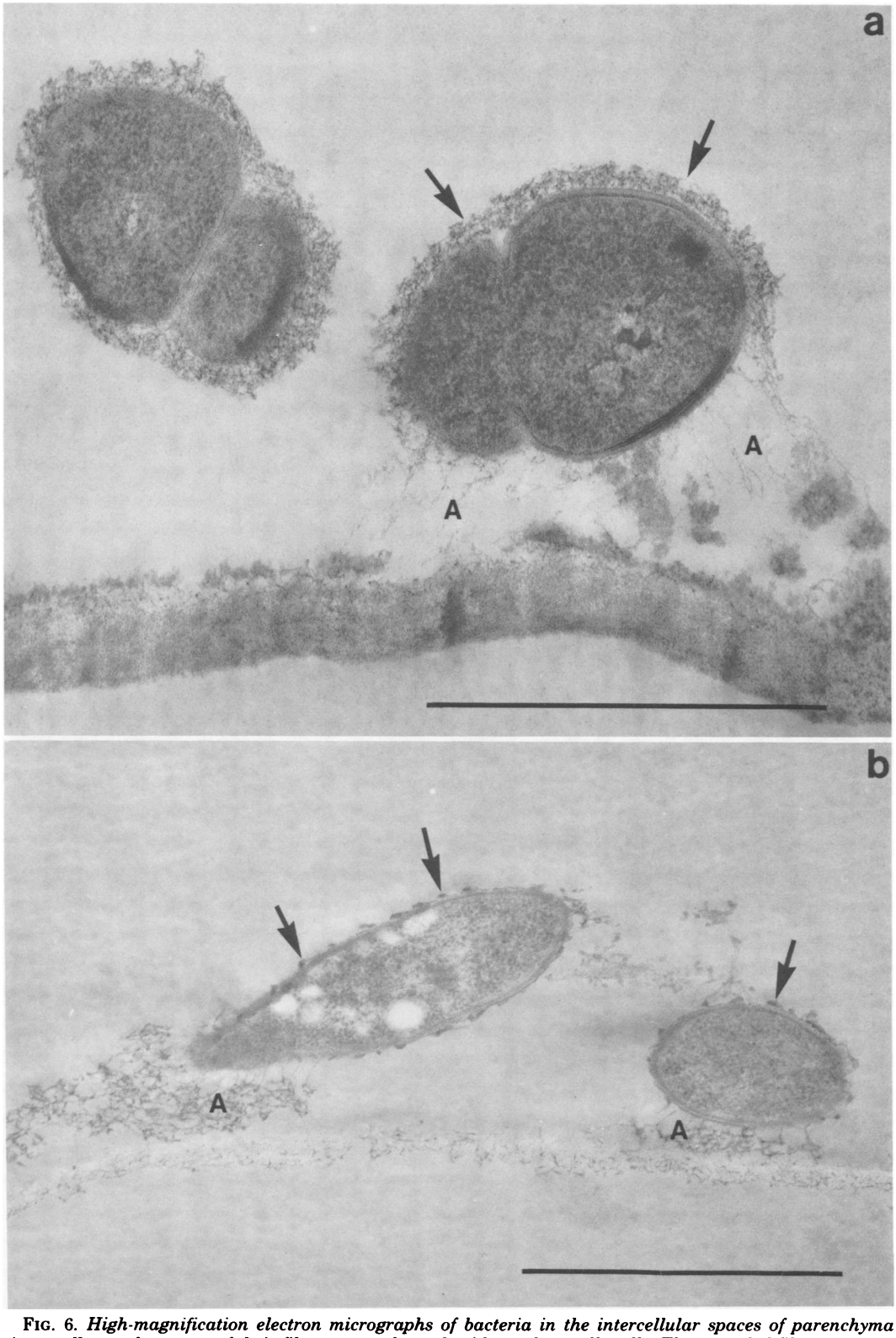

FIG. 6. *High-magnification electron micrographs of bacteria in the intercellular spaces of parenchyma tissue adherent by means of their fibrous exopolysaccharide to plant cell walls. The extended fibrous nature of the exopolysaccharide glycocalyx was maintained throughout the preparation for transmission electron microscopy. The fibers are anchored both on the bacterial cell and on the plant cell wall (A). The glycocalyx collapsed during dehydration when it is not actually connected to both surfaces and formed either a condensed fibrous mat (a, arrows) or a series of highly condensed electron-dense aggregates at the bacterial surface (b, arrows).*

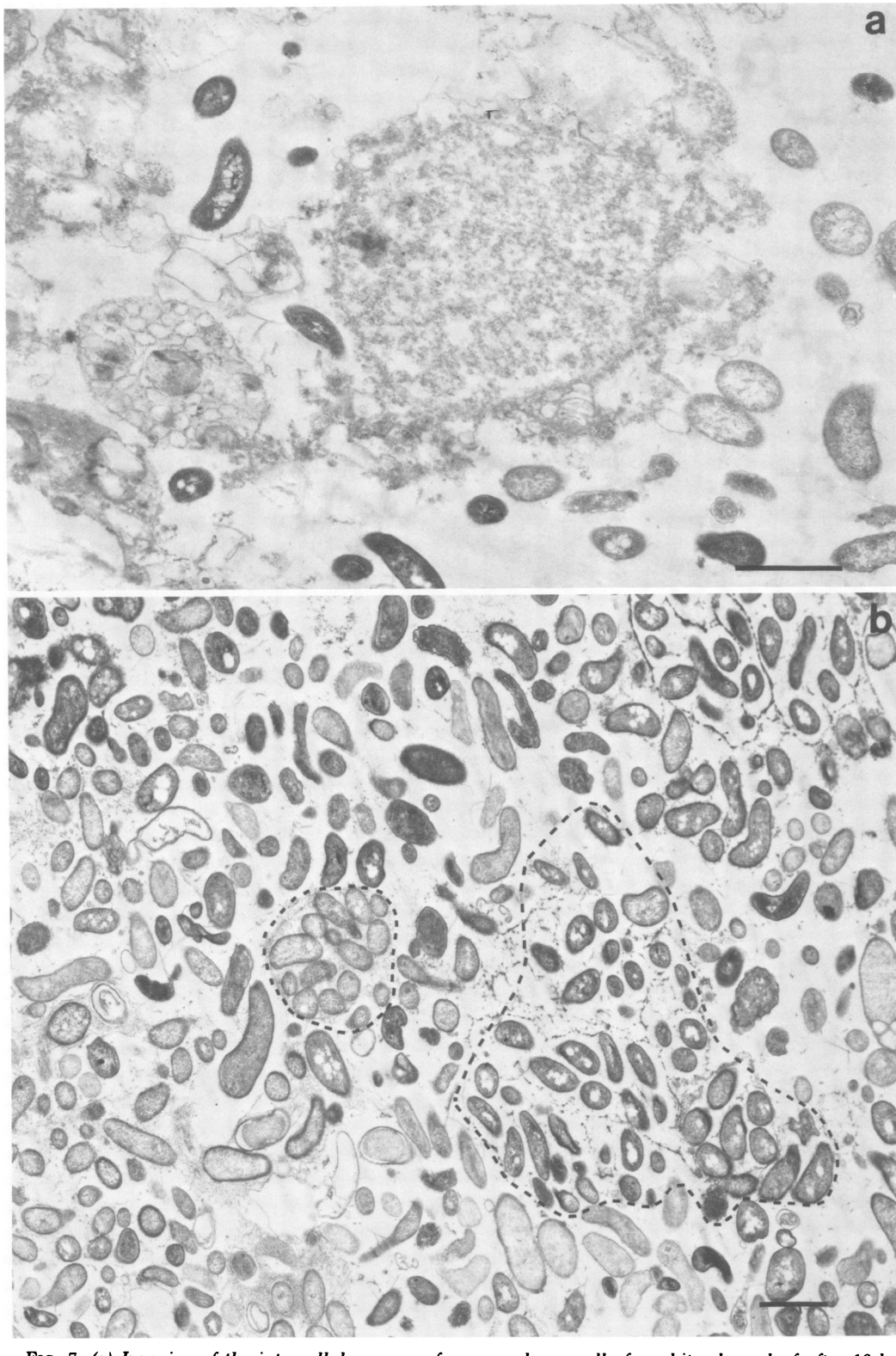

FIG. 7. *(a) Invasion of the intracellular space of a parenchyma cell of a white clover leaf after 16 h of incubation with rumen bacteria. The location is verified by the presence of plant cell cytoplasmic debris among a bacterial population of mixed morphological types. (b) Extensive proliferation of bacteria in the intercellular space of the parenchyma of an alfalfa leaf after 16 h of incubation with rumen bacteria. Note that this very extensive bacterial mass consists of easily discernible microcolonies (dotted lines).*

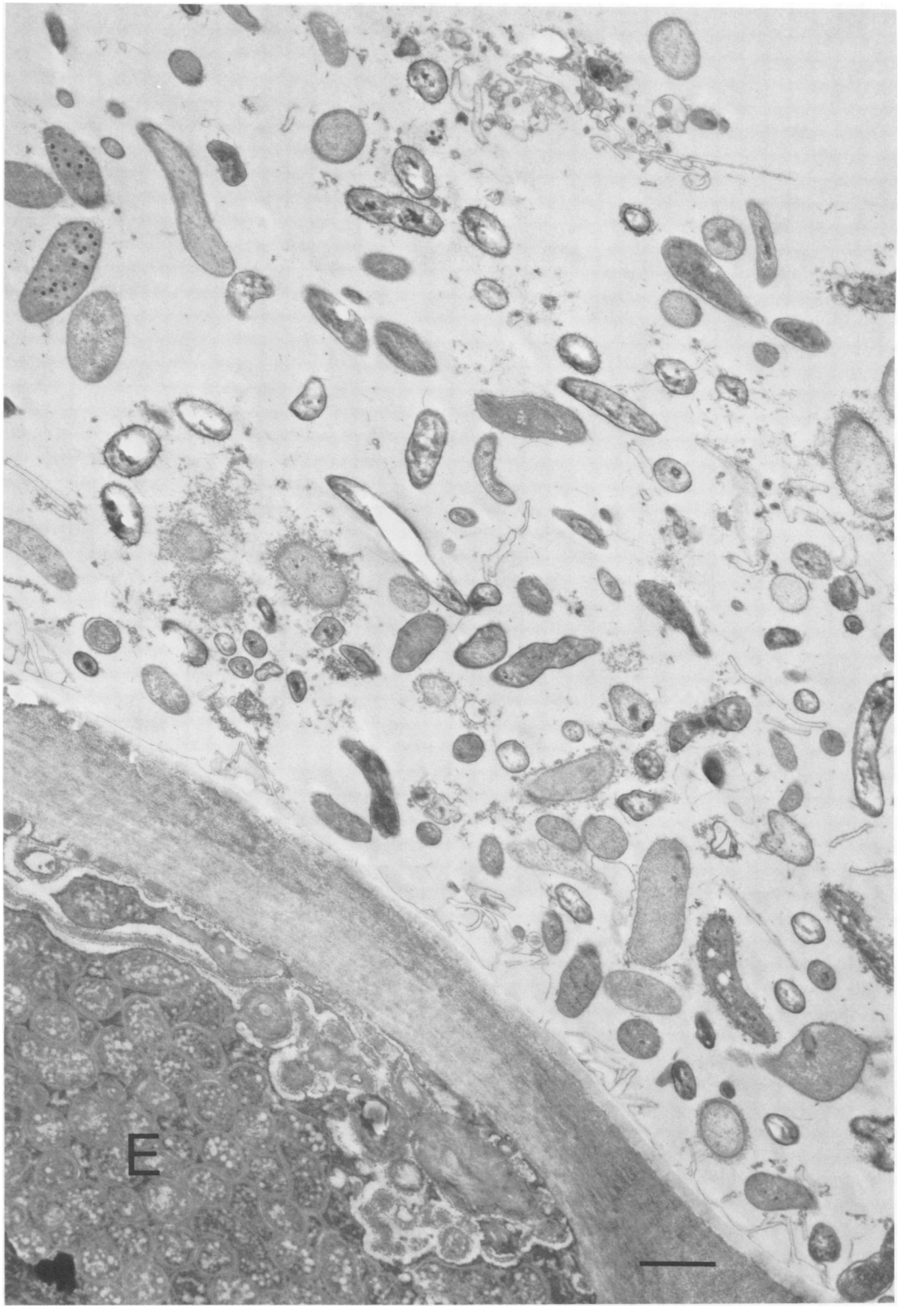

FIG. 8. *Thick adherent bacterial population that develops at the surface of a legume (alfalfa) leaf after prolonged (22-h) incubation with rumen bacteria. Note that the epidermal cell (E) seen in this micrograph has been invaded by bacteria and is filled by a very tightly packed mass of bacteria of a single morphological type.*

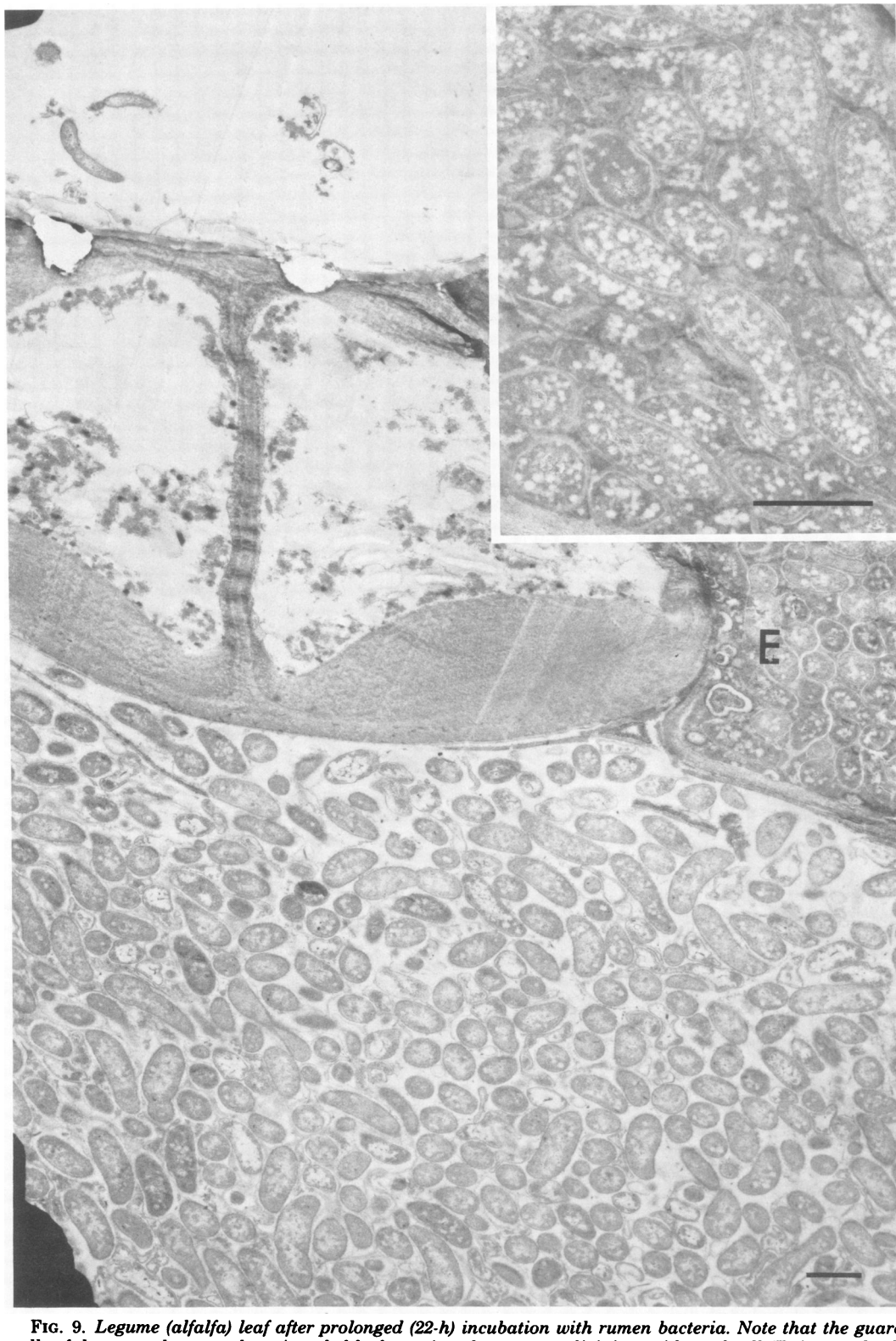

FIG. 9. *Legume (alfalfa) leaf after prolonged (22-h) incubation with rumen bacteria. Note that the guard cells of the stoma have not been invaded by bacteria, whereas an adjoining epidermal cell (E) is completely filled with bacteria, and the underlying intercellular space is also occupied by huge bacterial microcolonies. The inset shows the detailed structure of the tightly packed bacterial microcolony that completely fills the epidermal cell.*

other zones they may be just penetrating the stomata. Each sample used for electron microscopy was only a small point in this heterogeneous pattern, but a correct sequence of events of the bacterial invasion of leaf tissue could be built up when these events followed in a defined order in a very large number of such samples. Thus, in whole leaves, bacteria adhere to the leaf surface, proliferate at stomatal openings, invade the stomata, adhere to cell walls in the intercellular spaces, proliferate to form microcolonies, and finally invade the compartments of the plant cells. However, in leaves damaged by chewing, bacterial access to the underlying tissues would be much more rapid (6).

The true extent of bacterial digestion of plant material throughout this process may be underestimated by measurements of dry matter loss because plant biomass is converted to microbial biomass, which is retained within the structural skeleton of the plant tissue. However, in later stages of digestion, when bacterial activity has weakened the cellulosic framework of the plant tissue, parts of the tissue would be detached, and a measurement of dry matter lost would more closely approximate the true extent of bacterial digestion.

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