Effects of salinomycin and vitamin B₆ on in vitro metabolism of phenylalanine and its related compounds by ruminal bacteria, protozoa and their mixture

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An in vitro study was conducted to examine the effects of salinomycin (SL) and vitamin B₆ (B₆) on the production of phenylalanine (Phe) from phenylpyruvic acid (PPY) and phenylacetic acid (PAA) and of PAA from Phe and PPY by mixed rumen bacteria (B), mixed rumen protozoa (P) and their mixture (BP). Rumen microorganisms were collected from fistulated goats fed lucerne cubes (*Medicago sativa*) and a concentrate mixture (3:1) twice a day. Microbial suspensions were anaerobically incubated at 39°C for 12 h. Phe and some other related compounds in both supernatants and microbial hydrolysates of the incubations were analyzed by HPLC. When PPY was used as a substrate, it completely disappeared without additives and converted mainly to Phe and PAA on the average by 396 and 178, 440 and 189, and 439 and 147 µm in B, P and BP, respectively, during the 12 h incubation period. The rate of disappearance showed no significant differences between the microbial suspensions with and without SL and B₆ during the incubation period. The production of Phe from PPY with SL was enhanced ($p<0.05$) by 40, 20 and 19% in B, P and BP, respectively, while PAA production from PPY with SL was inhibited ($p<0.05$) by 35, 37 and 38% in B, P and BP, respectively, during the 12 h incubation period. On the other hand, with B₆, the production of Phe and PAA from PPY tended to be enhanced by 14 and 17, 9 and 11, and 7 and 22% in B, P and BP, respectively, during the 12 h incubation period. When PAA added as a substrate was incubated in the incubation medium without any additives, it disappeared by 483, 462 and 507 µm and converted mainly to Phe on the average by 231, 244 and 248 µm in B, P and BP, respectively. The disappearance of PAA with SL was inhibited ($p<0.05$) by 16, 15 and 20%, in B, P and BP, respectively, whereas the disappearance of PAA with B₆ was almost the same as that without B₆ in B and BP suspensions but tended to be enhanced by more than 9% in P suspensions during the 12 h incubation period. The production of Phe from PAA with SL tended to be inhibited by 12, 11 and 8% in B, P and BP, respectively, during the 6 h incubation period, but the inhibition was weakened during the 12 h incubation period, whereas Phe production from PAA with B₆ tended to be enhanced by 13, 16 and 8% in B, P and BP, respectively. When Phe was added as a substrate, the net Phe disappearance without additives was 549, 365 and 842 µm and converted mainly to PAA on the average by 254, 205 and 461 µm in B, P and BP, respectively. The net disappearance of Phe with SL was inhibited ($p<0.05$) by 38, 28 and 46%, whereas the net disappearance of Phe with B₆ was enhanced ($p<0.05$) by 9, 8 and 7% in B, P and BP, respectively. The production of PAA from Phe with SL was inhibited ($p<0.05$) by 73, 54 and 76% in B, P and BP, respectively. On the other hand, with B₆, PAA production from Phe was enhanced ($p<0.05$) by 19, 18 and 20% in B, P and BP, respectively. Based on these results, it seems that SL inhibited Phe disappearance and enhanced the synthesis of Phe from PPY, though not from PAA, and accumulated free Phe in the medium, whereas B₆ also enhanced Phe synthesis both from PPY and PAA, which could provide additional amino N for animals.

Key Words—phenylacetic acid; phenylalanine synthesis; phenylpyruvic acid; rumen bacteria; rumen protozoa; salinomycin; vitamin B₆

Several ionophores, such as salinomycin (SL), monensin, lasalocid, and tetronasin, have been shown to

favorably alter ruminal fermentation characteristics and metabolism (Bergen and Bates, 1984; Chen and Wolin, 1979; Newbold et al., 1988; Russell and Strobel, 1989; Russell et al., 1991; Schelling, 1984). The curative effects of SL on coccidiosis (Merchen and Berger, 1985) and feedlot bloat, negative effects on the in vivo total counts of Gram-positive cocci

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(Hoshino et al., 1986; Suda et al., 1995), mixed protozoa (Kobayashi et al., 1988; Suda et al., 1995), in vitro counts of mixed rumen fungi (Cann et al., 1993), effectiveness in preventing experimentally induced lactic acidosis (Nagaraja et al., 1985; Suda et al., 1995), improved rate of body weight gain (Bagley et al., 1988; Kobayashi et al., 1989) and feed efficiency (Reffett-Stable et al., 1989; Zhao et al., 1995) have been described. SL has been shown to alter ruminal volatile fatty acid proportions (Merchen and Berger, 1985; Suda et al., 1993) with a resultant increase of propionic acid and decrease of butyric and acetic acid production, similar to those effects produced by other ionophores (Bartley et al., 1979; Richardson et al., 1976).

Inhibition of the deamination of amino acids is of practical importance as a means of increasing ruminal escape of preformed dietary protein. Deamination determines the rate of ruminal amino acid degradation (Annison et al., 1959; Lewis, 1962). SL has been shown to decrease amino acid breakdown (Hoshino et al., 1992; Okuuchi et al., 1993), and this may be due to the inhibitory effect of deamination as has been observed with other ionophores (Hino and Russell, 1985; Newbold et al., 1990; Russell and Martin, 1984; Van Nevel and Demeyer, 1977; Wallace et al., 1981). In the case of tryptophan metabolism, Okuuchi et al. (1993) reported that tryptophan production from indolepyruvic acid in all the rumen bacterial and protozoal suspensions was greatly enhanced by SL, but skatole and indole production from tryptophan by SL were completely inhibited in the rumen bacterial suspensions. They also reported that indoleacetic acid production from tryptophan and indolepyruvic acid was inhibited by SL in the rumen microbial suspensions, and that indole production from tryptophan and indolepyruvic acid was stimulated by SL in the rumen protozoal suspensions.

On the other hand, it is well established that vitamin B₆ (B₆) acts as a coenzyme in many enzyme reactions that are involved in the metabolism of amino acids including transamination, decarboxylation, deamination and desulfhydration, and in the synthesis of amino acids (McDowell, 1989). Ruminants have no dietary requirements for B₆ because of microbial synthesis in the rumen, although a need for this vitamin by ruminants has been demonstrated (Johnson et al., 1950; Reid et al., 1945). B₆ was shown to effectively stimulate cellulose digestion by washed cell suspensions of mixed rumen bacteria (MacLeod and Murray, 1956), and cellulolytic rumen bacteria such as *Ruminococcus flavefaciens*, *Bacteroides (Fibrobacter) succinogenes* and so on require B₆. Several lactic acid bacteria require B₆ for growth in simple media (Broquist and Snell, 1949; Holden et al., 1951). However, there is no

evidence to show the effect of B₆ on the amino acid production by rumen microorganisms. Merricks and Salsbury (1974) reported that the dethiomethylation of methionine from the protozoa-rich fraction of rumen fluid was stimulated 2.5-fold by B₆ in the form of pyridoxal phosphate.

This study was undertaken to quantitatively investigate the effects of SL and B₆ on the production and degradation of phenylalanine and other related compounds by mixed rumen bacteria and protozoa. A quantitative study was also carried out to investigate and compare the effects of both on the production and degradation of these compounds in a rumen microbial ecosystem consisting of mixed rumen microorganisms with those of mixed bacteria and protozoa only.

Materials and Methods

Animals and their management. Three mature rumen-fistulated goats (Japanese native breed) with a mean live weight of 35(±5) kg, fed on a daily ration of lucerne (*Medicago sativa*) cubes (23 g DM/kg BW^{0.75}) and a concentrated mixture (8 g DM/kg BW^{0.75}) in two equal portions given at 9 am and 5 pm were used for the experiment. The goats were housed in individual pens under approximately constant environmental conditions. They had free access to fresh water.

Preparation of rumen microbial suspensions. Rumen contents were obtained from the fistulated goats before morning feeding and were strained through four layers of surgical gauze into a separating funnel which was gassed with a mixture of 95% N₂ and 5% CO₂. The contents were incubated at 39°C for up to 60 min to allow feed debris to float. The suspensions of mixed bacteria (B), mixed protozoa (P), and B plus P (BP) were prepared according to Onodera et al. (1992). The P suspensions always included 0.1 mg/ml each of chloramphenicol, streptomycin sulfate and penicillin G potassium to suppress the biochemical activity of the contaminating bacteria.

Incubation and sample treatments. Microbial suspensions (20 ml) were anaerobically (with a mixture of 95% N₂ and 5% CO₂) incubated at 39°C with and without 1 mM of phenylpyruvic acid (Aldrich Chemical Co. Inc., Milwaukee, U.S.A.), phenylacetic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) or L-phenylalanine (Nacalai Tesque Inc., Kyoto, Japan) as the substrate with (5 µg/ml, Okuuchi et al., 1993) and without salinomycin (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) or B₆ (10 µg/ml; see results) (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) in 30 ml Erlenmeyer flasks for up to 12 h. All incubations contained 0.5 mg/ml of rice starch.

Samples were collected (0.5 ml) at 0, 6 and 12 h, mixed with an equal volume of 4% (w/v) sulfosalicylic

acid for deproteinization and centrifuged at $27,000 \times g$ for 20 min. The supernatant was filtrated (0.45 μ m membrane) and stored at 4°C. Pellets were hydrolyzed with 6M HCl at 110°C for 20 h, filtrated through Whatman filter paper, and evaporated to dryness. About 1 ml of distilled water was added and evaporated again to remove the HCl. This treatment was repeated three times. The residue was then dissolved in 0.5 ml distilled water, filtrated through membrane filter (0.45 μ m) and stored at 4°C. The supernatants and hydrolysates were used to analyze Phe and its related compounds by HPLC (Amin et al., 1995).

In order to determine the protozoal numbers, 0.5 ml portions of the microbial suspensions were collected, mixed with 4.5 ml of methylgreen-formalin-salt (MFS) solution (Onodera et al., 1977) and kept at room temperature.

Analytical methods. Analyses of Phe and its related compounds were carried out by a HPLC method (Amin et al., 1995). The values of all of the compounds found in the supernatants and hydrolysates were expressed as the means of nine determinations and standard deviations of the differences between incubation with and without substrates and additives. Rumen protozoa were counted with the aid of a Fuchs-Rosenthal hemacytometer. Microbial nitrogen was determined using the Kjeldahl method (A. O. A. C., 1990). Analysis of variance (ANOVA) was carried out to check whether there was any significant differences between the mean values of the different groups. On the other hand, pairwise comparisons were also done using Student's *t*-test (Snedecor and Cochran, 1980).

Results

Choice of vitamin B₆ concentration

Phenylpyruvic acid (PPY) was incubated as a substrate and the production of phenylalanine (Phe) was examined with different concentrations of B₆ in bacterial and protozoal suspensions at the beginning of this experiment (Fig. 1). Phe production from PPY was enhanced by 11% in B and 9% in P suspensions with the addition of 10 μ g/ml of B₆, whereas the tendency of enhancing Phe production was up to 12 and 10% in B and P suspensions, respectively, with a higher concentration of B₆ (Fig. 1). This means that Phe production was increased only about 1% in both B and P suspensions with the addition of a higher concentration (up to 100 μ g/ml) as compared with the concentration of 10 μ g/ml of B₆. Finally, the lowest concentration of B₆ (10 μ g/ml) with relatively maximum production of Phe was chosen for subsequent experiments.

Fig. 1. Formation of phenylalanine (Phe) from phenylpyruvic acid by bacteria (B) and protozoa (P) during 12 h incubation with different concentrations of vitamin B₆.

Effects of salinomycin and vitamin B₆ on the metabolism of phenylpyruvic acid by rumen bacteria, protozoa and their mixture

Effects on the disappearance of phenylpyruvic acid.

Table 1 partly shows the disappearance of PPY with and without SL and B₆ in B, P and BP suspensions during a 12 h incubation period. PPY disappeared without additives by 652, 672 and 800 μ M in B, P and BP suspensions during a 6 h incubation period and completely disappeared during the 12 h incubation period. The rate of disappearance showed no significant differences between the microbial suspensions with and without SL and B₆ during the incubation period when checked by ANOVA (Table 1).

Effects on the synthesis of phenylalanine from phenylpyruvic acid. PPY was converted mainly to Phe and the production of it in B, P and BP suspensions with and without SL and B₆ is shown in Table 1. The production of Phe from PPY was almost similar to the results reported previously (Amin and Onodera, 1997b). In the case of PPY metabolism, only the values during the 6 h incubation period have been taken into consideration for discussion, because PPY was completely utilized by all the microbial suspensions during the 12 h incubation period. However, in some cases, the values during the 12 h incubation period were also used for discussion when necessary.

In B suspensions without additives, 286 and 404 μ mol/g microbial nitrogen (MN) (282 and 396 μ M)

Table 1. Disappearance of PPY and the production of Phe and PAA from PPY with and without SL and B₆ by B, P and BP during 12 h incubation.

	Incubation time (h)					
	B		P		BP	
	6	12	6	12	6	12
Disappearance of PPY (μM)						
Control	652 $\pm$ 38.8 ^a	1,028 $\pm$ 23.6 ^a	672 $\pm$ 25.7 ^a	1,011 $\pm$ 4.6 ^a	800 $\pm$ 45.3 ^a	1,037 $\pm$ 17.9 ^a
With SL	665 $\pm$ 39.4 ^a	1,016 $\pm$ 23.2 ^a	709 $\pm$ 34.1 ^a	1,023 $\pm$ 8.0 ^a	809 $\pm$ 51.2 ^a	1,017 $\pm$ 21.1 ^a
With B ₆	670 $\pm$ 55.5 ^a	1,031 $\pm$ 21.4 ^a	691 $\pm$ 30.0 ^a	1,030 $\pm$ 16.5 ^a	826 $\pm$ 11.8 ^a	1,026 $\pm$ 24.8 ^a
Production of Phe ($\mu\text{mol/g MN}$)						
Control	286 $\pm$ 29.5 ^a	404 $\pm$ 23.6 ^a	251 $\pm$ 11.7 ^a	372 $\pm$ 16.6 ^a	242 $\pm$ 13.0 ^a	299 $\pm$ 18.5 ^a
With SL	356 $\pm$ 40.5 ^b	564 $\pm$ 17.2 ^b	297 $\pm$ 12.0 ^b	447 $\pm$ 34.4 ^b	284 $\pm$ 19.9 ^b	357 $\pm$ 31.6 ^b
With B ₆	315 $\pm$ 28.6 ^{ab}	462 $\pm$ 7.6 ^c	276 $\pm$ 6.0 ^c	404 $\pm$ 12.1 ^{ab}	267 $\pm$ 12.5 ^{ab}	321 $\pm$ 7.0 ^{ab}
Production of PAA ($\mu\text{mol/g MN}$)						
Control	105 $\pm$ 19.4 ^{ab}	184 $\pm$ 41.1 ^{ab}	101 $\pm$ 13.7 ^{ab}	161 $\pm$ 18.5 ^a	94 $\pm$ 14.9 ^{ab}	101 $\pm$ 20.2 ^a
With SL	80 $\pm$ 23.0 ^a	119 $\pm$ 21.9 ^a	85 $\pm$ 11.0 ^a	102 $\pm$ 16.3 ^b	63 $\pm$ 13.0 ^a	63 $\pm$ 12.3 ^b
With B ₆	136 $\pm$ 26.2 ^b	216 $\pm$ 53.0 ^b	117 $\pm$ 15.6 ^b	179 $\pm$ 21.7 ^a	108 $\pm$ 11.8 ^b	123 $\pm$ 13.5 ^a

Phe, phenylalanine; PPY, phenylpyruvic acid; PAA, phenylacetic acid; SL, salinomycin; B₆, vitamin B₆; B, mixed bacteria; P, mixed protozoa; BP, mixed bacteria and protozoa; MN, microbial nitrogen.

^{a,b,c} Means in the same column with different superscripts differ significantly ($p < 0.05$).

of PPY were converted to Phe, while the production of Phe from PPY with SL was enhanced ($p < 0.05$) by 24.5 and 39.6% during the 6 and 12 h incubation periods, respectively. Phe was found both in the supernatants of the medium and in the hydrolysates of bacterial cells without SL, and increased Phe production was observed with SL both in the supernatants of the medium (61.7 and 95.0 μM during the 6 and 12 h incubation periods, respectively) and in the hydrolysates of bacterial cells (4.6 and 62.7 μM during the 6 and 12 h incubation periods, respectively), though the data were not shown in Table 1. On the other hand, the addition of B₆ to B suspensions showed that Phe production from PPY tended to be enhanced by 10.1% during the 6 h incubation period. The production of Phe was also increased with B₆, both in the supernatants of the medium (20.7 and 21.0 μM during the 6 and 12 h incubation periods, respectively) and in the hydrolysates of the bacterial cells (7.6 and 36.0 μM during the 6 and 12 h incubation periods, respectively).

In P suspensions without additives, 251 $\mu\text{mol/g MN}$ (297 μM) of PPY was converted to Phe, while the production of Phe with SL was enhanced ($p < 0.05$) by 18.3% during the 6 h incubation period. The addition of B₆ to P suspensions enhanced ($p < 0.05$) Phe production from PPY by 10.0% during the 6 h incubation period, as was the case of rumen bacteria.

The rumen microbial ecosystem mainly consists of bacteria and protozoa. In order to infer the effects of SL and B₆ on the synthesis of Phe and the production of PAA by natural rumen microorganisms, BP suspensions were also employed for incubation to speculate the real activities of the rumen microbial ecosystem

and to investigate the interactions between bacteria and protozoa, if any. In BP suspensions without additives, 242 $\mu\text{mol/g MN}$ (355 μM) of PPY was converted to Phe, whereas the production of Phe from PPY in suspensions with SL was enhanced ($p < 0.05$) by 17.4% during the 6 h incubation period. In the same BP suspensions, the concentration of Phe from PPY with SL was also found to be increased in the supernatants of the medium (46.4 and 61.6 μM during the 6 and 12 h incubation periods, respectively) and in the hydrolysates of microbial cells (14.9 and 24.3 μM during the 6 and 12 h incubation periods, respectively). On the other hand, Phe production from PPY with B₆ in BP suspensions tended to be enhanced by 10.3% during the 6 h incubation period. The concentration of Phe with B₆ was also found to be increased in the supernatants of the medium (26.2 and 3.0 μM during the 6 and 12 h incubation period, respectively) and in the hydrolysates of the microbial cells (10.5 and 29.0 μM during the 6 and 12 h incubation period, respectively).

Effects on the production of phenylacetic acid (PAA) from phenylpyruvic acid. The large amounts of PAA produced from PPY and the production of PAA in the B, P and BP suspensions with and without SL and B₆ are shown in Table 1. PAA production from PPY was almost similar to a result reported previously (Amin and Onodera, 1997b).

In B suspensions without additives, 105 $\mu\text{mol/g MN}$ (102 μM) of PPY was converted to PAA during the 6 h incubation period. The production of PAA from PPY with SL tended to be inhibited though not significantly (23.8%) during the 6 h incubation period. The produc-

Table 2. Disappearance of PAA and the production of Phe from PAA with and without SL and B₆ by B, P and BP during 12 h incubation.

	Incubation time (h)					
	B		P		BP	
	6	12	6	12	6	12
Disappearance of PAA (μM)						
Control	363 $\pm$ 27.1 ^a	483 $\pm$ 15.4 ^a	294 $\pm$ 26.5 ^a	462 $\pm$ 9.5 ^a	400 $\pm$ 54.2 ^a	507 $\pm$ 46.6 ^a
With SL	266 $\pm$ 35.2 ^b	404 $\pm$ 16.6 ^b	240 $\pm$ 4.6 ^b	393 $\pm$ 25.6 ^b	329 $\pm$ 30.9 ^a	406 $\pm$ 36.1 ^b
With B ₆	375 $\pm$ 25.4 ^a	511 $\pm$ 29.8 ^a	324 $\pm$ 31.3 ^a	502 $\pm$ 6.9 ^c	406 $\pm$ 64.0 ^a	518 $\pm$ 57.3 ^a
Production of Phe ($\mu\text{mol/g MN}$)						
Control	174 $\pm$ 12.3 ^a	240 $\pm$ 17.2 ^{ab}	132 $\pm$ 13.1 ^{ab}	208 $\pm$ 24.8 ^a	140 $\pm$ 8.1 ^{ab}	181 $\pm$ 10.9 ^{ab}
With SL	153 $\pm$ 6.8 ^b	231 $\pm$ 19.6 ^a	118 $\pm$ 19.7 ^a	193 $\pm$ 25.6 ^a	129 $\pm$ 9.2 ^a	167 $\pm$ 5.3 ^a
With B ₆	198 $\pm$ 10.4 ^c	272 $\pm$ 20.6 ^b	153 $\pm$ 15.9 ^b	241 $\pm$ 29.4 ^a	153 $\pm$ 8.7 ^b	196 $\pm$ 8.9 ^b

Abbreviations, see Table 1.

tion of PAA from PPY with B₆, different from the case of SL, tended to be enhanced by 29.5 and 17.4% in B suspensions during the 6 and 12 h incubation periods, respectively, but no significant differences were observed between those with and without B₆.

In P suspensions without additives, 101 $\mu\text{mol/g MN}$ (119 μM) of PPY was converted to PAA, while PAA production from PPY with SL tended to be inhibited by 15.8% during the 6 h incubation period, while significant inhibition ($p < 0.05$, 36.6%) was observed during the 12 h incubation period. The production of PAA from PPY with B₆ in P suspensions tended to be enhanced by 15.8% during the 6 h incubation period, different from the case of SL. However, no significant difference was observed between the P suspensions with and without B₆.

In BP suspensions without additives, 94 $\mu\text{mol/g MN}$ (137 μM) of PPY was converted to PAA, while the production of PAA from PPY with SL tended to be inhibited by 33.0% during the 6 h incubation period, and significant inhibition ($p < 0.05$, 37.6%) was observed during the 12 h incubation period. The production of PAA from PPY with B₆ tended to be enhanced by 14.9% during the 6 h incubation period in BP suspensions, but no significant difference was observed between the BP suspensions with and without B₆.

Effects of salinomycin and vitamin B₆ on the metabolism of phenylacetic acid by rumen bacteria, protozoa and their mixture

Effects on the disappearance of phenylacetic acid. The disappearance of PAA used as a substrate with and without SL and B₆ in B, P and BP suspensions during the 12 h incubation period is shown in Table 2. PAA disappeared without additives by 363 and 483, 294 and 462, and 400 and 507 μM , while the disappearance of PAA with SL was inhibited ($p < 0.05$) by 26.7 and 16.4, 18.4 and 14.9, and 17.8 and 19.9% during the 6 and 12 h incubation periods, in B, P and

BP suspensions, respectively.

On the other hand, the disappearance of PAA with B₆ was almost the same as that without B₆ during the 12 h incubation period in B and BP suspensions, whereas, in P suspensions, the disappearance of PAA with B₆, different from the result with SL, tended to be enhanced by 10.2 and 8.7% during the 6 and 12 h incubation periods, respectively. However, no significant differences were observed between those with and without B₆.

Effects on the synthesis of phenylalanine from phenylacetic acid. PAA was converted mainly to Phe, and the production of Phe in B, P and BP suspensions with and without SL and B₆ is shown in Table 2. The production of Phe from PAA was almost similar to results reported previously (Amin and Onodera, 1997b). In B suspensions without additives, 174 and 240 $\mu\text{mol/g MN}$ (168 and 231 μM) of PAA were converted to Phe, while the production of Phe with SL tended to be inhibited, though not significantly, by 12.1 and 3.8% during the 6 and 12 h incubation periods. The addition of B₆ to B suspensions showed that Phe production from PAA tended to be enhanced by 13.8 and 13.3% during the 6 and 12 h incubation periods, respectively.

In P suspensions without additives, 132 and 208 $\mu\text{mol/g MN}$ (156 and 244 μM) of PAA were converted to Phe, while Phe production from PAA with SL tended to be inhibited though not significantly, by 10.6 and 7.2% during the 6 and 12 h incubation periods, respectively. With B₆ in P suspensions, the production of Phe from PAA tended to be enhanced by 15.9% both in the 6 and 12 h incubation periods, different from the case with SL.

In BP suspensions without additives, 140 and 181 $\mu\text{mol/g MN}$ (193 and 248 μM) of PAA were converted to Phe, however, the production of Phe from PAA with SL tended to be inhibited by 7.9 and 7.7% during the 6 and 12 h incubation periods, respectively.

Table 3. Disappearance of Phe and the production of PAA from Phe with and without SL and B₆ by B, P and BP during 12 h incubation.

	Incubation time (h)					
	B		P		BP	
	6	12	6	12	6	12
Disappearance of Phe (μM)						
Control	369 $\pm$ 18.0 ^a	549 $\pm$ 10.0 ^a	283 $\pm$ 29.1 ^a	365 $\pm$ 34.6 ^a	498 $\pm$ 14.8 ^a	842 $\pm$ 15.0 ^a
With SL	238 $\pm$ 43.5 ^b	342 $\pm$ 26.7 ^b	207 $\pm$ 15.8 ^b	262 $\pm$ 15.6 ^b	280 $\pm$ 6.4 ^b	453 $\pm$ 14.0 ^b
With B ₆	392 $\pm$ 23.2 ^a	600 $\pm$ 22.1 ^c	305 $\pm$ 32.5 ^a	395 $\pm$ 52.0 ^a	552 $\pm$ 21.6 ^c	905 $\pm$ 32.1 ^c
Production of PAA ($\mu\text{mol/g MN}$)						
Control	187 $\pm$ 7.6 ^a	284 $\pm$ 12.7 ^a	151 $\pm$ 3.5 ^a	191 $\pm$ 8.1 ^a	218 $\pm$ 3.5 ^a	361 $\pm$ 18.0 ^a
With SL	56 $\pm$ 7.6 ^b	78 $\pm$ 10.5 ^b	70 $\pm$ 7.2 ^b	88 $\pm$ 3.2 ^b	57 $\pm$ 2.6 ^b	88 $\pm$ 3.8 ^b
With B ₆	222 $\pm$ 14.1 ^c	338 $\pm$ 16.5 ^c	175 $\pm$ 6.7 ^c	226 $\pm$ 14.8 ^c	268 $\pm$ 17.0 ^c	435 $\pm$ 34.0 ^c

Abbreviations, see Table 1.

The addition of B₆ to BP suspensions showed that Phe production from PAA tended to be enhanced by 9.3 and 8.3% during the 6 and 12 h incubation periods, respectively.

Effects of salinomycin and vitamin B₆ on the metabolism of phenylalanine by rumen bacteria, protozoa and their mixture

Effects on the disappearance of phenylalanine. Table 3 shows the disappearance of Phe used as a substrate with and without SL and B₆ in B, P and BP suspensions during the 12 h incubation period. The net Phe disappearance without additives was 369 and 549, 283 and 365, and 498 and 842 μM , while the net disappearance of Phe with SL was inhibited ($p<0.05$) by 35.5 and 37.7, 26.9 and 28.2, and 43.8 and 46.2% during the 6 and 12 h incubation periods in B, P and BP suspensions, respectively.

On the other hand, B₆ was effective for the disappearance of Phe in the B, P and BP suspensions during the 12 h incubation period. The net disappearance of Phe was enhanced by 6.2 and 9.3 ($p<0.05$), 7.8 and 8.2, and 10.8 and 7.5% ($p<0.05$) during the 6 and 12 h incubation periods in B, P and BP suspensions, respectively, different from the case of SL.

Effects on the production of phenylacetic acid from phenylalanine. Phe was converted mainly to PAA, and the production of PAA in B, P and BP suspensions with and without SL and B₆ is shown in Table 3. PAA production from Phe was almost similar to results reported previously (Amin and Onodera, 1997a). In B suspensions without additives, 187 and 284 $\mu\text{mol/g MN}$ (163 and 254 μM) of Phe were converted to PAA, while the production of PAA in the suspensions with SL was inhibited ($p<0.05$) by 70.0 and 72.5% during the 6 and 12 h incubation periods, respectively. When B₆ was added to the medium of B suspensions, PAA production from Phe was enhanced ($p<0.05$) by 18.7 and 19.0% during the 6 and 12 h incubation periods,

respectively, different from the case of SL.

In P suspensions without additives, 151 and 191 $\mu\text{mol/g MN}$ (161 and 205 μM) of Phe were converted to PAA, while the production of PAA from Phe in the suspensions with SL was inhibited ($p<0.05$) by 53.6 and 53.9% during the 6 and 12 h incubation periods, respectively, similar to the results of rumen bacteria. With B₆ in P suspensions, PAA production from Phe was enhanced ($p<0.05$) by 15.9 and 18.3% during the 6 and 12 h incubation periods, respectively, different from the case of SL.

In BP suspensions without additives, 218 and 361 $\mu\text{mol/g MN}$ (278 and 461 μM) of Phe were converted to PAA, whereas the production of PAA in the suspensions with SL was inhibited ($p<0.05$) by 73.9 and 75.6% during the 6 and 12 h incubation periods, respectively. When B₆ was added to the medium of BP suspensions, PAA production from Phe was enhanced ($p<0.05$) by 22.9 and 20.5% during the 6 and 12 h incubation periods, respectively.

Discussion

The rumen is a bioreactor in which various microorganisms are closely interrelated, and these microorganisms can contribute considerably to the nutrition of host animals. It is therefore very hard to modify a particular function without adversely affecting others. However, ionophores and B-vitamins appear to be compounds which can successfully manipulate ruminant metabolism without provoking serious problems in the nutritional system of ruminants.

In this experiment, a marked change of the effects of SL and B₆ were observed during the 12 h incubation period. When PPY was added as a substrate, Phe production from PPY in B and BP suspensions showed that a higher concentration of Phe in the supernatants and hydrolysates was found in the presence of SL and B₆. This means that both SL and B₆

can initiate rumen B and BP to accumulate extracellular Phe in the medium as well as in the body protein and free amino acid pool. It was shown that the tendency of SL to accumulate extracellular Phe from PPY was higher than the incorporation of Phe into the microbial cells, whereas B₆ stimulated to accumulate higher Phe in the microbial cells than that accumulated in the medium. This may be due to the fact that ionophores may inhibit the transport system to a certain limit to incorporate amino acids or peptides into the microbial cells, as mentioned with other ionophores like monensin (Van Nevel and Demeyer, 1977), while B₆ may enhance the production of amino acids in the microbial cells. Okuuchi et al. (1993) reported that rumen bacteria can accumulate higher extracellular tryptophan from indolepyruvic acid in the incubation medium. The production of tryptophan was enhanced with SL, similar to our present findings, though they did not determine tryptophan in the hydrolysate of the cell protein. In P suspensions, SL and B₆ also enhanced the production of Phe from PPY, and the production of Phe was found only in the supernatants of the medium because P did not grow in the present system mentioned previously in details (Amin and Onodera, 1997a,b). In this connection, tryptophan production from indolepyruvic acid with SL has been shown to be enhanced by the rumen P and BP suspensions (Okuuchi et al., 1993). The tendency to increase Phe production from PPY with SL and B₆ was lower in P and BP than that in B.

The production of PAA from PPY was shown to be inhibited by SL in all the microbial suspensions in this experiment. This means that rumen microorganisms are not so active in decarboxylating PPY in the presence of SL. The result was similar to that of Okuuchi et al. (1993) where indoleacetic acid production from indolepyruvic acid was shown to be inhibited by SL in all the microbial suspensions. The enhancement of Phe production and the inhibition of PAA production from PPY with SL indicated in this experiment suggest that rumen microorganisms might enhance the transamination of PPY to produce Phe and inhibit decarboxylation of PPY to produce PAA in the presence of SL. Thus, SL can change the metabolic flow of PPY toward transamination rather than decarboxylation in rumen bacteria and protozoa. This was the first demonstration as far as we are aware. On the other hand, B₆ was shown to increase PAA production from PPY in all the microbial suspensions, different from the case of SL. The tendency to increase PAA production was shown to be lowered after the 6 h incubation period, whereas Phe production was shown to be increased after incubation for 6 h with B₆. The enhancement of Phe and PAA production from PPY by B₆ in all the microbial suspensions was demonstrated for the

first time.

When PAA was used as a substrate, the disappearance of PAA was shown to be inhibited by SL in all the microbial suspensions. Thus, SL was shown to decrease PAA disappearance in rumen B, P and BP for the first time in this experiment. Okuuchi et al. (1993) reported that the disappearance of indoleacetic acid was completely inhibited by SL in B, P and BP suspensions. On the other hand, with B₆, no effect was found on the disappearance of PAA in B and BP suspensions, whereas in P suspensions, the disappearance of PAA tended to be enhanced, different from the results with SL.

Phe production from PAA was shown to be inhibited by SL in all the microbial suspensions in this experiment. In B and BP suspensions without SL, small amounts of Phe were found in the supernatants of the medium and large amounts were found in the hydrolysates of the microbial cells. The addition of SL decreased the production of Phe in the hydrolysates of the microbial cells during the incubation period and in the supernatants of the medium during the 6 h incubation period, though it was found to be increased in the supernatants of the medium during the 12 h incubation period, similar to the Phe production from PPY. In tryptophan metabolism by rumen B, the production of skatole from indoleacetic acid was completely inhibited by SL, and no tryptophan was found to be produced from indoleacetic acid in the supernatants of the P suspensions with or without SL as reported by Okuuchi et al. (1993). The observations of Allison (1965) and Kristensen (1974) reporting the production of Phe from PAA by rumen bacteria were again confirmed in this experiment, though it was also confirmed in our previous study (Amin and Onodera, 1997a). On the other hand, the production of Phe from PAA was shown to be enhanced by B₆ in all the microbial suspensions. In B and BP suspensions with B₆, the concentration of Phe was found to be increased both in the supernatants of the medium and in the hydrolysates of the microbial cells. This phenomenon seemed to be similar to SL in the supernatants of the rumen B and BP suspensions, though it was different in the hydrolysates of the microbial cells. Thus, it was shown in this experiment that B₆ may enhance the accumulation of both extracellular and intracellular Phe from PAA, different from the case of SL. This is the first report to demonstrate the ability of SL to inhibit and of B₆ to enhance the production of Phe from PAA in all the microbial suspensions.

When Phe was added as a substrate in the incubation medium, the net disappearance of Phe was shown to be inhibited by SL, whereas the disappearance of Phe was enhanced by B₆ in all the microbial suspensions, different from the case of SL. In this ex-

periment, the results from SL coincided with those of previous papers concerning other amino acids (Hoshino et al., 1992; Okuuchi et al., 1993), except for the fact that the degradation of tryptophan in P suspensions was slightly stimulated by SL (Okuuchi et al., 1993). SL caused a dramatic decline in deaminase activity (McAllister et al., 1994). Similar changes have been observed with other ionophores (Hino and Russell, 1985; Newbold et al., 1990; Russell and Martin, 1984; Van Nevel and Demeyer, 1977; Wallace et al., 1981; Yang and Russell, 1993a, b). Amino acid deamination has been discussed to be particularly sensitive to ionophores due partly to the elimination of a specific bacterial population of amino acid utilizers (Yang and Russell, 1993a) and to the decreased deaminative activity of ionophore-resistant bacteria (Newbold et al., 1990). Peptide and amino acid accumulation would be expected to be beneficial, firstly because rumen microorganisms would be able to assimilate preformed amino acids, saving the energy required for their biosynthesis, and secondly because some of these compounds may escape undegraded from the rumen and be utilized by the host (Wallace, 1994). The inhibition of amino acid degradation in the rumen is an obvious objective for manipulation. Even if the amino acids are not to pass undegraded from the rumen, if they were to be incorporated directly into microbial protein instead of being degraded to ammonia and then resynthesized, the energy cost of resynthesis would be saved. Therefore, amino acid breakdown may be nutritionally expensive not only because amino acids are lost, but also because of the high energetic costs of their breakdown. This is the first report to demonstrate the ability of SL to inhibit and of B₆ to enhance the deamination of Phe by rumen protozoa as well as rumen bacteria.

The production of PAA from Phe was shown to be inhibited by SL, whereas it was enhanced by B₆ in all the microbial suspensions, different from the case of SL. In tryptophan metabolism, Okuuchi et al. (1993) reported that indoleacetic acid production from tryptophan was inhibited with the addition of SL by over 90% in the suspensions of B and BP, whereas it was over 45% in the suspension of P during a 12 h incubation period. In this experiment, it was observed that the inhibition of PAA production from Phe was higher in the B and BP suspensions than in the P suspension. This means that the inhibition of Phe deamination by SL was more effective in B than in P. On the other hand, it was observed that the inhibition of PAA production from Phe was higher than the inhibition of PAA production from PPY in all the microbial suspensions. Thus, the higher inhibition of PAA production from Phe by SL is thought to have worked on the step from Phe to PPY, though the quantity of PPY was not

detected in this experiment.

This study concludes that SL inhibited Phe disappearance, and this inhibition, in turn, increased the concentration of dietary free Phe in the rumen. SL enhanced the synthesis of Phe from PPY, though not from PAA and accumulated a higher free Phe for incorporation into microbial cells, which could increase the flow of amino N to the lower gut. On the other hand, B₆ also enhanced Phe synthesis both from PPY and PAA and incorporated higher Phe for accumulation as free form, which could provide additional amino N for animals.

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