APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1976, p. 304-312
Vol. 31, No. 2

Printed in U.S.A.

Preservation of Gastrointestinal Bacteria and Their Microenvironmental Associations in Rats by Freezing

CHARLES P. DAVIS

Departments of Surgery and Medical Microbiology, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 10 September 1975

The use of frozen rat gastrointestinal tissue samples for both the recovery of viable bacteria and for observation of microbial communities associated with the tissue was investigated. A decrease of 1 log in lactobacilli, bifidobacteria, and anaerobes was observed when the numbers of bacteria recoverable from frozen tissue (stored 7 to 9 days) were compared to those recoverable from fresh nonfrozen tissue (zero time control). However, freezing did not appear to decrease the numbers of recoverable coliforms. Tissues, cleaved with razor blades after being frozen and stored for 7 to 9 days, showed bacterial communities situated on the mucosa and in the lumen of gastrointestinal specimens. This freezing technique preserved structures not previously observed in the gastrointestinal tract. This indicates that freezing is a good method to use to study such fragile microenvironments.

The murine gastrointestinal tract has microenvironments that include bacteria as integral components (6, 15). Some bacteria can form layers on the surface of the stomach, cecum, and large bowel, whereas others can attach to epithelial cell membranes (5, 7, 13-15, 17, 18). Other bacteria appear mainly in the lumen, and still others localize in crypts (3, 4).

During an investigation on the effect of freezing on rat intestinal bacteria, most of the lumenal contents were observed to adhere readily to the mucosal surface when pieces of frozen tissues were placed in a cold fixative. Often much of the lumenal contents and bacteria is removed from the mucosal surface when nonfrozen tissue is fixed in cold glutaraldehyde (4, 14). The results of the investigation on both the survival of intestinal bacteria and the preservation of the microecology within such frozen samples of the gastrointestinal tract are presented.

MATERIALS AND METHODS

Tissue preparation. Eight-week-old rats were anesthetized and killed by cervical dislocation as described previously (5). Approximately the same numbers of male and female rats were used in all the experiments. Segments (1 to 2 cm) of the proximal colon and 1- to 2-cm segments of the distal ileum were removed along with about 2 cm^2 of the keratinized region of the stomach. One piece of each tissue was placed in a separate vial, and the vial was immersed immediately in liquid nitrogen (-196 C). After 2 h, the vial was removed from the liquid nitrogen and placed immediately in a -70 C freezer.

Other pieces of stomach and colon from the same animals used to prepare frozen tissues were placed in prereduced tubes (9) of Trypticase soy broth (Difco), weighed, and introduced into an anaerobic glove box (80% N_2-10% H_2-10% CO_2) (1). The tissue pieces were homogenized as described previously (15) and then diluted in Trypticase soy broth. Still other pieces of fresh tissue were processed for light and scanning electron microscopy as described below. Frozen samples of tissue were processed identically as described above after storage at -70 C for 7 to 9 days (see below). All nonfrozen tissues (zero hour controls) were processed immediately after they were removed from the animals.

Microbiology. Anaerobes were enumerated on A2 agar (1) and incubated at 37 C anaerobically in the glove box, whereas coliforms were grown aerobically at 37 C on EMB agar (Difco). Anaerobes and coliforms were cultured from colonic homogenates. Lactobacilli and bifidobacteria were cultivated from stomach homogenates on lactobacillus agar (GIBCO) at 37 C in a 5% CO_2 atmosphere. A preliminary experiment (C. P. Davis, unpublished data) indicated by Gram reaction and volatile and nonvolatile fatty acid production that both of the latter genera were predominant in rat stomachs and produced distinctive colonies on lactobacillus agar. All colonies on anaerobic culture plates were counted after incubation for 1 week, whereas colonies on the other culture plates, incubated aerobically or in CO_2, were counted after 4 days. Fifteen predominant colony types from the anaerobically incubated plates of the frozen tissues were subcultured and then streaked onto blood agar and incubated anaerobically, aerobically, and in a CO_2 atmosphere. Pure cultures of bacteria that did not grow aerobically or in a CO_2 atmosphere were inoculated into PYG broth (9), and the genera to which they belonged was presumptively determined according to the Gram reaction and the volatile and nonvolatile fatty acids

they produced. Fatty acids were identified with a gas chromatograph (Dohrman, Mt. View, Calif.) (9).

Microscopy. After 7 or 9 days of storage at −70 C, each tissue sample was cleaved with a razor blade into a few pieces while still frozen. One piece was cultured for bacteria as described above. Another piece was placed in a cryostat, covered with 2% methyl cellulose in 0.85% saline, sectioned, and stained by the Gram method (15). A Zeiss Universal microscope was used for all light micrography.

For scanning electron microscopy, frozen pieces of stomach, colon, and ileum were placed in a 2.5% glutaraldehyde solution buffered with 0.1 M cacodylate (pH 7.4) at 4 C for 24 h. The tissues were rinsed twice in the buffer (4 C) and then postfixed in osmium tetroxide buffered with 0.1 M cacodylate (pH 7.4) at 4 C. Pieces of tissue with lumenal contents were gently handled in the fixation and dehydration procedure because the contents could be easily dislodged. The tissues were dehydrated and critical-point dried as described previously (14). Dried tissues were then fixed to aluminum stubs with silver conductive paint and coated with gold-palladium in a Denton 502 evaporator (Denton Vaccum, Cherry Hill, N.J.). Specimens were examined in a JEM U3 scanning electron microscope at 20 kV.

RESULTS

Recovery of bacteria from frozen samples. Total numbers of bacteria recovered from two regions of the rat gastrointestinal tract that were immediately cultured were compared with the total numbers of bacteria recovered from frozen samples stored for 7 or 9 days. Table 1 indicates that substantial numbers of bacteria were recovered from both stomach and colon of frozen and nonfrozen samples. Lactobacilli, bifidobacteria, and anaerobes showed a decrease of about 1 log between frozen and nonfrozen samples. However, the coliforms showed no significant differences in the numbers of viable bacteria isolated from the frozen and nonfrozen samples. Isolates from the frozen samples that were known to be strict anaerobes were tentatively identified by the morphological and biochemical characteristics that they showed in PYG broth. *Bacteroides, Lactobacillus, Fusobacterium,* and *Eubacterium* species were isolated.

Light microscopy. Frozen and nonfrozen tissue samples were tissue-Gram-stained and examined by light microscopy to see if any alterations had occurred in the various bacterial communities that are normally associated with the stomach, ileum, and colon of individual rats. In almost every tissue examined, there was little or no change in the bacterial communities that associated with the mucosa (Table 2). Gram-positive rods were found in layers of the keratinized epithelium of the stomach, whereas segmented filamentous microbes could be observed in the distal ileum (Fig. 1 and 2). Large numbers of fusiform-shaped bacteria were observed adjacent to the colonic epithelium in the frozen samples (Fig. 3). These observations paralleled those in the nonfrozen tissue sections from control animals.

However, the frozen ileal and colonic tissues appeared slightly different from the nonfrozen control tissues. The lamina propria in the frozen samples often showed fewer cells than those of the controls. In some cases, the length of the villi was shortened and epithelial cells were absent. The keratinized layer of the stomach was least affected by freezing. However, some sectons in the keratin layer of frozen stomach tissue showed larger clear areas than in the nonfrozen control. Although the tissue was modified somewhat by freezing in liquid nitrogen and storage at −70 C, the observed changes

TABLE 1. *Effect of freezing and storage on the viability of gastrointestinal bacteria*

Bacterial types	CFU/g[a]		Region of gastrointestinal tract cultured
	Frozen[b]	Fresh nonfrozen[c]	
Lactobacilli and bifidobacteria	2×10^{10} (3×10^{9}–5×10^{10})	10^{11} (10^{10}–7×10^{11})	Stomach
Coliforms	10^{9} (2×10^{8}–5×10^{9})	10^{9} (8×10^{7}–3×10^{9})	Colon
Anaerobes	4×10^{9} (3×10^{8}–10^{10})	2×10^{10} (10^{10}–4×10^{10})	Colon

[a] Average number of colony-forming units (CFU) per gram (wet weight) in 10 animals. Values in parentheses are the range of the average number of colony-forming units.

[b] Tissue samples (stomach and colon) from the animals were put in vials, and the vials then were placed in liquid nitrogen. The vials were then placed in a freezer at −70 C. After 7 to 9 days, frozen stomach and colon tissues were processed immediately by anaerobic methods and plated onto lactobacillus, EMB, or A2 agar.

[c] Tissue samples, adjacent to those removed for freezing, were immediately cultured; they were not frozen or stored.

TABLE 2. *Effect of freezing on the occurrence of bacteria associated with the mucosa in rat gastrointestinal tracts*

Method	Sample type[a]	Bacterial occurrence		
		Stomach[b]	Ileum	Colon
Light microscopy	Nonfrozen controls	5/5[c]	5/5[d]	5/5[e]
	Frozen	8/8	8/9	8/9
Scanning electron microscopy	Nonfrozen controls	2/2	2/2	2/2
	Frozen	10/10	10/10	10/10

[a] Both nonfrozen and frozen tissue were sectioned in a cryostat and stained with a tissue Gram stain for light microscopy. Also, both sample types were identically fixed, dehydrated, critically point dried, and metal coated for scanning electron microscopy.

[b] Area of gastrointestinal tract examined.

[c] Number of rat stomachs observed with a predominant population of gram-positive rods attached to the keratinized epithelium/total number of animals observed.

[d] Number of rat ilea observed with segmented filamentous microbes attached to epithelial cells/total number of animals observed.

[e] Number of rat colons observed with a layer of bacteria adjacent to the epithelium/total number of rat colons observed.

did not visibly affect the localization of bacterial communities in the gastrointestinal tract.

Scanning electron microscopy. All of the tissues examined with the scanning electron microscope showed that the bacterial communities observed with light microscopy remained intact (Table 2). Microbes observed in large numbers on the keratinized surface of the rat stomach were attached to each other and to the keratin layer of the stomach. The majority of the microbes were short and long rods (Fig. 4). These microbial types, sometimes viewed attached by their ends to the stomach epithelium, were most frequently seen attached by their side to the mucosa. Figure 5 shows the filamentous material that connects the bacterium and the mucosa. Furthermore, small indentations in the keratinized mucosa have about the same shape as the attaching bacteria and could be areas where bacteria once resided. Scattered among the long and short rods are at least two other less numerous microbial types, cocci and small tapered rods (Fig. 4 and 5). Also, probably at least three types of short rods are present. One has a coarse surface, another has a smooth surface, and the third is shorter and narrower than the first two types (Fig. 5). Much of the stomach lumenal content was lost when the section of stomach tissue was first removed from the rat. Consequently, a comparison of the lumenal- and keratin-associated populations was not done.

If a frozen piece of ileum is cleaved in cross section, the entire cross section of tissue and lumen can be viewed (Fig. 6). Such a section readily indicates that the microenvironment of segmented filamentous microbes is strictly limited to the surface of the mucosa (Fig. 7 and 8). These microbes were not observed in the center of the lumen. A low and variable population of microbes was observed in the lumen (Fig. 8).

The attachment site of these segmented filamentous microbes is easily recognizable in the frozen preparations (Fig. 7 and 9). In addition, another structure in the microenvironment was observed in these frozen preparations that was not viewed in previous studies by us or other investigators. This structure is the mucin layer. Usually it has been observed as isolated strands or flakes on the mucosal surface (12). In this study, it was observed to overlay the epithelial cell surface in the cleaved piece of ileum as a thin web or film with an occasional hole in it. To appreciate its expansive nature, however, the mucin layer was best observed when unobscured by the lumenal contents (Fig. 10). The latter was accomplished by viewing a region of the ileum where the contents had been gently removed prior to freezing. In these carefully prepared samples, such areas were found where the ileal tissue folded back on itself after it had first been cut out of the rat.

Freezing not only preserved a structure previously lost in other preparations but also allowed further insight into the microenvironment of segmented filamentous microbes. Figure 10 shows that most of these microbes are covered by the mucous layer. Thus these microbes, like the epithelial cells to which they are attached, exist separated from the lumenal environment by a mucous film.

Freezing allowed the thick layer of microbes present in colons to remain intact. The layer, juxtaposed to the colonic epithelium, extends far into the lumen. Examination of this layer reconfirms that a substantial mass of the colon

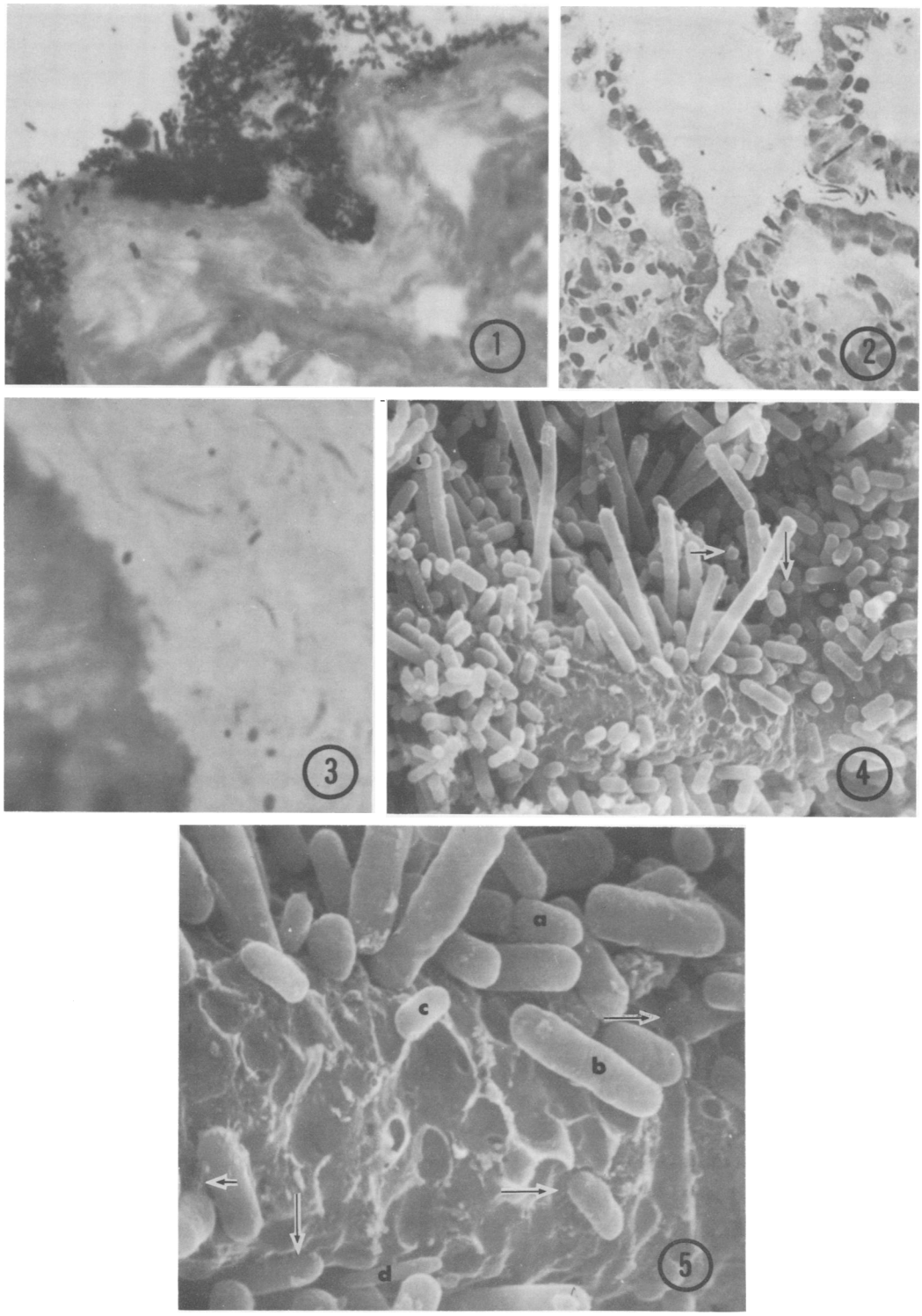


FIG. 1. *Tissue-Gram-stained section of frozen rat stomach with adjacent layer of gram-positive rods. Note vacant areas within keratin.* ×*1,360.*

FIG. 2. *Frozen rat ileum with segmented filamentous microbes. Some regions of the lamina propria and the epithelium of the villi are absent.* ×*554.*

FIG. 3. *Layer of bacteria on frozen colonic epithelium.* ×*3,499.*

FIG. 4. *Rat stomach epithelium with bacteria adhering to the keratin layer by their ends and sides. A few cocci (arrows) are also visible. Cocci are differentiated from end-on views of rods by rotating the specimen stage. Note the small indentations in the mucosa that are about the same size as adjacent bacteria.* ×*2,624.*

FIG. 5. *Four morphological types of rod-shaped bacteria. (a) Coarse surface; (b) smooth surface; (c) short and narrow rod with a smooth surface; (d) fusiform-shaped rod. Arrows show the filamentous material that connects the bacteria to the keratinized layer of the rat stomach.* ×*8,748.*

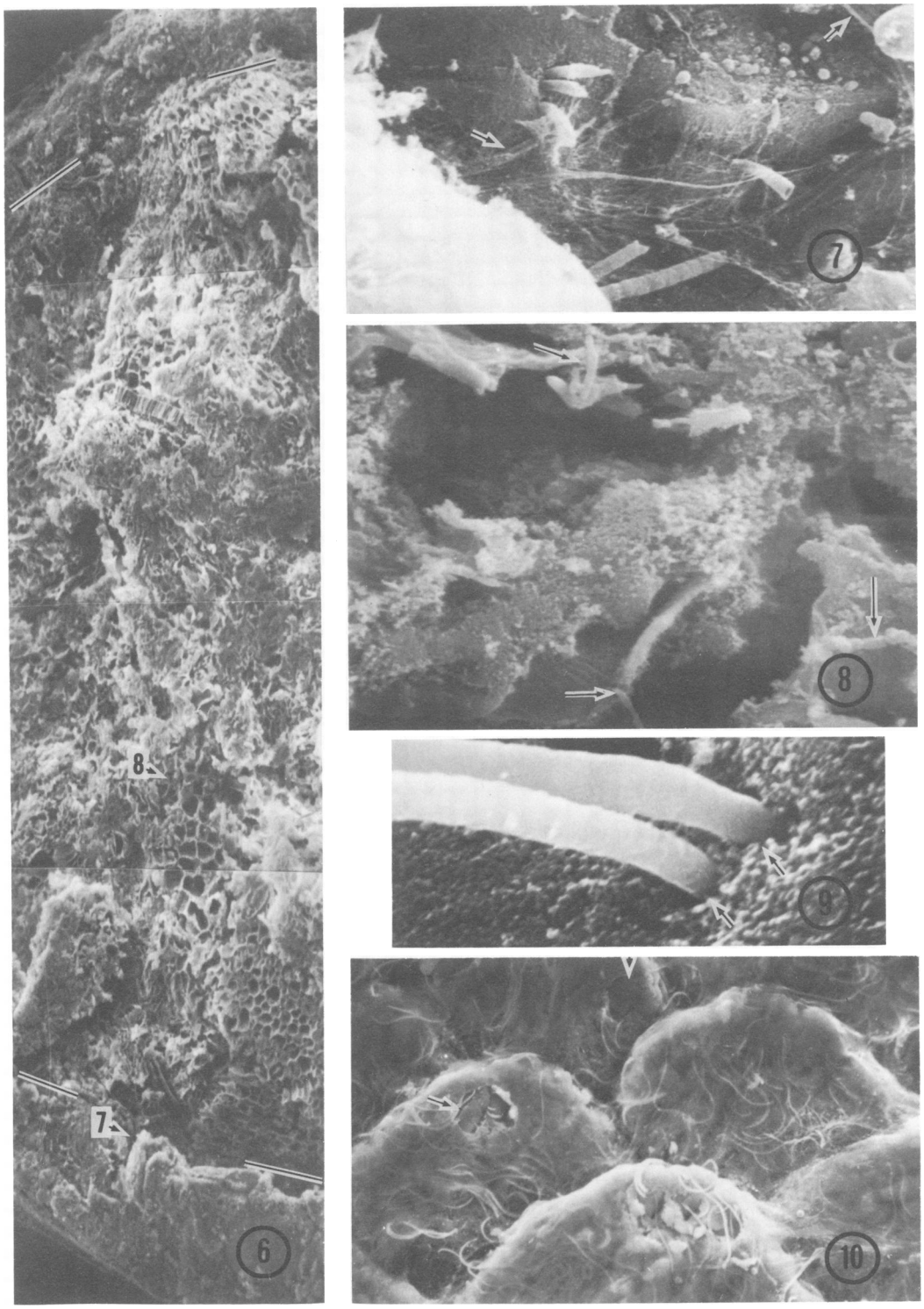


FIG. 6. *Montage of a frozen cross section of ileum. Bars show the inner border of the ileal tissue. Arrows indicate the areas where Fig. 7 and 8 were photographed.* ×68.

FIG. 7. *Attached segmented filamentous microbes on the rat ileum. Arrows show the film that often covers the villi and the microbes.* ×2,624.

FIG. 8. *The lumen of the ileum shows organisms (arrows) randomly arranged and adjacent to lumenal debris.* ×2,624.

FIG. 9. *Arrows indicate the attachment site of microbes attached to the ileal epithelial cell.* ×8,748.

FIG. 10. *An extensive film covers the ileal villi and segmented filamentous microbes. Note, however, that holes (arrows) are present in this film.* ×262.

is comprised of bacteria. The layer of bacteria is almost as thick as the colonic tissue (Fig. 11-13). Microbes in this layer are intertwined and apparently connected with each other and the epithelium by filaments.

At least six morphological types of microbes were observed in the rat colons (Fig. 14), although the most numerous microbial types were fusiform-shaped bacteria. Spiral- and rod-shaped microbes were also present in large numbers, whereas coccal-shaped organisms were observed infrequently.

As compared to the layer of bacteria on the epithelial surface, the lumenal region of the colon has only a slightly less dense bacterial population (Fig. 15). It was difficult to locate spiral-shaped bacteria in the lumen of the colon; however, it was easier to locate rod-shaped bacteria. This observation suggests that spiral-shaped bacteria localize preferentially in the mucosal layer and rod-shaped bacteria localize in the lumen.

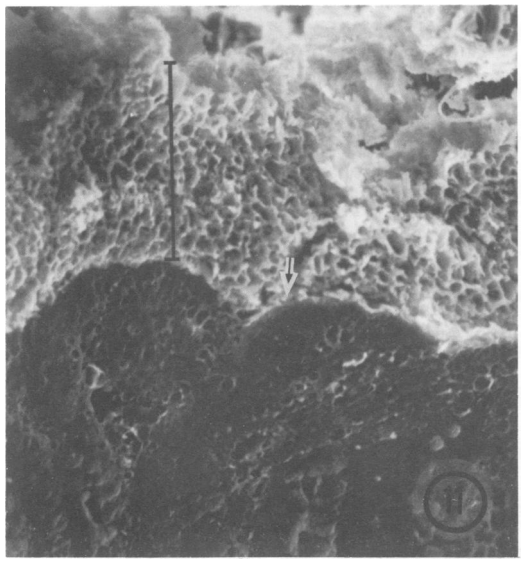
DISCUSSION

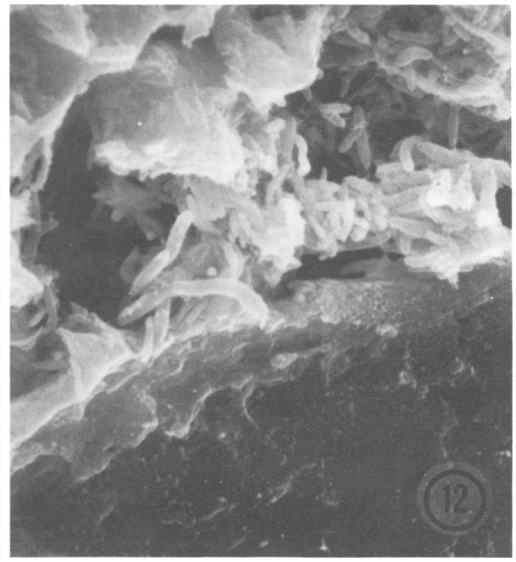
Crowther (2) studied the survival of bacteria in feces frozen and stored in glycerol broth. He found that freezing feces at −78 C or in liquid nitrogen (−196 C) was satisfactory for storage However, he noted a decrease of about 1 log in the total number of lactobacilli recoverable from frozen feces. Feces without glycerol added also showed a decrease of about 1 log in viable anaerobes (2). The results of my study, i.e., a comparison of the total numbers of lactobacilli, bifidobacteria, coliforms, and anaerobes from samples of frozen and nonfrozen tissue, are similar to those of Crowther. I observed a decrease of about 1 log in the total number of lactobacilli, bifidobacteria, and anaerobes, whereas the number of viable coliforms did not decrease after 7 to 9 days of freezing. Thus, it seems that storage of pieces of intestinal tissues at −70 C, after quick-freezing them in liquid nitrogen, allows for the recovery of a large number of bacteria associated with such samples. Since coliforms may overgrow slower replicating bacteria, one should remember that coliforms might obscure the recovery of some microbes that are numerically decreased by freezing if their number falls below the number of recoverable coliforms.

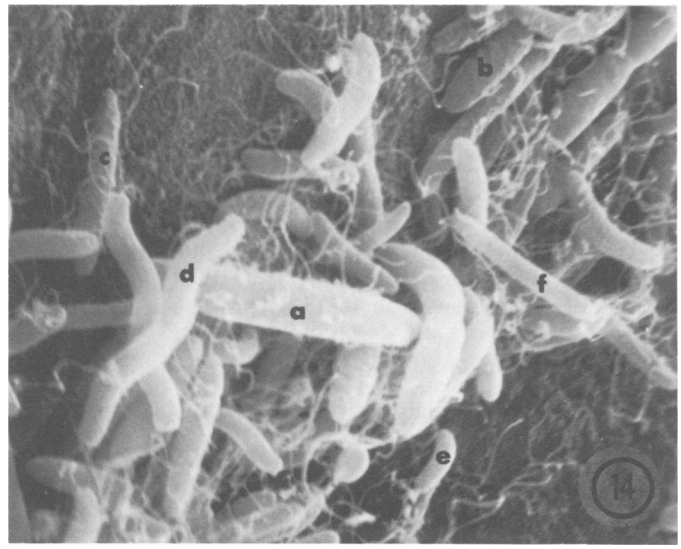
Several studies have indicated that rats and mice possess a microflora that is characteristic for each region of their gastrointestinal tract (3, 6, 14, 15, 17). Furthermore, there is evidence that dogs and chickens also have a characteristic microflora in their intestines (8, 10).

Recently, Savage and Blumershine (14) published scanning electron micrographs that showed different microbial communities located in the stomach, ileum, and large bowel of mouse gastrointestinal tracts. In this study it was shown, by the use of frozen samples, light microscopy, and scanning electron microscopy, that individual microbial communities are also located on the same three regions of rat gastrointestinal tracts. Each microbial community was similar, generally, in its morphology to those described in the mouse system. Furthermore, the results support and extend the culture and light microscope studies of other investigations of rat microbial communities (3, 13). However, some differences from the mouse flora were observed. For example, long tapering filamentous microbes composed of rod- or coccal-shaped bodies were not observed on rat stomachs, although they were reported on mouse stomachs (14). Also, filaments that often connect rod-shaped bacteria to keratin were not seen in scanning electron micrographs of mouse stomachs; however, they were observed in rat stomachs. This agrees with transmission electron microscopy results of Takeuchi and Savage which reported that the bacteria are connected to keratinized mouse stomachs by filaments (A. Takeuchi and D. C. Savage, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, M254, p. 115). Indentations in the keratin layer were observed in this study. I suggest that these observable indentations in the keratin were caused by bacteria. The rods, by digesting the keratin in connection with the filamentous material, could cause such indentations in the keratin. Similarly, extracellular coats and fibrous elements were shown to attach other bacteria to their substrates (11, 16).

The ultrastructures of microbial communities observed in the frozen ilea and colons were almost identical to those found in other investigations (5, 7, 14; C. P. Davis, Ph.D. thesis, University of Texas at Austin, p. 49-71, 1974). The ilea were populated by segmented filamentous microbes that selectively colonized the epithelial cells. As observed previously in mice and rats, the microbes were connected to epithelial cells by a segment at one end of the filament (5, 7). The results in this study confirm and extend the observations on the microenvironment of these bacteria. Segmented filamentous microbes and the ileal villi were separated from the lumen by a thin film of material that is probably mucus (12). This layer could serve to isolate the segmented filamentous microbes from the lumenal environment. The lumenal environment might contain enzymes or other



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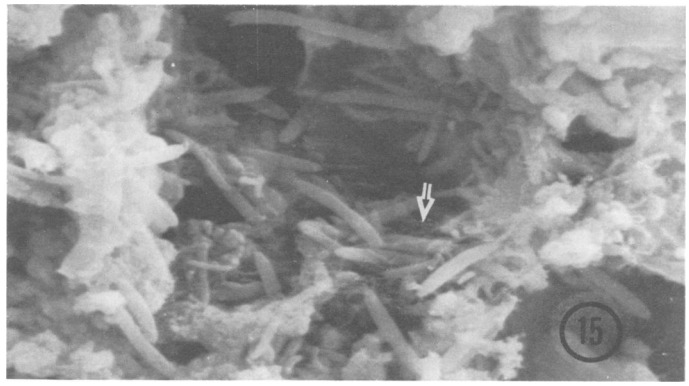

microbes inhibitory to their growth or survival. Furthermore, the layer could serve to keep the reproductive bodies of the segmented filamentous microbe away from the lumen, where they could easily be removed from their habitat, but adjacent to the villi, where they attach to complete their life cycle (5, 7).

Neither the extent to which this layer coats the ileum nor the size or frequency of holes in it is known. These holes could be the initial sites of gastrointestinal infections. It is well known that the ingestion of sufficient numbers of *Salmonella,* for example, will lead to an infection of the host via the gastrointestinal mucosa. Large numbers of organisms may be needed to initiate infection because such large numbers would increase the probability that a microbe would contact a hole in the mucus layer, thereby exposing susceptible tissue to the pathogen.

Although direct evidence is lacking, I believe that both the mucin layer and its observable holes are not artifacts caused by freezing. Such structures occasionally are found in conventionally prepared tissue (12). Since freezing obviously preserves lumenal contents and loosely or nonadherent bacteria in the samples, it is quite possible that a structure like the mucin layer would also be preserved. Recent observations show that mucin in glutaraldehyde-fixed germfree rat ilea is reduced or removed but that germfree rat ilea frozen before fixation retained mucin, yet showed larger holds than conventional rat ilea (C. P. Davis, E. Balish, and C. E. Yale, unpublished data). Again, these results suggest that freezing preserves structures that are lost with conventionally prepared tissues.

Massive numbers of bacteria were viewed on the surface of the colonic epithelium. The bacterial layers were easily observed in all rats examined, in contrast to a previous report that found the layers easily removed in the preparation of the tissue for scanning electron microscopy (14). Freezing preserved the bacterial population and showed that it occupies a region that is almost as thick as the colonic tissue. The heterogenous bacterial population is very similar to that found in mice. Savage and Blumershine (14) noted that fusiform- and spiral-shaped bacteria appeared to be connected to each other and to the colonic epithelium by weblike filaments. In this study, these filaments were observed in the rat, not only on the bacteria adjacent to the epithelium but also on bacteria located in the lumen. The nature of these filaments is not known, but they could be flagella, pili, mucous strands, or possibly a combination of all of these structures.

There is some loss of loosely associating material in samples even with freezing. Loss of some stomach contents was the major difficulty encountered with this technique. Large amorphous particles that resembled particles from the food pellets comprised the bulk of this material. Also, there was some minor loss of lumenal contents of the ileum and colon. Loss occurred mainly during fixation and the first few steps of dehydration. This loss can be minimized by quickly placing the frozen tissue into the fixative and by pipetting gently during the entire dehydration series. Also, because of the extremely complex surface area of the tissue and lumenal contents, there may be a few areas on the sample that charge under the electron beam. Usually, this can be reduced by recoating the sample.

Thus, freezing offers several advantages not available with other techniques for viewing microenvironments in the gastrointestinal tract. The frozen tissue can be stored at −70 C for at least 9 days before processing. Frozen tissue can be cleaved to yield a view of an ecosystem that is difficult or impossible to investigate with conventional preparations. When the tissue is frozen, loosely adherent structures are preserved that are probably lost or obscured by other techniques. I believe that this technique can be adapted for use in many other studies of microenvironments.

FIG. 11. *Cross section of a cleaved rat colon. The bar indicates the extensive layer of bacteria that extends toward the lumen. Below the bar is colonic tissue. Arrow indicates the area where Fig. 12 was photographed.* ×262.

FIG. 12. *This figure shows the mass of bacteria immediately adjacent to the colonic tissue. This dense heterogeneous population extends well into the lumen (see Fig. 11).* ×2,624.

FIG. 13. *Another view of the layer of microbes is shown in rat colon. The colon was cleaved at an oblique angle. It shows that the layer is composed almost entirely of bacteria.* ×875.

FIG. 14. *Various microbial types adjacent to the rat colonic mucosa and the filaments that intertwine the bacteria. There are at least six morphological types of bacteria observable: a, b, c (large, medium, and small fusiform-shaped rods); d (curved bacteria); e (small curved rods); and f (rod-shaped bacteria). Note that both the medium and small fusiform-shaped rods have a wavy surface.* ×8,748.

FIG. 15. *Colonic lumen with many microbes present in it, in contrast to the ileal lumen (see Fig. 8). Microbes in the lumen also possess filaments (arrow) that connect the bacteria to each other.* ×2,624.

ACKNOWLEDGMENTS

I thank Edward Balish for the helpful suggestions he gave during this work.

The author was supported by Public Health Service Medical Microbiology and Immunology training grant AI00451-04 from the National Institute of Allergy and Infectious Diseases during this study.

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