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Intestinal Microbial Flora after Feeding Phytohemagglutinin Lectins (*Phaseolus vulgaris*) to Rats

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Incorporation of purified phytohemagglutinin (PHA) lectins derived from red kidney beans (*Phaseolus vulgaris*) in the diet of weanling rats will cause growth failure, malabsorption of nutrients, and bacterial overgrowth in the small intestine. These effects are not caused by feeding a similar quantity of PHA to germfree rats. To define the morphological and bacterial changes on the mucosal surfaces of the jejunum, ileum, and cecum in greater detail, we pair fed two groups of weanling rats isocaloric, isonitrogenous diets with or without 0.5% PHA protein. On the jejunal surfaces of control rats, the mucous layer was a confluent covering with sparsely scattered bacteria and protozoa. In PHA-treated rats, the mucous layer was thin and discontinuous, and the microvillous surface of the tissue was extensively populated by bacterial cells of two distinct morphotypes—a gram-negative rod and a gram-positive coccobacillus. In all PHA-treated animals, these bacteria formed adherent monospecific or mixed adherent microcolonies on the tissue surface. Tissue damage was observed in PHA-exposed jejunal tissue as evidenced by vesiculation of the microvillous plasma membrane and by damage to the brush border membrane. On the ileal surfaces of control rats, there was a thick mucous layer within which small numbers of bacteria and protozoa were seen. Segmented filamentous bacteria were anchored in the tissue surface. In PHA-treated rats, the ileal surface was only incompletely covered by a mucous layer, and the overlying mucosal surface was extensively covered by large numbers of protozoan cells (predominantly *Hexamita muris*). Most of the ileal surfaces not covered by the mucous layer were occupied and virtually occluded by an overgrowth of these protozoan cells with occasional cells of *Giardia muris* and the tissue-associated segmented bacillus. In the ceca of control rats, the mucosa was incompletely covered by a discontinuous mucous layer and colonized by an unnamed *Spirillum* sp., other bacteria, and occasional protozoa. The cecal surfaces of PHA-treated rats retained most of their incomplete overlying mucous layer, which was heavily colonized by the same type of *Spirillum* sp. seen in untreated animals; intestinal crypts were colonized. These descriptive morphological studies demonstrate that exposure to purified PHA in the diet caused characteristic changes in the microbial ecology of the small intestine. The changes in microbial flora contributed to the malabsorption of nutrients in the small intestines of PHA-fed animals.

The development of preparative techniques for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) that allow the retention (33) and staining (24) of the mucous layer on the luminal surface of the intestinal mucosal tissue (microvillous layer) has facilitated the morphological examination of adherent microbial populations. Direct observation of the normal rat intestine has shown that, whereas certain morphologically distinct bacilli (6, 27) are directly adherent or embedded in the microvillous surface, the majority of tissue-associated intestinal bacteria and protozoa (7, 31, 34, 35) may actually live in the lumen or in the adjacent (10, 35) mucous layer. This complex layer (ca. 530 to 650 μm) of glycoproteins, proteins, glycolipids, bacteria, and secreted mucus (2, 37, 41) may move slowly over the tissue surface (7).

Several plant legumes contain soluble lectins which have been shown to cause weight loss and growth failure when fed to rats, mice, and chickens (3, 4, 14, 19, 20, 39, 40). This nutritional "toxicity" is a consequence of the lectin content of the beans (4, 40). Adverse effects of one of these lectins, phytohemagglutinin (PHA) derived from red kidney beans (*Phaseolus vulgaris*), are ameliorated by concurrent administration of antibiotics or feeding to germfree animals (4, 20) or both. Malabsorption of fat, nitrogen, and [^{57}Co]cobalamin observed in conventional rats fed PHA or crude red kidney bean extract was also improved with concurrent antibiotic administration (4). Other studies of the toxicity of rat green beans (fed to swine) and red kidney beans (fed to rats) showed that a proliferation of bacteria, including *Escherichia coli*, occurred in the small intestines of the lectin-fed animals (4, 20, 39, 40).

This study was undertaken to test the hypothesis that PHA may affect microbial ecology by changing the nature and distribution of certain bacteria and protozoa on the mucosal surface of the intestine. Purified PHA is composed of a mixture of several isolectins. Both mitogenic and erythroagglutinating isolectins are glycoproteins with specific binding affinity to the rat intestinal brush border membrane (3, 12, 14, 22). It is proposed that PHA, as a result of its adherence to the mucosal surface, may facilitate the proliferation and growth of microbial organisms in the bowel lumen and on the mucosal surface of the rat intestine.

During this study, purified PHA was fed to weanling rats in a complete nutritious, purified casein protein diet free of complex polysaccharides. Certain physiological parameters (cumulative weight change, fecal weight, nitrogen excretion, and [^{57}Co]cobalamin absorption) were measured in test (T) and pair-fed control (C) animals on isonitrogenous, isocaloric diets. Morphological observations of the microbial populations on the mucosal surfaces were made in the jejunum,

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ileum, and cecum of each animal at sacrifice to identify whether changes in microbial ecology might correlate with observed changes in absorption and nutrition.

MATERIALS AND METHODS

Preparation of PHA. Red kidney beans (*P. vulgaris*) were obtained from a single source (Laurelbrook Farms, Bel Air, Md.). Purified PHA was prepared from a saline extract of 100 g of the beans and extracted by thyroglobulin-Sepharose 4B affinity chromatography by the method of Felsted and coworkers (15). A single, discrete elution peak yielded 0.6 to 0.85 g of total protein as PHA from the 10 to 15 g of crude bean protein applied as saline extract. The peak had a high agglutination titer for human group O erythrocytes. Polyacrylamide gel electrophoresis carried out on 7% acrylamide–0.5% bisacrylamide at 4°C by the method of Reisfeld et al. (32) revealed five separate PHA isolectins with different and distinct mobilities. Individual isolectins contained various proportions of the L (mitogenic) and E (erythroagglutinating) subunits (23). Yields of PHA from several affinity column preparations were combined, analyzed for protein content, and mixed with the diet to provide the requisite PHA concentration.

Diet. Diets were established with Teklab purified diets (Teklab, Madison, Wis.) free of complex carbohydrate or fiber with casein protein as the sole nitrogen source. Purified PHA was incorporated in the test diet as 0.5% protein with 4.5% additional protein supplied as casein diet. C animals were pair fed an isocaloric, isonitrogenous 5% casein diet. The feeding period was conducted for 7 days after an initial 2 days on a control diet. Total protein intakes were low in both T and C groups on this regimen. This was necessitated by the need to extract and incorporate in the diet sufficient PHA, which was obtained in multiple yields by affinity column chromatography (4, 15). However, in other studies from our laboratory in which PHA was fed to animals with a normal dietary protein intake (3), absorptive and nutritional changes were identical to those encountered at the lower protein intake level.

Animals. Male outbred weanling Sprague-Dawley rats (50 to 70 g) were housed individually in mesh-bottom metabolic cages and maintained at a constant temperature with a 12-h light-dark cycle. Water was allowed ad libitum.

Study design. Two groups of 12 animals each were fed either the test diet containing PHA or the control casein diet for 7 days; they were then sacrificed by cervical dislocation, and tissue was processed for morphological study within 60 s of sacrifice. Sample segments of jejunal and ileal tissue for bacteriological, immunofluorescent, and microscopic analyses were obtained from standard sites 10 cm distal to the ligament of Treitz and 5 cm proximal to the ileocecal valve. Cecal tissue was obtained by longitudinal incision of the resected sac with sharp scissors.

Morphological techniques. Lengths (1 cm) of jejunum and ileum were longitudinally slit with scissors before being fixed by immersion in 0.1 M cacodylate buffer (pH 7.2) containing 5% glutaraldehyde. The fixative solution was prepared from 70% glutaraldehyde stored in ampoules under argon gas. After overnight fixation at 4°C, the tissues were washed six times with cacodylate buffer. They were then postfixed with 2% osmium tetroxide in cacodylate buffer for 3 h. Tissues were made conductive by the modified procedure of Malick and Wilson (25) for thiocarbohydrazide. After the thiocarbohydrazide procedure, tissues were transferred to a 10-step ethanol-water dehydration series ending in 100% ethanol. They were then transferred into a 10-step Freon 113-ethanol series ending with 100% Freon 113, in which they were stored. We allowed 30 min for equilibration at each step in both infiltration series. For SEM, the tissues were critical-point dried (9), mounted on stubs, and examined without metal coating. A Hitachi model 450 scanning electron microscope was used for SEM at an accelerating voltage of 20 kV, and Ilford FP4 panchromatic film was used to photograph the specimens. The course traverse control of the instrument was calibrated to facilitate the recording of the actual area scanned in any specimen examination.

When TEM was also performed, duplicate specimens of tissue were put through the same procedures, except that ruthenium red (0.05%) was included in all solutions. The duplicate specimens of tissue were fixed with osmium tetroxide and washed with cacodylate buffer containing 0.05% ruthenium red. They were then dehydrated in a 30 to 100%, five-step acetone-water dehydration series in which ruthenium red (0.05%) was included in the first two steps, ending in two immersions in propylene oxide. Tissue was then embedded by the method of Spurr (38). Sections were cut on a LKB Ultratome III (LKBO Producter AB) with a glass knife. Microscopy was performed with an A.E.I. EM801 electron microscope at an accelerating voltage of 60 kV, and specimens were photographed with Eastman Kodak microscope film. The area of sectioned material examined at any instance was estimated by counting grid squares >50% occupied by the sections and calculating the area examined.

Because examination with a dissecting microscope showed that even solution changes during preparation for electron microscopy tore and removed the coherent, fixed mucous layer from the tissue surface, we developed solution vials that contained a wire mesh at the bottom of the vessel, and we changed solutions so that fluid shear forces were minimized. Throughout this study, removal of the mucous layer was prevented by these techniques as monitored by low-power light microscopy (33). Immunofluorescence studies were performed on each tissue to confirm our previous observations of PHA adherence to the brush border membranes of PHA-treated animals (3). Cryostat-frozen sections were also obtained from control tissues and were variously layered with PHA (either L or E isolectins or phosphate buffer). Each specimen was stained with either of two antilectins previously tested for monospecificity or with normal rabbit serum (3). Fluorescein-labeled goat anti-rabbit immunoglobulin was used as the second antibody. Casein protein did not adhere to the mucosal surface when similar immunofluorescence studies with anticasein antisera were performed (3).

Brush border disaccharidase enzyme activities were measured on jejunal tissue homogenates, and [^{57}Co]cobalamin absorption was measured by methods previously described (4) in a group of animals on an identical dietary regimen. Viable counts of the aerobic bacteria adherent to the mucosal surface were measured. Adherent bacteria were defined as those still attached to the tissue after washing of the intestinal lumen four times with phosphate-buffered saline at 4°C. After homogenization of washed intestinal tissue in a VirTis homogenizer (The VirTis Co., Inc., Gardiner, N.Y.), 10-fold serial dilutions of a broth culture medium were made onto blood agar plates incubated for 48 h. The number of specific colony-forming organisms was counted and expressed as the logarithm to the base 10 of the mean colony counts on plates containing 1 to 1,000 organisms. Luminal washings of a group of T and C rats were centrifuged at 200 g for 20 min to sediment protozoa.

TABLE 1. Dietary and metabolic data for weanling rats fed an isonitrogenous, isocaloric purified casein diet with or without 0.5% purified PHA[a]

Animal group	Dietary regimen	Dietary Consumption (g/rat per day)	N_2 intake (mg/rat per day)	Mean wt change (g/rat per 7 days)	Total aerobic colony counts (log_{10} organisms/g of tissue [wet/wt])	Mucosal disaccharidase activity (U)			[^{57}Co]cobalamin absorption (%)
						Lactase	Sucrase	Maltase	
T	0.5% PHA + 4.5% casein protein	3.8 ± 0.9	37.2 ± 1.9	−5.9 ± 0.8	7.02 ± 0.40[b]	0.95 ± 0.57[c]	46.2 ± 4.4[b]	295.6 ± 24.3[b]	25.6 ± 2.0[b]
C	5% Casein protein	3.9 ± 0.4	33.2 ± 3.0	−2.1 ± 1.5	3.30 ± 0.78	14.1 ± 2.3	77.0 ± 5.6	608 ± 36.9	61.2 ± 1.5

[a] All results are presented as the mean ± the standard error of the mean for six animals.
[b] $P < 0.001$.
[c] $P < 0.01$.

Protozoa were stained with trichrome and Giemsa stains for identification and differential counting.

RESULTS

T animals received an isonitrogenous, isocaloric diet equivalent to that fed to C animals. Mean body weight loss was greater in T animals during the study period ($P < 0.01$). However, both T and C animals appeared to remain healthy and active, although fecal pellets were softer and less formed in T animals. Total aerobic bacterial colony counts were increased in washed jejunal tissue homogenates of T animals ($P < 0.001$). Jejunal disaccharidase activity was reduced in T animals ($P < 0.01$). [^{57}Co]cobalamin absorption with and without supplementary purified rat gastric intrinsic factor, was diminished in T animals ($P < 0.001$) (Table 1).

Morphological observations. (i) Jejunum. When at least 8 mm^2 of the surface of the jejunum of each of the C rats was examined by SEM, the microvillous surface of this tissue was almost completely covered by the apparent amorphous residue of the mucous layer. A small number of bacteria and protozoa were seen throughout the overlying structure, and some segmented filamentous bacteria (6, 35) could be seen indistinctly through the mucous layer. These implanted bacilli, assorted bacteria, and occasional *Giardia muris* (27) were the only microbial morphotypes seen on the microvillous surface in these occasional areas where the mucus was discontinuous. Examination of tissue from C rats by TEM revealed features similar to those described in the mouse (33), with retention of the mucous layer in places. Bacteria were identified which were separated by large areas of mucus.

Direct examination by SEM of at least 10 1-mm^2 areas of the surfaces of the jejuna of all T rats showed discontinuities in the mucous layer. These discontinuities exposed the microvilli, which were extensively colonized by bacteria (Fig. 1 and 2). Most of the jejunal microvillous surfaces of T animals were not covered by the mucous layer, and these surfaces were extensively colonized by a confluent layer of rod-shaped bacteria (Fig. 3) between whose cells vesicular elements of the microvilli protruded. The microvilli were altered in the colonized areas, and two distinct morphotypes wee seen among the adherent bacteria (Fig. 1 and 2). Rod-shaped (ca. 1.2 by 3 μm) and coccobacillary cells (ca. 1.0 by 1.2 μm) characterized by well-defined division furrows (Fig. 1, arrows) were embedded in or deformed the microvilli of the brush border membrane separately, and in other areas, cells of the two morphotypes formed mixed adherent microcolonies (Fig. 2). Examination by SEM showed a continuous cover of coccobacilli that completely occluded both the rod-shaped bacteria and the tissue surface (Fig. 4, inset) over an area of at least 1.5 mm^2. Examination at a higher magnification showed both their characteristic division furrows (Fig. 4, arrows) and their tendency to grow in confluent masses connected by the amorphous residues of the extracellular matrix. In four animals selected for intensive examination, this bacterial overgrowth was observed in >90% of the total 30 mm^2 examined at 8 to 10 randomly selected locations on the tissue surface.

TEM preparations of these same jejunal tissues showed that their microvillous surfaces were extensively colonized by rod-shaped bacteria (Fig. 4), and examination of these cells at high magnification showed that they had a gram-negative cell wall structure (11). Vesicular elements of apparently damaged microvilli (Fig. 3 and 4) were interspersed among the adherent bacteria. Gram-negative rod-shaped bacteria were also seen adjacent to the luminal surface of goblet cells (Fig. 3, G) in the jejunum. This bacterial overgrowth was seen in >75% of the total of 6.5 mm^2 of sections from the jejuna of six representative animals. In one T animal (Fig. 4), the continuous layer of gram-negative rod-shaped bacteria was covered, over much of the surface of this tissue, by a very thick (ca. 25 μm) accretion of coccobacillus-shaped cells, whose examination at higher magnification showed clearly that they had a gram-positive cell wall structure (11). The spaces between these gram-positive coccobacilli contained fibrous ruthenium red-staining residues of an anionic exopolysaccharide material (Fig. 4) that appeared to have constituted an extracellular matrix.

Of the 12 T animals, all showed at least partial colonization of the jejunal microvillous border by either rod-shaped and coccobacillary bacteria or both. Two animals showed a confluent colonization of this tissue by rod-shaped gram-negative bacteria, and one showed confluent colonization by an inner layer of gram-negative rod-shaped bacteria and a thick, superficial accretion of gram-positive coccobacilli.

(ii) Ileum. Examination of the ileal surfaces of C rats by SEM showed that the preparative methods retained the mucous layer on the microvillous surfaces of these tissues. The tissue surfaces were not directly visualized except in tears and fortuitous discontinuities, where they were not colonized except by segmented filamentous bacilli (6). Small numbers of bacteria and protozoa were seen within the mucous layer when large areas (>8 mm^2 in four locations in six animals) were examined at higher magnification. Examination by TEM revealed that the mucosal surface of C rat ileum was colonized by segmented filamentous bacilli, as

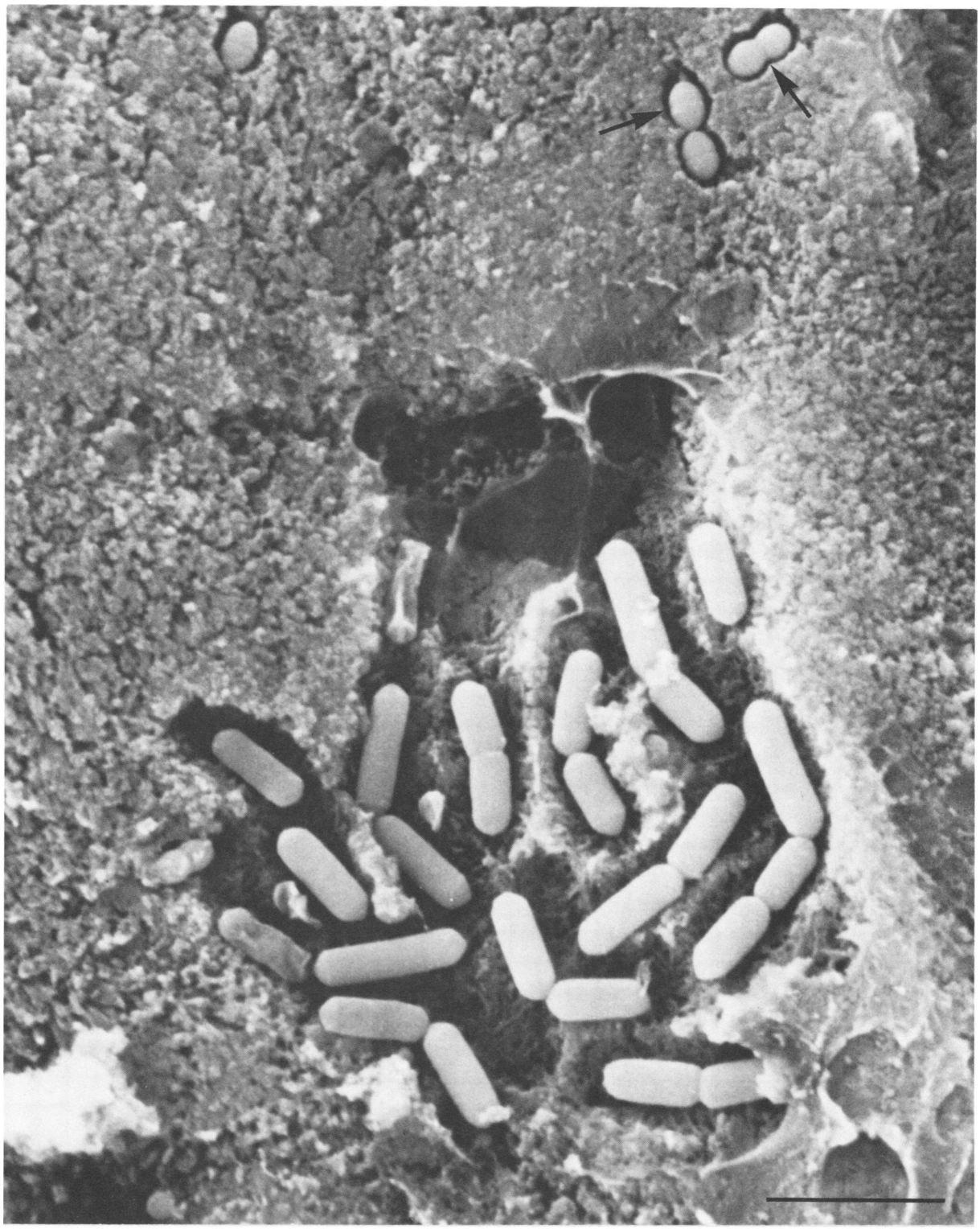

FIG. 1. SEM of the surface of the jejunum of a T rat 7 days after the initiation of lectin feeding showing details of the colonization of the jejunal surface by rod-shaped cells (ca. 1.2 by 3 μm) and coccobacillary cells (ca. 1.0 by 1.2 μm) with distinct division furrows (arrows). These two distinct morphotypes formed separate adherent microcolonies in this instance, and the bacterial cells appear to grow in depressions in the microvillous surface of the colonized tissue. Bar, 5 μm.

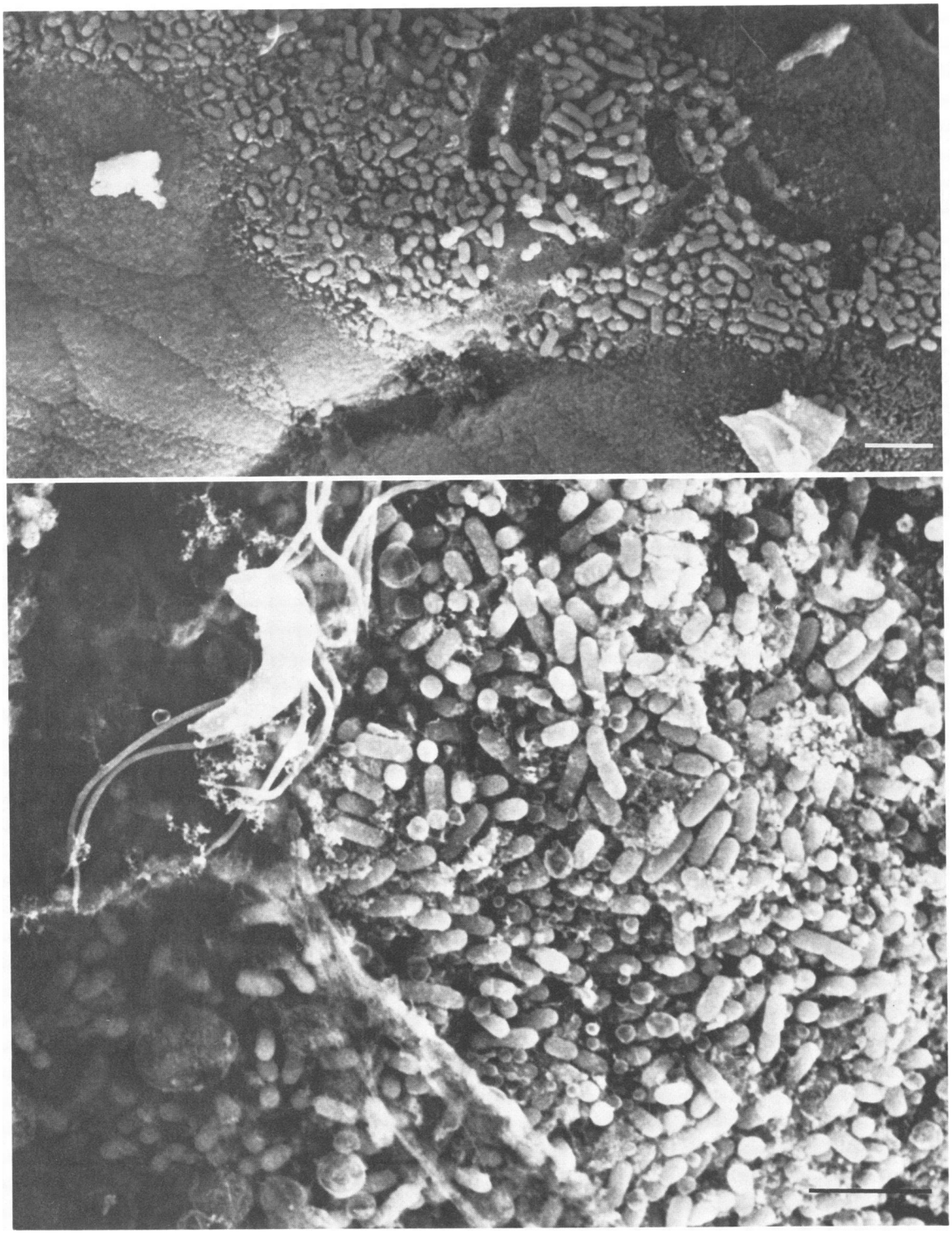

FIG. 2. SEM of the microvillous surface of the jejunum of a T rat. (Top) Areas of the tissue surface were colonized by two morphotypes of adherent bacteria (rods and coccobacilli) that sometimes formed mixed bacterial microcolonies. (Bottom) Most of this tissue surface was heavily colonized by bacteria and was devoid of a mucous layer, but some areas retained a very thin layer through which the heavily colonized tissue surface could still be resolved. Bars, 5 μm.

observed by others (6, 29, 33), and small numbers of bacteria and protozoa in the mucous layer or adjacent to the mucosa.

Direct examination of at least 4 mm^2 of the ileal surface of each of the T animals showed incomplete coverage of this tissue by the mucous layer (Fig. 5). In some areas (Fig. 5, top), mucus covered the tissue surface except at tears caused by condensation during dehydration, in some areas the ileal surface was exposed (Fig. 5, I), and in others the

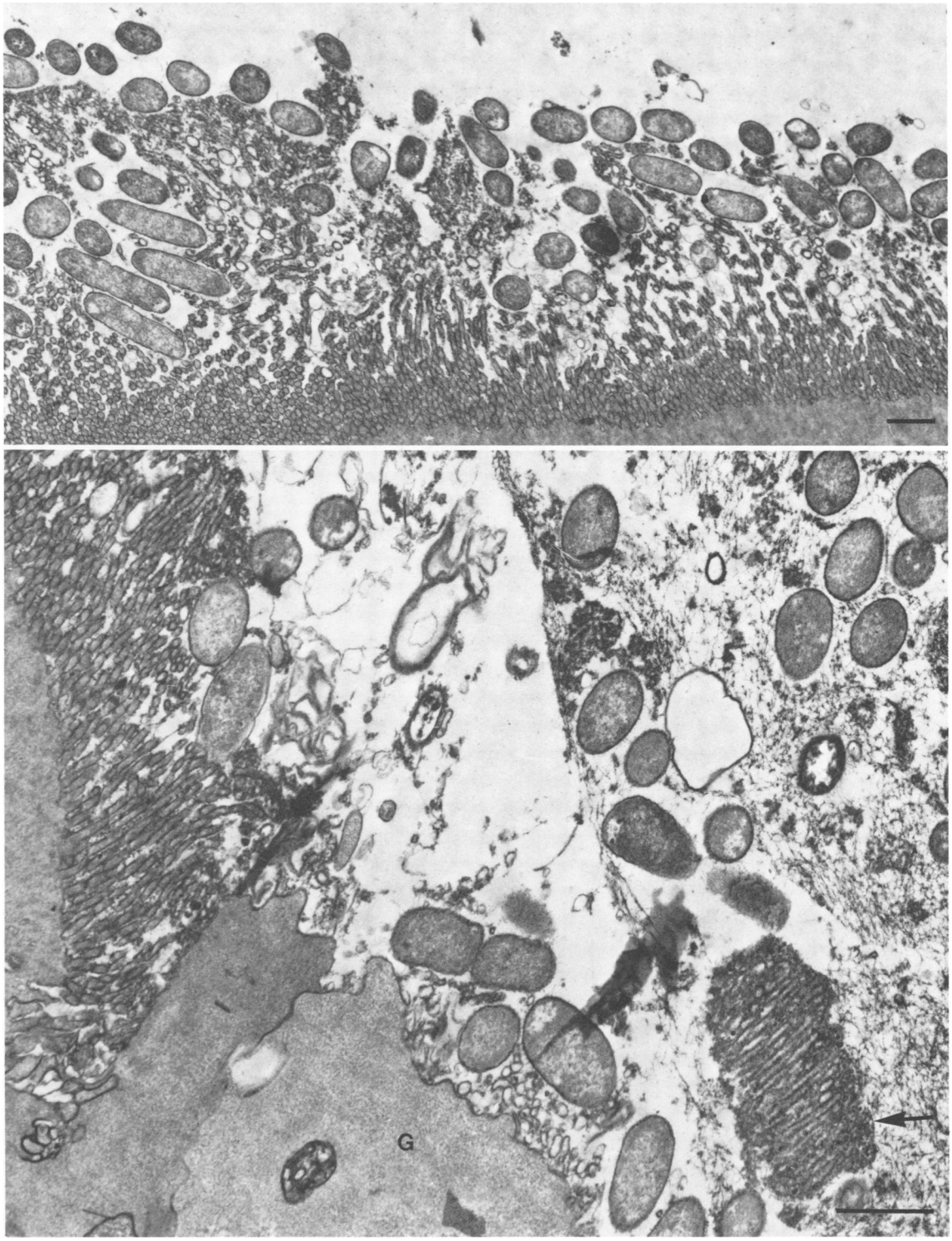

FIG. 3. TEM of the microvillous surface of the jejunum of a T rat. (Top) The epithelial surface was very heavily colonized by rod-shaped bacteria whose cell envelopes, at higher magnification, were of the gram-negative type (7). The plasma membranes of the colonized epithelial cells appear to be deranged, with the formation of large numbers of detached vesicles. Here the protozoa of the predominant morphotype, *H. muris*, are clearly seen, as are occasional cells of *G. muris* and the filamentous bacteria inserted into the microvillous tissue surface. (Bottom) Note that bacteria are associated with the microvillous border of epithelial cells, with the luminal surface of goblet cells (G), and with luminal cellular debris, including a segment (arrow) of detached brush border membrane.

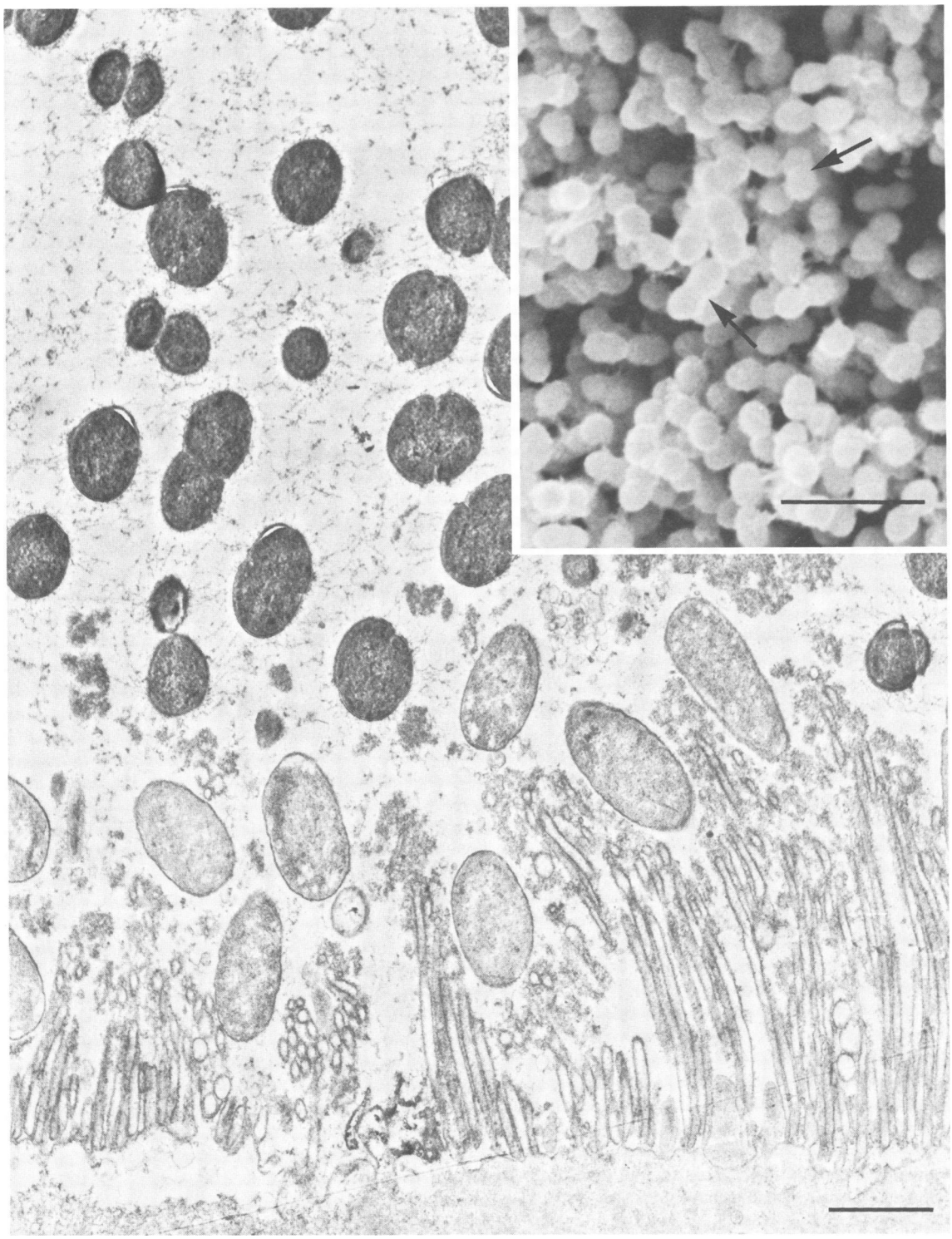

FIG. 4. TEM of the microvillous surface of the jejunum of a T rat. The tissue surface was heavily colonized by rod-shaped gram-negative cells. Distal to this immediately juxtaposed layer of adherent gram-negative rod-shaped bacteria, a matrix-enclosed biofilm of coccobacilli can be seen. Bar, 1 μm. (Inset) SEM of the same luminal surface as above, showing that both the tissue surface and the inner layer of adherent bacteria were completely occluded by a thick layer of coccobacilli with prominent division furrows (arrows). Bar, 5 μm.

microvillous surface was covered by a confluent mass of protozoa (Fig. 5, P). When the mucous layer was examined at higher magnification (Fig. 6), a large proportion of this overlying structure was seen to be occupied by protozoa whose cellular outlines could be clearly distinguished within the condensed mucous layer. At the edge of the area covered by the mucous layer (Fig. 5), protozoan cells could be visualized directly and a small number of rod-shaped and

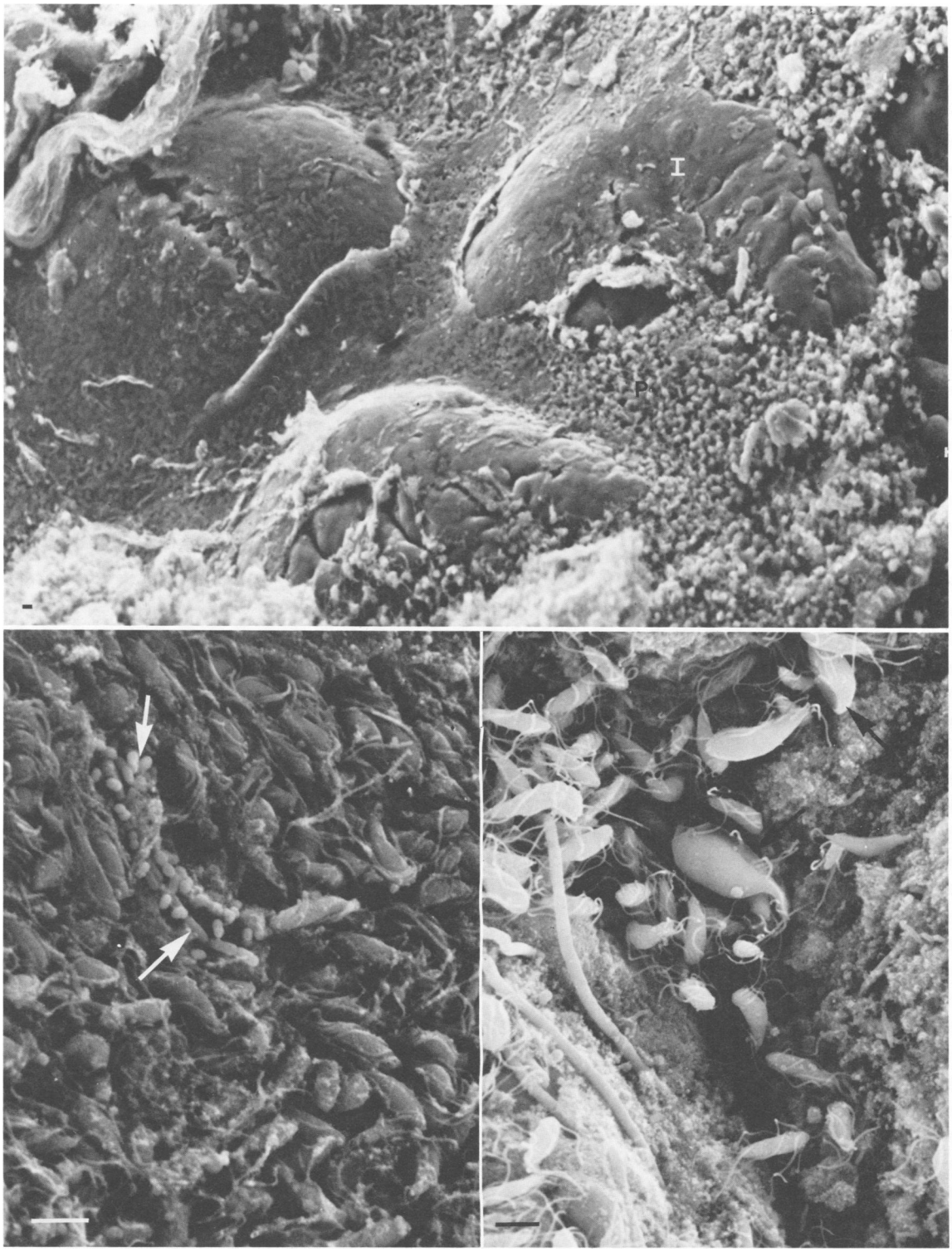

FIG. 5. (Top) SEM of the mucosal surface of the ileum of a T rat. In some areas this tissue surface was covered by the mucous layer, whereas in others it was exposed (I) or covered with confluent masses of protozoan cells (P). (Bottom, right) Protozoa formed a confluent mass that actually occluded the tissue surface (arrows demonstrate *G. muris*) and (bottom, left) aggregates of coccobacillary bacteria were occasionally seen (arrows) within this layer of protozoa. Bars, 5 μm.

coccobacillary bacteria could be discerned in occasional aggregates (Fig. 5, bottom left, arrows). Where the mucous layer became very thin and covered the tissue only incompletely, large numbers of protozoa (*Hexamita muris*) were seen, as were occasional cells of *G. muris* (Fig. 5, bottom right) and of the filamentous bacillus described previously (6, 26, 27, 35). Very few bacterial cells were seen in the thin residue of mucus (Fig. 5). In the areas of the ileal

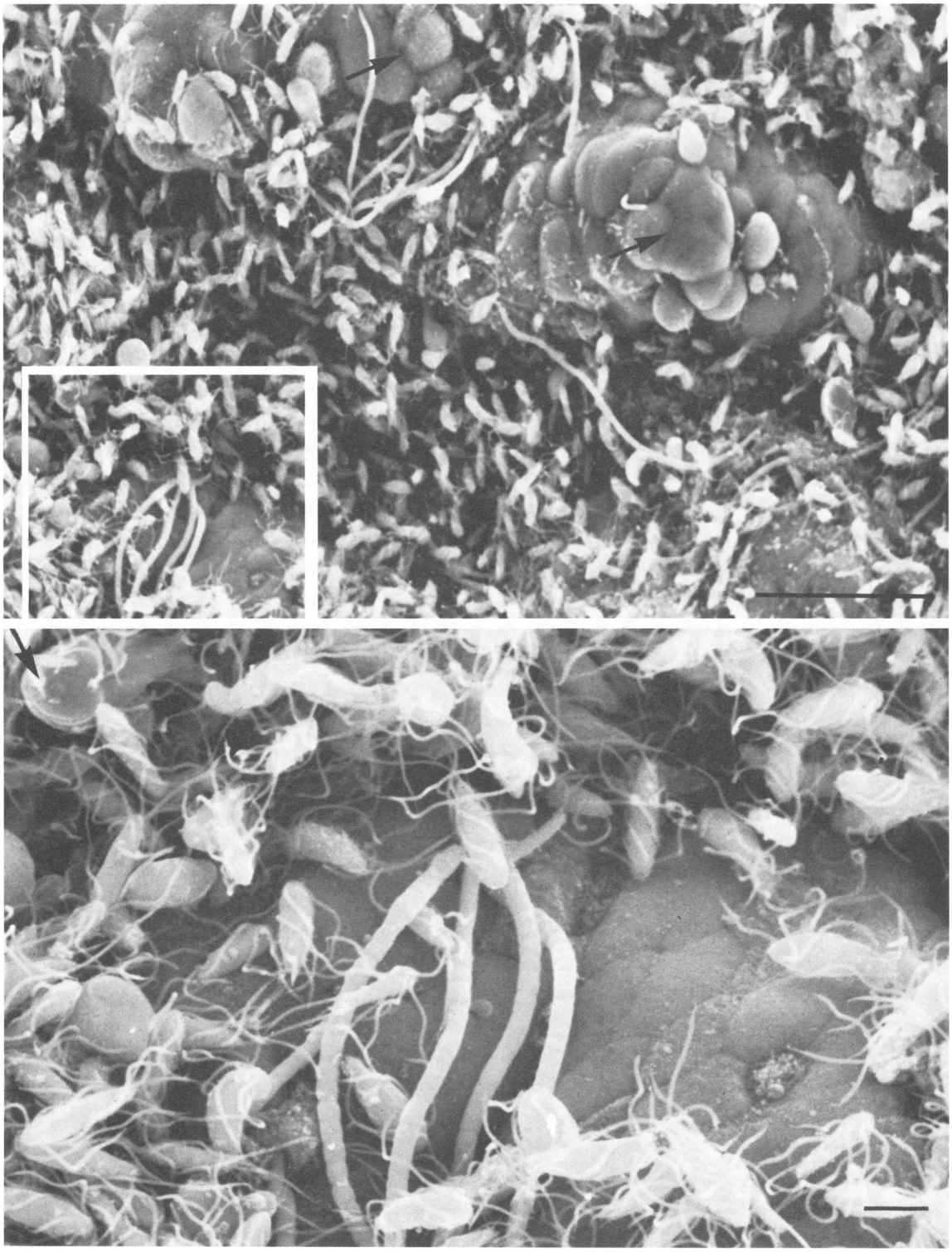

FIG. 6. SEM of the ileal surface of a T rat. (Top) The surface of this tissue was almost completely covered by a confluent mass of protozoa (*H. muris*), but the desquamating tips of the villi were clearly discernible (arrows), as were tissue-associated filamentous bacteria. (Bottom) Higher magnification of the area designated by the box, showing the lack of mucus covering the ileal surface, which was very heavily colonized by *H. muris*, with occasional *G. muris* (arrow) and tissue-associated filamentous bacteria. Bars, 5 μm.

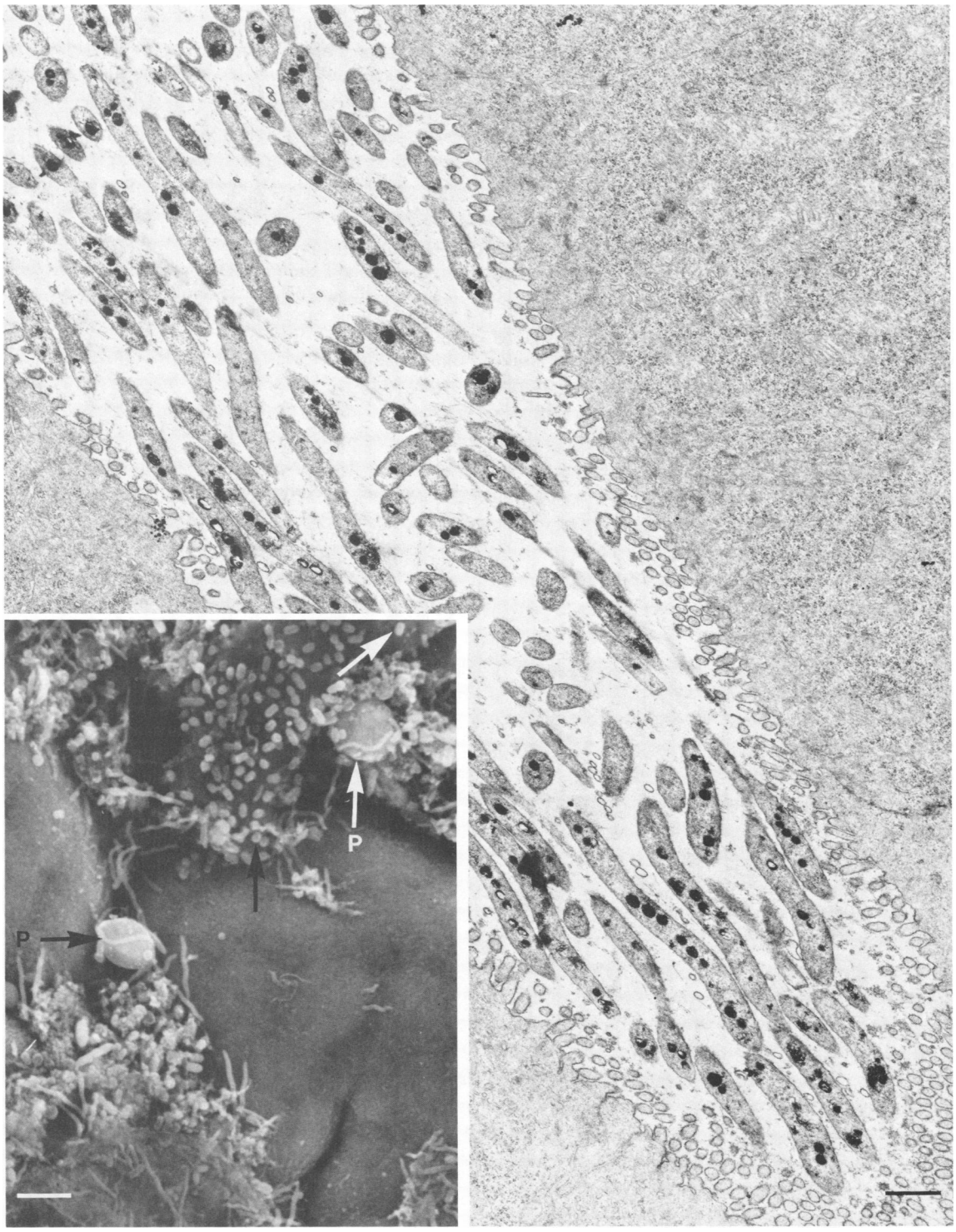

FIG. 7. TEM of a crypt in the cecal surface of a T rat. The crypt was occupied by spirilla with thin, irregular cell walls and prominent, electron-dense cytoplasmic inclusions. Bar, 1 μm. (Inset) SEM of the cecal surface of a C rat, showing the occurrence of sinuous spirilla within the incomplete mucous blanket and the occasional presence of protozoa (P) and rod-shaped bacteria within slablike amorphous matrices (arrows). Bar, 5 μm.

surface occupied almost exclusively by confluent masses of protozoal cells (Fig. 6), the desquamating cells on the tips of the villi could be clearly discerned (Fig. 6, top, arrows), as could segmented filamentous bacteria protruding from the tissue. Examination of these areas at higher magnification (Fig. 6, box) showed *H. muris*, *G. muris*, (Fig. 6, bottom, arrow), and adherent segmented filamentous bacteria but very few other microorganisms. Direct microscopic examination of centrifuged luminal contents of T animals revealed 22 *H. muris* organisms per *G. muris* organism per high-

power field; only rare protozoa were identified in similar contents of C animals.

Extensive TEM examination of sections of ruthenium red-stained preparations from four animals confirmed data yielded by SEM in that >60% of the ileal surfaces of T rats were covered by protozoa. Very exhaustive examination of sectioned material by TEM revealed only a few bacteria, spiral organisms visible in the crypts and, with the exception of the segmented filamentous organisms, unattached to the microvillous surfaces of the ilea of C and T rats.

(iii) Cecum. Direct examination of >4-mm^2 areas of the surfaces of the ceca of C rats by SEM showed them to be only partially covered by a mucous layer. An unnamed *Spirillum* sp. (28, 30) predominated on the microvilli of these tissues (Fig. 7) and these sinuous organisms were often concentrated in condensed residues of mucus. Rod-shaped bacteria (Fig. 7, inset, arrows) were associated with slablike amorphous residues and unstructured aggregates on the tissue surface, and occasional protozoa (Fig. 7, inset, P) were associated with the microvilli of these tissues. TEM of sections of ruthenium red-stained preparations of the cecal epithelia of C rats showed the presence, within a fibrous matrix, of bacteria with thin, crenulated cell walls and electron-dense cytoplasmic structures. Other areas of the tissue surface were occupied by a mixture of spirilla and other bacterial morphotypes.

The microvillous surfaces of the ceca of T rats retained partial coverage by the mucous layer. All elements of this overlying structure were very heavily populated by spirilla of the same morphotype seen in C animals; large populations were especially associated with the intestinal crypts. TEM sections of ruthenium red-stained preparations of the cecal tissue of T rats (Fig. 7) showed the spirilla to be also in crypts apparently filled with these organisms. Examinations at higher magnification showed the presence of electron-dense cytoplasmic structure and the very thin (ca. 8 nm) dimensions and irregular contours of their cell envelopes, as described by other workers (28, 30).

Immunofluorescent staining of jejunal, ileal, and cecal tissues from T animals demonstrated specific adherence of PHA to the mucosal surface of the villus. No immunofluorescence was observed in the crypt region (Fig. 8). No binding of the only other dietary protein, casein, to the mucosal surface was observed by utilizing fluorescent antibodies (3).

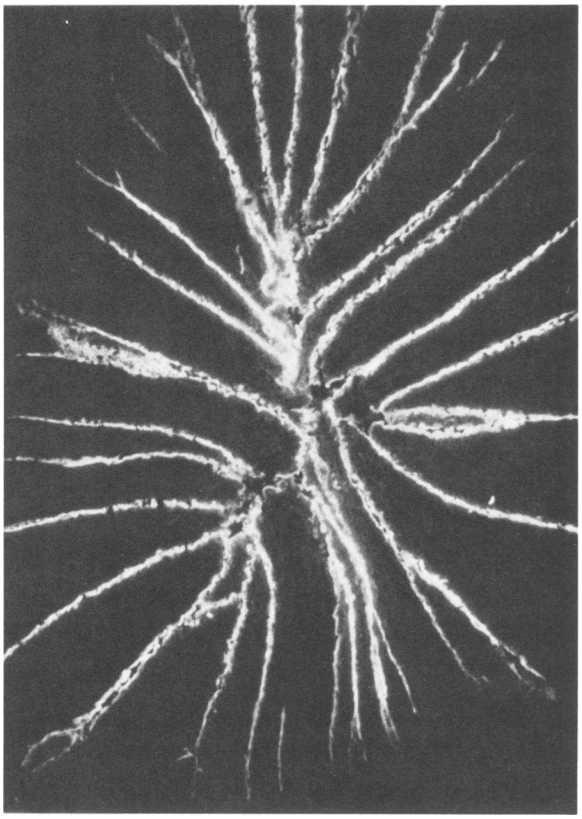

FIG. 8. Fluorescent photomicrograph of a cross-section of jejunal lumen stained with anti-PHA fluorescent antibody. The lectin is seen as a continuous pattern which delineates the intestinal villi (magnification, ×63). Tissue from casein-fed C animals demonstrated an absence of fluorescence.

DISCUSSION

These morphological studies characterized changes in the microbial flora of the rat small intestine and cecum which accompany feeding with PHA lectin. They provide additional evidence to support the findings of previous studies (3, 4) in which an intraluminal overgrowth of bacteria developed in the jejuna and ilea of rats fed diets containing PHA and red kidney beans, which was associated with malabsorption of lipid, nitrogen, and [^{57}Co]cobalamin.

In T rats the jejunal mucous layer was thinned and populated by a gram-negative rod and a gram-positive coccobacillus; changes of some degree were observed in all of the animals studied. However, bacteria were not continuous over the entire mucosal surface, which may partially account for the variability of damage to the microvillous surface observed on histological examination of tissue from T animals in other studies (4, 22). Mucosal microvillous damage was most likely a consequence of the bacterial overgrowth. Microvillous membrane damage and reduced disaccharidase activity have been observed in association with other forms of bacterial colonization (21, 31). Purified PHA fed to germfree animals did not cause morphological defects in the intestinal mucosa (3). The bacterial flora were developed in association with PHA feeding was characteristic for T animals and differed from that of C animals in magnitude as well as in the types of colonizing organisms observed.

It is likely that the gram-negative bacteria were coliform organisms. *E. coli* was identified in high concentration in cultures of jejunal fluid from PHA- or red kidney bean-treated animals in previous studies (4, 20, 39, 40). Moreover, cultures of washed, homogenized tissue in our laboratory have grown an aerobic gram-positive enterococcus in significantly higher concentration from PHA-exposed tissue (J. G. Banwell and R. Howard, unpublished observations), which may account for the other bacterial form identified in these studies.

The nature of the association between the two bacterial species and the intestinal brush border membrane remains to be defined. PHA will bind to rat jejunal brush border membranes (4). Adherence of PHA to the intestine may have facilitated the colonization by *E. coli*. Other lectins, concanavalin A, and *Helix pomatia* lectin will bind to *E. coli* via the lipopolysaccharide of the cell wall (31). PHA has a major role in facilitating bacterial attachment in the rhizobium-legume symbiosis (13). Adherence of many enteropathogenic *E. coli* is dependent on surface characteristics of their fimbriae and on other specific pathogenic characteris

tics such as enterotoxin production, hemagglutination, and serotype (18). Genetic determinants of all these features frequently depend on plasmid-associated extrachromosomal material and are transferable to other bacterial strains. A role for such factors in PHA-induced bacterial colonization is possible but as yet undefined. Coaggregation between specific pairs of bacteria has been demonstrated to occur in the mouth (8).

Changes in the microbial ecology of T animals were different in the distal small intestine. Although some rod-shaped and coccobacillary bacterial aggregates were observed in T animals, overgrowth by the protozoan *H. muris* was the major finding. This organism is known to be a commensal in the rat, but whether it is truly host-species specific is uncertain, since protozoa of similar morphology are found in the golden hamster, house mouse, and various wild rodents. Epithelial brush border damage was not observed in the ilea of *H. muris*-infected T animals. It is of interest that *G. muris* was identified only occasionally in this study and in both C and T animals (29). Cause for the PHA-induced proliferation of *H. muris* is unknown. Many protozoa have been identified as predators of bacteria in biological systems (1). Therefore, it is possible that their abundance may have been related to the concomittant upper intestinal bacterial proliferation which might have provided an improved nutritional source for their growth and proliferation (1). However, a direct effect of PHA on protozoan proliferation and colonization may also have been important.

In the cecum, the predominant organism was an unnamed *Spirillum* sp. (29) with morphological features similar to those described by other workers (6, 29). Further characterization by quantitative morphometric techniques (28) would be necessary before PHA-induced overgrowth of this organisms can be ensured.

A mucous layer is normally present on the mucosal surface of mouse and rat intestine in vivo and in vitro (2, 37, 41). Some mucus discharge from goblet cells onto the surface might have also occurred as a result of the preparative techniques that were employed. Discharge of mucus has been observed after washing with saline, but this was not done in this study (17). Discontinuity of the mucous layer may appear with SEM as an artifact of fixation and dehydration. However, in T animals its absence can best be attributed to PHA exposure and the associated accumulation of bacteria on the mucosa. Glycosidase production and degradation of goblet cell mucin by colonizing bacteria are consequences of overgrowth of bacteria in the blind-loop syndrome (21, 31); mucinase, sialidase, and a variety of proteases can be identified in culture supernatants of pathogenic enteric organisms which are able to penetrate the mucous biofilm and attach to the intestinal brush border membrane (16, 36).

Alterations in the nutrient composition of the diet and starvation are known to cause changes in the distribution of the intestinal microflora of the mouse (5, 31). However, these factors cannot by themselves explain the striking microbial changes associated with PHA ingestion. Although both T and C pair-fed groups were provided diets lower in protein than normal, changes in microbial flora were only observed in the T group.

Although exogenous PHA enhanced the proliferation of bacteria and protozoal growth, the mechanism by which it caused these changes in the enteric microbial flora remain uncertain. PHA may have had additional effects other than acting as an adherence factor (4, 30). These effects may have been exerted on mucus synthesis or secretion, host defense mechanisms, or cell membrane turnover, all of which could have altered the ecological environment for the bacterial flora (7, 34). Nevertheless, these changes which accompany PHA feeding may represent a unique way for changing the intestinal microflora. Better understanding of the ecological changes may be achieved by further time-sequence studies of microbial colonization (35) of the intestinal epithelium.

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