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Dissimilation of Tryptophan and Related Indolic Compounds by Ruminal Microorganisms In Vitro[1]

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Intraruminal doses of L-tryptophan cause acute pulmonary edema and emphysema in cattle. The D and L isomers of tryptophan and 22 related indolic compounds were incubated with ruminal microorganisms in vitro. Incubation of L-[U-benzene ring-^{14}C]tryptophan with ruminal microorganisms for 24 h resulted in 39% of the added radioactivity being incorporated into skatole, 7% into indole, and 4% into indoleacetate (IAA). D-Tryptophan was not degraded to any of these metabolites. The major pathway of skatole formation from L-tryptophan appeared to be by the decarboxylation of IAA. Incubation of [2-^{14}C]IAA with ruminal microorganisms for 24 h resulted in 38% incorporation into skatole. L-[5-Hydroxy]tryptophan was degraded to 5-hydroxyskatole and 5-hydroxyindole, whereas 5-hydroxyindoleacetate was degraded to only 5-hydroxyskatole. Incubation of indolepyruvate, indolelactate, and indolealdehyde with ruminal microorganisms resulted in the formation of both skatole and indole. Under similar conditions, indoleacetaldehyde was converted to IAA and tryptophol. The addition of increasing concentrations of glucose (0 to 110 mM) reduced the formation of both skatole and indole from L-tryptophan and resulted in the accumulation of IAA. Antibiotics reduced the degradation of L-tryptophan to skatole and indole, with kanamycin and neomycin particularly effective in reducing the decarboxylation of IAA to skatole.

Previous studies on the ruminal degradation of tryptophan have established that several metabolites are formed by ruminal microorganisms. Indole, indoleacetate (IAA), and indolepropionate were detected in washed cell suspensions of ruminal microorganisms incubated with tryptophan (22). Short incubations of L-[3-methylene-^{14}C]tryptophan with ruminal microorganisms resulted in the radioactivity being incorporated into IAA (30). Indole and skatole were formed in vitro and in vivo from DL-tryptophan (23, 24). More recently, tryptamine has been detected in in vitro incubations of DL-tryptophan with ruminal microorganisms (29). Several studies also report the natural occurrence of low concentrations of indole and skatole in ruminal fluid (9, 24, 31).

Renewed interest in the ruminal metabolites of tryptophan degradation was prompted by recent studies that demonstrate that the intraruminal administration of large doses of tryptophan or IAA will induce pulmonary edema and emphysema in cattle (10, 11, 14). Because tryptophan induces the disease when it is given intraruminally but not when given intravenously or intraperitoneally, it suggested that a metabolite of tryptophan fermentation by ruminal microorganisms might be the causative agent (10). This investigation was conducted to identify ruminal metabolites of tryptophan which may be responsible for pulmonary edema and emphysema in cattle.

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MATERIALS AND METHODS

Preparation of ruminal fluid. Ruminal fluid used in the in vitro studies was obtained from two steers maintained on an alfalfa-grass hay diet. About 1 liter of fluid was withdrawn 4 h after the morning feeding and transported to the laboratory in vacuum bottles. The fluid was strained through four layers of cheesecloth to remove debris and used immediately in the experiments.

Incubation conditions. Incubations were conducted in 50-ml Erlenmeyer flasks fitted with rubber sleeves. The substrates (5 to 10 mg) were dissolved in 1 ml of 0.1 M NaOH and added to the incubation flask along with 24 ml of strained ruminal fluid. Addition of the NaOH increased the pH of the ruminal fluid to 7.2 to 7.6, but the pH returned to normal (6.8) within an

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hour. Each flask was flushed for 30 min by inserting a syringe needle, attached to a cylinder of carbon dioxide, through the rubber sleeve and using another needle as an exhaust. After the flasks were flushed with CO_2, the exhaust needle was removed first, and positive pressure was attained by removing the gassing needle. Incubations were conducted at 37 C in a reciprocal water bath shaker for 24 h. Substrate controls, using both boiled (20 min) ruminal fluid and distilled water, were incubated at the same time.

Incubations were terminated by the addition of three drops of 85% phosphoric acid. The entire incubation mixture was centrifuged at 20,000 × *g* for 30 min. The pellet was washed twice with 10 ml of 0.05 M potassium phosphate buffer (pH 7.0) and recentrifuged. The two washings were combined with the original supernatant and saved for analysis. The final pellet was discarded. When ^{14}C-labeled substrates were incubated, the pellet fraction contained less than 2% of the initial radioactivity.

Extraction of metabolites. Indolic metabolites were extracted with methylene chloride from the supernatant under acidic, basic, and neutral conditions (27). The pH of the supernatant (45 ml) was adjusted to 2.0 with 85% phosphoric acid and extracted three times with 3 volumes of methylene chloride to remove the neutral and acidic indolic metabolites. The basic metabolites were not extracted into the organic phase. The methylene chloride phase was drawn off and washed three times with distilled water adjusted dropwise to pH 8.0 with saturated K_2CO_3. The acidic metabolites partitioned into the aqueous phase. Readjustment of the pH for the two remaining aqueous phases permitted the extraction of the basic and acidic metabolites by methylene chloride. The separated acidic, basic, and neutral fractions were taken to dryness in a warm water bath (35 C) by using a rotary evaporator and brought back to a volume of 5 ml with methylene chloride.

Thin-layer chromatography. Acidic, basic, and neutral methylene chloride fractions were spotted on precoated Silica Gel G plates (20 by 20 cm) (E. Merck AG, Darmstadt, Germany). The solvent systems used for development were: 2-propanol-ammonia-water (20:1:2 vol/vol) (solvent 1), 1-butanol-acetic acid-water (12:3:5 vol/vol) (solvent 2), benzene-chloroform (6:94 vol/vol) (solvent 3), and cyclohexane-chloroform-diethylamine (4:5:1 vol/vol) (solvent 4). Skatole and indole, inseparable in the acidic (solvent 1) and basic (solvent 2) solvent systems, could be partially resolved in 6% benzene-chloroform (solvent 3) and completely separated in the cyclohexane-chloroform-diethylamine system (solvent 4) (18). Three 1-h developments of the same chromatographic plate with solvent 4 gave a retardation factor (R_f) value of 0.65 for skatole and 0.54 for indole.

Indolic metabolites were located with several reagent sprays: Ehrlich, Van den Bergh, Pauly, Van Urk, and Gibb (18, 21, 26). Color reactions with these reagents for the various indolic metabolites mentioned in the text are: blue, brown, blue green, blue gray, gray brown, orange, purple, purple brown, pink, red, red brown, violet, violet brown, yellow, and yellow brown. Identification was based on the R_f values of the metabolite in the four solvent systems and its color reactions with the various reagent sprays against authentic compounds spotted on the same chromatographic plate.

The presence of oxindole, catechol, and other phenolic compounds in the L-tryptophan and IAA incubations was tested by using thin-layer chromatography (TLC) in two solvent systems: benzene-methanol-acetic acid (45:8:4 vol/vol) and chloroform-methanol-water-formic acid (100:20:19:0.8 vol/vol) (13). Reagent sprays used for detection were: nitrite-molybdate reagent (2), Gibb reagent (21), and 6% ferric chloride-ferric cyanide reagent (5).

When ^{14}C-labeled substrates were used, metabolites were first visualized on chromatograms with Ehrlich reagent and then scraped into scintillation vials containing 10 ml of Bray solution (8). A Nuclear Chicago-Corp. liquid scintillation spectrometer (model 720) was used. Counting efficiency determined by using [^{14}C]toluene as the internal standard was between 40 to 60%, depending on the color complex formed between the indolic compounds and Ehrlich reagent.

Gas-liquid chromatography. Skatole and indole were determined by gas-liquid chromatography (GLC) by using a Beckman (model GC-5) gas chromatograph equipped with a hydrogen flame ionization detector (7). A stainless-steel column (2 m by 2 mm) packed with 20% Carbowax 4000 on Chromosorb W, DMCS, (60 to 80 mesh) was used. An isothermal column temperature of 180 C was used with helium as the carrier gas. The peak area determined by the height times width at half-height was measured against a standard curve established with authentic skatole and indole.

Preparation of protozoa enrichments. Crude protozoa enrichments were obtained by sedimentation and low-speed centrifugation of ruminal fluid (12). Freshly collected ruminal fluid (1 liter) was allowed to stand for 1 h in 250-ml separatory funnels kept at 38 C. The sedimented protozoa were drawn off, and the remaining ruminal fluid was centrifuged at 121 × *g* for 10 min to bring down the smaller protozoa. The two protozoal fractions were combined, washed, and resuspended four times in a buffer solution containing 0.1% $NaHCO_3$, 0.5% NaCl, 0.01% $MgSO_4$, 0.01% $CaCl_2$, and 0.1% KH_2PO_4 (17). The protozoa were divided into two 24-ml portions and incubated in the same buffer solution with or without penicillin and streptomycin. Twenty-four ml of the original ruminal fluid and the centrifuged ruminal fluid, freed of protozoa, were incubated at the same time. Incubation was for 24 h at 37 C under CO_2.

Skatole and indole in ruminal fluid in vivo. Ruminal fluid (50 ml) was collected just before feeding and at 4-h intervals over a period of 24 h from two steers fed a hay diet once daily. The fluid was strained through cheesecloth, and the fine debris that passed through the cloth was permitted to settle to the bottom. Two ml of ruminal fluid essentially free of debris was extracted three times with 20 ml of methylene chloride by using a Burrell shaker. Three extractions were sufficient to remove all of the skatole and indole as determined by TLC and GLC of

ruminal fluid spiked with a standard skatole and indole mixture. The extract was evaporated to approximately 1 ml over a water bath (25 C) with a rotary evaporator. The concentrated extract was transferred to a 3-ml conical test tube with four 0.5-ml washes of methylene chloride and evaporated at room temperature to 0.1 ml with N_2 gas. Samples of 2 μliters were analyzed for skatole and indole by GLC as described above.

Substrates. DL-[U-benzene ring-^{14}C]tryptophan (95 mCi/mmol) and [2-^{14}C]IAA (58 mCi/mmol) were obtained from Amersham-Searle Corp., Arlington Heights, Ill. L-[U-^{14}C]tryptophan (592 mCi/mmol) was from New England Nuclear Corp., Boston, Mass. Examination by TLC in two solvent systems showed these compounds to have a radiochemical purity of greater than 98%. In some instances L-[U-benzene ring-^{14}C]tryptophan was obtained from DL-[U-benzene ring-^{14}C]tryptophan by paper chromatography, using a solvent system of methanol-1-butanol-benzene-water (20:10:10:1 vol/vol), modified by substituting 1-butanol for 1-pentanol (25). 5-Hydroxyskatole was chemically prepared from skatole (19).

L-Tryptophan, D-tryptophan, IAA, and other indolic substrates were obtained from several commercial sources and were of reagent grade.

RESULTS

In vitro incubations with indolic compounds. Table 1 shows the R_f values and color reactions of identifiable metabolites detected in the 24-h incubations of L-tryptophan and other indolic compounds with ruminal microorganisms in vitro. L-Tryptophan was degraded to three indolic metabolites identified from their R_f values and color reactions in solvents 1, 2, and 4 as skatole, indole, and IAA (Table 1). Incubation of L-[U-benzene ring-^{14}C]tryptophan with ruminal microorganisms and analysis by TLC in solvent 4 confirmed that these are the major metabolites of L-tryptophan degradation (Fig. 1). Skatole and indole were not degraded to oxindole, catechol, or other phenolic compounds by ruminal microorganisms. No other metabolites were detected in the acidic and neutral extracts when using the various solvent systems and detection sprays. The basic extract from the L-tryptophan incubations showed a faint spot on chromatographic plates corresponding to tryptamine when sprayed with Ehrlich reagent. When L-[U-benzene ring-^{14}C]tryptophan was incubated, no radioactivity was detected in this spot.

When 5-hydroxy-L-tryptophan was incubated with ruminal fluid, two metabolites with R_f values and color reactions distinctly different from skatole and indole were formed. One of these metabolites resulted in an immediate blue-green color with Ehrlich reagent, turning dark blue after 24 h. Its R_f values and color reactions were similar to that of synthetic 5-hydroxyskatole. By comparison with authentic compounds, the other metabolite formed from 5-hydroxy-L-tryptophan was identified as 5-hydroxyindole. Although 5-hydroxyindoleacetate was not detected as an intermediate in the incubation of 5-hydroxy-L-tryptophan, the incubation of this compound with ruminal microorganisms resulted in its further degradation to 5-hydroxyskatole.

IAA was degraded to a neutral, Ehrlich-positive metabolite with R_f values and color reactions identical to skatole. The metabolite was extracted from incubations under neutral conditions and was shown to also have the same retention time as skatole with GLC. Incuba-

TABLE 1. *R_f values and color reactions of L-tryptophan and metabolites detected in the incubations of indolic compounds with ruminal microorganisms in vitro*

Compounds	R_f values[a]				Color reactions[b]				
	Solvent 1	Solvent 2	Solvent 3	Solvent 4	Ehr	VB	Pa	VU	G
Skatole	0.68	0.76	0.60	0.65	P → B	Y	RBr	Y	BGy → Br
Indole	0.67	0.74	0.54	0.54	PK	RBr	Y	Or	RBr
IAA	0.22	0.70	0.00	0.00	P	R	B	P	GyBr
5-Hydroxyskatole	0.65	0.68	0.13	0.22	BG → B	YBr	RBr	YBr	Br
5-Hydroxyindole	0.65	0.72	0.08	0.18	Br → Y	Br	RBr	Br	Br
Tryptophol	0.64	0.71	0.05	0.18	P	Y	Y	Y	VBr
L-Tryptophan	0.23	0.39	0.00	0.00	P	Y	Y	Y	PBr

[a] Solvent systems used for development of the indolic compounds are described in the Materials and Methods section.

[b] Color reactions of the indolic compounds were obtained by spraying the chromatograms with various spray reagents as also described in the text. Abbreviations for spray reagents: Ehrlich (Ehr), Van den Bergh (VB), Pauly (Pa), Van Urk (VU), and Gibb (G). Color reaction abbreviations: blue (B), brown (Br), blue green (BG), blue gray (BGy), gray brown (GyBr), orange (O), purple (P), purple brown (PBr), pink (PK), red (R), red brown (RBr), violet (V), violet brown (VBr), yellow (Y), and yellow brown (YBr).

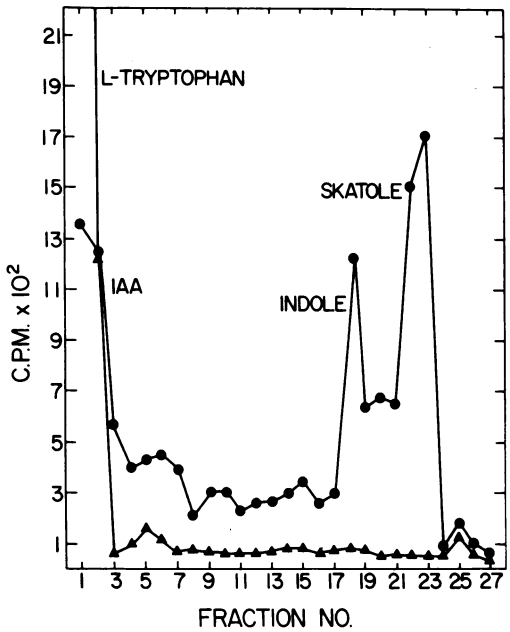

FIG. 1. *Incubation of* L-[*U-benzene ring-*^{14}C]*tryptophan with ruminal fluid. Ruminal fluid (24 ml) was incubated with* L-[*U-benzene ring-*^{14}C]*tryptophan (9.8* $\times$ 10^{-4} *M, 0.021 mCi/mmol) dissolved in 1 ml of 0.1 M NaOH. Incubation was for 24 h at 37 C under* CO_2. *Metabolites were extracted with* CH_2Cl_2 *and separated by TLC (solvent 4). Fractions of 0.5 cm were scraped from the plate and counted by liquid scintillation spectrometry. Symbols: ruminal fluid incubation (●); boiled ruminal fluid control (▲).*

tion of [2-^{14}C]IAA with ruminal microorganisms and analysis by TLC in solvent 1 confirmed that skatole was the only metabolite formed (Fig. 2).

Both skatole and indole were formed during the incubation of indolepyruvate, indolelactate, and indolealdehyde with ruminal microorganisms. Indoleacetaldehyde was converted to IAA and tryptophol.

D-Tryptophan was not degraded to either skatole, indole, or IAA by ruminal microorganisms under the experimental conditions. Other indolic compounds that were not degraded by ruminal microorganisms in vitro included: 5-methoxy-DL-tryptophan, *N*-acetyl-tryptophan, 5-methoxyindoleacetate, indolecarboxylate, indolepropionate, indolebutyrate, indoleacetonitrile, indoleacrylate, indoleglyoxalate, indoleacetamide, tryptophol, 5-hydroxytryptamine (creatinine sulphate complex and hydrogen oxalate salt), and tryptamine-HCl.

Incorporation and time sequence studies. In six experiments, the incubation of L-[U-benzene ring-^{14}C]tryptophan (9.8 $\times$ 10^{-4} M, 0.021 mCi/mmol) with ruminal microorganisms for 24 h resulted in 39 $\pm$ 4% standard error mean (SEM) of the added radioactivity being incorporated in skatole, 7 $\pm$ 2% SEM into indole, and 4 $\pm$ 0.5% SEM into IAA. Similarly, when [2-^{14}C]IAA (1.1 $\times$ 10^{-3} M, 0.018 mCi/mmol) was incubated, 38 $\pm$ 8% SEM of the added radioactivity was found in skatole. The formation of [^{14}C]skatole from [2-^{14}C]IAA suggested a decarboxylation reaction with the ^{14}C label located on the methyl carbon of skatole. The formation of nonradioactive indole by the cleavage of acetate from [2-^{14}C]IAA was a possibility. However, examination of IAA metabolites by TLC as well as the metabolites of the incubation of [U-benzene ring-^{14}C]IAA with ruminal microorganisms indicated that this reaction does not occur.

The in vitro degradation of L-tryptophan to skatole and indole is only apparent with long incubations. The results summarized in Table 2 indicate that at 6 h of incubation only 0.1% of the added radioactivity from L-[^{14}C]tryptophan was incorporated into skatole. This increased to 1.8% with 12 h of incubation, 10.3% with 18 h, and 15.0% at 24 h. The incorporation of ^{14}C label into indole increased from 0.1% with 6 h

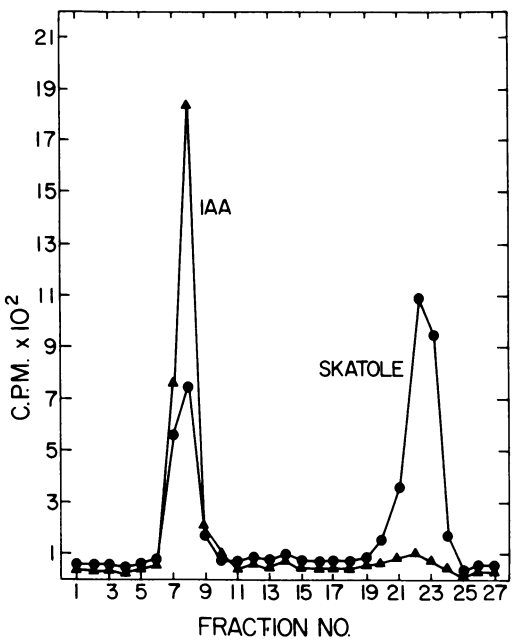

FIG. 2. *Incubation of* [2-^{14}C]*IAA with ruminal fluid. Ruminal fluid (24 ml) was incubated with* [2-^{14}C]*IAA (1.1* $\times$ 10^{-3} *M, 0.018 mCi/mmol) dissolved in 1 ml of 0.1 M NaOH. Incubation was for 24 h at 37 C under* CO_2. *Metabolites were extracted by* CH_2Cl_2, *separated by TLC (solvent 1), and counted as described under Fig. 1. Symbols: ruminal fluid incubation (●); boiled ruminal fluid control (▲).*

of incubation to 2.3% with 24 h of incubation. Incorporation into IAA showed a reverse trend with 8.2% of the added radioactivity being associated with this metabolite at 6 h, increasing to a maximum of 12.1% at 12 h, then decreasing to 9.3 and 2.6% by 18 to 24 h. The aqueous fractions, containing the undegraded L-[^{14}C]-tryptophan and other metabolites not extracted by methylene chloride, decreased from 7.8 to 1.4% over the 24-h incubation period. Total radioactivity found in the aqueous fraction and in the metabolites examined could only account for 16.2% of the added radioactivity at 6 h, 19.7% at 12 h, 23.5% at 18 h, and 21.3% at 24 h.

Influence of ruminal protozoa and bacteria on the formation of metabolites. Incubation of L-[U-benzene ring-^{14}C]tryptophan with a crude preparation of ruminal protozoa for 24 h resulted in only 0.1% of the added radioactivity incorporated into skatole, 3% into indole, and 8% into IAA (Table 3). L-[^{14}C]tryptophan was not removed from the incubation by methylene chloride extraction, and it represented 70% of the added radioactivity remaining in the aqueous fraction. Addition of antibiotics, penicillin, and streptomycin (400 μg/ml) to the incubation decreased IAA but had no effect on skatole, indole, or the aqueous fraction. Incubation of L-[^{14}C]tryptophan with ruminal fluid, freed of protozoa, resulted in 13% incorporation into skatole, 4% into indole, and 50% into IAA. Only 16% of the added radioactivity was found in the aqueous fraction. The use of untreated ruminal fluid resulted in 26% incorporation into skatole, 4% into indole, and 3% into IAA. Only 44% of the added radioactivity could be accounted for by skatole, indole, IAA, and the aqueous fraction.

TABLE 2. *Time sequence of metabolites formed from L-tryptophan by ruminal microorganisms in vitro*[a]

Incubation time (h)	Added radioactivity incorporated into:[b] (%)				
	Aqueous	Skatole	Indole	IAA	Total
6	7.8	0.1	0.1	8.2	16.2
12	5.5	1.8	0.3	12.1	19.7
18	2.6	10.3	1.3	9.3	23.5
24	1.4	15.0	2.3	2.6	21.3

[a] The complete system contained L-[U-benzene ring-^{14}C]tryptophan (4.9×10^{-4} M, 0.083 mCi/mmol) dissolved in 1 ml of 0.1 M NaOH and 49 ml of strained ruminal fluid.

[b] Samples of 10 ml were removed, acidified, extracted with CH_2Cl_2, separated by TLC, and counted as described in the text.

Effect of glucose concentration on the formation of metabolites. The addition of increasing concentrations of glucose (0 to 110 mM) to the incubations resulted in a progressive decrease in the incorporation of L-[U-^{14}C]tryptophan activity into skatole and indole but an increase in incorporation into IAA (Table 4). With the exception of the 17 mM glucose concentration, the incorporation of radioactivity into skatole fell from 26% in the control to less than 2% with increasing glucose concentration. Similarly, the incorporation of radioactivity into indole fell from 5% to 0.1%. IAA increased from 3 to 7%. The aqueous fraction, containing the undegraded L-tryptophan and other metabolites not extracted by methylene chloride, increased from 14% in the control to 63% in the flask containing the highest glucose concentration (110 mM). Final pH values obtained at the termination of the 24 h incubation showed a decrease from 6.1 in the control to 4.2 in the flask containing the highest glucose concentration.

Effect of antibiotics on the formation of metabolites. With the exception of streptomycin, all of the antibiotics tested at 400 μg/ml reduced the conversion of L-tryptophan to skatole (Table 5). Polymyxin B, chlorotetracycline, and oxytetracycline were most effective with only 0.2, 1.2, and 2.0% incorporation into

TABLE 3. *Formation of L-tryptophan metabolites by ruminal bacteria and protozoa preparations*[a]

Preparation	Added radioactivity incorporated into:[b] (%)				
	Aqueous	Skatole	Indole	IAA	Total
Protozoa	69.6	0.1	3.0	7.8	80.5
Protozoa + antibiotics	72.0	0.1	3.3	4.7	80.1
Bacteria	15.7	12.6	3.9	50.4	82.6
Ruminal fluid	10.8	26.3	4.0	2.5	43.6

[a] Each flask contained L-[U-benzene ring-^{14}C]tryptophan (9.8×10^{-4} M, 0.021 mCi/mmol) dissolved in 1 ml of 0.1 M NaOH and 24 ml of strained ruminal fluid or buffer. Values reported are the means of two experiments.

[b] Metabolites were extracted with CH_2Cl_2, separated by TLC (solvent 4), and counted by liquid scintillation spectrometry.

TABLE 4. *Effect of increasing glucose concentration on the formation of metabolites from L-tryptophan by ruminal microorganisms in vitro*[a]

Glucose concentration (mM)	pH	Added radioactivity incorporated into:[b] (%)				
		Aqueous	Skatole	Indole	IAA	Total
Control	6.1	14.0	25.5	4.9	2.6	47.0
6	6.0	32.8	16.0	4.7	2.9	56.4
11	5.8	34.5	15.7	4.1	2.2	56.5
17	5.6	15.3	24.4	4.4	2.6	46.7
28	5.5	45.7	9.0	2.3	2.7	59.7
56	5.1	50.2	5.1	0.5	7.1	62.9
78	4.5	56.8	2.5	0.1	6.5	65.9
110	4.2	62.9	1.5	0.1	6.5	71.0

[a] Each flask contained L-[U-benzene ring-^{14}C]tryptophan (9.8×10^{-4} M, 0.021 mCi/mmol) dissolved in 1 ml of 0.1 M NaOH, the appropriate amount of glucose, and 24 ml of strained ruminal fluid. Incubation was for 24 h at 37 C under CO_2. Values reported are the means of two experiments.

[b] Metabolites were extracted, separated, and counted as described in the text.

TABLE 5. *Effect of antibiotics on the formation of metabolites from L-tryptophan by ruminal microorganisms in vitro*[a]

Antibiotic	Added radioactivity incorporated into:[b] (%)				
	Aqueous	Skatole	Indole	IAA	Total
Control	35.6	13.7	5.1	3.1	57.5
Chlorotetracycline	69.5	1.2	1.1	4.4	76.2
Kanamycin	31.9	4.7	4.3	31.6	72.5
Neomycin	18.7	6.7	5.3	40.9	71.6
Oleandomycin	59.3	4.7	2.4	6.3	72.7
Oxytetracycline	55.4	2.0	2.0	17.1	76.5
Penicillin G	51.3	5.4	5.4	7.4	69.5
Polymyxin B	64.6	0.2	5.5	5.2	75.5
Streptomycin	47.6	14.7	4.0	2.4	68.7

[a] Each flask contained L-[U-benzene ring-^{14}C]tryptophan (9.8×10^{-4} M, 0.021 mCi/mmol) dissolved in 1 ml of 0.1 M NaOH, 10 mg of antibiotic (400 μg/ml), and 23 ml of strained ruminal fluid. Incubation was for 24 h at 37 C under CO_2. Values reported are the means of two experiments.

[b] Metabolites were extracted, separated, and counted as described in the text.

skatole, respectively. The untreated control showed 14% of the added radioactivity of L-[^{14}C]tryptophan incorporated into skatole. Incorporation into indole was also reduced by the tetracyclines, but not by neomycin, penicillin G, and polymyxin B. The addition of the aminoglycosidic antibiotics (kanamycin and neomycin, and to a lesser extent, oxytetracycline) reduced the incorporation of radioactivity into skatole and increased (10- to 13-fold) the radioactivity into IAA. The undegraded L-tryptophan, represented by the radioactivity remaining in the aqueous fraction after methylene chloride extraction, ranged from 19% (neomycin) to 70% (chlorotetracycline) compared with 36% for the control.

Skatole and indole concentrations in ruminal fluid. Skatole and indole were detected in ruminal fluids in variable but low concentrations over several months. Quantitative analysis of ruminal fluid obtained at 4-h intervals for 24 h from two steers fed once daily showed that skatole was present from 0.09 to 0.59 μg of ruminal fluid per ml (Fig. 3). Measurable concentrations of indole were present in the ruminal fluid of only one steer at 0 and 4 h after feeding, with values of 0.15 and 0.30 μg/ml, respectively. Trace amounts (<0.001 μg/ml) of indole were detected in samples obtained from both steers at longer intervals after feeding.

DISCUSSION

The data indicate that skatole, indole, and

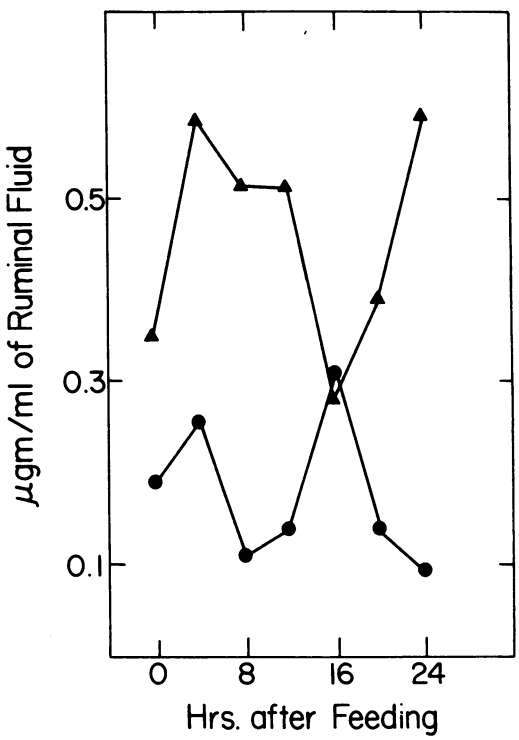

FIG. 3. *Concentration of skatole in ruminal fluid. Steers were fed at zero time, and 50-ml samples of ruminal fluid were collected at 4-h intervals. Strained ruminal fluid (2 ml) was extracted three times with 20 ml of CH_2Cl_2, evaporated to 0.1 ml, and analyzed by GLC. Values are reported as micrograms of ruminal fluid per milliliter. Steer no. 1 (●); steer no. 2 (▲).*

IAA are the major indolic metabolites of L-tryptophan fermentation by ruminal microorganisms in vitro and that skatole is the chief indolic metabolite produced. IAA has previously been shown to be a product of tryptophan degradation in short-term in vitro incubations with ruminal microorganisms (30). Another study demonstrated that IAA was incorporated into microbial cells and that strains of *Ruminococcus albus* synthesize tryptophan from this metabolite (1). Our results (Table 2, Fig. 2) indicate that the major route by which skatole is formed from L-tryptophan is by a two-step process involving (i) the initial formation of IAA from L-tryptophan, and (ii) the subsequent decarboxylation of IAA to skatole. The second reaction in this sequence is only apparent in vitro with long-term incubations, as indicated in Table 2.

This study also indicates that other indolic compounds are converted to skatole and indole by ruminal microorganisms. Besides tryptophan and IAA, indolepyruvate, indolelactate, and indolealdehyde may serve as precursors for the formation of skatole and indole in the rumen. Based on the indolic compounds that are degraded by ruminal microorganisms to identifiable metabolites, it is possible to derive a tentative scheme for the fermentation pathways occurring in ruminal fluid in vitro (Fig. 4).

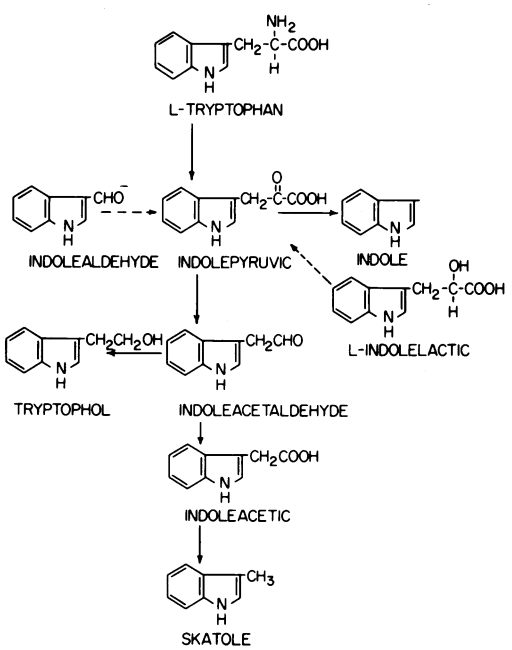

FIG. 4. *Tentative pathway for the fermentation of L-tryptophan and related indolic compounds by ruminal microorganisms in vitro.*

It is clear from several of the studies conducted, especially Table 2, that the majority of the labeled tryptophan incubated with ruminal microorganisms cannot be accounted for by the formation of the indolic metabolites, skatole, indole, and IAA. Methylene chloride extraction to remove the indolic metabolites from the aqueous fraction will not remove tryptophan. The radioactivity remaining in the aqueous fraction is, therefore, due to undegraded tryptophan and other labeled metabolites not extracted by methylene chloride. Even in considering the radioactivity remaining in this fraction, all of the added radioactivity cannot be accounted for. It is possible that some of this loss may be attributed to assimilation by microbial cells and complete degradation to labeled carbon dioxide.

The study conducted with crude preparations of ruminal bacteria and protozoa indicates that in the absence of protozoa, L-tryptophan can be degraded to skatole, indole, and IAA. It does not exclude the possibility that ruminal protozoa may also be important in the formation of these metabolites on a quantitative basis. The difference between the incorporation of label into metabolites by the bacteria preparation and untreated ruminal fluid could be explained by the treatment of the ruminal fluid to remove the protozoa prior to incubation. Ruminal bacteria are strict, obligate anaerobes and may have been affected during the separation procedure. The inhibitory effects of the antibiotics tested (Table 5) would strongly suggest that the bacterial population of ruminal fluid is responsible for the formation of these metabolites.

Previous studies (6, 16) demonstrate that the formation of indole by tryptophanase can be induced by tryptophan and repressed by glucose in pure cultures. Addition of increasing amounts of glucose to in vitro incubations of ruminal microorganisms resulted in a progressive decrease in the incorporation of L-[^{14}C]tryptophan label into both skatole and indole. It is, therefore, conceivable that like indole, the formation of skatole can be induced by the appropriate substrate and repressed by glucose. One cannot, however, rule out the effects of the decrease in pH observed in this study.

Although antibiotics will effectively control the degradation of L-tryptophan to skatole and indole, no attempt was made to assess their effects on the total microbial population in ruminal fluid. Because these antibiotics were tested at a relatively high level and may have had multiple effects, any assessment of their usefulness in preventing the tryptophan-

induced emphysema in cattle must take this factor into account. The aminoglycosidic antibiotics, kanamycin and neomycin, were most effective in reducing the decarboxylation of IAA to skatole.

Quantitative analysis by GLC and qualitative analysis by TLC of ruminal fluid obtained from two steers indicate that skatole is the predominant naturally occurring indolic metabolite in vivo. We have also shown that the intraruminal administration of large doses of L-tryptophan (0.35 g/kg BW) resulted in relatively high skatole concentrations (4.4 μg/ml) in ruminal fluid (unpublished data). Ruminal fluid periodically obtained over several months from the steers used in these studies showed that the concentration of skatole and indole did vary. Other studies (9, 24, 31) indicate that indole is the predominant metabolite in the rumen. No immediate explanation is apparent for the observed variations in skatole concentration of ruminal fluid between animals or with respect to time after feeding. Although both animals were housed together and received the same diet, the ruminal fluid from steer 2 was consistently higher in skatole concentration.

The specific bacteria responsible for the degradation of L-tryptophan to skatole, indole, and IAA in ruminal fluid remain to be isolated and identified. We have obtained in pure culture from ruminal fluid a bacterium capable of degrading L-tryptophan to only indole and IAA. A mixed culture that we are also maintaining will only effect the decarboxylation of IAA to skatole. These metabolites are usually associated with the lower intestinal tract and fecal material.

Skatole and indole are toxic to rumen ciliate protozoa (3, 15) and to *Tetrahymena* (4, 20), resulting in cell immobility and disintegration of membranous structure. Skatole and indole also show a bacteriostatic action on gram-negative enteric bacteria (32, 33). Although the exact mechanism of this toxicity is not known, it is believed to be related to the lipophilic properties of these compounds (15, 28).

In other studies, we have examined skatole as a possible causative agent of tryptophan-induced pulmonary edema and emphysema. Our findings indicate that when administered intraruminally and intravenously to cattle and intraruminally to goats, skatole will induce emphysema similar in clinical signs and lung lesions to the tryptophan-induced disease (11). We now believe skatole to be the metabolite of L-tryptophan fermentation in the rumen that is responsible for the experimentally induced emphysema.

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LITERATURE CITED

1. Allison, M. J., and I. M. Robinson. 1967. Tryptophan biosynthesis from indole-3-acetic acid by anaerobic bacteria from the rumen. Biochem. J. **102**:36.
2. Arnow, L. E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531-537.
3. Bailey, R. W., and B. H. Howard. 1962. Preparation of enzymes from rumen protozoa by indole disintegration. Arch. Biochem. Biophys. **99**:299-303.
4. Bailey, R. W., and G. B. Russell. 1965. Disruption of protozoa by indole and related compounds. Nature (London) **208**:1001-1002.
5. Barton, G. M., J. A. F. Gardner, and R. S. Evans. 1952. Paper chromatography of phenolic substances. Nature (London) **170**:249-250.
6. Boyd, W. L., and H. C. Lichstein. 1955. The effect of carbohydrates on the tryptophanase activity of bacteria. J. Bacteriol. **69**:584-589.
7. Bradley, B., and J. R. Carlson. 1974. A gas-liquid chromatographic procedure for the determination of indole and 3-methylindole in bovine plasma. Anal. Biochem. Accepted for publication.
8. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279-285.
9. Cappa, V. 1955. Presenza di indolo nel contenuto del rumine di bovini. Ann. Fac. Agr. Ser. **1**:392-396.
10. Carlson, J. R., I. A. Dyer, and R. J. Johnson. 1968. Tryptophan-induced interstitial pulmonary emphysema in cattle. Amer. J. Vet. Res. **29**:1983-1989.
11. Carlson, J. R., M. T. Yokoyama, and E. O. Dickinson. 1972. Induction of pulmonary edema and emphysema in cattle and goats with 3-methylindole. Science **176**:298-299.
12. Chalupa, W., and A. J. Kutches. 1968. Biohydrogenation of linoleic-1-^{14}C acid by rumen protozoa. J. Anim. Sci. **27**:1502-1508.
13. Dagley, S., and D. T. Gibson. 1965. The bacterial degradation of catechol. Biochem. J. **95**:466-474.
14. Dickinson, E. O., G. R. Spencer, and J. R. Gorham. 1967. Experimental induction of an acute respiratory syndrome in cattle resembling bovine pulmonary emphysema. Vet. Rec. **80**:487-489.
15. Eadie, J. M., and A. E. Oxford. 1954. A remarkable disintegrative effect of skatole upon certain rumen ciliate protozoa. Nature (London) **174**:973.
16. Evans, W. C., W. C. R. Handley, and F. C. Happold. 1942. The tryptophanase-tryptophan reaction. Possible mechanisms for the inhibition of indole production by glucose in cultures of *B. coli*. Biochem. J. **36**:311-318.
17. Gutierrez, J., P. P. Williams, R. E. Davis, and E. J. Warwick. 1962. Lipid metabolism of rumen ciliates and bacteria. I. Uptake of fatty acids by *Isotricha prostoma* and *Entodinium simplex*. Appl. Microbiol. **10**:548-551.
18. Heacock, R. A., and M. E. Mahon. 1963. Paper and thin-layer chromatography of the hydroxy skatoles. Can. J. Biochem. Physiol. **41**:487-496.
19. Heacock, R. A., and M. E. Mahon. 1963. The hydroxylation of skatole. Can. J. Biochem. Physiol. **41**:2381-2390.
20. Hogg, J. R., and A. M. Elliot. 1951. Comparative amino acid metabolism of *Tetrahymena geleii*. J. Biol. Chem. **192**:131-139.
21. Jepson, J. B. 1960. Indoles and related ehrlich reactors, p. 183-211. *In* I. Smith (ed.), Chromatographic and electrophoretic techniques, vol. 1. Interscience Publishers, New York.

22. Lacoste, A. M. 1961. Dégradation du tryptophane par les bactéries de la panse de ruminants. C. R. Acad. Sci. Paris **252**:1233-1235.
23. Lewis, T. R., and R. S. Emery. 1962. Intermediate products in the catabolism of amino acids by rumen microorganisms. J. Dairy Sci. **45**:1363-1368.
24. Lewis, T. R., and R. S. Emery. 1962. Metabolism of amino acids in the bovine rumen. J. Dairy Sci. **45**:1487-1492.
25. Loh, H. H., and C. P. Berg. 1971. Production of D-kynurenine and other metabolites from D-tryptophan by the intact rabbit and by rabbit tissue. J. Nutr. **101**:465-475.
26. Myer, C. S. 1950. The co-determination of indole and skatole. Ass. Off. Agric. Chem. **33**:971-976.
27. Perley, J. E., and B. B. Stowe. 1966. The production of tryptamine from tryptophan by *Bacillus cereus* (KVT). Biochem. J. **100**:169-174.
28. Rogers, K. S. 1969. Rabbit erythrocyte hemolysis by lipophilic aryl molecules. Proc. Soc. Exp. Biol. Med. **130**:1140-1142.
29. Schatzmann, H. J., and H. Gerber. 1972. Production of tryptamine from tryptophan by ruminal fluid *in vitro*. Zentralbl. Veterinaermed. Reihe A **19**:482-489.
30. Scott, T. W., P. F. V. Ward, and R. M. C. Dawson. 1964. The formation and metabolism of phenyl-substituted fatty acids in the ruminant. Biochem. J. **90**:12-24.
31. Spisna, D., and V. Cappa. 1954. Presenza di indolo nel contenuto del rumine di bovini. Boll. Soc. Ital. Sper. **30**:1418-1419.
32. Tittsler, R. P., and L. A. Sandholzer. 1935. The bacteriostatic action of indole on gram-negative enteric bacteria and on certain cocci. J. Infect. Dis. **57**:64-69.
33. Tittsler, R. P., L. A. Sandholzer, and E. T. Callahan. 1935. The bacteriostatic action of skatole on gram-negative enteric bacilli. J. Infect. Dis. **57**:57-60.