Effect of Microflora on the Free Amino Acid Distribution in Various Regions of the Mouse Gastrointestinal Tract

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The distribution of free amino acids in the contents of various regions of the gastrointestinal tract (stomach, upper small intestine, lower small intestine, cecum, upper colon and lower colon) was studied in germfree and conventionalized mice. Particular emphasis was placed on the conversion of tryptophan to indole as a probe for studying intermicrobial interactions and microbe-host interactions in vivo. Great differences were observed in the free amino acid content of the various regions of the digestive tract in each type of mouse and also in any one region between germfree and conventionalized mice. As would be expected, there were fewer differences in amino acid distribution between the types of mice in both regions of the small intestine. This correlates with a much lower population of microorganisms in these regions. The changes in free amino acid content and distribution produced by microflora are great enough to serve as a good probe for studying the interactions of a limited number of species of microbes in gnotobiotic animals and assign possible specific functions to each species.

To approach an understanding of the metabolic and physiological interactions between different species of intestinal microflora and between the microflora and the host animal we have focused on a single aspect because of the complexity of these interactions. We are concentrating on amino acid metabolism, particularly that of tryptophan, as influenced by the microbrial enzyme tryptophanase.

In animal cells tryptophan enters primarily two pathways; one leading to the production of serotonin, the other leading to kynurenine. Because of the importance of these metabolites to the host animal, we wanted to determine just what role the intestinal microflora play in controlling the amount of tryptophan available for further metabolism by the host.

The rate of absorption of individual amino acids by the intestine is influenced by the concentration of the various amino acids (6), competition for transport enzymes (24, 26), and ratios of the different amino acids to each other (23).

Because of the complexities of studying microbrial interactions in vivo, we decided to elucidate in vitro some of the bacterial metabolic functions, particularly amino acid metabolism. Preliminary investigations of in vitro metabolism by two species of enteric bacteria have revealed that each organism can markedly alter the other's growth and amino acid metabolism (D. D. Whitt, R. D. DeMoss, and J. Roehrig, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, p. 138).

However, for in vitro studies of amino acid metabolism to be meaningful, the conditions should duplicate as nearly as possible those in the host animal. Many workers have investigated the effect of microflora on the gastrointestinal tract of animals. However, very little information is available on the free amino acid content of various regions of the gastrointestinal tract in the presence and absence of microflora. For that reason, we have determined the free amino acid content of various regions of the gastrointestinal tract of germfree and conventionalized mice.

MATERIALS AND METHODS

Mice. The mice used in these studies were all half- or full siblings born and reared under germfree conditions in this laboratory. The parents were originally obtained from Charles River Breeding Laboratory, Wilmington, Mass. All mice, both germfree and conventionalized, were maintained in plastic film-type isolators (G-F Supply Division, Standard Safety Equipment Co., Palatine, Ill.)

Diet. All germfree and conventionalized mice were maintained on the same sterile food purchased from Charles River Breeding Laboratory. They were provided both food and sterilized water ad libitum.

Conventionalization of germfree mice. The germfree mice were segregated by sex and three to five mice were maintained in metal cages. Three weeks before sacrifice, one conventional female mouse from the colony of the Department of Microbiology, University of Illinois was placed in each cage.
All mice thus treated became conventionalized as indicated by the size of the cecum.

Preparation of samples for analysis. At the time of analysis, the mice were 6 to 7 weeks old. They had been starved for 14 to 17 h but provided water ad libitum during this period. The mice were sacrificed by CO₂ narcosis. The gastrointestinal tract was removed immediately and divided into six sections: stomach, upper small intestine, lower small intestine, cecum, upper colon, and lower colon. The contents were gently expressed from each section. The contents of each section were pooled for each group of germfree mice and for each group of conventionalized mice. The pooled contents were weighed.

Amino acid extraction and analysis. Each of the pooled samples was suspended in 10 ml of cold glass-distilled water and agitated vigorously. The samples were allowed to stand on ice for 30 min during which period they were agitated several times. They were then centrifuged at 0 C for 45 min at 19,000 x g. The resulting supernatants were decanted and filtered through several layers of cheesecloth, then frozen overnight. To 2.5 ml of the thawed supernatant was added 0.5 ml of 30% trichloroacetic acid. After mixing, the samples were allowed to stand for 30 min and then centrifuged for 30 min at 0 C at 19,000 x g. The resulting supernatant was passed through a membrane HAWP 01300 filter, 0.45 μm (Millipore Corp.). The filtrate was lyophilized, then dissolved in 0.2 M sodium citrate buffer, pH 2.2, and analyzed on a Beckman Spinco model 120 amino acid analyzer by the method of Moore and Stein (17) and Moore et al. (16).

Tryptophan determination. The amount of tryptophan present in the samples used for amino acid analysis was determined enzymatically by using tryptophanase which had been partially purified from Vibrio K-12. However, prior to the assay the samples (which were taken before treatment with trichloroacetic acid) were boiled for 10 min to partially inactivate the remaining digestive enzymes which otherwise rapidly inactivate tryptophanase. Three 0.5-ml portions of each sample were boiled. After boiling 0.11 ml of reaction mix (0.5 M potassium phosphate buffer, pH 8.0, plus 1 mg of bovine serum albumin/ml plus 2.5 x 10⁻⁴ M pyridoxal-5'-phosphate) was added to each tube. To one series 0.02 ml of partially purified tryptophanase and 0.1 ml of 0.02 M L-tryptophan were both added to determine how much inhibition remained after boiling. Only 0.02 ml of partially purified tryptophanase was added to the second series of samples to detect the amount of tryptophan present. The third series was used as a control for the amount of indole already present in the samples. The samples were incubated at 37 C for 30 min, which was sufficient time for the reaction to go to completion. Two-tenths milliliter of 2.5 N NaOH was added. The indole produced was extracted into 2.0 ml of toluene. A volume of 3.0 ml of color reagent (14.7 g of p-dimethylaminobenzaldehyde plus 52 ml of H₂SO₄, plus 948 ml of 95% ethanol) was added to 1.0 ml of the toluene layer. After 20 min of incubation at room temperature the samples were centrifuged at 0 C for 15 min at 19,000 x g. The absorbance of the supernatant was read at a wavelength of 568 nm on a Gilford 300-N spectrophotometer.

Indole determination. The amount of free indole present in the aqueous extracts of the intestinal contents was measured after extracting the indole in 1.0 ml of sample into 2.0 ml of toluene. One milliliter of the toluene extract was added to 3.0 ml of the color reagent and treated as described under the section on tryptophan determination.

RESULTS

The data presented here are based on three experiments in which a total of 19 germfree and 17 conventionalized mice were used.

The relative molar ratios of 18 free amino acids and indole (isoleucine = 1.0) from each of the six regions of the gastrointestinal tract are presented in Fig. 1. The data are averages from the three experiments. The vertical line represents the standard deviation. Indole is present in such small amounts it is difficult to evaluate the data from Fig. 1, so the mean nanomoles of indole per gram of intestinal contents, the ratio to isoleucine, and the standard deviation for each region are presented in Table 1.

In Fig. 2 the data are represented as the ratio of the amount of any particular amino acid in germfree mice to that in conventionalized mice (i.e., a ratio of 1.0 represents equal amounts of the amino acid in both types of mice).

The total concentration of free amino acids in the various regions of the gastrointestinal tract and in the diet is presented in Table 2.

DISCUSSION

Large differences were observed in the free amino acid content of various regions of the gastrointestinal tract in each type of mouse (Fig. 1) and also in any one region between germfree and conventionalized mice (Fig. 2).

Because the mice had been starved for at least 14 h before sacrifice, one would expect little difference in the values obtained for the stomach contents of the two types of mice due to the rapid emptying of gastric contents after ingestion (8). However, the differences observed (Fig. 2a) may be due to coprophagy, which was not prevented, or to different rates of gastric emptying since it has been shown that the presence of microflora stimulates gastric emptying.

**Fig. 1.** Molar ratio of individual free amino acids to isoleucine (ile) in the gastrointestinal contents of germfree and conventionalized mice. A, Stomach; B, upper small intestine; C, lower small intestine; D, cecum; E, upper colon; F, lower colon. The vertical bar represents one standard deviation on each side of the mean.
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A

GERMFREE
CONVENTIONALIZED
nmoles ile/g:
germmfree = 150.8
conventional = 249.9

B

GERMFREE
CONVENTIONALIZED
nmoles ile/g:
germmfree = 5055.4
conventional = 4820.8

C

GERMFREE
CONVENTIONALIZED
nmoles ile/g:
germmfree = 2043.7
conventional = 5831.5

D

GERMFREE
CONVENTIONALIZED
nmoles ile/g:
germmfree = 245.4
conventional = 274.0

E

GERMFREE
CONVENTIONALIZED
nmoles ile/g:
germmfree = 248.8
conventional = 365.6

F

GERMFREE
CONVENTIONALIZED
nmoles ile/g:
germmfree = 246.1
conventional = 482.1
emptying (7). The microflora population of the stomach is quite high as compared with the small intestine (18). The higher concentration of certain amino acids (arg, thr, ser, glu, tyr, and trp) in germfree animals may result from more extensive hydrolysis of proteins due to their remaining in the stomach of these animals for a greater period of time.

As is evident from the figures (1b and 2b) the concentration of each of the free amino acids in the upper half of the small intestine is quite similar in both types of animals. This may be due to several factors. The concentration of free amino acids in this region is much greater than in the stomach. Most of these amino acids are from protein of endogenous origin (19) because of the rapid absorption of dietary protein (5, 7). Exogenous protein is more digestible than endogenous protein (5, 7). Although the amounts of trypsin and chymotrypsin are greater in

| Region of gastrointestinal tract | Indole (nmol/g) | Ratio of indole to isoleucine |
|---------------------------------|-----------------|-----------------------------|
| Stomach                         | 4.8             | 0.025 ± 0.02                |
| Upper small intestine           | 1.9             | 0.00045 ± 0.00007           |
| Lower small intestine           | 3.6             | 0.00007 ± 0.0003            |
| Cecum                           | 40.1            | 0.14 + 0                    |
| Upper colon                     | 33.2            | 0.075 ± 0.007               |
| Lower colon                     | 54.5            | 0.11 ± 0.04                 |

**TABLE 1. Distribution of indole in various regions of the gastrointestinal tract of conventionalized mice**

**Fig. 2.** Molar ratio of individual free amino acids in the gastrointestinal contents of germfree compared to conventionalized mice. The dashed line represents a ratio of 1.0 (i.e., equal amounts of an amino acid in both types of mice). A, Stomach; B, upper small intestine; C, lower small intestine; D, cecum; E, upper colon; F, lower colon. The vertical bar represents one standard deviation on each side of the mean.
TABLE 2. Total concentration of free amino acids in the diet and in various regions of the gastrointestinal tracts of germfree and conventionalized mice

| Region          | Total free amino acids (nmol/g) | Germfree | Conventionalized |
|-----------------|---------------------------------|----------|------------------|
| Stomach         | 6,486                           | 6,891    |                  |
| Upper small intestine | 99,063                        | 116,652  |                  |
| Lower small intestine   | 37,692                         | 120,202  |                  |
| Cecum           | 11,652                          | 11,782   |                  |
| Upper colon     | 18,112                          | 12,634   |                  |
| Lower colon     | 14,515                          | 11,820   |                  |
| Diet            | 27,792                          |          |                  |

Germfree than in conventional mice (21), there is also little autodigestion of these digestive enzymes in this region. The presence of peptides arising from hydrolysis of dietary protein exert a protective effect on trypsin and chymotrypsin (23). In addition, the proteases themselves are least active in this region of the small intestine. Any amino acids which are released here are likely to remain because in the upper portion of the small intestine absorption of amino acids is not maximal (20).

The first point where the presence of microflora can be observed to have a pronounced effect on the free amino acid composition of the gut contents is the lower small intestine. The concentration of each of the amino acids is approximately three times greater in the conventionalized mice than in the germfree mice (Fig. 2c). However, the ratios of the amino acids to isoleucine were essentially the same in the two types of mice (Fig. 1c). The concentrations of the free amino acids in the gut contents of the conventionalized animals were the same in the lower small intestine as in the upper small intestine, but the concentrations in the lower small intestine of the germfree mice were three-fold lower than in the upper small intestine.

There are a variety of physiological parameters that may contribute to these differences. The lower concentrations in the distal small intestine of the germfree mice may reflect a greater rate of absorption of free amino acids by this region. A number of factors can influence the rate of absorption of amino acids by the gut, among which is water uptake (14). Since the water content of both the intestinal wall and the intestinal contents is greater in germfree than conventional mice (3), this may well have an effect on the concentration of amino acids remaining in the lumen of the intestine. There is a higher concentration of endogenous protein in the lower small intestine of germfree rats (21) which implies a greater ratio of undigestible protein, therefore a lower concentration of free amino acids.

Not only does the intestinal microflora exert an indirect effect on protein digestion, as evidenced by the work of Combe and Pion (4) in which the absence of flora retarded digestion of endogenous protein, but its presence also has a more direct effect. Marcus and Lengemann (13) showed that the digestion of endogenous protein occurs to a great extent in the ileum through the combined action of bacteria and the host's digestive enzymes. Loesche (12) also demonstrated that microbial enzymes are necessary for recovery of amino acids from endogenous protein.

The cecum is perhaps the best-studied region of the gastrointestinal tract as far as comparisons of germfree and conventional animals are concerned. Earlier workers have shown that the cecal contents of germfree mice weigh up to seven times as much as those of conventional mice and also contain 10 times as much total protein (11). The accumulation of free amino acids and water in the germfree cecum is in part due to the fact that its mucosa absorbs water and amino acids much more slowly than that of conventional animals, but excretion through the wall is similar (3). These observations are consistent with the data obtained in this study. We found that although the total amount of free amino acids in the cecum was several-fold higher in germfree animals, the overall concentration of free amino acids was approximately the same.

The much greater amount of free amino acids in the germfree cecum is also attributable to other factors. In conventional mice approximately 90% of the nitrogen in the lower small intestine is resorbed before reaching the cecum, but in germfree animals the nitrogen passes to the cecum (12). The nature of the material in the ceca of the two types of animals differs. In germfree mice the material represents primarily protein and nitrogen of endogenous origin, e.g., trypsin, chymotrypsin, and intestinal sloughings, whereas in conventional animals the cecal material is primarily undigested and unab sorbed residues (11). There are more free amino acids, degraded mucoproteins, small peptides, and urea in the cecum of germfree animals than in conventional animals (7, 11).

Our observation that the overall concentration of free amino acids is similar in the ceca of germfree and conventionalized animals but that the relative concentrations of the individual amino acids are markedly different (Fig. 1d and 2d) is consistent with the notion that the type of proteins present in the two kinds of mice...
are of significantly different nature. Also, the work of Bayley et al. (1), which indicates that amino acids may be fermented in the cecum of conventional animals to amines and ammonia, would explain further differences in the types of amino acids present in germfree as opposed to conventionalized mice. One example of the microbial transformation of a free amino acid to a compound that can no longer be utilized by the host is the conversion of tryptophan to indole. Intestinal microbes are also capable of converting tryptophan to skatole and indoleacetic acid (22, 25, 27), but because the host tissues are capable of carrying out the same conversion, we were interested in the conversion of tryptophan to indole since this reaction can only be carried out by the bacteria in the gut, not by the host tissues. A considerable amount of indole is formed in the cecum of conventionalized mice (Table 1). No indole was present in the cecum of germfree mice.

Although the contents of the upper and lower colon were analyzed separately, the data obtained from the two regions were so similar for each type of mouse that they will be considered together (see Fig. 1e, f, 2e, f). The overall concentration of free amino acids was essentially the same in both types of mouse, as well as being quite similar to that of the cecum. The latter observation is not surprising in view of the fact that proteolytic activity is approximately the same in the cecum and colon (10); the observation is also consistent with the data of Cho and Bayley (2) which showed that the protein concentration was the same in the cecum and colon.

The different distribution of free amino acids in the two types of mice is probably due to several factors. Not only does the microflora cause modification of some nitrogenous compounds in this region of the intestinal tract (7), but several amino acids are resistant to fermentation by microflora removed from this part of the digestive tract (15). The microflora is also capable of anabolizing certain forms of nitrogen, including certain amino acids, into bacterial protein (9), thus changing the pattern of free amino acids. Also, as mentioned in the discussion of the data from the cecum, the microflora is capable of transforming significant amounts of tryptophan in the colon to indole (Table 1).

In general, the data presented in this paper indicate that the changes in free amino acid content and distribution produced by the microflora in different regions of the gastrointestinal tract of mice are great enough to make feasible a study of the interactions of a limited number of species of microbes in gnotobiotic animals and to assign probable specific functions to each species. The significant production of indole in the lower regions of the gut is of specific interest. This metabolic conversion will serve as a good probe for studying not only in vivo microbial interactions but also the interaction between the intestinal microflora and the host animal.

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