

## Intracolonic Tensions of Oxygen and Carbon Dioxide in Germfree, Conventional, and Gnotobiotic Rats<sup>1</sup> (39229)

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Gases are present in the gastrointestinal tract of animals and man. Oxygen and nitrogen are transported by the circulatory system and reach the intestinal lumen by diffusion from capillaries of the intestinal mucosa. Other gases in the intestine, especially carbon dioxide, methane, and hydrogen, are products of bacterial metabolism. Swallowed air may also account for some of the gases in the alimentary tract (1). The intestinal mucosa exhibits an oxygen tension ( $P_{O_2}$ ) on the order of 40 mm Hg, which is similar to that of venous blood (2). Oxygen diffuses across the intestinal epithelium in a diminishing  $P_{O_2}$ -gradient between the mucosa and the lumen (3). Flatus usually has a  $P_{O_2}$  of less than 15 mmHg (4). Utilization of intraluminal  $O_2$  by colonic bacteria is thought to lower the  $P_{O_2}$  to the level present in flatus (5).

We have recently shown that intracolonic gaseous tensions of  $O_2$  and  $CO_2$  in germfree and conventional rats were significantly different, and reflected the absence of a microflora in germfree animals (6). Conventional rats had a lower intracolonic  $P_{O_2}$  owing to bacterial consumption of  $O_2$ , and a higher intracolonic  $P_{CO_2}$  owing to the additional  $CO_2$  produced by bacterial metabolism. The present report renews the investigation of germfree and conventional rats to provide a basis for expanding our studies to germfree rats colonized with one and two different bacterial species, and examines the relation of intracolonic gaseous tensions to cecal size and bacterial populations in feces.

**Material and methods.** Ten conventional, six germfree, and 43 gnotobiotic rats were

studied. All were of the Fischer 344 CDF strain (originally obtained from Charles River Breeding Laboratory, Wilmington, Mass.), and were bred at the Louisiana State University Germfree Facility. Rats weighed  $207 \pm 45$  g (mean  $\pm$  standard deviation); most were female. They were reared in clear plastic cages on corncob bedding and were fed Purina 5010-C diet (Ralston Purina Co., St. Louis, Mo.) and water *ad libitum*. The diet was autoclaved in standard sterilizing cylinders for 25 min at 121 C following three prevacuums. Germfree rats were maintained in flexible plastic isolators; conventional rats were maintained in the open laboratory. Germfree and gnotobiotic rats were removed from their isolators immediately before study.

One group of germfree rats was monoassociated with a strain of *Bacteroides fragilis* isolated from rat feces. Colonizations, monoassociate as well as diassociate, with this organism and with a strain of *Escherichia coli* isolated from a human infection were achieved simply by adding the microorganism to drinking water (7).

Rats in two other germfree isolators became colonized accidentally due to contamination during routine maintenance, and were used as gnotobiotics. Those in one isolator were monoassociated with a strain of *Staphylococcus epidermidis*; those in the other became diassociated with strains of *Bacillus macerans* and an aerobic diphtheroid.

Simultaneous and continuous *in vivo* measurements of  $P_{O_2}$  and  $P_{CO_2}$  were made with a Medspect mass spectrometer (Scientific Research Instruments Corp., Baltimore, Md.) by means of a gas-sampling cannula positioned in the colon and connected to the mass spectrometer (8). The cannula was a Teflon-coated, 22-gauge stainless tube; the Teflon at the sampling tip was

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expanded into a bulb 2.5-cm long and 1.3-mm wide. Each rat was lightly anesthetized with ether. Germfree rats were observed to succumb to ether more quickly and more deeply than conventional rats. The sampling tip was inserted into the anus and passed 5 cm into the lumen of the colon. Once positioned, the cannula was anchored to the base of the rat's tail with tape, and the rat was placed in a restraining cage with the tail and attached cannula emerging. Although the rat usually awoke while being restrained, an additional 10 min was allotted for the mass spectrometer to equilibrate. Therefore, all measurements of gaseous tensions were monitored in unanesthetized rats. Curves depicting gaseous tensions at 5-min intervals were plotted. Statistical comparison of cumulative grand means of gaseous tensions at 5-min intervals along each curve were based on Student's *t* test for unpaired experiments.

Animals were killed by ip injection of 3% sodium pentobarbital, and removed from the restraining cage. The abdomen was prepared with ethanol and opened aseptically. The position of the cannula in each rat was verified and the colon examined to determine if it had been perforated. Quantitative bacteriological studies, employing methods previously described (6), were conducted upon specimens of feces removed from the colon in the vicinity of the measuring tip of the cannula. The cecum of each rat was removed and weighed along with its contents.

**Results.** Germfree and conventional rats were monitored to obtain baseline intracolonic tensions of  $O_2$  and  $CO_2$  for this study (Fig. 1). The upper pair of curves show the mean  $P_{CO_2}$  at 5-min intervals. The curve for  $P_{CO_2}$  in conventional rats was usually fluctuant; the curve for  $P_{CO_2}$  in germfree rats was relatively smooth. The fluctuations in conventional rats were thought to be due to high levels of  $P_{CO_2}$  near fecal pellets passing the gas-sampling tip of the cannula. However, measurements with conventional rats fasted for 24 hr and for 48 hr prior to study showed similar variations. The lowest pair of curves in Fig. 1 show the mean  $P_{O_2}$  in germfree rats to be slightly greater than in conventional rats. The curve for germfree rats was smoother.

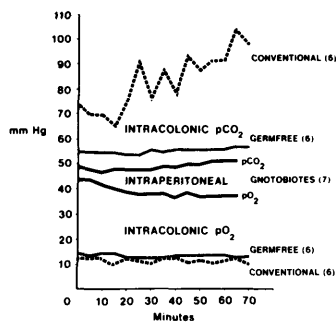


FIG. 1. Mean intracolonic gaseous tensions of  $O_2$  and  $CO_2$  in rats. The upper pair of curves show  $P_{CO_2}$ ; the lower pair show  $P_{O_2}$ ; the central pair show ip tensions. The number of rats is indicated.

Figure 1 also shows the mean ip  $P_{CO_2}$  and  $P_{O_2}$  in rats whose colons became perforated during monitoring. Rats usually struggled while restrained, and there was always the possibility that the rigid cannula would perforate the colon. This complication occurred in 12% of our animals, and, although not planned, allowed us to obtain measurements in the peritoneal cavity adjacent to the site regularly measured intracolonic. The ip  $P_{O_2}$  was significantly greater than intracolonic  $P_{O_2}$ , and was so elevated that we could diagnose perforation of the colon with precision if it had occurred. Nevertheless, the location of the cannula and perforation of the colon were always verified at necropsy. Intraperitoneal gaseous tensions were independent of germfree, conventional, or gnotobiotic status of rats.

The cumulative gaseous tensions at 5-min intervals, calculated from curves such as just shown, are listed in Table I along with a summary of statistical comparisons. Intraperitoneal gaseous tensions were significantly different from those in the colons of germfree and conventional rats. Intraperitoneal tensions in rabbits have recently been reported by Klossner and associates (9), who employed a polarographic method of analysis. Their mean ip measurements of  $P_{O_2}$  and  $P_{CO_2}$  (42 and 48 mmHg, respectively) agree with ours (Table I).

Although the grand means for intracolonic  $P_{O_2}$  in conventional and germfree rats were of similar magnitude, the slight difference of 1.7 mmHg between these means was significant ( $P < 0.001$ ) at 28 degrees of freedom. The greater difference in means

TABLE I. CUMULATIVE GASEOUS TENSIONS IN GNOTOBIOTIC RATS.

Site of measurement and status of rats	Number of rats	Grand mean $\pm$ standard deviation		Statistical significance of means relative to intracolonic tensions			
				Germfree rats		Conventional rats	
		Po <sub>2</sub> (mm Hg)	Pco <sub>2</sub> (mm Hg)	Po <sub>2</sub>	Pco <sub>2</sub>	Po <sub>2</sub>	Pco <sub>2</sub>
Intraperitoneal	7	38.9 $\pm$ 2.3 (14) <sup>a</sup>	49.1 $\pm$ 1.8 (14)	S <sup>b</sup>	S	S	S
Intracolonic							
Conventional	6	11.1 $\pm$ 1.0 (15)	83.4 $\pm$ 11.5 (15)	S	S	—	—
Germfree	6	12.8 $\pm$ 0.8 (15)	54.6 $\pm$ 0.9 (16)	—	—	S	S
<i>Staphylococcus epidermidis</i>	7	13.1 $\pm$ 2.0 (12)	48.1 $\pm$ 2.4 (13)	NS <sup>b</sup>	S	s <sup>b</sup>	S
<i>Escherichia coli</i>	5	14.9 $\pm$ 1.3 (13)	63.1 $\pm$ 3.9 (13)	S	S	S	S
<i>Bacteroides fragilis</i>	6	9.3 $\pm$ 0.8 (20)	55.8 $\pm$ 1.4 (21)	S	S	S	S
<i>E. coli</i> + <i>B. fragilis</i>	6	19.4 $\pm$ 0.8 (16)	48.7 $\pm$ 1.5 (16)	S	S	S	S
<i>Bacillus macerans</i> + aerobic diphtheroid	6 <sup>c</sup>	9.5 $\pm$ 1.4 (13)	57.7 $\pm$ 1.1 (12)	S	S	s	S
<i>Bacillus macerans</i> + aerobic diphtheroid	6 <sup>d</sup>	14.4 $\pm$ 1.4 (16)	57.3 $\pm$ 2.1 (16)	S	S	S	S

<sup>a</sup> Bracket ( ) designates number of readings at 5-min intervals.

<sup>b</sup> S indicates  $P < 0.001$ ; s indicates  $P < 0.005$ ; NS indicates  $P > 0.05$ .

<sup>c</sup> Colonized for 4 to 12 weeks.

<sup>d</sup> Colonized for 20 weeks.

for Pco<sub>2</sub> (28.8 mmHg) was also significant.

**Monoassociated rats.** Intracolonic Po<sub>2</sub> values for gnotobiotics ranged from 9.3 to 19.4 mmHg, and appeared to be independent of the oxygen requirements of the colonizing microorganisms. The mean Po<sub>2</sub> in rats monoassociated with *S. epidermidis* was not significantly different from that in germfree rats, but was different from that in conventional rats. The mean Po<sub>2</sub> in rats monoassociated with either *E. coli* or *B. fragilis* was significantly different from that in germfree and conventional rats. However, all differences in Po<sub>2</sub> values were slight, and could be discriminated only by statistical analysis. The mean Pco<sub>2</sub> in monoassociated rats was significantly different from those in germfree and conventional rats, but closer numerically to germfree than to conventional rats.

**Diassociated rats.** The mean Po<sub>2</sub> in rats diassociated with *E. coli* and *B. fragilis* was significantly greater than that for germfree and conventional rats and that for rats monoassociated with each species. The mean Pco<sub>2</sub> was significantly less than that for germfree rats and the respective monoassociates. The large difference (34.8 mmHg) in Pco<sub>2</sub> between these diassociates and conventional rats was also significant.

The difference in mean Po<sub>2</sub> of rats diassociated with *B. macerans* and a diphtheroid

for 4 to 12 weeks and for 20 weeks was significant ( $P < 0.001$ ), and appeared to indicate the range of normal variation in Po<sub>2</sub> values in gnotobiotic rats. In contrast, the mean Pco<sub>2</sub> in these two groups was not significantly different ( $P > 0.05$ ).

**Cecal size.** The influence of colonization on cecal size is summarized in Table II. "Conventionalization" of a germfree rat by removing it from the germfree isolator and maintaining it in a cage previously occupied by conventional rats caused the size and weight of the cecum to become diminished to that found in conventional rats. However, mono- and diassociation of germfree rats did not in general alter the enlarged cecum (averaging 6 to 10% of body weight) characteristic of germfree rodents (10). Although the cecal size of rats monoassociated with *E. coli* was significantly smaller than that of germfree rats, it was also significantly greater than that of conventional rats, and was within the range for germfree rodents.

**Bacteriological studies.** The bacterial population in feces at or near the site monitored for intracolonic gaseous tension is presented in Table III, which compares the fecal flora of mono- and diassociated rats with that of conventional rats. Counts of each bacterial species studied were smaller as the microorganism progressed from monoassociate to diassociate to a component of the normal

TABLE II. CECAL SIZE OF GNOTOBIOTIC RATS.

Status of rats	Number of rats	Duration association (weeks)	Cecum as percentage of body weight (mean $\pm$ SD)	Statistical significance relative to germ-free rats
Germfree	4	—	10.6 $\pm$ 1.4	—
Conventional	7	—	2.7 $\pm$ 1.1	$P < 0.001$
"Conventionalized"	1	12	2.3	$P < 0.02$
<i>Staphylococcus epidermidis</i>	7	2-9	10.4 $\pm$ 1.6	NS
<i>Escherichia coli</i>	6	3-5	7.4 $\pm$ 0.4	$P < 0.001$
<i>Bacteroides fragilis</i>	6	6	10.7 $\pm$ 1.5	NS
<i>E. coli</i> + <i>B. fragilis</i>	6	4-6	11.3 $\pm$ 1.0	NS
<i>Bacillus macerans</i> + aerobic diphtheroid	6	4-12	11.9 $\pm$ 1.4	NS
<i>Bacillus macerans</i> + aerobic diphtheroid	6	20	12.6 $\pm$ 2.7	NS

<sup>a</sup> NS indicates  $P > 0.05$ .

TABLE III. FECAL FLORA OF GNOTOBIOTIC RATS.

Microorganism	Bacterial colonies per gram of feces		
	Monoassociated rats	Diassociated rats	Conventional rats (3)
<i>Staphylococcus</i>	8.91 $\pm$ 0.20 (3) <sup>a</sup>		5.10 $\pm$ 0.50
<i>Escherichia coli</i>	9.23 $\pm$ 0.30 (5)	8.87 $\pm$ 0.40 (6)	5.49 $\pm$ 0.83
<i>Bacteroides</i>	10.21 $\pm$ 0.33 (6)	9.45 $\pm$ 0.31 (6)	8.31 $\pm$ 0.32
<i>Bacillus macerans</i>		8.96 $\pm$ 0.22 (5)	
Aerobic diphtheroid		8.62 $\pm$ 1.73 (5)	5.03 $\pm$ 4.36
Aerobic lactobacillus			8.67 $\pm$ 0.91
<i>Streptococcus</i>			4.35 $\pm$ 3.70
Enterococcus			7.10 $\pm$ 1.52
<i>Peptostreptococcus</i>			8.46

<sup>a</sup> Arithmetic mean of log  $\pm$  standard deviation; brackets indicate the number of rats.

flora. However, only in the case of *B. fragilis* was the same strain known to be indigenous to our rats. The diminution of *E. coli* in the progression from mono- to diassociate was not significant ( $P > 0.10$ ), but that of *B. fragilis* was different ( $P < 0.005$ ).

**Discussion.** These data confirm our previous finding that intracolonic tensions of O<sub>2</sub> and CO<sub>2</sub> in germfree and conventional rats were significantly different (6). It seems reasonable to expect conventional rats to have a lower intracolonic Po<sub>2</sub> than germfree rats owing to bacterial consumption of circulatory oxygen diffusing into the intestine. However, study of additional rats has narrowed the difference in mean Po<sub>2</sub> between germfree and conventional rats from 66% (14.1 vs 8.5 mmHg, respectively) previously (6) to 15% (12.8 vs 11.1 mmHg, respectively) in the present study. Levels of intracolonic Po<sub>2</sub> were less than 15 mmHg (4), but differences in Po<sub>2</sub> among germfree, conventional, and gnotobiotic rats were slight.

In addition to the CO<sub>2</sub> produced by bacte-

rial metabolism, CO<sub>2</sub> present in the alimentary tract also arises by release from reaction between acids and the bicarbonate of gastrointestinal secretions and the carbonates of food; smaller amounts are available from metabolic activity of the gut itself and from the diet (1). Study of additional rats has not affected drastically the difference in mean Pco<sub>2</sub> between germfree and conventional rats. The difference was 50% (37.5 vs 74.4 mmHg, respectively) initially (6), and is now 35% (54.6 vs 83.4 mmHg, respectively). Thus, intracolonic Pco<sub>2</sub> is a parameter directly related to the normal intestinal flora. The mean Pco<sub>2</sub> in gnotobiotics diassociated with *B. macerans* and a diphtheroid was unchanged when measured between 4-12 weeks and again after 20 weeks (Table I). Pco<sub>2</sub>, therefore, also appears to be a more stable indicator of a normal intestinal flora than does Po<sub>2</sub>.

Cecal size was inversely proportional to intracolonic Pco<sub>2</sub> and was smallest for conventional rats, but of similar magnitude in

gnotobiotic and germfree rats (Table II). Both cecal size and intracolonic  $P_{CO_2}$  were related to the presence of a normal flora. The germfree rat is characterized by several deviations from normal gastrointestinal morphology and function directly related to the absence of an intestinal microflora. These include a cecum enlarged by an elevated colloidal osmotic pressure due to the absence of proteolytic and hydrolytic enzymes of microbial origin (11); a more positive (oxidized) cecal oxidation-reduction potential than in conventional rats since the normal flora produces a more reduced environment by its metabolic activities (12); and thinner walls of the small intestine with smaller villi and consequently less absorptive surface (13). The enlarged cecum of germfree rodents persists during the life span (10).

The population of a given bacterial species in monoassociates was progressively smaller in diassociates and in conventional rats (Table III). Freter and colleagues (7, 14) have investigated this phenomenon in mice, and have reported that the population of *E. coli* in the murine intestine and cecal size are dependent upon diet and the presence of at least 95 different species and strains of anaerobes. Intracolonic tensions of  $O_2$  and  $CO_2$  also correlate with a normal intestinal flora. Of the two,  $P_{CO_2}$  is the more sensitive indicator.

**Summary.** The relation of intracolonic gaseous tension to fecal microflora was investigated by mass spectrometric measurements of intracolonic  $O_2$  and  $CO_2$  in unanesthetized germfree, conventional, and gnotobiotic rats; ip measurements were obtained in rats whose colons became perforated accidentally; fecal bacterial flora and cecal size were also determined. Gnotobiotics were monoassociated with *Escherichia coli*, *Bacteroides fragilis* and *Staphylococcus epidermidis*, and were diassociated with *E. coli* plus *B. fragilis* and with *Bacillus macerans* plus an aerobic diphtheroid. Mean intracolonic  $P_{O_2}$  in conventional rats (11.1 mmHg) was significantly lower than in germfree rats (12.8 mmHg); mean intracolonic  $P_{CO_2}$  in conventionals (83.4 mmHg) was greater

than in germfree rats (54.6 mmHg). Differences of  $P_{O_2}$  values among all rats were slight. However, intracolonic  $P_{CO_2}$  values were directly related to  $CO_2$  production by the normal intestinal flora, and were thus significantly lower in both germfree and gnotobiotic rats. Intraperitoneal tensions were independent of the status of rats, and the mean ip  $P_{O_2}$  and  $P_{CO_2}$  (38.9 and 49.1 mmHg, respectively) agreed with values in the literature. The enlarged cecum, characteristic of germfree rats, was also present in gnotobiotic rats. Counts of each bacterial species in feces of monoassociated rats were  $10^8$  to  $10^{10}/g$ ; counts were progressively smaller in feces of diassociated rats and conventional rats ( $10^8$  to  $10^9/g$  and  $10^5$  to  $10^8/g$ , respectively). Intracolonic gaseous tensions of  $CO_2$  clearly reflected the presence of a normal flora in conventional rats, and were inversely proportional to cecal size.

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