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3-Methylindole (Skatole) and Indole Production by Mixed Populations of Pig Fecal Bacteria

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Pig fecal slurries converted added L-tryptophan either to indole without detectable intermediates or to 3-methylindole (skatole) via indole-3-acetate. The initial rate of production of 3-methylindole was greatest at pH 6.5 and less at pH 5.0 and 8.0; the initial rates of indole production were similar at pH 6.5 and 8.0. More than 80% of the tryptophan added was converted to 3-methylindole at pH 5.0; at pH 8.0 85% was converted to indole. Both pathways had similar K_m values for tryptophan and similar maximum rates. Indole-3-carbinol and indole-3-acetonitrile completely inhibited the production of 3-methylindole from indole-3-acetate but had no effect on the reactions involving L-tryptophan.

Anoxic metabolism of L-tryptophan can lead to the production of two alternative volatile lipophilic compounds, indole and 3-methylindole (skatole). These compounds are produced in the rumen and in the ceca and colons of monogastric animals by microbial degradation of L-tryptophan which originates from the diet or from material derived from the upper part of the intestine (20). The production of 3-methylindole has practical consequences in farm animal production since this compound accumulates in the fat of uncastrated male pigs and contributes to an unpleasant taste and odor in cooked boar meat (15).

The production of 3-methylindole in the rumen has been extensively studied (1a), whereas monogastric animals have been almost ignored. The D isomer of tryptophan is not degraded, and the L isomer can be either degraded directly to indole or converted to indole-3-acetic acid and then to 3-methylindole (19). The reactions are mediated by bacteria and are sensitive to antibiotics (5, 10, 19).

Many types of intestinal bacteria are capable of producing indole from L-tryptophan. In contrast, production of 3-methylindole has been reported in strains of only two of the genera containing common intestinal bacteria, the genera *Clostridium* and *Lactobacillus*. A *Lactobacillus* strain that produces 3-methylindole has been isolated from a bovine rumen and has been partially characterized; this organism forms 3-methylindole by decarboxylating indole-3-acetic acid, but is not able to form 3-methylindole directly from L-tryptophan (21, 22). Other bacteria that have been reported to produce 3-methylindole are a strain of *Lactobacillus helveticus* (14), *Clostridium scatologenes*, and *Clostridium nauseum* (4, 16, 18). In contrast to the *Lactobacillus* rumen strain and two unidentified isolates obtained from pig intestines (10a), *C. scatologenes* DSM 757 was able to generate 3-methylindole from L-tryptophan (1).

The aim of this study was to investigate the kinetics of formation of indole and 3-methylindole by a mixed bacterial population from the large intestines of pigs by using in vitro techniques.

Incubation of fecal slurries. Fresh feces (total weight, 200 g) were collected from pigs fed a standard barley-soy bean-based diet and were suspended in sterile anaerobic mineral salt medium which contained (per liter) 5.0 g of $NaHCO_3$, 0.9 g of NaCl, 0.9 g of $(NH_4)_2SO_4$, 0.45 g of KH_2PO_4, 0.45 g of $K_2HPO_4 \cdot 3H_2O$, 0.03 g of $CaCl_2 \cdot 2H_2O$, 0.02 g of $MgCl_2$, 0.01 g of $MnSO_4 \cdot 4H_2O$, 0.01 g of $CoCl_2 \cdot 6H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 1.0 g of cysteine to give a 10% (wt/vol) fecal slurry. This suspension was transferred to a CO_2-flushed sterile plastic bag and homogenized with a stomacher (Seward Medical, London, United Kingdom) under CO_2 for 2 min. The resulting homogenate was filtered through sterile cheesecloth to remove crude particulate material. All further manipulations were done under a constant flow of O_2-free carbon dioxide.

Sterile serum bottles (working volume, 125 ml) were inoculated with 20 ml of the 10% fecal slurry. The bottles were sealed with sterile butyl rubber stoppers kept in place with metal screw caps. Immediately after inoculation the gas phase of each serum bottle was changed to O_2-free CO_2 by three successive cycles of evacuation and refilling with CO_2 in which we used a manifold connected to a vacuum pump and a cylinder of CO_2. The serum bottles were connected to the manifold through 18-gauge needles. The CO_2 was scrubbed free of O_2 by passing it over a hot catalyst (type R3-11; BASF). The bottles were removed from the manifold under a slight overpressure of CO_2 and placed in a reciprocating shaker (60 rpm, 5-cm throw) in a water bath at 38°C, the pressure was adjusted to atmospheric pressure by using a 25-gauge needle, and substrate and/or inhibitors were added as indicated below. Samples were periodically removed from the bottles with plastic syringes, and pH and 3-methylindole and indole contents were measured by gas chromatography or high-performance liquid chromatography (HPLC).

For measurements at constant pH values 1,800-ml aliquots of 10% (wt/vol) fecal slurries were added to bioreactors (9) with a working volume of 2.0 liters. L-Tryptophan or indole-3-acetic acid was added to a final concentration of 250 μM. The incubation temperature was maintained at 38°C by using a circulating water bath. The culture pH was maintained at the desired value by using a pH controller, 5 M NaOH, and 5 M HCl. Anoxic conditions were maintained by sparging cultures with high-purity nitrogen gas.

To measure the activities in different fractions, feces were fractionated by centrifugation at 20,000 × *g* for 10 min. The supernatant was removed, and the pelleted material was resuspended in salt medium so that the volume was the same as the

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supernatant volume. The fractions and the original fecal suspension were incubated as described above. L-Tryptophan and indole-3-acetic acid were each added at a concentration of 50 mg/liter.

Analysis of indole compounds. For the gas chromatography analysis, 1 ml of medium was transferred to a centrifuge tube with a conical bottom, diluted with 1 ml of distilled water, and made alkaline with 2 drops of 4 M NaOH. An internal standard (50 μl of 5-methylindole [100 mg/liter] in methanol) was added, and 1 ml of chloroform was added for extraction. The mixture was vortex mixed until an emulsion was formed. To break the emulsion, centrifugation at 2,700 × *g* for 10 min in a bench centrifuge was necessary. The chloroform phase was transferred to autosampler vials for gas chromatography. Analyses were performed as described by Jensen and Jensen (13).

For the analysis of nonvolatile indole compounds by HPLC, 1 ml of medium was transferred to a centrifuge tube with a conical bottom, 1.94 ml of HPLC grade methanol was added, and indole-2-carboxylic acid and 2-methylindole (60-μl portions of 1-mg/ml stock solutions in methanol) were added as internal standards. The mixture was vortex mixed, placed in a freezer at −21°C for 15 min to accelerate precipitation of the particulate matter, and centrifuged at 2,700 × *g* for 10 min in a bench centrifuge. A 1-ml sample was transferred to a 1.5-ml microtube and centrifuged in a bench centrifuge at 15,000 × *g* for 10 min. A 125-μl sample was transferred to a fresh tube, and 4.875 ml of high-purity water containing 550 μl of 5% chlorhexidine (HIBITAN; ICI PHARMA) per liter as a preservative was added. The chromatographic conditions used were the conditions described by Hansen-Møller (6).

Metabolism of added L-tryptophan and indole-3-acetic acid in different fractions obtained from fecal slurries. To clarify the reaction pathways for the conversion of L-tryptophan to indole and 3-methylindole, fecal slurries were incubated either with no addition or with L-tryptophan or indole-3-acetic acid added at a concentration of 50 μg/g, which corresponded to the mean concentration of 3-methylindole in feces. The L-tryptophan concentration decreased rapidly at a constant rate to zero within 3 h (Fig. 1), while the indole-3-acetic acid concentration increased transiently to a maximum value at 2 h, which was followed by a decrease at 6 h. The indole concentration increased rapidly at a constant rate to a maximum value at 5 h and then decreased slightly. Production of 3-methylindole started with a lag phase that was followed by a more rapid burst and then slower production. The maximum rate of production of 3-methylindole was lower than the maximum rate of production of indole. When fecal slurries were incubated with indole-3-acetic acid (Fig. 1), the production of 3-methylindole was practically the mirror image of the degradation of indole-3-acetic acid, and no production of indole was observed. Production of indole-3-propionic acid was not observed in any experiment.

The resuspended pellets obtained after centrifugation at 20,000 × *g* and the original fecal suspension exhibited the same level of activity and essentially the same kinetics for the breakdown of L-tryptophan and the degradation of indole-3-acetic acid to 3-methylindole. The supernatants exhibited much lower levels of activity. These observations demonstrate that the reactions which occurred were the result of microbial activity. The same pattern of degradation of L-tryptophan and indole-3-acetic acid to 3-methylindole and indole was found in incubation mixtures containing feces from several different pigs (data not shown).

In all cases the sum of the total amounts of indole compounds detected was constant or decreased only slightly, showing that appreciable conversion to other metabolites (for example, metabolites involving breakdown of the indole group) did not occur to an appreciable extent. Our results show that the metabolism of L-tryptophan by the bacterial populations in pig large intestines is similar to the metabolism of L-tryptophan in rumina (19) and results in the formation of two volatile products, indole and 3-methylindole. Which of these compounds is produced depends on the primary fate of L-tryptophan, which may be converted to indole or deaminated to indole-3-acetic acid. Indole-3-acetic acid did accumulate as a transient intermediate under the conditions used in our experiments, but it was finally converted quantitatively to 3-methylindole.

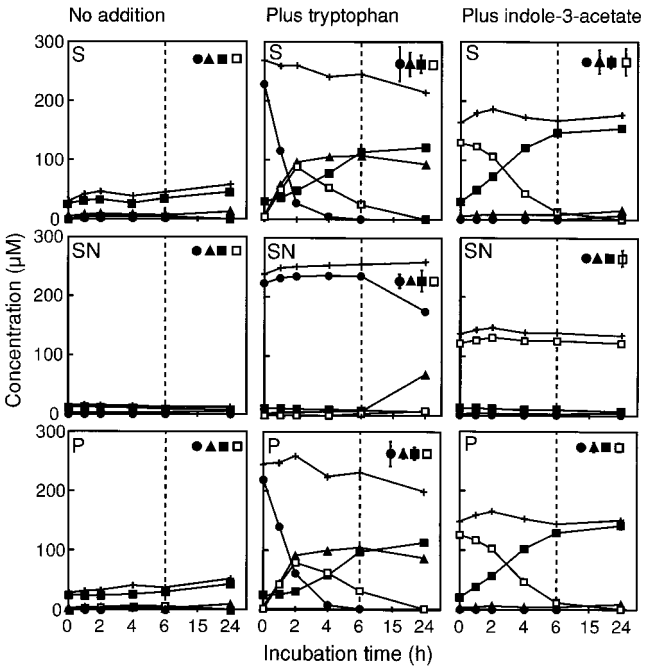

FIG. 1. Production of indole compounds by different fractions from pig fecal slurries in the absence of added substrate and after addition of L-tryptophan and indole-3-acetic acid. The pH ranged from pH 6.0 to 7.0. Analyses were performed by using HPLC. Each point is the mean from two replicate incubations in which the slurries were derived from the same sample of feces. Error bars are shown for each component and indicate to the maximum difference between the two values obtained. S, untreated suspension; SN, supernatant; P, pellet after centrifugation at 20,000 × *g* for 10 min. Symbols: ●, L-tryptophan; ▲, indole; ■, 3-methylindole; □, indole-3-acetic acid; +, total indole compounds.

Effect of pH. The effects of different pH values on microbial production of 3-methylindole from indole-3-acetic acid were investigated by using bioreactors in which the pH was kept constant. The highest rate of production was observed between pH 6.0 and 7.0, and less than one-half of the maximal activity was observed at pH 5.0 or 8.0 (data not shown). The effects of pH on the amount of L-tryptophan converted to 3-methylindole and indole were investigated at pH 5.0, 6.5, and 8.0, pH values which cover the normal pH range in the ceca and colons of pigs. The results are shown in Fig. 2. The pH had dramatic effects on the relative production of indole and 3-methylindole. At pH 6.5, 40% of the L-tryptophan was converted to 3-methylindole and 60% was converted to indole after 24 h of incubation. At pH 5.0, 80% of the added L-tryptophan was converted to 3-methylindole and 20% was converted to indole, while at pH 8.0 only 15% of the added L-tryptophan was converted to 3-methylindole and 85% was converted to indole. Our results show that the relative amounts of the two end products can be altered by changing the environmental condi-

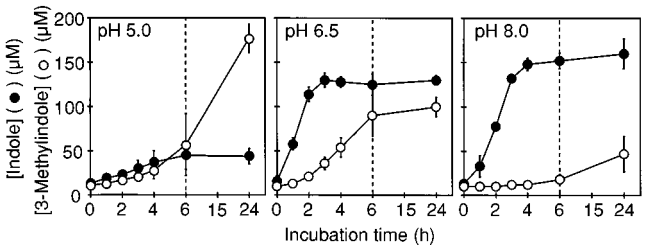

FIG. 2. Production of indole and 3-methylindole from added L-tryptophan by pig fecal slurries incubated in bioreactors at constant pH values. The initial concentration of L-tryptophan was 250 μM. Analyses were performed by gas chromatography. Each point shows the mean and standard deviation from three independent experiments performed with fecal samples from different pigs fed the same diet. Symbols: ●, indole; ○, 3-methylindole.

tions. High pH values favor the production of indole, while low pH values favor the production of 3-methylindole.

Kinetics of indole and 3-methylindole formation. Figure 3 shows the effect of substrate concentration on the initial rate of conversion of indole-3-acetic acid to 3-methylindole and the initial rate of conversion of L-tryptophan to indole or indole-3-acetic acid (as measured by the production of indole-3-acetic acid plus 3-methylindole). All three reactions can be fitted to hyperbolic kinetics. Table 1 summarizes the Michaelis-Menten constants obtained in several experiments in which we incubated slurries of feces obtained from different pigs fed the same standard barley-soy bean diet. The K_m values for the two reactions involving L-tryptophan are similar. The maximum rates of metabolism (V_{max} values) are close to the V_{max} for the conversion of indole-3-acetic acid to 3-methylindole, but this reaction has a higher K_m for the substrate than the two reactions involving L-tryptophan, which is consistent with the observation that indole-3-acetic acid accumulates as a transient intermediate during incubation. The kinetic parameters for the three reactions that we were interested in varied considerably with fecal slurries obtained from different experimental animals, but the general pattern is clear. Under in vivo conditions the steady-state concentration of L-tryptophan is normally much lower than the observed values for K_m, so the rates are

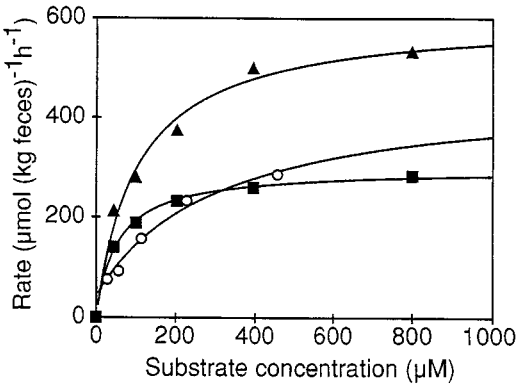

FIG. 3. Effect of substrate concentration on the production of indole and 3-methylindole by pig fecal slurries. The pH ranged from pH 6.0 to 7.0. Analyses were performed by HPLC. The lines represent the best fit of the experimental points to the Michaelis-Menten equation. Symbols: ○, indole-3-acetic acid to 3-methylindole (the best fit values were K_m = 105 μM and V_{max} = 610 μmol kg of feces^{-1} h^{-1}); ▲, L-tryptophan to indole (the best fit values were K_m = 55 μM and V_{max} = 300 μmol kg of feces^{-1} h^{-1}); ■, L-tryptophan to indole-3-acetic acid (measured as the sum of the production of indole-3-acetic acid and the production of 3-methylindole; the best fit values were K_m = 310 μM and V_{max} = 440 μmol kg of feces^{-1} h^{-1}).

TABLE 1. Kinetic parameters for reactions in the metabolism of tryptophan to indole and 3-methylindole by pig fecal slurries[a]

Reaction	K_m (μM)		V_{max} (μmol kg of feces^{-1} h^{-1})	
	Median	Interquartile range	Median	Interquartile range
Indole-3-acetate→3-methylindole	136	78–168	450	295–525
L-Tryptophan→indole	34	28–76	415	295–850
L-Tryptophan→indole-3-acetate[b]	52	25–88	580	400–875

[a] Values for fecal samples from at least eight pigs fed the same diet.
[b] Values were determined by measuring the production of indole-3-acetate plus the production of 3-methylindole.

determined by k_{cat} (V_{max}/K_m) and are limited by the amount of L-tryptophan available. The k_{cat} values are similar for the two reactions involving L-tryptophan and are about three times lower for the reaction in which indole-3-acetic acid is the substrate. This allowed us to predict that indole-3-acetic acid should accumulate in situ at steady-state concentrations that are somewhat higher than the steady-state concentration of L-tryptophan.

Our data for the activity of indole-3-acetic acid decarboxylation by mixed populations of pig intestinal bacteria can be compared with the data obtained by Honeyfield and Carlson (8) by using a particulate preparation from a *Lactobacillus* strain capable of converting indole-3-acetic acid to 3-methylindole. These authors obtained similar pH profiles for activity, with maximum activity at pH 7.5 rather than between pH 6.5 and 7.0 as in our results, and obtained a K_m of 140 μM, which is within the range of values which we obtained.

Metabolism of indole derivatives. To investigate whether other indole derivatives were capable of acting as substrates for the formation of 3-methylindole or indole, we tested a series of commercially available compounds with different side chains at position 3 (Table 2). L-Tryptophan, indole-3-acetic acid, and indole-3-pyruvate were substrates for the formation of indole and 3-methylindole, whereas indole-3-aldehyde, indole-3-carbinol, indole-3-acetonitrile, and 3-indolylacetate inhibited the formation of 3-methylindole.

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TABLE 2. Effects of indole compounds on 3-methylindole and indole production by pig fecal slurries

Compound[a]	Rate of production (μmol kg of feces^{-1} h^{-1}) of:	
	3-Methylindole	Indole
None	10	20
Indole-3-acetic acid	333	34
L-Tryptophan	125	343
D-Tryptophan	10	20
Indole-3-pyruvate	63	129
3-Dimethylaminomethylindole	22	0
Indole-3-lactate	20	34
Indole-3-carboxylic acid	12	26
Indole-3-ethanol	10	20
Indole-3-propionate	9	23
Indole-3-aldehyde	3	40
3-Indolylacetate	0	55
Indole-3-carbinol	0	26
Indole-3-acetonitrile	0	20

[a] All compounds were added at a concentration of 50 mg/liter.

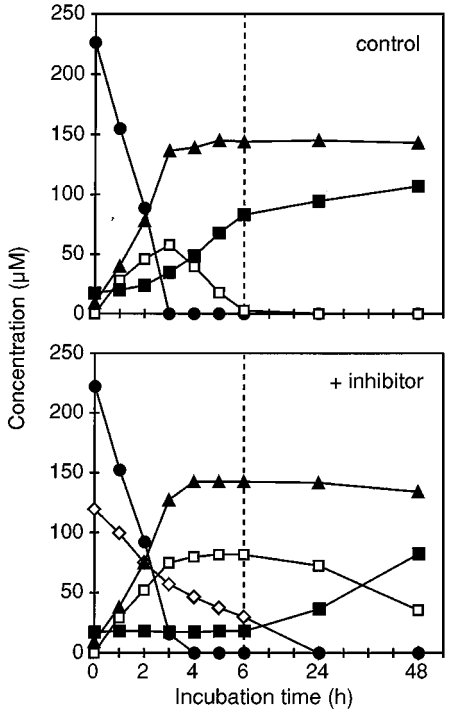

FIG. 4. Effect of indole-3-carbinol on the metabolism of added L-tryptophan by pig fecal slurries. Indole-3-carbinol was added at an initial concentration of 340 μM. The pH was 6.5. Analyses were performed by HPLC. Symbols: ●, L-tryptophan; ▲, indole; ■, 3-methylindole; □, indole-3-acetic acid; ◇, indole-3-carbinol.

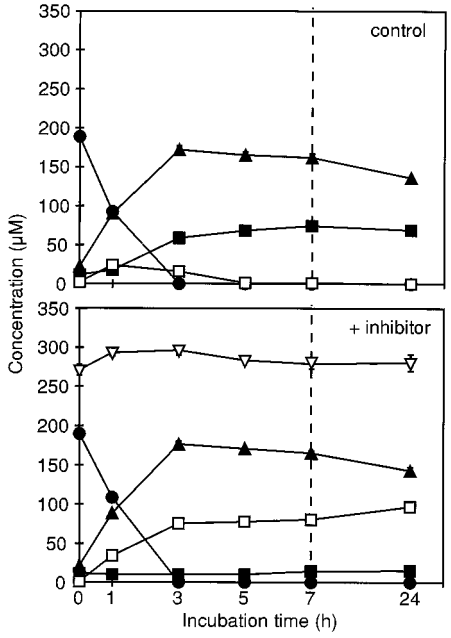

FIG. 5. Effect of indole-3-acetonitrile on the metabolism of added L-tryptophan by pig fecal slurries. Indole-3-acetonitrile was added at an initial concentration of 320 μM. The pH ranged from pH 6.0 to 7.0. Analyses were performed by HPLC. Results are the means and standard deviations from three replicate incubations in which the same feces sample was used. Symbols: ●, L-tryptophan; ▲, indole; ■, 3-methylindole; □, indole-3-acetic acid; ▽, indole-3-carbinol.

indole-3-pyruvic acid, and the metabolism of indole-3-propionic acid were studied in more detail by determining the metabolite concentrations by HPLC. During a 24-h incubation, indole-3-lactate at an initial concentration of 250 μM was converted to indole-3-propionate (77%), indole (8%), and 3-methylindole (5%). The same concentration of indole-3-pyruvate was converted to indole-3-propionate (43%), indole (15%), and 3-methylindole (14%) in 24 h. Indole-3-propionic acid was not metabolized during a 24-h incubation.

Indole-3-pyruvic acid is a potential intermediate in the conversion of L-tryptophan to indole-3-acetic acid. In our experiments added indole-3-pyruvic acid and indole-3-lactic acid were used preferentially as reductant sinks, resulting in the production of indole-3-proprionic acid, which was not further metabolized over a 24-h period. Since indole-3-propionic acid was not observed as a breakdown product of L-tryptophan, it is clear that L-tryptophan did not function as the electron acceptor in a Stickland reaction under our conditions. Our results are in contrast to those of Chung et al. (2), who found that indole-3-pyruvic acid accumulated as an intermediate in the conversion of L-tryptophan to indole-3-acetic acid by rat feces and two isolated strains of intestinal bacteria. Our results are still compatible with the occurrence of indole-3-pyruvic acid as an intracellular intermediate in the metabolism of L-tryptophan.

Gramine (3-dimethylaminomethylindole) occurs naturally in several plant species (17) and can reach concentrations as high as 10 mg/g (dry weight) in barley shoots (7). Since barley is a common ingredient of pig feed, our observation that this compound increases the production of 3-methylindole may be of practical significance.

Inhibition of the conversion of L-tryptophan to indole-3-acetic acid, 3-methylindole, and indole by indole derivatives. As shown in Table 2, indole-3-carbinol inhibited the production of 3-methylindole but did not affect the production of indole. We studied the effect of this inhibition in more detail (Fig. 4) and found that indole-3-carbinol had no effect on the disappearance of L-tryptophan, but inhibited the conversion of indole-3-acetic acid to 3-methylindole. Indole-3-carbinol is unstable, especially at low pH values. Control experiments without added L-tryptophan (data not shown) demonstrated that indole-3-carbinol was not converted to a detectable indole derivative. It is noteworthy that the production of 3-methylindole recovered as soon as the indole-3-carbinol had disappeared.

Increasing amounts of indole-3-carbinol result in increased inhibition of the reaction from indole-3-acetic acid to 3-methylindole; the degree of inhibition depends on the concentration of indole-3-acetic acid present. With 450 μM indole-3-acetic acid, 35 μM indole-3-carbinol inhibited the reaction by 65% and 140 μM caused 90% inhibition. Since indole-3-carbinol was unstable during incubation, it was not possible to draw any conclusions about the type of inhibition from the results.

The effect of addition of indole-3-acetonitrile on the metabolism of L-tryptophan is shown in Fig. 5. Indole-3-acetonitrile resembles indole-3-carbinol as it inhibits the reaction step between indole-3-acetic acid and 3-methylindole. From our results it is clear that indole-3-acetonitrile is much more stable than indole-3-carbinol, and production of 3-methylindole was completely inhibited throughout the 24-h incubation.

It is desirable to minimize the amount of 3-methylindole produced in pigs since accumulation of this compound in the fat has negative effects on the quality of the meat. Our observation that indole-3-carbinol and indole-3-acetonitrile act as specific inhibitors of indole-3-acetic acid decarboxylation opens the possibility of inhibiting 3-methylindole production by using these compounds as dietary supplements. Indole-3-

acetonitrile seems to be the more useful of the two compounds because of its greater stability. Alternatively, our results show that at least in principle, 3-methylindole production can be decreased by raising the pH of the intestinal contents. Claus et al. (3) have claimed that raising the pH of the intestinal contents of pigs by adding bicarbonate to the diet reduces the amount of 3-methylindole in feces and backfat, although experimental details are lacking. However, we have observed that addition of dietary constituents which increase microbial activity in the colon, such as carbohydrates which are not taken up in the small intestine, decreases the intestinal production of 3-methylindole even though the pH of the intestinal contents is lower because of increased production of volatile fatty acids (11, 12). In this case the increased biomass production and the availability of alternative substrates lead to a decrease in L-tryptophan breakdown which more than compensates for the effects of the lower pH.

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