***In Vitro* Metabolism of Tryptophan by Ruminal Protozoa and Bacteria : The Production of Indole and Skatole and their Effects on Protozoal Survival and VFA Production**

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Abstract An *in vitro* study was conducted to confirm the production of indole (Ind) and skatole (Skt) from L-tryptophan (Trp) by mixed rumen protozoa (P suspensions), mixed rumen bacteria (B suspensions) and their combinatorm (BP suspensions), and to examine the effects of salinomycin (SL) on the production of Ind and Skt from Trp by those microorganisms. The effects of Ind and Skt on the survival of protozoa and the production of volatile fatty acids (VFA) by those microorganisms were also examined. Rumen microorganisms were collected from a fistulated goat. P suspensions produced only Ind from Trp during a 12-h incubation period. SL stimulated the production of Ind by P suspensions. Both B and BP suspensions produced Skt and Ind. SL almost completely inhibited the production of Skt and Ind by those microbial suspensions. Low concentrations of Ind and Skt (2 mM) kept protozoa alive in BP suspensions for a long time, stimulated the total VFA production in B and BP suspensions, and increased the molar percentages of propionate and butyrate in BP suspensions.

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Ever since 3-methylindole (skatole) was specified as a causative factor of tryptophan-induced pulmonary edema and emphysema in cattle and goats by CARLSON *et al.*⁵⁾, tryptophan metabolism by rumen microorganisms has been studied in detail mainly by CARLSON and his co-workers. A tentative pathway for the fermentation of L-tryptophan by mixed

rumen microorganisms *in vitro* was shown by YOKOYAMA and CARLSON¹⁸⁾. They showed, using cheese-cloth strained rumen contents from steers maintained on an alfalfa-grass hay diet, that 39% of the initial radioactivity of the added L-[U-benzen ring-¹⁴C] tryptophan was incorporated into skatole, 7% into indole and 4% into indoleacetate after incubation for

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24 h, and that 38% of the initial radioactivity of the added [2-¹⁴C] indoleacetate was incorporated into skatole during a 24-h incubation period. They thought, therefore, that the main path for the formation of skatole from L-tryptophan was by a two-step process involving the initial formation of indoleacetate from tryptophan and the subsequent decarboxylation of indoleacetate to skatole. However, they omitted describing that indole formation might probably be due to the tryptophanase in rumen microbes, as shown in other tryptophanase^{3,9)}, and the tentative pathway they offered for the fermentation of L-tryptophan and related indolic compounds by ruminal microorganisms *in vitro* did not include the path for the formation of indole by tryptophanase. Their results also showed that incubation of the same ¹⁴C-labelled tryptophan with ruminal fluid freed of protozoa for 24 h resulted in 50% incorporation into indoleacetate, 13% into skatole and 4% into indole, while incubation of the same radioactive compound with a crude preparation of ruminal protozoa for 24 h resulted in 8% of the added radioactivity incorporated into indoleacetate, 3% into indole and only 0.1% into skatole. They also mentioned that the bacterial population of ruminal fluid was responsible for the formation of these indolic metabolites, though it did not exclude the possible role of the ruminal protozoa in the formation of these metabolites. Later, rumen protozoa were also shown to produce mainly indoleacetate (30 to 50% of the degraded tryptophan) from L-tryptophan by means of paper chromatography as an analytical technique⁸⁾.

In terms of the prevention of acute pulmonary edema and emphysema in cattle, ionophore antibiotics such as monensin and salinomycin (SL) have been shown to have an inhibitory effect on the production of skatole by *in vitro* and *in vivo* rumen microorganisms^{10-12, 15)}, but their effects on indole production by mixed rumen microbes and on indolic

compounds production by protozoa have not been examined.

The present *in vitro* study was conducted to confirm the production of indole and skatole from L-tryptophan by mixed rumen bacteria, mixed rumen protozoa and their combinations, and to examine the effects of SL on the production of indole and skatole from tryptophan by those microorganisms. The effects of indole and skatole on the survival of protozoa and the production of volatile fatty acids (VFA) in those microbial suspensions were also examined.

Materials and Methods

1. Rumen microbial suspensions

Rumen microorganisms were obtained from a fistulated Japanese-Saen goat (female, 40 kg) fed on a daily ration consisting of lucerne (*Medicago sativa*) cubes (23 g dry matter/kg body-weight^{0.75}) and concentrate feed (formula feed: alphanatbeef, Chubu-Shiryo, Japan; 8 g dry matter/kg body-weight^{0.75}) in two equal portions given at 0900 and 1700 h.

Rumen contents were taken before the morning feed and were strained through four layers of surgical gauze. Samples of strained rumen fluid were then poured into a separating funnel that had been gassed with O₂-free 95% N₂-5% CO₂. The funnel and contents were maintained at 39°C for up to 60 min to allow fine feed particles to float. Most of the lower portion was then anaerobically transferred to centrifuge tubes, cooled in ice cold water (about 4°C) and centrifuged at 270 × *g* for 3 min. The supernatant fluid thus obtained was microscopically examined and found to be free from protozoa. Since it consisted mainly of mixed rumen bacteria, it was used for bacterial suspensions and designated "B suspension". The centrifuged pellet (mainly protozoa) was washed five times with MB 9 buffer solution¹⁶⁾ and then divided into two portions. One portion was anaerobically added to the B suspension at about 4% (vol/vol) so as to

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prepare almost the same microbial densities as those original strained rumen digesta, and was used for mixed microbial suspensions of bacteria and protozoa (BP suspension). The other portion was anaerobically resuspended in MB 9 buffer solution at about 4% (vol/vol) to produce protozoal suspension (P suspension). The latter always included 0.1 mg/ml each of dihydrostreptomycin sulfate and penicillin G potassium to suppress the biochemical activity of contaminating bacteria.

2. Incubation and sample treatments

Microbial suspensions (30 ml) were anaerobically incubated, in triplicate, in 50 ml Erlenmeyer flasks for up to 12 h at 39°C. All incubations contained 0.5 mg/ml of rice starch. L-tryptophan (1 µmol/ml, Wako Pure Chemical Industries Ltd., Japan), salinomycin (SL, 5 µg/

ml, Kaken Pharmaceutical Co. Ltd., Japan), indole or skatole (both dissolved in minimal volumes of ethanol with 1 ml of the ethanol solution added to 30 ml incubation) were added to various incubations.

Samples (1 ml) of the incubations were removed throughout the incubation periods. Some were prepared for HPLC analysis by the addition of an equal volume of 4% (wt/vol) sulfosalicylic acid, centrifugation at $27,000 \times g$ for 20 min, filtration (0.45 µm membrane) and storage at 4°C. Others were prepared for VFA analysis by mixing them with 1 ml 10% metaphosphoric acid (in 2N H₂SO₄).

In order to determine protozoal numbers, 0.5-ml portions of the microbial suspensions were collected, mixed with 4.5 ml of MFS (methylgreen-formalin-salt) solution¹⁷⁾ and

Table 1. Effect of salinomycin (SL) on the degradation of tryptophan and production of indole and skatole by mixed rumen protozoa (P), mixed rumen bacteria (B) and their mixture (BP)

Rumen		Incubation time (h)				
microorganisms		0	3	6	9	12
Tryptophan existed (nmol/ml) ^a						
P suspension	Without	1,025±10	998±15 ^b	974±6 ^b	976±29 ^b	958±13 ^b
	With	988±12	955±12 ^b	960±12 ^b	941±19 ^b	876±48 ^c
B suspension	Without	997±14	814±11 ^c	687±10 ^c	492±11 ^c	335±17 ^d
	With	999±13	935±35 ^b	903±6 ^b	925±62 ^b	890±13 ^c
BP suspension	Without	1,021±15	766±18 ^c	583±20 ^c	370±22 ^d	214±17 ^e
	With	978±22	919±24 ^b	866±9 ^b	850±8 ^b	843±20 ^c
Indole produced (nmol/ml) ^a						
P suspension	Without	0	14.1±1.5 ^f	32.2±1.6 ^f	53.2±6.3 ^f	62.8±1.7 ^f
	With	0	6.5±1.1 ^g	16.7±1.3 ^f	34.0±2.2 ^f	117.1±23.6 ^g
B suspension	Without	0	5.1±0.3 ^g	4.8±0.3 ^g	11.0±3.2 ^g	18.3±5.8 ^h
	With	0	0 ^h	0 ^h	0 ^h	0 ⁱ
BP suspension	Without	0	3.7±0.4 ^g	15.2±4.0 ^f	22.6±1.9 ^g	32.0±8.2 ^h
	With	0	0 ^h	0 ^h	0 ^h	0 ⁱ
Skatole produced (nmol/ml) ^a						
P suspension	Without	0	0	0 ^j	0 ^j	0 ^j
	With	0	0	0 ^j	0 ^j	0 ^j
B suspension	Without	0	5.9±1.8	7.2±2.4 ^k	25.4±3.9 ^k	44.3±20.1 ^k
	With	0	0	0 ^j	0 ^j	4.8±0.7 ^j
BP suspension	Without	0	0	32.3±3.5 ^l	109.1±1.1 ^l	213.0±63.0 ^l
	With	0	0	0 ^j	0 ^j	13.9±0.4 ^m

^a Values are means±SE.

^{b, c, d, e, f, g, h, i, and j, k, l, m} Means in the same column with different superscript differ (P<0.01).

Table 2. Effects of indole and skatole on the rate of survival (%) of rumen protozoa in mixed protozoal (P) suspension and in mixed bacterial and protozoal (BP) suspension

Addition	Incubation time (h)		
	0	3	6
In P suspension ^a			
None	100	96.2±0.2	79.3±11.8
Indole 2 mM	100	24.8±6.7 ^b	6.9± 0.7 ^b
Indole 10 mM	14.5± 1.2	0 ^b	0 ^b
Skatole 2 mM	86.2± 4.1 ^b	9.6±0.1 ^b	2.0± 0.6 ^b
Skatole 10 mM	19.8± 2.1 ^b	0 ^b	0 ^b
In BP suspension ^a			
None	100	91.0±3.4	91.1± 3.9
Indole 2 mM	100	88.6±6.3 ^b	86.2± 4.7 ^b
Indole 10 mM	81.5± 7.5 ^b	13.2±1.2 ^b	0.8± 0.7 ^b
Skatole 2 mM	100	91.5±1.8 ^b	71.5±10.5 ^b
Skatole 10 mM	80.9±20.8 ^b	6.4±3.5 ^b	0 ^b

^a Values are means±SE.^b Significantly different from control (P<0.01).

kept at room temperature.

3. Analytical methods

Analyses of tryptophan, indole and skatole were carried out by HPLC¹⁴⁾. VFA concentrations were measured by gas chromatography¹³⁾. Rumen protozoa were counted with the aid of a Fuchs-Rosenthal hemacytometer. Statistically significant differences between mean values were tested for via the Student's t-test.

Results

1. Degradation of tryptophan

Table 1 shows that in P suspensions (93% of *Entodinium*, 6% of Diplodiniinae and 1% of Isotrichidae), the addition of SL increased the degradation of tryptophan from 6.5% of the amount added to 11.3% during a 12-h incubation. By contrast, in B and BP suspensions without SL, 66.4 and 79.0% respectively of the tryptophan was degraded, whereas when SL was added these values were decreased to only 10.9 and 13.8%.

2. Production of indole

Table 1 also shows that in P suspensions without SL, indole was produced in significantly larger concentrations than in B and BP

Table 3. Effects of indole and skatole on the production of total volatile fatty acids (μmol/ml) by mixed rumen protozoa (P), mixed rumen bacteria (B) and their mixture (BP)

Addition	Incubation time (h)	
	3	6
In P suspension ^a		
None	4.68±0.10	7.62±0.62
Indole 2 mM	2.04±0.09 ^b	2.49±0.31 ^b
Indole 10 mM	0 ^b	0 ^b
Skatole 2 mM	1.19±0.60 ^b	1.39±1.13 ^b
Skatole 10 mM	0 ^b	0 ^b
In B suspension ^a		
None	4.36±0.54	7.46±0.97
Indole 2 mM	5.59±0.71	8.00±0.64
Indole 10 mM	1.45±0.39 ^b	0.88±0.51 ^b
Skatole 2 mM	5.41±1.28	8.71±3.48
Skatole 10 mM	0.69±0.35 ^b	0.83±0.18 ^b
In BP suspension ^a		
None	6.87±3.02	14.28±2.76
Indole 2 mM	8.41±1.23	18.07±2.50
Indole 10 mM	3.01±1.77 ^b	1.86±0.36 ^b
Skatole 2 mM	9.25±2.35	16.46±2.06
Skatole 10 mM	1.63±1.53 ^b	3.15±2.46 ^b

^a Values are means±SE.^b Significantly different from control (P<0.01).

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Table 4. Effects of indole and skatole on the molar percentages of volatile fatty acids produced in the mixed rumen bacterial (B) suspension and in the mixed bacterial and rotozoal (BP) suspension after incubation for 6 h

Addition	Molar percentage (%)		
	Acetate	Propionate	Butyrate
In B suspension ^a			
None	58.5±14.7	29.3±12.1	8.2± 1.6
Indole 1 mM	62.9± 8.3	24.9± 9.6	7.5± 2.2
Indole 2 mM	59.0±12.7	28.2±12.2	7.7± 0.5
Indole 4 mM	62.0± 9.5	25.2± 8.9	8.1± 0.8
Skatole 1 mM	65.4± 9.7	23.4± 8.7	7.2± 0.9
Skatole 2 mM	64.7±10.2	23.7±10.3	8.6± 0.7
Skatole 4 mM	78.9±22.8	6.9± 9.5	9.2±11.9
In BP suspension ^a			
None	61.1± 2.5	17.0± 3.1	14.1± 0.9
Indole 1 mM	55.8± 4.5	18.2± 2.8	15.5± 1.0
Indole 2 mM	54.7± 0.8 ^b	18.3± 1.5	15.7± 2.0
Indole 4 mM	50.1± 2.5 ^b	21.0± 2.1	16.0± 1.9
Skatole 1 mM	53.0± 4.5 ^b	18.5± 2.6	16.8± 3.1
Skatole 2 mM	50.4± 0.6 ^b	21.5± 2.9	16.7± 1.6
Skatole 4 mM	50.6± 6.8 ^b	21.4± 3.5	16.8± 3.2

^a Values are means±SE.

^b Significantly different from control ($P<0.01$).

suspensions. The effect of adding SL was to increase the indole production after 12 h in P suspensions, but to inhibit completely its production in both B and BP suspensions.

3. Production of skatole

P suspensions, either with or without SL, produced no skatole from tryptophan during 12-h incubation periods (Table 1). By contrast, B and BP suspensions produced as much as 44.3 and 213.0 nmol skatole per ml respectively in 12 h. However, when SL was added to these suspensions, skatole production was completely repressed for 9 h and was only 4.8 and 13.9 nmol/ml after 12 h of incubation.

4. Effects of indole and skatole on the survival of rumen protozoa

These results are shown in Table 2. In P suspensions, 2 mM indole destroyed 75% protozoa, whereas 10 mM completely annihilated them after 3 h. Skatole produced a similar pattern. In BP suspensions, the effects were similar, though both 2 mM indole and skatole

appeared to exert a less toxic effect on the protozoa.

5. Effects of indole and skatole on the production of VFA

Results in Table 3 show that in P suspensions, 2 mM indole and skatole reduced the total VFA production at 6 h to 32.7 and 18.2% of the control value, respectively. Ten mM indole and skatole completely depressed VFA production. In B suspensions, 2 mM indole and skatole tended to enhance VFA production, while 10 mM indole and skatole strictly depressed VFA production. In BP suspensions, the control values of VFA production over a 6 h period were about two times higher than those in P and B suspensions. In this case, 2 mM indole and skatole also tended to enhance the VFA productions. Both 10 mM indole and skatole severely decreased VFA production.

Table 4 shows the effects of indole and skatole on the molar percentages of VFA produced. In B suspensions, indole and skatole

tended to decrease the molar percentages of propionate and to increase those of acetate, when compared with the control values. In BP suspensions, however, both indole and skatole tended to increase the molar percentages of propionate and butyrate, and to decrease the percentage of acetate, when compared with the control values.

Discussion

1. Effects of salinomycin on the production of indole and skatole

In P suspensions without SL, 67 nmol/ml (6.5%) of the added tryptophan was degraded and 62.8 nmol/ml of indole (equivalent to about 6.1% of the added tryptophan) was produced in the medium during a 12-h incubation period (Table 1). Thus the molar amount of the degraded tryptophan and that of the produced indole were proximate. An intriguing feature was that indole production in P suspensions was significantly higher ($P < 0.01$) than those in B and BP suspensions. Skatole was not produced in P suspensions. As described in the "Introduction", YOKOYAMA and CARLSON¹⁸⁾ showed that 8% of the radioactivity of ¹⁴C-labelled tryptophan was incorporated into indoleacetate, 3% into indole and 0.1% into skatole, after incubation of a crude preparation of rumen protozoa for 24 h. Another report⁸⁾ also showed that a main metabolite of L-tryptophan by rumen protozoa was indoleacetate. In the present experiments, indoleacetate was not determined, but since the molar amount of the degraded tryptophan was almost equal to that of the produced indole, indole seemed to be the only product or at least a principal metabolite of rumen protozoa, which is different from previous result obtained by other investigators^{8,18)}.

In P suspensions with SL, indole production tended to be lower than in P suspensions without SL during a 9-h incubation period, but at 12 h, indole production with SL was significantly ($P < 0.01$) (1.86 times) higher than

that without SL (Table 1). Thus SL did not severely affect indole production by rumen protozoa and in a later incubation period it stimulated indole production by protozoa, which was different from the inhibitory effect of ionophores on indole and skatole production by rumen bacteria (see Table 1 and References 10 and 11). This is the first observation of the effect of SL on the tryptophan metabolism by rumen protozoa, as far as we are aware. Though we can not speculate with confidence how SL acts on the production of indole from tryptophan by protozoa, SL appears to stimulate the transport of tryptophan into the cells of protozoa, or to activate an enzyme to produce indole. However the latter idea is improbable, because SL completely inhibited the production of indole in B and BP suspensions.

In B suspensions without SL, 1.84 and 4.44% of the added tryptophan were converted to indole and skatole, respectively, after incubation for 12 h, while the degraded tryptophan at 12 h was 66.4% of the added tryptophan (Table 1). Thus about 60% of the added tryptophan could be converted to other metabolites among which indoleacetate could be a main product, because skatole has been revealed to be produced from tryptophan by indoleacetate as an intermediate¹⁸⁾, though indoleacetate was not determined in the present experiment. These results showed a tendency similar to the results of the previous report by other investigators¹⁸⁾ in which indoleacetate, indole and skatole were 50, 4 and 13% of the initial molar amount of tryptophan, respectively, after incubation for 24 h. In B suspensions, SL completely inhibited the indole production during a 12-h incubation period and severely inhibited skatole production, being only 0.48% of the added tryptophan at 12 h. However, the degraded tryptophan was 10.9% of the added tryptophan, so about 10% should be other metabolites like indoleacetate. These results suggest that SL inhibited at the level

of tryptophan conversion to indoleacetate as well as the description by HAMMOND and CARLSON¹¹⁾ and at the same time at the level of indoleacetate conversion to skatole.

In BP suspensions without SL, 3.13 and 20.9% of the added tryptophan were converted to indole and skatole, respectively, after incubation for 12 h, while the degraded tryptophan at 12 h was 79.0% of the added tryptophan (Table 1). Therefore, about 55% of the added tryptophan could be other forms of metabolites (maybe mainly indoleacetate). In the experiment by YOKOYAMA and CARLSON¹⁸⁾, 7 and 39% of the added radioactivity of ¹⁴C-tryptophan were incorporated into indole and skatole after incubation for 24 h, and these results had a tendency similar to the present experiment, but in their experiment only 4% of the added radioactivity incorporated into indoleacetate after incubation for 24 h.

A notable point was that the amount of skatole (213.0 nmol/mL) produced in BP suspensions without SL during a 12-h incubation period was significantly ($P < 0.01$) (ca. 5-fold) higher than that (44.3 nmol/mL) produced in B suspensions without SL during the same incubation period, despite the fact that protozoa never produced skatole in the present experiment. According to the report of YOKOYAMA and CARLSON¹⁸⁾, this point was also true, namely the percentage of skatole produced in mixed suspensions of protozoa and bacteria was about 3-fold higher than that produced in only bacterial suspensions. Complicated interactions between protozoa and bacteria probably resulted in a value more than an arithmetic or mechanical sum of single values (metabolic activities) of protozoa and bacteria. The existence of protozoa in the rumen microbial ecosystem might stimulate in some way, skatole production by bacteria. Ecological points like this should be analyzed in near future.

In BP suspension with SL (Table 1), indole was not produced during a 12-h incubation

period. Skatole was not produced till 9 h, though at 12 h it was produced by 1.42% of the added tryptophan. Since the degraded tryptophan was 13.8% of the added tryptophan, about 12% of the added tryptophan was thought to be other metabolites like indoleacetate. These results also suggest that SL inhibited both at the level of tryptophan conversion to indoleacetate and at the level of indoleacetate conversion to skatole, as mentioned for B suspensions.

Thus in B and BP suspensions, SL was confirmed to be effective in inhibiting the production of metabolites like indoleacetate, skatole and indole. SL probably inhibited the transport of tryptophan into bacterial cells.

2. Effects of indole and skatole on protozoa and VFA production

The disruptive effects of indole and skatole on rumen protozoa were shown firstly by EADIE and OXFORD⁷⁾ and the nature of indole was applied for preparing some enzymes of protozoa themselves, because it was selective for protozoa and any associated bacteria were not broken^{1,2)}. The concentration of indole they used for the disruption of protozoa was about 0.1% (about 8.5 mM). In the present experiment, 10 mM indole and skatole also destroyed almost all protozoal cells in P suspensions immediately after it was added (Table 2).

It is of interest that in BP suspensions, both indole and skatole significantly ($P < 0.01$) lowered the molar percentage of acetate and had tendencies to enhance the molar percentages of propionate and butyrate (Table 4). Indole and skatole at this level might shift slightly the flow of electrons more in the direction of propionate and butyrate as electron sink products. As far as we are aware, these observations have never been reported.

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In vitro におけるルーメンプロトゾアおよびバクテリアによる
トリプトファン代謝：インドールおよびスカトールの
生成ならびにそれらの化合物がプロトゾアの生存
および VFA の生成に及ぼす影響

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In vitro でのルーメンプロトゾア (P 懸濁液), ルーメンバクテリア (B 懸濁液) およびそれらの混合懸濁液 (BP 懸濁液) による L-トリプトファン (Trp) からのインドール (Ind) およびスカトール (Skt) の生成を確認するとともに, これらの微生物懸濁液による Trp からの Ind および Skt 生成に及ぼすサリノマイシン (SL) の影響を検討する目的で本研究を計画した. また, 同時に, Ind および Skt がプロトゾアの生存および上記の各種微生物懸濁液中における揮発性脂肪酸 (VFA) の生成に及ぼす影響も検討した. ルーメン微生物は, フィスチュラ装着山羊から採取した. その結果, P 懸濁液は, 12 時間の培養中に Trp から Ind のみを生成した. SL は P 懸濁液における Ind の生成を促進した. B および BP 懸濁液中では, とともに Trp から Skt および Ind が生成された. そして, これらの生成は SL によりほぼ完全に抑制された. 低濃度 (2 mM) の Ind および Skt 存在下では, BP 懸濁液中のプロトゾアは長時間生存した. そして, B および BP 懸濁液中における総 VFA の生成を促進し, また, BP 懸濁液中におけるプロピオン酸と酪酸のモル百分率を増加させた.

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