EFFECTS OF NEOMYCIN AND PENICILLIN ADMINISTRATION ON MUCOSAL PROLIFERATION OF THE MOUSE SMALL INTESTINE

WITH MORPHOLOGICAL AND FUNCTIONAL CORRELATIONS*,‡

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The administration of the antibiotic neomycin has been associated with a reversible intestinal malabsorption syndrome (1–5); shorter, broader villi and decreased levels of intestinal enzymes have been reported both in humans (6–11) and in experimental animals (12–14). The severity of the intestinal morphologic and physiologic alterations appears to increase with dose and time (15–17).

Neomycin also reduces the quantity of intestinal bacteria, the intestinal environment becoming analogous to that in the germfree animal. The morphology of the gut of the germfree animal also differs from that of the conventional animal (18–21), but with longer, thinner villi and enhanced intestinal absorption of monosaccharides and amino acids (22, 23). The absence of bacterial flora may account for the differences in gastrointestinal structure and function of the germfree animal from that of its conventional counterpart. Neomycin, on the other hand, may directly affect the epithelial cells of the small bowel, counteracting any villus alteration or enhanced absorptive capacity secondary to microflora reduction.

The present study was designed to assess the effect of an antibiotic regimen on fecal microflora and small intestinal function and morphology. Mice were fed neomycin and penicillin in order to evaluate small intestinal cell population kinetics, intestinal transport of an amino acid, and intestinal enzymes by disaccharidase assay of the brush border and histochemical staining reactions of epithelial cell enzyme systems.

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Materials and Methods

Intestinal Transport.—16 male and 16 female CFW mice (Carworth Farms, New City, N. Y.), 70 days old and with an average weight of 27 g, were randomly divided into two groups of 8 males and 8 females each. All mice were fed a standard animal diet[1] which does not contain antibiotics; food and water were allowed ad lib. Mice were housed on San-i-cel bedding in plastic cages in a conventional animal room. One group received an oral antibiotic regimen daily while the second served as a control. Potassium penicillin (E. R. Squibb and Sons, New York) and neomycin sulfate (mycifradin sulfate)[2] were dissolved in distilled water, 2.4 g (4 million units) and 10 g respectively per liter. Fresh antibiotic solution was prepared twice a week and kept at 4°C until it was administered. Animals were fed the antibiotic solution as drinking water. Consuming, on the average, 5 ml of water per day, each mouse received 50 mg of neomycin and 12.0 mg (20,000 units) of penicillin per day. The control mice were on tap water. Weight of a constant sampling of both the control and the antibiotic-treated mice was recorded twice weekly.

Three control and three experimental mice were sacrificed by a snap of the neck 8, 16, 22, 29, and 36 days after the initiation of the antibiotic regimen. Intestinal transport of an amino acid was evaluated by the everted gut sac technique (24). Immediately after sacrifice, the small intestine was isolated, stripped of its mesentery, and rinsed with Krebs-bicarbonate buffer. The intestine was everted and then divided into three approximately equal segments. Crystals of L-methionine-methyl-^{14}C, in a concentration of 1×10^{-3} M, were dissolved in buffer and 1.0 ml was introduced into each everted sac. Each sac was subsequently placed in an Erlenmeyer flask containing 5.0 ml of buffer with an identical concentration of the labeled methionine as that instilled on the mucosal side. The flasks were gassed with a mixture of 95% oxygen–5% carbon dioxide and incubated in a Dubnoff shaker at 37°C for 1 hr. At the end of this period, each sac was drained, the volume was recorded and each segment was then weighed. Aliquots of the mucosal and serosal fluids were analyzed for radioactivity in a Packard Tri-Carb liquid scintillation counter. Results were calculated and expressed as micromoles of methionine transported per gram wet tissue weight of intestine per hour.

Radioautography.—Small intestinal cell proliferation kinetics were studied in 45 antibiotic-treated mice and 16 control mice of the same strain (CFW mice, Carworth Farms). 30 μc of sterile thymidine-methyl-^{3}H (New England Nuclear Corp., Boston, Mass.), 6.7 c/mM in 0.3 ml of sterile water, were injected intraperitoneally. Groups of nine antibiotic-treated animals were injected at weekly intervals up to 5 wk after initiation of treatment. Eight control animals were injected after 1 wk and the remaining eight on the 5th wk of the experiment, at the same times as the treated mice.

Segments of the small bowel were taken 5 cm proximal to the ileocecal junction as the mice were sacrificed under ether anesthesia according to the schedule outlined in Table I.

The intestinal specimens were rinsed with Krebs buffer and fixed in 10% neutral formalin. The specimen was divided into three segments, embedded in Paraplast, and cross-sectioned at 4 μ at three comparable levels separated by at least 100 μ. The slides were then coated with Kodak NTB-2 nuclear track emulsion by the dipping method (25, 26) at 80% humidity and 28°C. Slides were stored with the tissue section up in black boxes and maintained in a dark room at 4°C. After 33 days of exposure, the slides were developed and stained with hematoxylin-light green. After the slides were coded, 20–30 "ideally" (midlongitudinally) sectioned villi were selected for quantitation from each animal. At a magnification of 400 the total

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[1] Purina Lab Chow, Special Formula 5010C, Purina Labs., St. Louis, Mo.

[2] Mycifradin sulfate, 70% as neomycin base, Upjohn, Kalamazoo, Mich. 11.35 mM (700 mg/100 ml) neomycin used.

number of cells lining one side of the section 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 per cent value of total cells of the villi labeled was then calculated.

Bacteriology.—Qualitative and quantitative bacteriologic cultures of the stools of three randomly selected control mice and of three randomly selected experimental mice, prior to initiation of treatment, were performed on selective media. 0.1 g of the stool sample was dispersed and diluted in 9.9 ml of norite 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 quantiative count of total aerobes, total anaerobes, coliforms, *Streptococci* sp., *Bacteroides* sp., *Clostridia* sp. and total microaerophilic and anaerobic lactobacilli was obtained. The details of these methods are described elsewhere (27). Thereafter, fecal cultures were performed prior to the time of sacrifice on all the antibiotic-treated

TABLE I

Schedule of Sacrifices after Thymidine-^3H Injection

Time after injection of thymidine-^3H	No. of animals studied	
	Control	Antibiotic
hr		
24	4	2
48	4	3
72		2
96		2

animals used in the transport studies. The first transport control group, sacrificed on the 8th day of the experiment, was similarly cultured. Ceca of the control and of the antibiotic-treated mice were compared for size at time of sacrifice.

Histochemistry.—Enzymes of the small intestine brush border and epithelial cells were evaluated by histochemical staining techniques (28). 8 control and 45 mice treated with antibiotics for 1–5 wk were anesthetized with ether, after which two segments of the proximal bowel (about 5 cm from the pylorus) and two segments of the distal bowel (about 4 cm from the ileocecal junction) were removed and rinsed with Krebs buffer. A portion of the left lobe of the liver was also removed. Sections of gut from each animal were placed on the portion of that animal's liver, which served not only as support of the intestinal sample but also as control for the enzymes studied. The specimens were immediately immersed in liquid nitrogen, and stored at −70°C in an airtight polyethylene bag.

Histochemical reactions were carried out on cryostat sections cut 4 μ thick at −20°C and picked up on a cover slip. The following enzymes were studied: acid and alkaline phosphatase, nonspecific esterase, NADH[3] dehydrogenase, NADPH dehydrogenase, lactic dehydrogenase, and succinic dehydrogenase. The histochemical reactions were performed simultaneously on both control and experimental animals and then compared for the enzymes studied. The slides were coded and the intensities of the stain were evaluated on a 0 to 5 scale by two investigators and one technician.

[3] NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

Disaccharidase Assay.—At the time tissue was obtained for the histochemical studies, adjacent portions of the proximal gut were also obtained for assay of disaccharidases. The small intestine was cut open and the mucosa scraped off with a glass slide onto a piece of previously weighed cork. The specimen was weighed and then homogenized with four times

TABLE II

Transport of L-Methionine by the Proximal, Middle, and Distal Intestine in Antibiotic-Treated and Control Mice

Level of small intestine	Control	Antibiotic	*P**
Proximal	0.8 ± 0.5‡	0.7 ± 0.7	<0.4
Middle	1.5 ± 0.6	1.4 ± 0.9	<0.4
Distal	2.7 ± 0.7	1.9 ± 1.2	<0.05

* By Student's *t*-distribution.

‡ Micromoles of methionine transported per gm gram of tissue weight per hour ± SD.

TABLE III

Radioautography in Control and Antibiotic Mice 24 hr after Injection of Labeled Thymidine

Sex	Measurement	Control	Antibiotic 1–5 wk	*P*	Antibiotic 3–5 wk	*P*
Both	No. of labeled cells/villus	23.8 ± 4.3	30.3 ± 9.7	<0.05	35.0 ± 7.0	<0.0025
	Total cells/villus	57.6 ± 5.5	63.5 ± 3.6	<0.01	63.8 ± 3.4	<0.025
	Per cent*	41.5 ± 6.9	48.2 ± 16.8	<0.2	55.4 ± 13.9	<0.025
♂	No. of labeled cells/villus	24.2 ± 6.0	28.9 ± 14.1	<0.30	37.9 ± 9.8	<0.05
	Total cells/villus	60.5 ± 2.4	63.8 ± 3.9	<0.10	63.8 ± 4.1	<0.15
	Per cent	39.7 ± 8.4	45.8 ± 24.4	<0.35	60.4 ± 19.8	<0.10
♀	No. of labeled cells/villus	23.4 ± 2.8	31.9 ± 1.9	<0.0005	32.1 ± 1.8	<0.005
	Total cells/villus	54.8 ± 6.5	63.3 ± 3.7	<0.025	63.8 ± 3.6	<0.05
	Per cent	43.2 ± 5.7	50.5 ± 5.4	<0.05	50.4 ± 4.4	<0.10

* No. of labeled cells per villus divided by total No. of cells per villus × 100.

its weight of distilled water. Assays were performed for lactase and sucrase content by the Dahlqvist method (29). A group of eight germfree mice (Carworth Farms) of the same strain was similarly studied. Results were recorded as micromoles disaccharide hydrolyzed per minute per gram of wet tissue weight.

RESULTS

Transport.—Total transport of L-methionine in the antibiotic-treated animals (1.6 ± 1.2)[4] did not differ significantly when compared to that of the

[4] ±, standard deviation. Values expressed as micromoles of methionine transported per gram of wet tissue weight per hour.

control mice (1.8 ± 1.0) ($P < 0.25$). When transport in all the three segments of intestine was compared for each week studied, the only significant difference found was after the 4th wk of therapy. At this time, the transport in the experimental group was reduced (0.7 ± 0.5) compared to the control (1.7 ± 0.6) ($P < 0.005$). Methionine transport was also calculated for each third of gut studied (Table II). Only the distal third of the gut of the experimental group transported less amino acid than that of the control group.

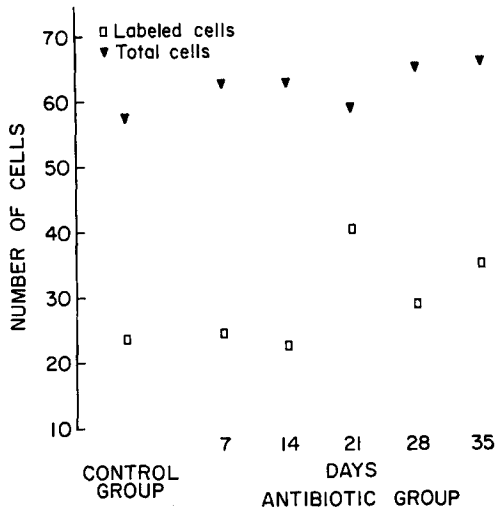

FIG. 1. Total and labeled cells of villi in all control and antibiotic-treated mice 24 hr after thymidine-^{3}H injection.

Radioautography.—After the 1st and 2nd wk of antibiotic therapy, there were no significant differences in intestinal cell proliferation between the experimental and the control groups. Cell counts done 24 hr after the injection of labeled thymidine in animals on the antibiotic regimen for 3, 4, and 5 wk revealed a significant increase in the height of labeled cells (Table III); that is, more epithelial cells were labeled in the villi of the antibiotic-treated group (35.0 ± 7.0 to 23.8 ± 4.3 in controls, Fig. 1). When the results were separated by the animals' sex, it was observed that the treated males had a greater number of cells labeled than the treated females (37.9 ± 9.8 to 32.1 ± 1.8), and likewise each group had more than their corresponding controls (24.2 ± 6.0 and 23.4 ± 2.8 respectively). There was a greater variance, however, in the number of labeled cells in the males (Fig. 2). A smaller increase in the total number of epithelial cells in the villi of the antibiotic-treated animals was present compared to the controls (63.8 ± 3.4 to 57.6 ± 5.5). Therefore, the per cent of labeled cells (number over the total cells) was greater for the antibiotic group 3–5 wk after therapy than for the controls (55.4 ± 13.9 to 41.5 ± 6.9) (Figs. 3 and 5).

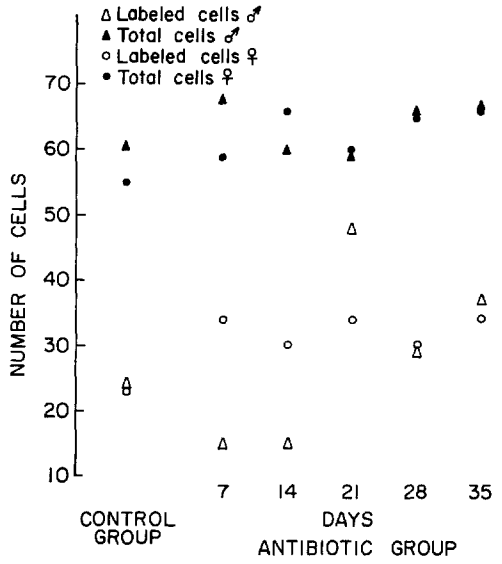

FIG. 2. Total and labeled cells of villi in male and female control and antibiotic-treated mice 24 hr after thymidine-^{3}H injection.

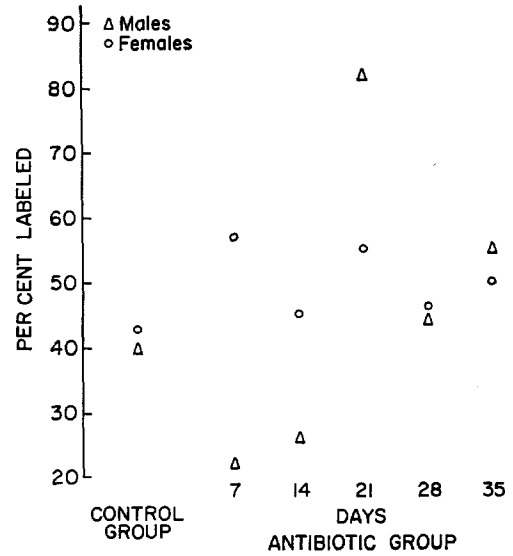

FIG. 3. Per cent of villus labeled (number of labeled cells per villus divided by the total number of cells per villus × 100) in male and female control and antibiotic-treated mice 24 hr after thymidine-^{3}H injection.

Only the sections taken at 24 hr after injection of the labeled thymidine were counted for comparative purposes since, in those taken 48 hr and thereafter in both groups, all the epithelial cells in the villi were labeled. The total number of epithelial cells per villus, however, was again significantly greater in the experimental group.

Bacteriological Flora.—The quantitative and qualitative bacterial recoveries were similar in the two groups at the start of the experiment. 8 days after antibiotic therapy and thereafter, only yeast organisms were recovered from the stools with the culture media employed (Table IV).

Enlarged ceca developed by the 1st wk of antibacterial treatment and the

TABLE IV

*Results of Quantitative and Qualitative Stool Cultures in Control and Antibiotic Mice**

Days on antibiotics	0	0	8	8	16	22	29	36
State of animal	Control	Experimental	Control	Experimental	Experimental	Experimental	Experimental	Experimental
Total aerobes	1.1×10^8	7.9×10^6	5.0×10^7	—	Yeast	Yeast	—	Yeast
Total anaerobes	2.0×10^9	2.7×10^8	3.7×10^8	—	—	Yeast	—	—
Coliforms	2.0×10^6	8.6×10^5	3.0×10^4					
Streptococci	1.0×10^8	4.0×10^7						
Lactobacilli	4.0×10^8	5.0×10^8	3.0×10^8					
Bacteroides	5.2×10^5	3.0×10^5	2.6×10^8					
Yeast					2.0×10^7	5.0×10^5	1.4×10^5	4.0×10^5

* Selective culture media were also employed for Staphylococci, Veillonella, Clostridia, and Diphtheroids, but none were recovered in any of the stools cultured.

ceca remained enlarged for the remaining 4 wk (Fig. 4). Cecal contents were fluid. Watery stools, similar to those seen in the germfree state, also developed by 1 wk of treatment and persisted throughout the experiment.

Histochemistry.—Histochemical assay of nonspecific esterase and NADH dehydrogenase levels in the proximal gut showed decrease in staining reaction in the antibiotic-treated mice when compared to control mice (Fig. 6). These differences were not apparent in the specimens of the distal gut. Stains for acid and alkaline phosphatase, NADPH dehydrogenase, lactic dehydrogenase, and succinic dehydrogenase in both proximal and distal gut were similar in both groups of animals.

Lactase and Sucrase Activity. The activity of sucrase of the antibiotic-treated mice (11.4 ± 1.3)[5] was increased as compared to that of the controls (9.7 ± 2.8) ($P < 0.1$). Lactase activity (1.2 ± 0.4), on the other hand, was not significantly decreased when compared to the controls (1.5 ± 4) ($P < 0.25$). A

[5] Values expressed as micromoles of disaccharide hydrolyzed per minute per gram.

reduction of the lactase levels was more apparent after 16 days of treatment (0.8 ± 0.3) ($P < 0.1$).

In the axenic mice, however, significantly increased levels of sucrase (13.1 ± 2.6) were present compared to their controls (8.0 ± 2.3) ($P < 0.05$), while lactase activity was only slightly higher (1.6 ± 0.4 compared to 0.9 ± 0.7 in the controls) ($P < 0.1$).

Weight.—At the onset of the experiment, the control mice weighed 27.5 ± 2.9 g which increased to 30.5 ± 2.0 g over the 35 day period. Antibiotic-treated mice went from a weight of 27.0 ± 4.8 g to 31.5 ± 4.2 g over the same period. The experimental animals thus gained weight comparably to the control group.

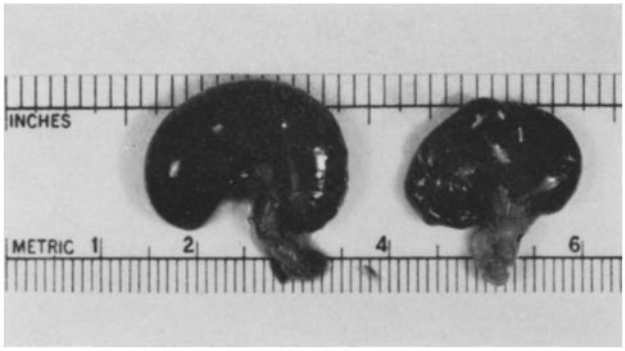

FIG. 4. The 2 g cecum on the left comprised 8% of the body weight of a mouse on antibiotics for 38 days. The ceca of antibiotic-treated mice consistently appeared two to three times larger than the conventional mice ceca which, like the one on the right, comprised 1–2% of the body weight.

DISCUSSION

In the present study, the oral administration of neomycin and penicillin was associated with a reduction in the intestinal bacterial flora. Neomycin, however, may have direct adverse effects on the gut, accounting for the observed alterations in the small intestine.

Neomycin may cause a malabsorption syndrome (1–5). In humans, as with the animals, this reversible effect increases with time and dose (15–17). Light and electron microscopic changes in the crypt cells, blunting of villi, and inflammatory cell infiltration of the lamina propria have been reported (11, 30). Loss of microvilli may also be present (30). In humans, neomycin induced steatorrhea is more severe when the drug is given orally or when instilled directly into the jejunum than when instilled directly into the lower bowel (5). The magnitude of the observed azotorrhea, however, is the same whether the drug is given orally or instilled directly into jejunum or ileum (5). In addition to its adverse effects on the small intestine, neomycin has been shown to inhibit lipolysis in vitro (31)

and to bind bile salts by forming a precipitate at both the bile salt concentrations and the pH usually found in the duodenum (3, 4, 9). The administration of bile salts and pancreatic enzymes concurrent with the antibiotic does not consistently ameliorate the steatorrhea of neomycin malabsorption (5). Thus the steatorrhea of neomycin therapy in humans appears to be a combination of maldigestion and malabsorption.

In rats treated with large doses of neomycin, an apparent increase in the uptake of L-phenylalanine by ileal slices may have been related to the associated starvation described in these animals (12, 32). In another study, this antibiotic depressed the absorption of glucose in monkeys with Thiry-Vella loops of mid small intestine (33). This time and dose-dependent effect of neomycin on absorption occurred in the absence of morphologic alterations (33). High doses of neomycin in rats have also caused an impairment in the absorption of carbohydrates, also without changes in the intestine (13, 16). The only change in transport of methionine occurred in the animals treated for 4 wk; in all the treated animals, only the distal segments of gut showed this decrease. On the other hand, in some species (34) diets containing other antibiotics such as penicillin or tetracycline have been followed by an enhanced absorption of the nutrient tested (34–36). Excess weight gain has been described in some of these animals (37). This improved growth may be related to alterations in the intestinal bacterial flora, concomitant with an enhanced intestinal absorption of nutrients. In our study, however, no significant differences in weight were noted.

The intestinal absorption of monosaccharides and of amino acids is greater in germfree animals than in their conventional counterparts (20, 22, 23). Monocontamination of germfree animals decreases this absorptive capacity (23). In addition, conventionalizing germfree animals induces changes in small intestinal morphology, altering the height and width of villi and increasing inflammatory cells in the lamina propria (20, 21, 23). The antibiotic regimen used in the present study removed recoverable organisms from the gut, except for yeast. The gross morphologic findings of thin intestinal walls, enlarged ceca, and watery stools in the treated mice are also encountered in animals reared in germfree environments (18, 21, 38–40). The administration of various antibiotics to adult mice (41) has also induced enlarged ceca with fluid contents within a day of antibacterial therapy (38). Alterations in the mechanisms of water transport may result from the changes in the intestinal microflora, as the aforementioned characteristics occur in both germfree and antibiotic-treated animals. Differences from the usual microbial flora occurred with each antibiotic employed, but after two days a secondary microflora developed in all the treated animals (38). The absence of intestinal bacteria in our mice is related to the administration of the two antibiotics.

In some studies, decreased levels of various intestinal enzymes following neomycin administration have been reported (17); in other studies no such alter-

ations have been noted (33, 42). In man, histochemical studies have demonstrated decreased staining reactions of succinic dehydrogenase and ATPase (17). Histochemical evaluation of six enzymes were not altered, however, in monkeys with Thiry-Vella fistula (33). Nonspecific esterase and NADH dehydrogenase-staining reactions were decreased in our treated mice, compatible with direct injury of the intestinal epithelial cells. This finding is also compatible with the observed increase in the rate of cellular proliferation, for less mature intestinal epithelial cells may have less well-developed enzyme systems (43). More mature epithelial cells on villi where lower rates of cell migration are present, as in germfree animals, may have well-developed enzyme systems, as suggested by stronger histochemical-staining reactions (43).

Disaccharidase activity in the brush border of the small intestine is decreased both in association with neomycin therapy (8, 10) and in intestinal malabsorptive syndromes (44, 45). In humans, disaccharidases were reduced after 3 days of treatment with a daily dose of 8 g of neomycin (30). In the present study, lactase activity in the antibiotic-treated animals was diminished, a finding compatible with the observed high cellular proliferation rate. The diminution of lactase, an enzyme which is sensitive to intestinal injury, could also reflect a direct toxic effect of neomycin on the gut. Sucrase levels, however, tended to be higher in the treated than in the controls. These changes are not reflections of adaptations to the diet since both groups received the same diet.

In one study, neomycin was reported to cause an increase in mitotic count, explained as mitotic arrest of undifferentiated crypt cells (11). The present study suggests that this antibiotic increases cell proliferation and migration rates. Despite the absence of intestinal microorganisms, except for yeast, the mitotic and migration rates did not resemble those reported for germfree animals, but rather approximated the changes observed after enteric infections (46). It is suggested that neomycin induces these changes in dynamic intestinal morphology independent of the reduction of microflora. The effect may be mediated by a hormonal factor. This seems to be the case in massive small bowel resection on the basis of parabiotic observations (47). Hormonal effects may also explain the differences in cellular kinetics observed between the males and females.

The cell proliferation rate of the mucosa of the small bowel and the migration rate of the epithelial cells of the villi may be viewed as a spectrum. On the one end is the low rate of the germfree state, producing very mature cells on the villi, which appear to contain greater enzymatic activity (42), and whose intestine has an enhanced transport capacity (23). At the other end is the inflamed gut with a high cellular proliferation rate (46, 48, 49) and young cells with less enzymatic activity. In these animals, absorptive capacity may be reduced. The conventional animal may thus represent the middle portion of this spectrum. Grossly, the gut of antibiotic-treated animals may resemble that

of germfree animals; however, the direct effects of neomycin on the intestine may cancel whatever effect the reduction of the intestinal microflora may have on morphology and function of the small intestine.

SUMMARY

The effects of an oral neomycin and penicillin regimen on intestinal bacteriology and on morphology and function of the small intestine of mice were investigated. Quantitative and qualitative stool cultures on selective media of the treated animals revealed only growth of yeast organisms. The treated animals developed enlargement of the ceca with fluid contents and watery stools, resembling characteristics of germfree animals.

Radioautography with tritiated thymidine revealed an increased epithelial cell migration rate in the mice treated with the antibiotics for 3 to 5 wk. A slight increase in villus height was also noted. The treated male mice showed greater variance than the treated females in epithelial cell migration rates.

Histochemical staining reactions showed a decrease in nonspecific esterase and in NADH dehydrogenase activity in the proximal gut of the antibiotic animals. Stains of distal gut and those for acid and alkaline phosphatase, NADPH dehydrogenase, lactic dehydrogenase, and succinic dehydrogenase were similar to the controls. A slight increase in sucrase activity and a slight decrease in lactase activity in the antibiotic animals was observed in contrast to control animals. Germfree mice, however, had greater sucrase and lactase activity. Transport of L-methionine was slightly reduced in the distal segment of the treated animals.

Since the direction of these changes is away from the intestinal state observed in germfree animals, they are probably the result of the direct action of the antibiotics on the gut.

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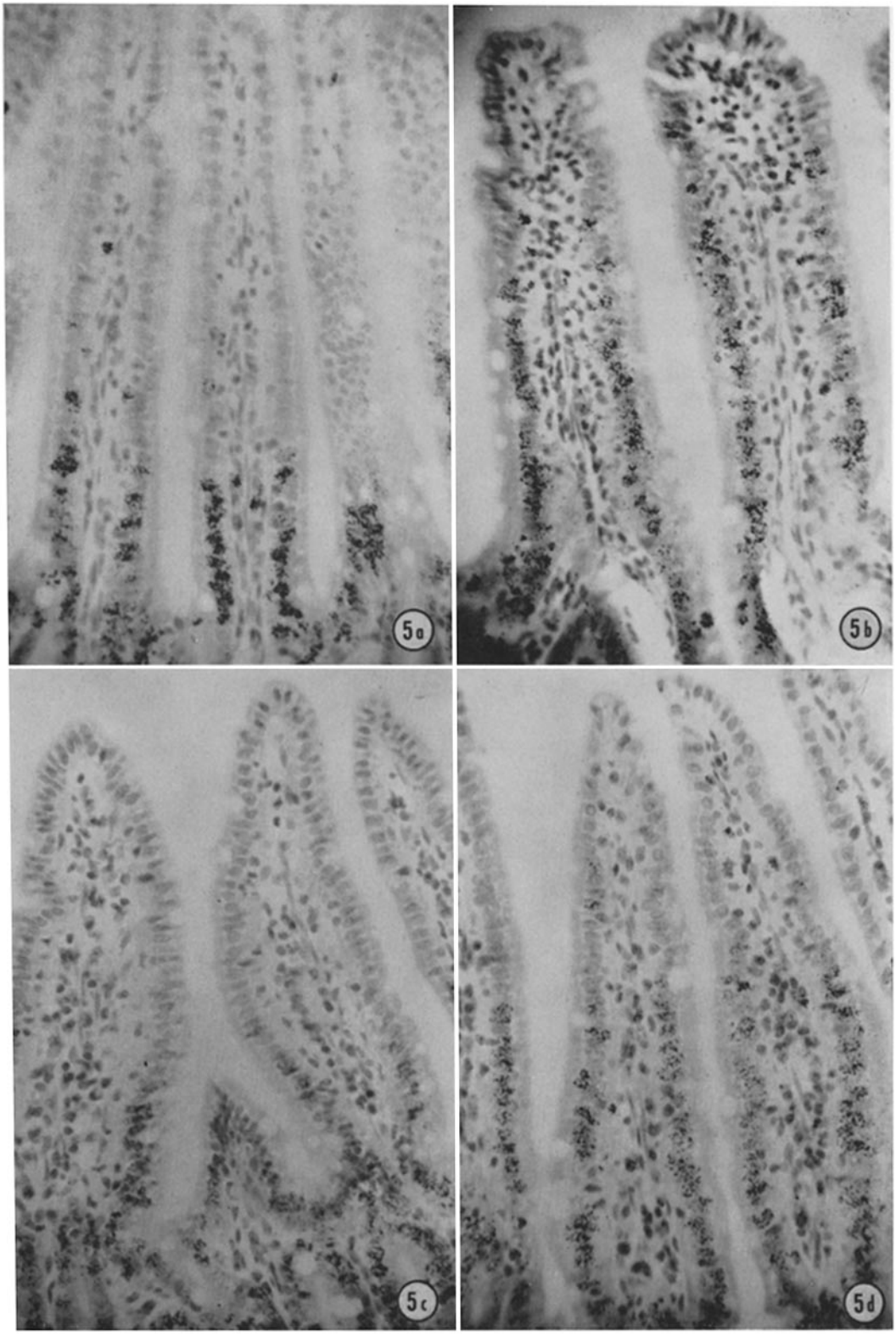
FIG. 5. Radioautography in control and antibiotic-treated mice 24-hr after thymidine-^{3}H injection. (*a*) Female control; (*b*) female on antibiotics for 22 days; (*c*) male control; (*d*) male on antibiotics for 36 days.

Note the greater height of the labeled column in the treated animals. Hematoxylin–light green. × 400.


5a
5b
5c
5d

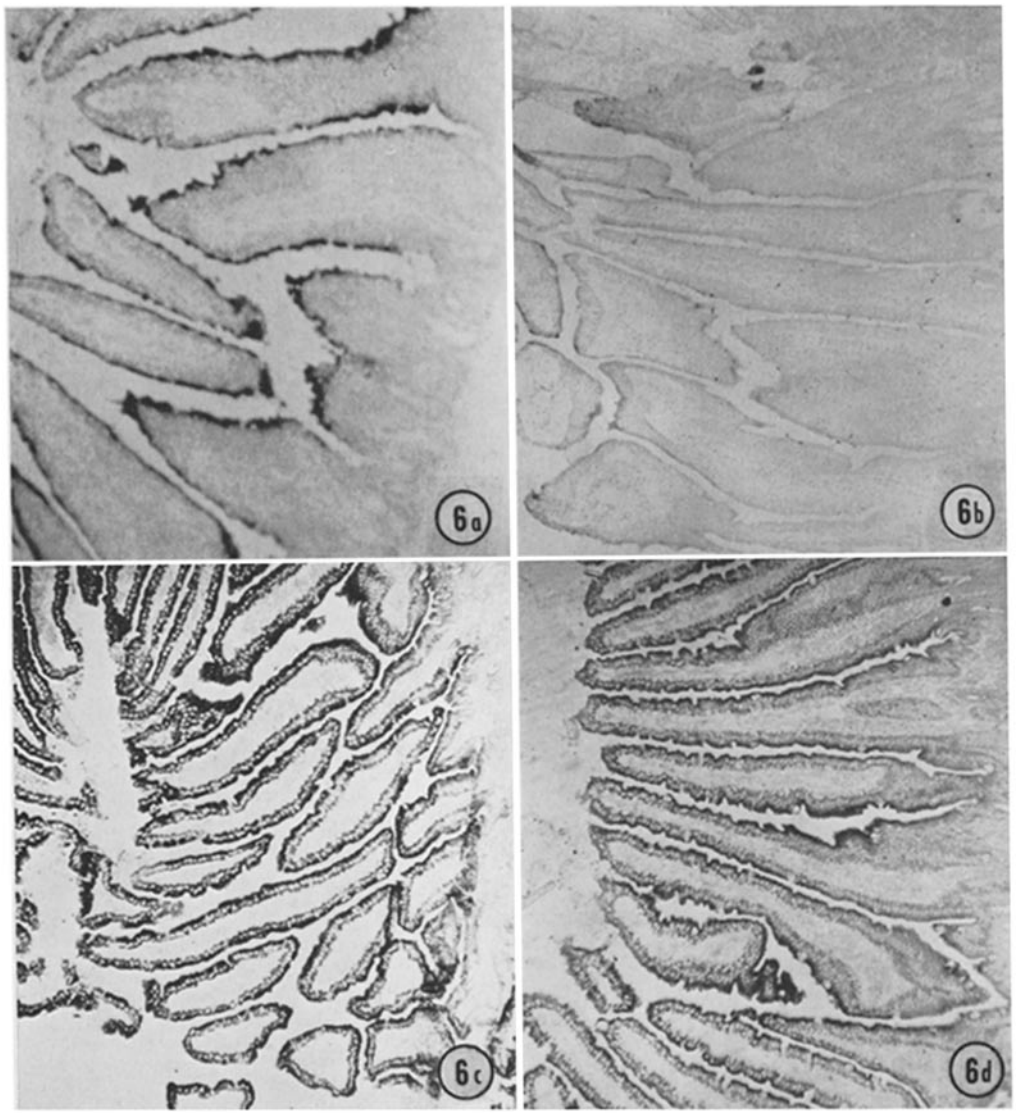


FIG. 6. Photomicrographs of the intestine of control and antibiotic-treated mice. (*a*) NADH reaction, control; (*b*) NADH reaction, treated 30 days; (*c*) nonspecific esterase reaction, control; (*d*) nonspecific esterase reaction, treated 32 days.

Staining reactions are diminished in the treated animals. × 100.