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Effect of Colon Flora and Short-Chain Fatty Acids on Growth In Vitro of *Pseudomonas aeruginosa* and *Enterobacteriaceae*

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Heat-stable antibacterial activity in the following suspensions was demonstrated against *Pseudomonas aeruginosa* at pH 6.5, 6.0, and 5.5: (i) pooled colon contents of normal mice; (ii) an anaerobic, 48-h culture of normal mouse feces; and (iii) anaerobic, 48-h cultures of different bacteria from human colon flora (*Escherichia coli*, *Bacteroides fragilis*, *Klebsiella pneumoniae*, and *Proteus mirabilis*). The lower the pH of the medium, the greater was the antibacterial activity of these suspensions. The antibacterial activity of five fatty acids (propionic, butyric, isobutyric, acetic, and formic acids) was greater against *P. aeruginosa* than against three *Enterobacteriaceae* (*E. coli*, *K. pneumoniae*, and *P. mirabilis*) at all fatty acid concentrations (0.16 M to 0.005 M) and at the 3 pH values studied (5.5, 6.0, and 6.5). As the pH value increased, the antibacterial activity decreased. Antibacterial activity was greater at higher fatty acid concentrations, and at each pH value it was greatest for the fatty acids having high pK_a values. Lactic acid, with the lowest pK_a, exhibited little or no antibacterial activity. Acetic and butyric acids, two of the three predominant volatile fatty acids determined by gas chromatography in the mouse colon contents and in the anaerobic culture of mouse feces, occurred in vivo in concentrations which inhibited growth of *P. aeruginosa* in vitro at the pH of the mouse cecum. These results suggest that undissociated short-chain fatty acids produced by the colon flora may be a mechanism of intestinal resistance to colonization by *P. aeruginosa*.

A striking feature of the bacterial ecology of the gastrointestinal tract is its resistance to colonization by enteric pathogens such as *Salmonella* and *Shigella* after their oral administration (4, 10, 12), whereas *Bacteroides fragilis* and bifidobacteria and, to a much lesser extent, *Escherichia coli* uniformly proliferate in the large bowel to high titers (9, 13).

Resistance to *Salmonella* and *Shigella* has been attributed in part to volatile fatty acids which inhibit in vitro growth of these enteric pathogens at the pH level (1, 6, 11) and the reduction-oxidation potential of the colon (1, 11).

The normal intestinal tract is also quite resistant to colonization by *Pseudomonas aeruginosa;* 10^6 or more *P. aeruginosa* must be ingested by normal men (2) and mice (5) before *P. aeruginosa* can be demonstrated in feces, and fecal excretion is only transient.

The experiments reported here investigated in vitro the role of the normal colon flora and certain of its metabolic products in the resistance of the large intestine to colonization by *P. aeruginosa*.

MATERIALS AND METHODS

CF-1 white male mice (Carworth Farms, New City, N.Y.) were used in the experiments. One strain of *P. aeruginosa* (*P. aeruginosa*-1) was originally isolated from the blood of a patient and passed three times intraperitoneally in mice. Another strain (*P. aeruginosa*-2) was isolated from the feces of a mouse on arrival in the laboratory. The other organisms (*E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *B. fragilis*) were isolated from human feces. Stock cultures were maintained by storing samples of a culture (grown for 18 h) in Trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Md.) at −20 C. Inocula for each experiment were prepared by subculture in Trypticase soy broth and incubation

at 37 C for 18 h. The subculture was then serially diluted in saline solution to yield approximately 10^4 bacteria/ml.

The following suspensions were tested for antibacterial activity against *P. aeruginosa:* (i) pooled colon contents of normal mice; (ii) a chopped meat-broth culture of normal mouse feces incubated for 48 h anaerobically at 37 C; and (iii) cultures of different bacteria from human colon flora (a strain of *E. coli*, *B. fragilis*, *K. pneumoniae*, and *P. mirabilis*) incubated for 48 h anaerobically at 37 C. These suspensions were diluted in equal volumes of 0.2 M phosphate-buffered nutrient broth and centrifuged, and the supernatant fluids were used unheated or after heating for 15 min in a boiling-water bath. The supernatant fluids were adjusted to pH 5.5, 6.0, and 6.5 by addition to concentrated hydrochloric acid and sterilized by passing through a membrane filter (0.45-μm pore size, The Nalge Co., Rochester, N.Y.). Immediately thereafter, 0.1 ml of a saline solution containing approximately 10^3 *P. aeruginosa* was inoculated into 1 ml of each supernatant fluid and incubated at 37 C aerobically and anaerobically. An anaerobic environment was achieved with the Gaspak system (Baltimore Biological Laboratories). Sodium nitrate (0.1%) was added to the buffered nutrient broth to permit anaerobic growth of *P. aeruginosa*. At the start and end of each incubation period pH was determined with a ceramic frit junction combination electrode (Beckman Co., Fullerton, Calif.). Controls were cultures of 10^3 *P. aeruginosa* per ml in buffered nutrient broth diluted in equal volumes of distilled water at the three pH values and incubated at 37 C aerobically and anaerobically. The 50% buffered nutrient broth used in control cultures had been passed through a membrane filter (0.45-μm pore size) to control for possible toxic antibacterial substances in the filter. The number of viable *P. aeruginosa* per ml in each suspension was determined by spreading 0.1 ml of each suspension and of 10-fold dilutions of each suspension on blood agar plates immediately, and after 4 and 20 h of incubation.

Six short-chain fatty acids, (Table 1) were tested for antibacterial activity. These were propionic, isobutyric, butyric, acetic, formic, and lactic acids. Each fatty acid was serially diluted in distilled water to yield concentrations of 0.32 to 0.01 M fatty acid. Each dilution was added to an equal volume of 0.2 M phosphate-buffered nutrient broth. Each fatty acid dilution was adjusted to pH 5.5, 6.0, and 6.5 by addition of concentrated hydrochloric acid, and sterilized by passing through a membrane filter (0.45-μm pore size). Immediately thereafter, 0.1 ml of a saline solution containing approximately 10^3 *P. aeruginosa*, *E. coli*, *P. mirabilis*, or *K. pneumoniae* was inoculated into 1 ml of each fatty acid solution and incubated at 37 C aerobically and anaerobically. Control cultures that were in buffered nutrient broth diluted in equal volumes of distilled water at the three pH values and incubated aerobically and anaerobically at 37 C were also studied. At the start and end of each incubation period, pH was determined. The number of viable bacteria per ml was determined as described above for *P. aeruginosa*.

TABLE 1. *pK_a and percentage of un-ionized fraction of short-chain fatty acid at pH 6.5, 6.0, and 5.5*

Fatty acid	pK_a	Percent un-ionized		
		pH 6.5	pH 6.0	pH 5.5
Propionic acid	4.87	2.3	6.9	18.9
Isobutyric acid	4.84	2.1	6.4	17.9
Butyric acid	4.81	2.0	6.0	16.9
Acetic acid	4.75	1.7	5.3	15.1
Formic acid	3.75	0.2	0.6	1.7
Lactic acid	3.08	0.04	0.1	0.4

Volatile fatty acid analysis was performed by gas chromatography on colon contents of normal mice and on an anaerobic 48-h chopped meat-broth culture of normal mouse feces. A Glowall chromatograph with a flame ionization detector and temperature programmer (Glowall Corp., Willow Grove, Pa.) was used with a 6-foot (about 1.83 meter) coiled glass column containing 2% neopentylglycol succinate on a 60/80 mesh ChromoSorb WAW support (Supelco, Inc., Bellefonte, Pa.). The flow rate of nitrogen carrier gas was 40 ml/min. The starting column temperature was 65 C and it rose to 150 C at a rate of 5 C/min. A sample (1 ml) was acidified to pH 2 or lower with 0.1 ml of concentrated hydrochloric acid and extracted in 1 ml of ethyl ether. A sample (3 μliters) of the ether extract was injected onto the column. By using this technique, nonvolatile short-chain fatty acids (e.g., lactic and succinic acids) could not be detected. Quantitative analysis of fatty acids in the samples was determined from areas under the curves compared to areas under curves of known concentrations of each fatty acid in an ether solution acidified with concentrated hydrochloric acid to pH 2 or lower.

The pH of cecal contents was determined in 12 mice anesthetized with ether. The abdomen was opened and, as quickly as possible, a ceramic frit junction combination pH electrode (Beckman Co., Fullerton, Calif.) was inserted into the center of a mass of cecal material in situ.

RESULTS

During all of the incubations, pH remained at the initial level.

Figure 1 shows the log number per milliliter of viable *P. aeruginosa*-1 at the start and after 4 and 20 h of aerobic incubation in filtrates of mouse colon contents and in filtrates of an anaerobic culture of mouse feces at pH 6.5, 6.0, and 5.5. The data for anaerobic incubation were almost identical. The results with *P. aeruginosa*-2 were similar. In 50% buffered nutrient broth alone at the three pH values, there were more than 10^8 *P. aeruginosa*/ml after 20 h of incubation, whereas multiplication of *P. aeruginosa* was inhibited at pH 6.5, 6.0, and 5.5 by colon contents and the anaerobic culture of feces. The antibacterial activity was affected by

pH; the lower the pH, the greater was the antibacterial activity. Inhibition at the lower pH levels was not due simply to the acidity of the medium, because broth controls buffered at pH 5.5 never inhibited multiplication of *P. aeruginosa*.

The antibacterial activity was also heat stable. Preparations of heated supernatant fluids of pooled colon contents and of an anaerobic fecal culture revealed similar pH-sensitive inhibitory activity against *P. aeruginosa*.

Figure 2 shows the log number of viable *P. aeruginosa*-1 per ml at the start and after 4 and 20 h of aerobic incubation in filtrates of anaerobic cultures of different human fecal bacteria. Results of anaerobic incubation of *P. aeruginosa*-1 in these filtrates were similar. In these experiments, filtrates of cultures of *E. coli*, *B. fragilis*, *K. pneumoniae*, and *P. mirabilis* exhibited similar heat-stable, pH-sensitive inhibitory activity against *P. aeruginosa*.

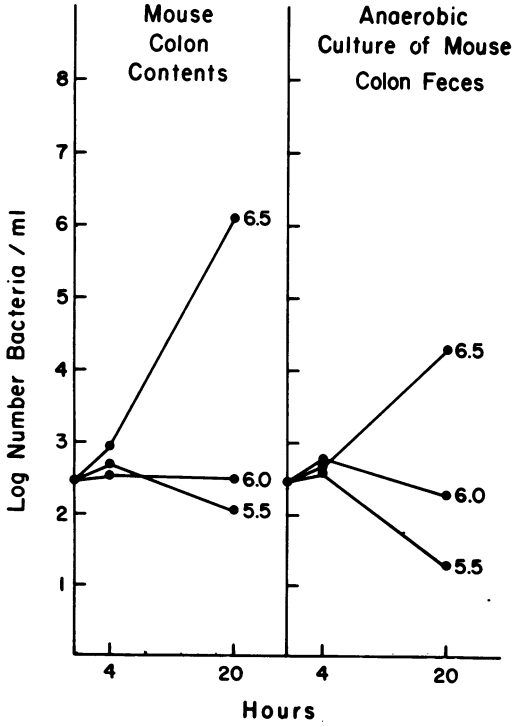

FIG. 1. *Log number of viable P. aeruginosa per milliliter at the start and after 4 and 20 h of aerobic incubation at 37 C in unheated filtrate of mouse colon contents (left), and in an unheated filtrate anaerobic 48-h chopped meat-broth culture of mouse feces (right) buffered at pH 6.5, 6.0, and 5.5. In 50% buffered broth alone at these pH values, there were more than 10^8 P. aeruginosa/ml after 20 h of incubation.*

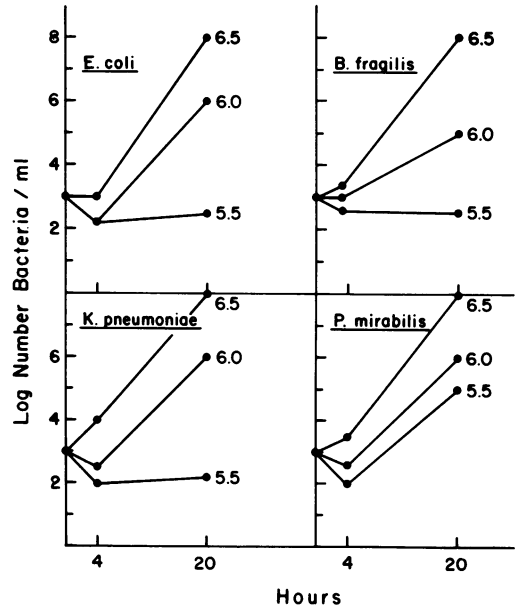

FIG. 2. *Log number of viable P. aeruginosa per milliliter at the start and after 4 and 20 h of aerobic incubation at 37 C in filtrates of cultures of human fecal strains of E. coli, B. fragilis, K. pneumoniae, and P. mirabilis buffered at pH 6.5, 6.0, and 5.5. In 50% buffered broth alone at these pH values, there were more than 10^8 P. aeruginosa/ml after 20 h of incubation.*

The predominant volatile fatty acids in mouse colon contents were acetic, propionic, and butyric acids. The same fatty acids were predominant in the anaerobic chopped meat-broth culture of mouse feces. In the mouse colon contents the concentrations were 0.03 M ± 0.01 SD acetic acid, 0.005 M ± 0.001 SD propionic acid, and 0.004 M ± 0.002 SD butyric acid.

The data for the log change in the number of viable *P. aeruginosa* per milliliter during 20 h of anaerobic incubation for the six concentrations of the fatty acids at pH 6.5, 6.0, and 5.5 are presented in Fig. 3. Data for 4 h of incubation are omitted because there was little change in the number of viable *P. aeruginosa*-1 per milliliter after 4 h of incubation in any of the fatty acids. Data for aerobic incubation were almost identical. At pH 6.5, 0.005 M butyric, 0.02 M propionic, and 0.04 M acetic acids partially inhibited growth of *P. aeruginosa* after 20 h of incubation. At pH 6.0 and 5.5, 0.02 M or more propionic, butyric, and acetic acids completely inhibited multiplication of *P. aeruginosa* after 20 h of incubation. Higher concentrations, especially of butyric acid, were bactericidal at pH 6.0 and 5.5. The antibacterial activity was dependent on the pH of the medium; the lower

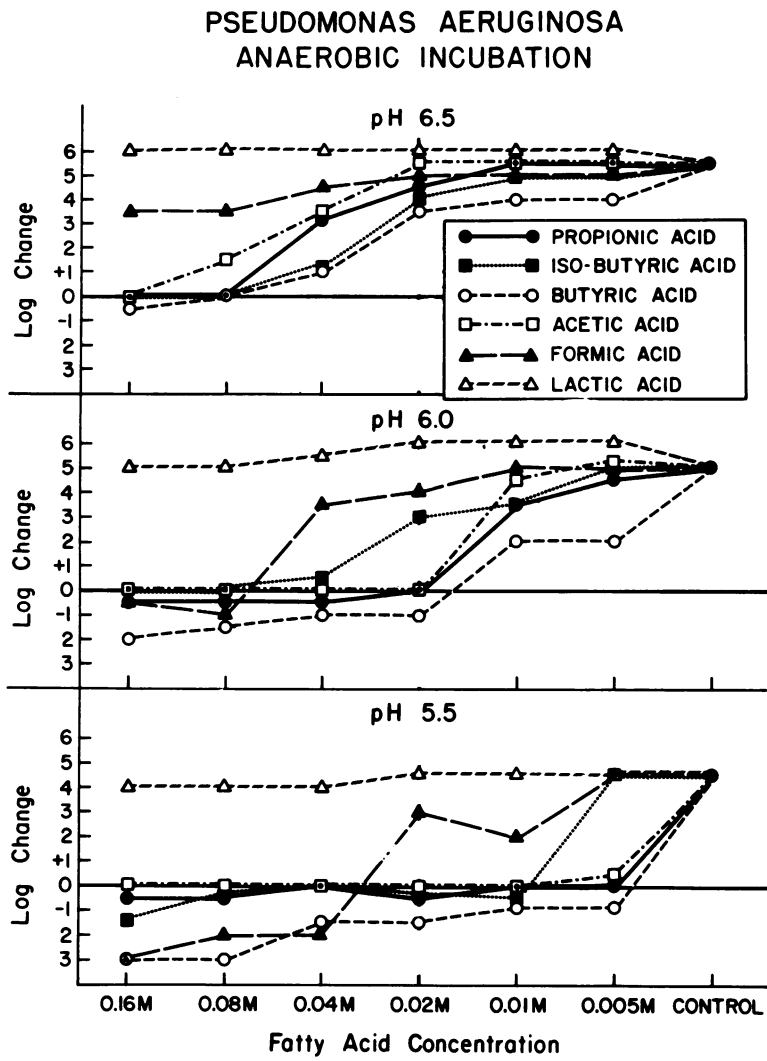

FIG. 3. *Log change in the number of viable P. aeruginosa/ml after 20 h of anaerobic incubation in various concentrations of lactic, formic, acetic, butyric, isobutyric, and propionic acids at pH 6.5, 6.0, and 5.5.*

the pH, the greater was the antibacterial activity of the fatty acid. In addition, the antibacterial activity was greatest at each pH value for the acids having high pK_a values and least for lactic acid, which has the lowest pK_a, value.

Three strains of *Enterobacteriaceae* (*E. coli*, *P. mirabilis*, and *K. pneumoniae*) were also tested for sensitivity to fatty acids in the same manner as *P. aeruginosa*. Figure 4 shows the log change in concentration of the three *Enterobacteriaceae* after 20 h of anaerobic incubation in propionic, butyric, and acetic acids (the three most antibacterial fatty acids) as compared to that of *P. aeruginosa*-1 at pH 6.0. The average pH of cecal contents of 12 mice was 6.25 ± 0.45 SD. *P. aeruginosa* was clearly the most sensitive organism to the three fatty acids; growth was completely inhibited in fatty acid concentrations of 0.02 M or more, and growth was partially inhibited in as little as 0.01 M. In these experiments the four organisms were inhibited most by propionic acid, the fatty acid with the highest pK_a, and were inhibited least by acetic acid, the fatty acid with the lowest pK_a.

Formic acid, which has a lower pK_a than acetic acid, was less inhibitory than the other three fatty acids against the organisms. It was more inhibitory against *P. aeruginosa* than against the *Enterobacteriaceae*. Lactic acid, with the lowest pK_a, had no inhibitory effect

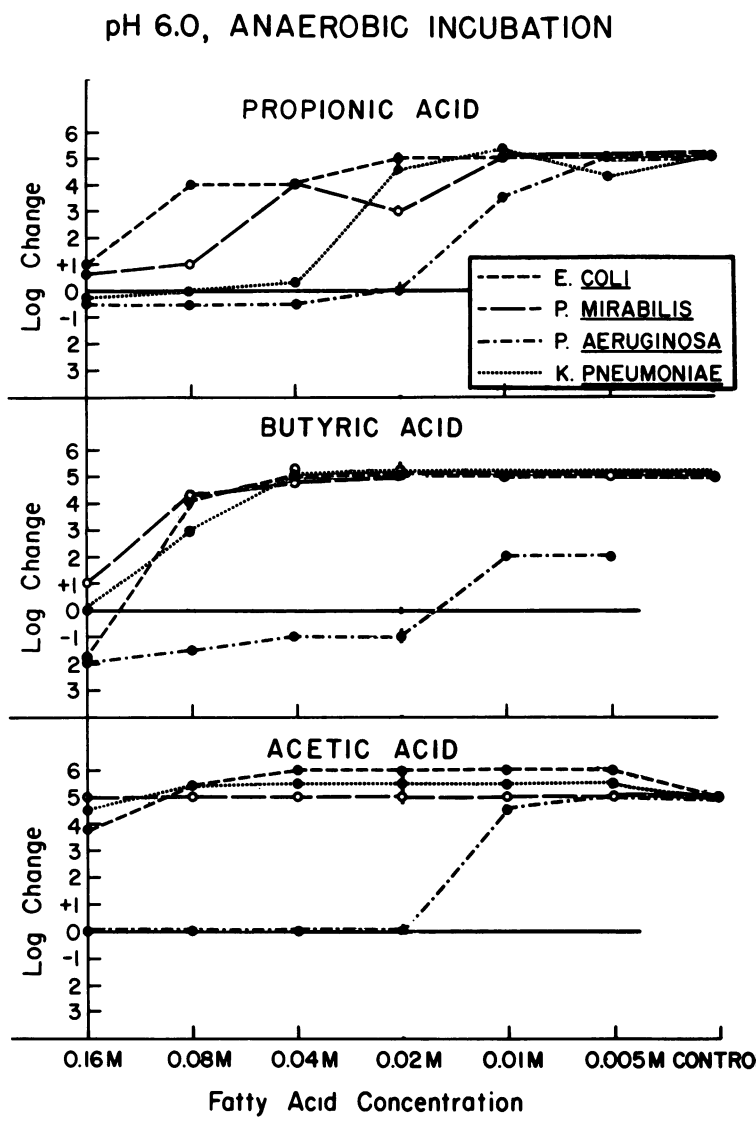

FIG. 4. *Log change in the number of viable E. coli, P. mirabilis, K. pneumoniae, and P. aeruginosa per milliliter after 20 h of anaerobic incubation in various concentrations of propionic, butyric, and acetic acids at pH 6.0.*

against any of the four organisms at pH 6.0. Results after aerobic incubation were similar to those after anaerobic incubation.

DISCUSSION

The heat stability of the inhibitory activity of colon contents and of the anaerobic culture of feces suggests that bacteriocins were not the inhibitory agents, as bacteriocins are heat-labile. In addition, the observation that culture filtrates of three species of *Enterobacteriaceae* and one species of *Bacteroidaceae* were inhibitory to *P. aeruginosa* also suggests that the inhibitory agents are not bacteriocins, because bacteriocins are antagonistic only to organisms closely related to the one that produces them.

The effect of pH on the inhibitory activity suggests that weak acids, such as the short-chain fatty acids produced by the metabolism of fecal microorganisms, were the antibacterial substances. Acetic, propionic, and butyric acids were the predominant short-chain fatty acids in mouse colon contents and in an anaerobic culture of mouse feces. Acetic and butyric acids

were found in vivo in concentrations which were inhibitory in vitro at the pH of the mouse cecum. As in the present study, Bohnhoff et al. (1) found in normal mouse colon contents 0.02 to 0.04 M acetic acid, 0.008 to 0.02 M butyric acid, and a pH of < 6.3 in 66% of the animals. The fatty acid concentrations, pH, and Eh of the large bowel also probably account for the inhibitory activity of large bowel contents in vitro against *Salmonella* and the resistance to intestinal colonization by this organism (1, 11).

In addition, a recent study showed that the appearance of anaerobic fusiform bacilli in the large intestinal lumen of 2-week-old mice and germfree mice exposed to normal mice was correlated with the appearance of volatile fatty acids, especially butyric acid, and with a 10^4-fold decline in numbers of coliform bacilli in the large intestine (8). Penicillin feeding of normal adult mice eliminated the anaerobic fusiforms from the large intestine. This was associated with the disappearance of significant levels of butyric acid and with a 10^6-fold increase in numbers of coliform bacilli (8). This suggested that butyric acid produced by the anaerobic fusiform bacilli suppresses growth of coliform bacilli in vivo in the large intestine.

In the present study the dependence of the inhibitory effect on the pK_a of the fatty acid and on pH suggests that the un-ionized acid molecule is the inhibitory agent for *Pseudomonas*. Table 1 shows the percentage of un-ionized molecules for each of the fatty acids studied at the three pH values. The acids with the highest pK_a (propionic, isobutyric, butyric, and acetic acids) have the greatest percentages of un-ionized molecules, which increases with decreasing pH (11). Among the first four acids, there are only small differences in pK_a values and percentages of un-ionized molecules at each pH. Similarly, there are only small differences in antibacterial activity among the first four fatty acids.

Possible mechanisms for the antibacterial activity of fatty acids may be uncoupling of oxidative phosphorylation and inhibition of adenosine triphosphate-inorganic PO_4 exchange, which have been produced by short-chain fatty acids in vitro (3, 7).

The fatty acids inhibit growth of *P. aeruginosa* to a greater degree than the *Enterobacteriaceae* tested in these studies. This inhibitory activity may explain the normal resistance of the intestinal tract to colonization by *P. aeruginosa*. Persistence of *E. coli* in high titers in the normal intestinal tract may be explained by the relative insensitivity of *E. coli* to concentrations of fatty acid which are inhibitory to *P. aeruginosa*.

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