SMALL INTESTINAL MUCOSAL CELL PROLIFERATION AND BACTERIAL FLORA IN THE CONVENTIONALIZATION OF THE GERMFREE MOUSE*

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Intestinal mucosal cells, originating in the crypts, migrate up the villi of the small intestine and slough off into the lumen (1, 2). Studies of the kinetics of these events have revealed a differential rate of cellular renewal between the intestinal mucosal cells of germfree animals and those of conventional animals (3, 4). The time for migration of the mucosal cells from the crypt to villus tip is twice as long in the germfree animal (3, 4). The absence of intestinal bacterial flora in the germfree animal may account for this difference in dynamic morphology.

Recent advances in intestinal and fecal culturing techniques have provided information about the intestinal bacterial flora under normal and experimental conditions (5–7). The changing patterns of intestinal microorganisms have been followed both in newborn animals (8–11) and after contamination in germfree animals (12).

The present study was designed to follow the changes in intestinal cellular renewal and microbial flora as a function of time in going from a germfree system to a conventional one. Experiments involving both germfree and conventional animals have utilized conventionalized (formerly germfree) animals as the control subjects; however, the possibility of altering cellular kinetics of the intestinal epithelium and the span necessary for the alteration have not previously been investigated.

Materials and Methods

Animals.—35 male and 35 female CFW mice (Carworth Farms, New City, N. Y.) born and reared under standard germfree conditions were randomly divided into two groups,

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each containing equal numbers of males and females. One group of 16 animals was maintained in plastic cages in a Snyder (Trexler type) flexible film isolator (Snyder Manufacturing Co., New Philadelphia, Ohio) under standard germfree conditions (13). They were given sterilized drinking water and fed a sterilized commercial diet (Purina Laboratory chow, special formula 5010 C) ad libitum. In order to ascertain that a germfree environment was maintained, bacterial cultures of the isolators, the bedding, and the animals' fecal droppings were taken both on arrival of the animals and upon each entrance into the isolators.

Another group of 54 mice was removed from the germfree isolators and placed in a conventional animal room. These animals received the same sterilized diet and drinking water ad libitum as the germfree animals. Fresh stools of conventionalized mice were obtained daily for culture of both anaerobic and aerobic microorganisms on selective media.

TABLE I

Schedule of Injections and Sacrifices in Germfree and Conventionalized Animals

Day	Conventionalized		Germfree	
	Injections	Sacrifices	Injections	Sacrifices
0	1, 2, 3, 4, 5, 6		1–16	
1	7, 8, 9, 10, 11, 12	1, 2		1–3
2	13, 14, 15, 16, 17, 18, 19, 20	3, 4, 7, 8		4–7
3	21, 22, 23, 24, 25, 26, 27, 28	5, 9, 10, 13, 14, 15		8–12
4	29, 30, 31, 32, 33, 34	6, 11, 16, 17, 18, 21, 22, 23		13–16
5	35, 36, 37,* 38, 39, 40	12, 19, 24, 25, 26, 29, 30		
6	41, 42, 43, 44, 45	20, 27, 31, 32, 35, 36		
7	46, 47, 48, 49, 50	28, 33, 34, 37,* 38, 39, 41, 42		
8	51, 52, 53, 54	40, 43, 44, 46, 47		
9		45, 48, 49, 51, 52		
10		50, 53, 54		

* Animal 37 died 1 day after conventionalization.

Radioautography.—30 μc of sterile thymidine-methyl-^{3}H (New England Nuclear Corp., Boston, Mass.) in 0.3 ml of water was administered intraperitoneally to each animal. The injection of the germfree mice was performed in the isolator by admitting the sterile thymidine vials through a transfer lock sterilized with 2% peracetic acid. Animals were removed from the isolators only immediately before sacrifice.

The schedule of the labeled thymidine injections is detailed in Table I. On day 0, for instance, all the germfree mice were injected, as were Nos. 1–6 of the animals taken from the germfree environment. On day 1, 24 hr after conventionalization, mice 7–12 were similarly injected. On day 2, and subsequently, other animals in the conventionalized group were injected, until the eight day. In the same table are listed the times after injection when the appropriate mice were sacrificed and specimens removed for study. Thus, for each day after conventionalization, radioautography was done 24, 48, and 72 hr after the administration of the labeled thymidine. In this manner, cell population kinetics in the distal small intestine of the mouse were studied from 1 to 10 days after removal of the animal from a germfree environment.

Each mouse was sacrificed with ether anesthesia. A 5 cm segment of distal small bowel

was then removed 5 cm from the ileocecal junction; these were first rinsed with Krebs' buffer and then fixed in a solution of 10% neutral formalin. Each gut specimen was divided into three equal parts, embedded in Paraplast, and sectioned at 4 μ at three comparable levels separated by at least 100 μ. The slides were then coated with Kodak NTB-2 nuclear tract emulsion by the dipping method (14, 15) at 80% humidity and 28°C. The slides were stored, with the tissue sections facing up, in black boxes and maintained in a dark room at 4°C. After 33 days of exposure, the slides were developed and then stained with hematoxylin–light green. After the slides were coded, 20–30 ideally longitudinally sectioned villi were selected for quantitation from each animal. At 400× magnification the total number of cells lining one side of the sec-

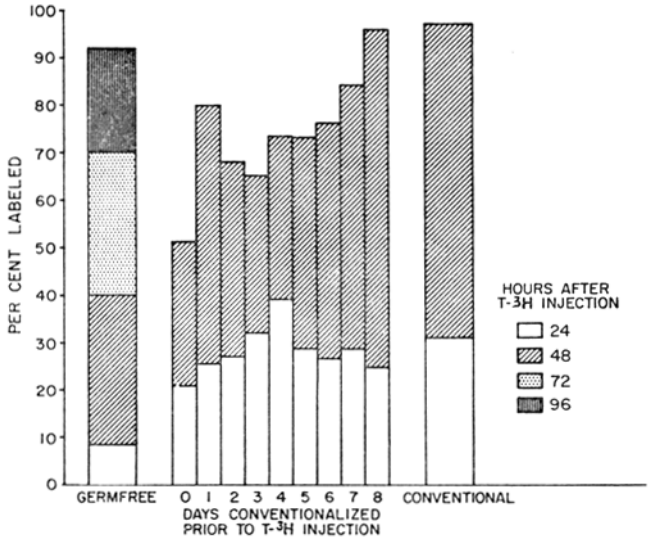

FIG. 1. Percentage of villi labeled (number of labeled cells divided by total number of cells × 100) after thymidine-^{3}H (T-^{3}H) injection in germfree, conventionalized, and conventional mice. Values for conventionalized animals at 72 hr were also determined, and all showed 100% labeling.

tion of each villus was counted from crypt to tip. The number of cells behind the leading edge of the labeled epithelium was also counted. Thus, an average villus height and an average labeled height, in terms of epithelial cells in a single-file column, were determined for each animal. The percentage value of total cells of the villi labeled was then calculated.

Bacteriology.—Alterations in the bacterial flora in the conventionalized mice were followed by qualitative and quantitative cultures of fecal samples on selective culture media. 0.1 g of stool sample was dispersed and diluted in 9.9 ml of Norit A charcoal–water, and serial dilutions were made. A calibrated platinum loop (delivering 0.01 ml) was used to streak a series of eight culture media from the tube dilutions so that a final quantitative count of total aerobes, total anaerobes, coliforms, *Streptococcus* sp., *Bacteroides* sp., *Clostridium* sp., and total microaerophilic and anaerobic lactobacilli was obtained. The details of these methods are described elsewhere (16).

RESULTS

The proportion of labeled cells per villus, 24 hr after administration of ^{3}H-thymidine, was 9% in the germfree animal as contrasted with 31% in the

conventional mouse (Fig. 1). 48, 72, and 96 hr after administration of the ^{3}H-thymidine to the germfree animals, 40, 70, and 92% of the cells, respectively, were labeled (Fig. 2). In the conventional animals, however, 97% of the cells were labeled by 48 hr (Fig. 3). Fig. 1 shows the results of the radioautography studies in the conventionalized animals, up to the 8th day after removal from the germfree environment. This graph reveals that there was a progressive increase in the percentage of cells labeled, beginning on the first day of conventionalization. By the 4th day, the percentage of cells labeled 24 hr after administration of the ^{3}H-thymidine was similar to that of the conventional animal. However, the extent of labeling of cells 48 hr after administration of

TABLE II

*Results of Quantitative and Qualitative Stool Cultures in Germfree, Conventionalized, and Conventional Mice**

Organisms	Days after conventionalization							Conventional mice
	1	2	3	4	5	6	7	
Total aerobes	1.9×10^{11}	1.0×10^{9}	3.0×10^{7}	1.1×10^{8}	3.2×10^{7}	4.9×10^{7}	3.7×10^{8}	8.0×10^{7}
Total anaerobes	2.0×10^{11}	1.0×10^{9}	2.2×10^{8}	1.0×10^{9}	1.4×10^{9}	1.9×10^{8}	1.0×10^{9}	1.2×10^{9}
Coliforms	3.3×10^{11}	1.0×10^{8}	5.4×10^{9}	8.9×10^{7}	4.1×10^{7}	7.7×10^{6}	7.0×10^{6}	1.0×10^{6}
Streptococci	6.0×10^{9}	1.0×10^{8}	4.1×10^{7}	2.0×10^{8}	4.0×10^{6}	1.3×10^{7}	$<1.0 \times 10^{4}$	$<1.0 \times 10^{4}$
Lactobacilli	6.6×10^{9}	1.8×10^{10}	5.0×10^{7}	1.1×10^{8}	5.3×10^{8}	3.3×10^{7}	4.0×10^{8}	3.5×10^{8}
Bacteroides	$<1.0 \times 10^{4}$	$<1.0 \times 10^{4}$	$<1.0 \times 10^{4}$	$<1.0 \times 10^{4}$	6.0×10^{6}	1.0×10^{6}	2.0×10^{5}	1.3×10^{8}

* There was no microbial growth in the stools of the germfree animals. 8 hr after conventionalization only flavobacteria and yeast (7.8×10^{5} cells) were cultured from the animals' stools. Selective culture media were also employed for staphylococci, *Veillonella*, and diphtheroids, but none of these organisms were recovered in any of the stools cultured.

^{3}H-thymidine reached 90% of the value in the conventional animal on the eighth day of conventionalization (Fig. 4).

Bacteriological studies of the germfree housing and fecal matter revealed no bacterial growth in the culture media employed. Flavobacteria and yeast were the only organisms recovered in the stools of the animals cultured 8 hr after conventionalization. After 24 hr, significant numbers of aerobic and anaerobic organisms were cultured from the stools of previously germfree mice (Table II). After conventionalization, predominantly coliform organisms were recovered, but by the eighth day of conventionalization the coliform bacteria decreased to approximate the numbers recovered from the stools of our normal mice. Streptococci were found in large numbers only in the first 4 days, while the lactobacilli established themselves by the 1st day of conventionalization but persisted at levels comparable to those obtained in the conventional animals. In contrast, the organisms of the *Bacteroides* group were first recovered in significant numbers on the 5th day; however, by the 8th day they still remained quantitatively below the levels found in the stools of normal mice (Table II).

DISCUSSION

When animals reared under standard germfree conditions were placed in a conventional environment, proliferation of intestinal bacteria occurred within a day. A stable bacterial pattern was cultured in the stool by the fourth day of conventionalization, and by the eighth day it remained qualitatively and quantitatively similar to that of the conventional mice, except that the *Bacteroides* organisms had not attained the number ordinarily recovered in the normal mice. Temporally related to the changes in intestinal bacteria was the observation that the epithelial cell migration rate doubled over the 8 day period from the beginning of conventionalization. Thus, as far as dynamic small intestinal morphology is concerned, the conventionalized animals used in germfree research must be considered to be in a state of transition for the first 8 days. At this time epithelial cell migration and intestinal microflora become similar to those of animals reared under standard conditions. Since the maturity of epithelial cells and their enzyme systems relate to the rate of migration in the villi (17), these changes may be significant in terms of intestinal digestive and absorptive mechanisms.

Various factors (18–21) and substances (22–24) may affect the mitotic and the migration rates of intestinal epithelial cells. In older animals, production of cells in the crypts is decreased and the generation time is increased, while the migration rate on the villus is decreased (19). In the short period of time involved in this study, age probably did not play a role in affecting intestinal cell kinetics.

Germfree animals have a decreased intestinal epithelial cell migration rate, as compared to their conventional counterparts. An experimentally induced enteric infection with *Salmonella typhimurium* caused an increase in ileal cell renewal rate and a decrease in the transit time of the epithelial cells from the crypt to the tip of the villus (18). Theoretically, elimination of intestinal bacteria with antibiotics could change small intestinal cell kinetics in the conventional animal toward that in the germfree; however, when neomycin is one of the antibiotics employed, direct effects on the small bowel occur, decreasing the rate of epithelial cell migration (22).

A number of studies have been concerned with the changes which may be induced by the microflora of the gut (8, 12, 25, 26). The present study demonstrates alterations in intestinal cell mitotic and migration rates when bacteria are introduced into animals in a germfree environment. Intestinal bacteria or, alternatively, products of the bacteria may directly affect small intestinal epithelial cell age. But it is probably the interaction of the bacteria with the gut which causes these changes in the conventional animal, for germfree animals receive food which is autoclaved and contains inert bacteria and bacterial products.

The role of the normal intestinal bacteria in affecting intestinal epithelial cell turnover and inflammatory responses in the lamina propria and, conversely, the

effect of the secretions of the gut on the intestinal microflora need further elucidation. The lamina propria of the germfree animal has few inflammatory cells, and their circulating immunoglobulin levels are lower than in normal animals. The introduction of bacteria by placing these animals in a conventional environment increases the number of immunologically competent cells and antibody levels in blood, probably as a result of antigenic stimuli from bacteria or bacterial products. The histology of the normal small intestine thus represents a response to a "chronic infection," in contrast to the germfree state. Further alterations in epithelial cell kinetics and increases in inflammatory cells occur in conventionalized animals after enteric infections. This situation may also pertain in the human in conditions said to be associated with greater numbers of bacteria in the proximal small bowel and may account for the differences in small intestinal morphology encountered in various areas of the world (27–30).

SUMMARY

The relationship between intestinal colonization and the small bowel mucosal cellular proliferation rate during conventionalization of the germfree mouse was examined. 16 mice were maintained under standard germfree conditions, and 54 others were conventionalized. Migration of the small bowel epithelial cells was followed by radioautography with administration of tritiated thymidine. Colonization was followed by qualitative and quantitative bacteriological fecal analyses. The percentages of the villi labeled (as determined by cell count) 24, 48, and 72 hr following thymidine administration showed immediate progression in the conventionalized animals from the germfree villus migration time (4 days) toward the conventional villus migration time (2 days). The epithelial migration rate of animals conventionalized for 8 days was comparable to that of conventional animals.

After conventionalization, aerobic and anaerobic organisms undergo a period of extensive multiplication; however, 72 hr later the number of these microorganisms cultured in the stool decrease and are similar to those recovered from normal animals. Coliforms and streptococci are recovered in large numbers only in the first days after conventionalization, while the *Bacteroides* are first recovered in significant numbers on the fifth day of conventionalization. Except for smaller numbers of *Bacteroides*, the bacterial populations in the stools of the conventionalized animals are qualitatively and quantitatively similar by the eighth day of conventionalization to those of true conventional mice.

Adaptive balance between cell proliferation and sloughing, and thus migration rate, begins immediately after conventionalization of germfree animals as bacterial populations establish themselves throughout the gastrointestinal tract, and results in a doubling of the mucosal cell turnover after 8 days. At this time both the small intestinal epithelial cell migration rate and the intestinal microflora are similar to those of conventional animals.

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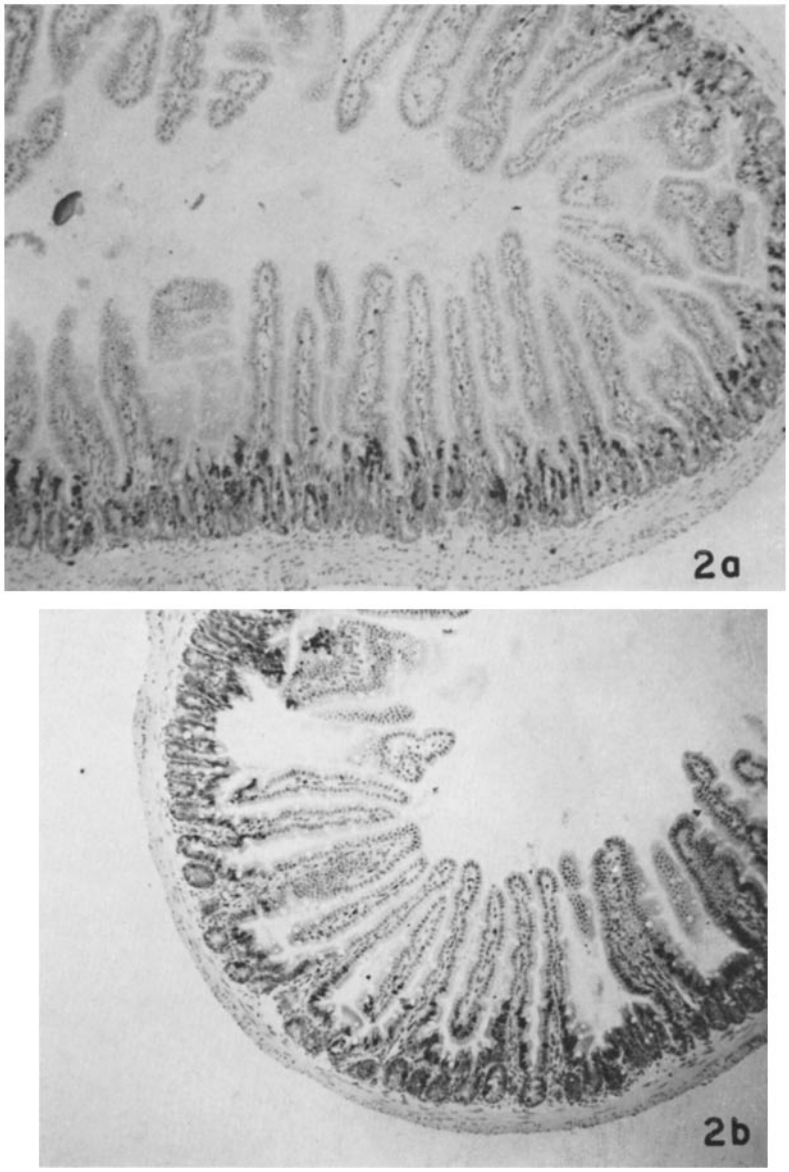
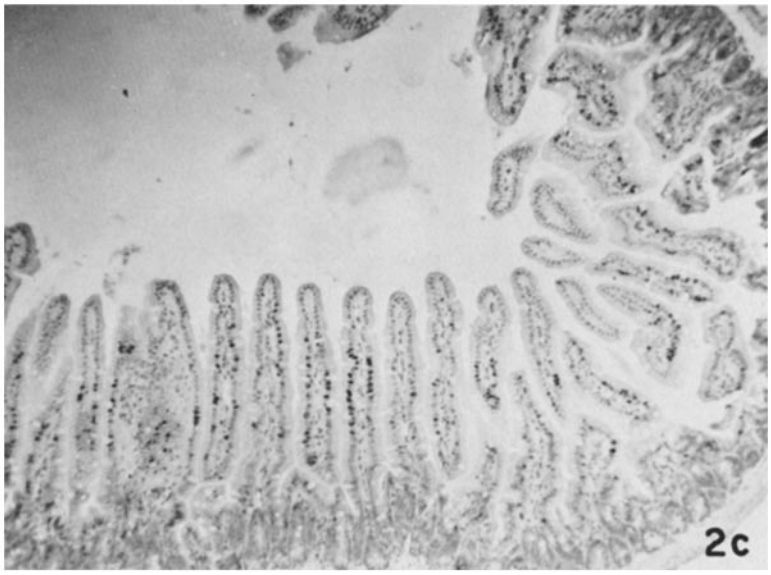


FIG. 2. Radioautography in germfree mice after thymidine-^{3}H injection. a, 24 hr after injection; b, 48 hr after injection; c, 72 hr after injection; d, 96 hr after injection. The labeled column approaches total villus height after 4 days. Hematoxylin–light green, × 100.



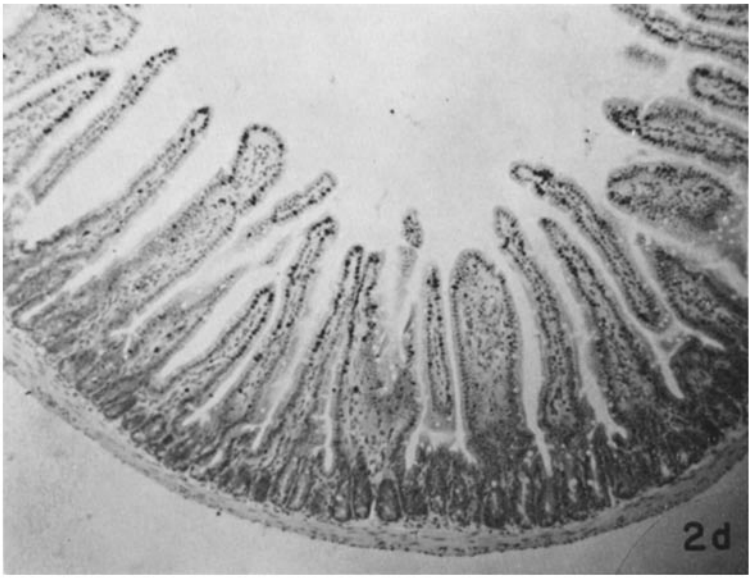


FIG. 2. See page 667 for legend.

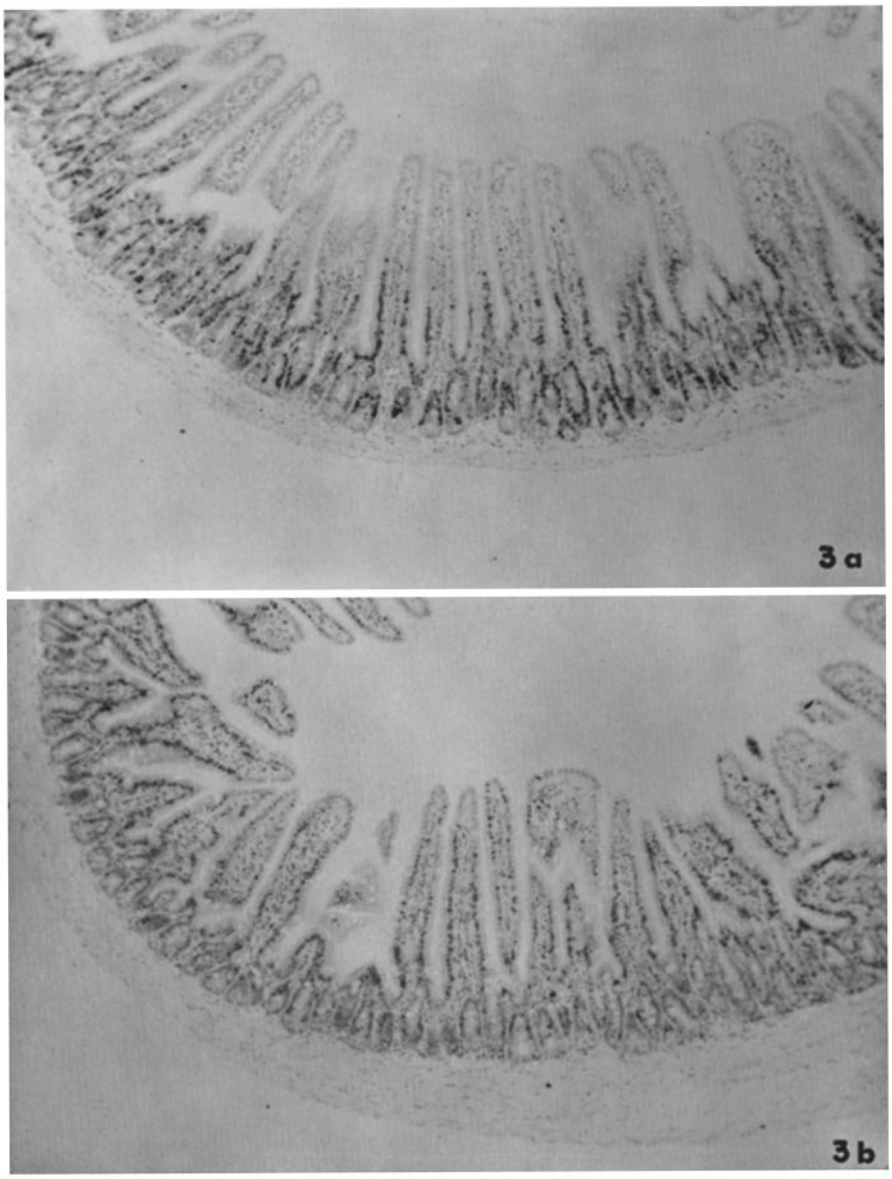


FIG. 3. Radioautography in conventional mice after thymidine-^{3}H injection. a, 24 hr after injection; b, 48 hr after injection. The labeled column approaches total villus height after 2 days. Hematoxylin–light green, × 100.

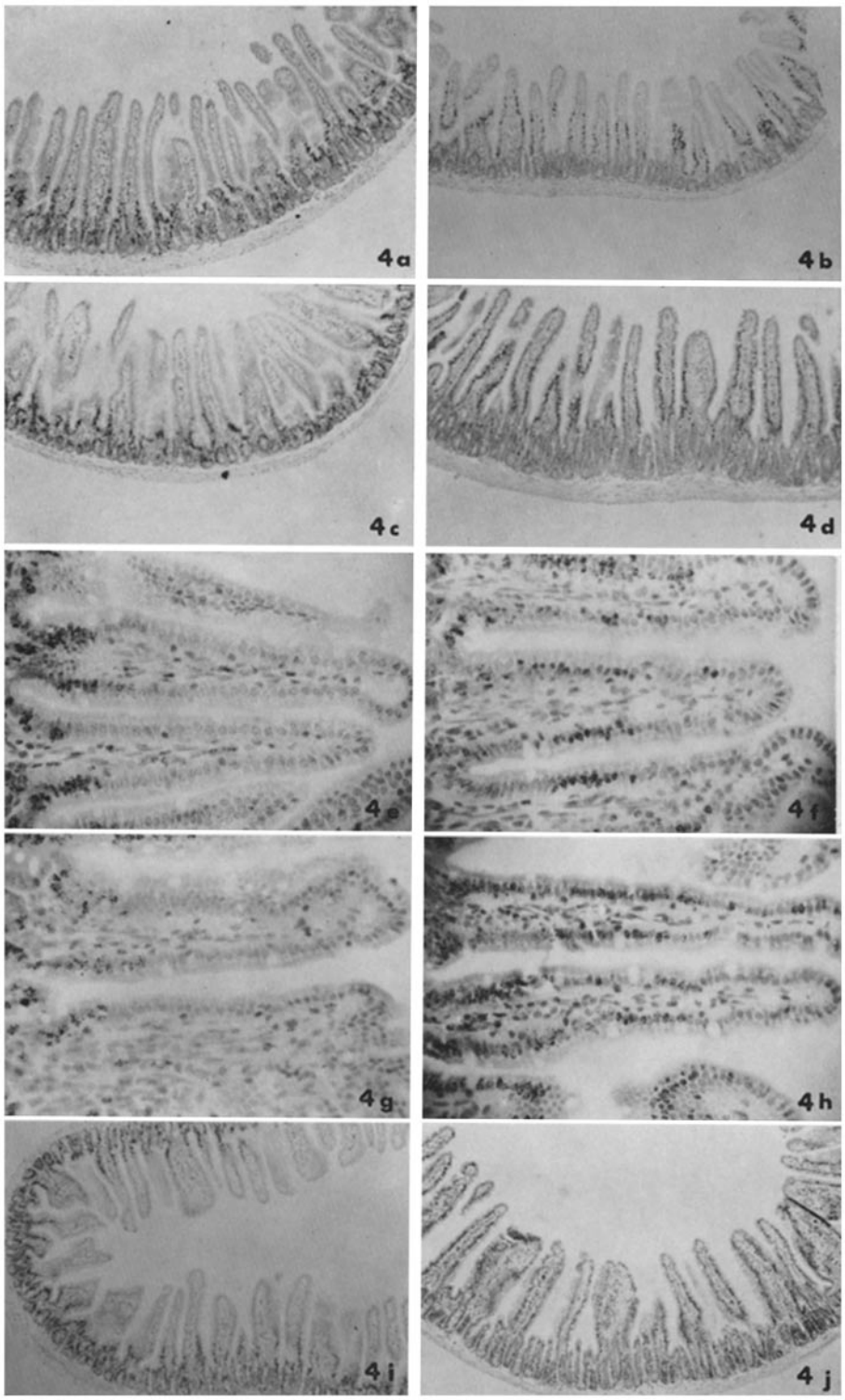


FIG. 4. Radioautography in conventionalized mice after thymidine-^{3}H injection. a and b, conventionalized 0 days prior to injection (× 78); c and d, conventionalized 2 days prior to injection (× 78); e and f, conventionalized 4 days prior to injection (× 310); g and h, conventionalized 6 days prior to injection (× 310); i and j, conventionalized 8 days prior to injection (× 78). a, c, e, g and i, 24 hr after injection; b, d, f, h, and j, 48 hr after injection. Hematoxylin–light green.