Effects of salinomycin and vitamin B₆ on the in vitro synthesis of lysine from the stereoisomers of 2,6-diaminopimelic acid by mixed rumen protozoa, bacteria, and their mixture

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An in vitro study was conducted to examine the effects of salinomycin (SL) and vitamin B₆ (pyridoxine hydrochloride) (B₆) on the production of lysine from the three stereoisomers of 2,6-diaminopimelic acid (DAP-SI) by mixed rumen protozoa (P), mixed rumen bacteria (B), and their mixture (PB). P, B, and PB were isolated from the rumen of goats given a concentrate mixture and lucerne cubes, separately incubated for 12 h with and without DAP-SI (5 mM) as a substrate and SL (5 µg/ml) and/or B₆ (10 µg/ml) as additives. In P suspensions, SL and B₆ reduced the amount of DAP-SI by 2.1 times ($p < 0.001$, where p is probability) and 19.9% ($p < 0.05$), respectively, and also increased the production of lysine by 2.4 times ($p < 0.001$) and 26.8% ($p < 0.05$), respectively, during 12 h incubation. In B suspensions, the reductions of DAP-SI with a single addition of SL or B₆ were 8.5% ($p < 0.001$) and 2.7%, respectively, and lysine production increased by 54.3 and 32.9% ($p < 0.001$), respectively, during 12 h incubation. In PB suspensions, the reductions of DAP-SI were 21.9 and 11.7% ($p < 0.001$) with a single addition of SL or B₆, respectively, and the production of lysine increased by 81.4 and 39.4% ($p < 0.001$), respectively, during 12 h incubation. When SL and B₆ were added together to the P, B, and PB suspensions, lysine production further increased by 12.3, 21.3, and 12.4% more than the cases of adding SL only during 12 h incubation, respectively. SL and B₆ were demonstrated to enhance the production of lysine from DAP-SI by mixed rumen protozoa, mixed rumen bacteria and their mixture in this study.

Key Words—effect of salinomycin; effect of vitamin B₆; lysine synthesis; rumen bacteria; rumen protozoa; stereoisomers of diaminopimelate

Monensin and salinomycin (SL), kinds of ionophores (polyether antibiotics), have been shown to favorably alter ruminal fermentation characteristics and metabolism (Bergen and Bates, 1984; Schelling, 1984). Several other ionophores such as lasalocid, lysocellin, narasin, and tetronasin have also been shown to provide similar benefits. Of these ionophores, SL is produced by *Streptomyces albus* (Kinashi et al., 1973). There have been many reports concerning its curative effect on feedlot bloat and negative effects on the in vivo total counts of Gram-positive cocci (Hoshino et al., 1986) and mixed rumen protozoa (Onodera et al., 1986), effectiveness in preventing experimentally induced lactic acidosis (Nagaraja et al., 1985), and improved rates of body weight gain

(Merchen and Berger, 1985) and feed efficiency (McClure et al., 1980; Merchen and Berger, 1985; Miyazaki et al., 1994; Onodera et al., 1986; Owens et al., 1982; Turgeon et al., 1983). Furthermore, total amino acid concentration in the air-dried matter of rumen contents of goats has been shown to extremely increase by feeding SL (Onodera et al., 1987). SL has also been shown to decrease amino acid breakdown in mixed sheep rumen microbes in vitro (Hoshino et al., 1992). This may be due to the inhibitory effect of deamination as has been observed with other ionophores (Newbold et al., 1990). Okuuchi et al. (1993) have shown, in their in vitro study, that tryptophan production from indolepyruvate by rumen microorganisms increased when SL was added to the incubation media.

On the other hand, it has generally been thought that a vitamin B complex (B vitamins) was produced in the rumen of ruminants, and that it was unnecessary

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to give B vitamins to ruminant animals. In earlier papers, however, vitamin B₆ (B₆) was shown to effectively stimulate cellulose digestion by washed cell suspensions of mixed rumen bacteria (MacLeod and Murray, 1956) and the growth of cellulolytic rumen bacteria such as *Ruminococcus flavefaciens*, *Bacteroides (Fibrobacter) succinogenes* and so on. There have been no reports to show the effect of B₆ on lysine production by rumen microorganisms. In our previous studies, we demonstrated, in detail, lysine production from the three stereoisomers of 2,6-diaminopimelic acid (DAP-SI) by mixed rumen protozoa and bacteria (El-Waziry and Onodera, 1996; El-Waziry et al., 1995). In this study, we examine the effects of SL and B₆ on the in vitro synthesis of lysine from DAP-SI by mixed rumen protozoa, bacteria, and their mixture.

Materials and Methods

Animals. Three mature rumen fistulated goats (Japanese native breed) with a mean live weight of 35(±5) kg, fed on a daily ration consisting of lucerne cubes (23 g DM/kg BW^{0.75}) and concentrate 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 obtained from the fistulated goats before morning feeding were strained through four layers of surgical gauze into a separatory 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 protozoa (P), bacteria (B), and their mixture (PB) 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 activities of contaminating bacteria.

Incubation and sample treatments. Buffer solution (MB9) (Onodera and Henderson, 1980) and all tools used in this study were previously autoclaved, and MB9 buffer was gassed with N₂+CO₂ again under germ-free conditions. DAP-SI solutions, as substrates, were prepared dissolving a mixture of three stereoisomers of 2,6-diaminopimelic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) in MB9 buffer. The microbial suspensions (20 ml) prepared above were incubated with and without DAP-SI (5 mM, due to Onodera and Takashima (1989) and Onodera et al. (1991)) and salinomycin (SL) (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) (5 µg/ml) (Okuuchi et al., 1993) and/or B₆ (pyridoxine hydrochloride) (Daiichi Pharmaceutical Co.,

Ltd., Tokyo, Japan) (10 µg/ml) (Amin and Onodera, 1995) in 30 ml Erlenmeyer flasks at 39°C for up to 12 h. All incubation mixtures contained 0.5 mg/ml rice starch. Samples collected (1 ml) at 0, 6, and 12 h were mixed with an equal volume of 20% trichloroacetic acid (TCA) for deproteinization, left overnight and centrifuged at 27,000×g for 20 min. The supernatant was washed three times with diethyl ether to remove TCA and evaporated to remove diethyl ether. Sediments of bacteria and bacteria plus protozoa were hydrolyzed with 6 M HCl at 110°C for 20 h in sealed tubes. After cooling, the contents were filtered through filter paper (Whatman No. 2) and washed three times with pure water. The filtrate was evaporated to dryness, washed three times with pure water to remove HCl, dissolved again in 1 ml of pure water, and then filtered again through a 0.45 µm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) before analysis. All samples were subjected to analyses for DAP-SI and lysine.

Analytical methods. The analyses for DAP-SI and lysine were carried out by a HPLC method according to El-Waziry et al. (1996). Concentrations of these compounds were expressed by the differences between the figures of incubations with and without substrates. A statistical analysis of the data was performed using the Fisher test (F-test) (Snedecor and Cochran, 1980).

Results and Discussion

The effect of SL or B₆ on lysine production by mixed rumen protozoa, bacteria, and their mixture

In the case of P suspensions, only the supernatant fluid of the incubation mixture was provided for the analyses of DAP-SI and lysine, because in this incubation, protozoa cannot grow and accumulate any lysine in the body protein.

Table 1 shows the reduction of each DAP-SI and the increase in lysine in the supernatant of the P suspensions after incubation for 6 and 12 h with a mixture of three DAP-SI. The DAP-SI used consisted of 63.3% *meso*-, 17.5% *LL*-, and 19.2% *DD*-forms. DAP-SI content, as a whole, decreased by 0.251 and 0.518 mM on average, and converted mainly to lysine by 0.182 and 0.411 mM (34.9 and 79.3% of the reduction in DAP-SI) on average by incubation for 6 and 12 h, respectively, when neither SL nor B₆ were added. The rest (0.069 and 0.107 mM) of the missing DAP-SI may have been converted to other undetermined compound(s) like pipercolic acid as an end product of lysine metabolism in rumen protozoa (Onodera and Kandatsu, 1972). With the single addition of SL or B₆, the reductions in DAP-SI content were an average of 0.603 and 0.286 mM (2.40 and 1.14 times higher than that without SL, $p < 0.001$ and B₆, $p < 0.05$, p is probability) after

Table 1. Effects of salinomycin (SL) and vitamin B₆ (B₆) on the breakdown of the three stereoisomers of 2,6-diaminopimelic acid (DAP) and lysine production by mixed rumen protozoa.^a

	Incubation time (h)					
	DAP only		DAP+SL		DAP+B ₆	
	6	12	6	12	6	12
Decrease in substrate						
meso-DAP (mm)	0.122±0.021 ^b	0.295±0.027	0.321±0.057	0.771±0.079	0.141±0.021	0.341±0.043
LL-DAP (mm)	0.064±0.007	0.128±0.013	0.151±0.034	0.181±0.023	0.080±0.016	0.170±0.015
DD-DAP (mm)	0.065±0.014	0.095±0.015	0.131±0.016	0.160±0.012	0.065±0.021	0.110±0.011
Total DAP (mm)	0.251±0.020	0.518±0.027	0.603±0.059*	1.112±0.079*	0.286±0.031**	0.621±0.043**
Increase in lysine						
Lysine (mm)	0.182±0.011	0.411±0.044	0.491±0.032*	0.982±0.028*	0.251±0.021**	0.521±0.028**
Lysine (nmol/mg protozoal N)	231.3±14.0	522.2±55.9	623.9±40.7*	1,247.8±35.6*	318.9±26.7**	662.0±35.6**

^a Mixture of three stereoisomers of 2,6-diaminopimelate (DAP) was added to the protozoal suspensions as substrate (5 mm), with and without SL (5 µg/ml) or B₆ (10 µg/ml). Values are shown as the differences between those with and without substrate after incubation at 39°C.

^b Mean±SD (n=3). *, significantly different from DAP only at $p<0.001$; **, significantly different from DAP only at $p<0.05$.

Table 2. Effects of salinomycin (SL) and vitamin B₆ (B₆) on the breakdown of the three stereoisomers of 2,6-diaminopimelic acid (DAP) and lysine production by mixed rumen bacteria.^a

	Incubation time (h)					
	DAP only		DAP+SL		DAP+B ₆	
	6	12	6	12	6	12
Decrease in substrate						
meso-DAP (mm)	0.191±0.012 ^b	0.316±0.052	0.281±0.047	0.383±0.064	0.252±0.021	0.281±0.043
LL-DAP (mm)	0.081±0.007	0.152±0.002	0.062±0.014	0.136±0.038	0.071±0.016	0.130±0.015
DD-DAP (mm)	0.069±0.005	0.117±0.010	0.111±0.016	0.116±0.019	0.080±0.021	0.190±0.011
Total DAP (mm)	0.341±0.022	0.585±0.064	0.454±0.049*	0.635±0.078*	0.403±0.031	0.601±0.043
Increase in lysine						
Supernatant (S)	0.021±0.010	0.061±0.011	0.031±0.011	0.172±0.042	0.021±0.010	0.136±0.010
Hydrolysates (H)	0.121±0.021	0.173±0.020	0.170±0.031	0.189±0.010	0.160±0.110	0.175±0.091
Lysine in S+H (mm)	0.142±0.024	0.234±0.033	0.201±0.032*	0.361±0.015*	0.181±0.021*	0.311±0.028*
Lysine (nmol/mg bacterial N)	269.5±50.1	488.5±68.9	419.6±66.8*	753.7±31.3*	377.9±43.8*	649.3±58.5*

^a Mixture of three stereoisomers of 2,6-diaminopimelate (DAP) was added to the bacterial suspensions as substrate (5 mm), with and without SL (5 µg/ml) or B₆ (10 µg/ml). Values are shown as the differences between those with and without substrate after incubation at 39°C.

^b Mean±SD (n=3). *, significantly different from DAP only at $p<0.001$.

6 h incubation, and 1.112 and 0.621 mm (2.15 and 1.20 times higher than that without SL, $p<0.001$ or B₆, $p<0.05$) after 12 h incubation. The increases in lysine were an average of 0.491 and 0.251 mm (2.70 and 1.38 times higher than that without SL, $p<0.001$ or B₆, $p<0.05$) after 6 h incubation, and 0.982 and 0.521 mm (2.39 and 1.27 times higher than that without SL, $p<0.001$ or B₆, $p<0.05$) after 12 h incubation. The average protozoal nitrogen of the P suspensions in this study was 0.787 ± 0.008 mg/ml. The amounts of lysine produced after 6 and 12 h per milligram of protozoal nitrogen, therefore, were 231.3 and 522.2 nmol without the addition of SL or B₆, while that with the addition of SL or B₆ were 623.9 and 318.9 nmol after 6 h

incubation, and 1247.8 and 662.0 after 12 h incubation, respectively.

In the case of B suspensions, both the supernatant fluid and the hydrolysate of the bacterial cells in the medium were analyzed, because bacteria can grow in this incubation medium and accumulate lysine in the body protein.

Table 2 shows the reduction of each DAP-SI and the increase in lysine in the supernatant and hydrolysates of the bacterial cells in the B suspensions incubated for 6 and 12 h with a mixture of three DAP-SI. Each stereoisomer of DAP-SI used had the same composition as that used for the P suspensions. The reductions in DAP-SI content, as a whole, were 0.341

and 0.585 mm on average and the production of lysine was 0.142 and 0.234 mm (41.6 and 40.0% of the reduction in DAP-SI) for 6 and 12 h incubation, respectively, when neither SL nor B₆ were added to the incubation mixture. The rest (0.199 and 0.351 mm) of the missing DAP-SI may have been partially used for constructing cell walls and/or partially degraded to other undetermined compounds like acetic and butyric acids and ammonia (Dohner and Cardon, 1954; Onodera and Kandatsu, 1975). With the single addition of SL or B₆, the reductions in DAP-SI were 0.454 and 0.403 mm on average (33.1 and 18.2% higher than that without SL, $p < 0.001$ or B₆) after 6 h incubation, and 0.635 and 0.601 mm (8.5 and 2.7% higher than that without SL, $p < 0.001$ or B₆, almost the same as that without B₆) after 12 h incubation. The increases in lysine were 0.201 and 0.181 mm on average (41.5 and 27.5% higher than that without SL or B₆, $p < 0.001$) after 6 h incubation, and 0.361 and 0.311 mm (54.3 and 32.9% higher than that without SL or B₆, $p < 0.001$) after 12 h incubation, respectively. The average bacterial nitrogen in B suspensions in the experiment was 0.479 ± 0.008 mg/ml, and the amount of lysine produced after 6 and 12 h per milligram of bacterial nitrogen was 269.5 and 488.5 nmol, respectively, without the addition of either SL or B₆, while that with the addition of SL or B₆ was 419.6 and 377.9 nmol for 6 h incubation, and 753.7 and 649.3 nmol for 12 h incubation, respectively.

When lysine production by protozoa and bacteria were compared, the lysine production by the protozoa with the addition of SL was 65.6% higher than that of the bacteria, but that by the protozoa with the addition of B₆ was almost the same as that by the bacteria.

The mixed suspensions of rumen protozoa and bacteria were incubated for 12 h, which is close to the natural microbial ecosystem of the rumen. Table 3 shows

the reduction of each DAP-SI and the increase in lysine in the supernatant and hydrolysates in the PB suspensions after 12 h incubation. The DAP-SI used had the same composition of each stereoisomer as that used for the P and B suspensions. The reduction in DAP-SI content, as a whole, in the PB suspensions was 0.770 mm and the production of lysine was 0.452 mm (58.7% of the missing DAP-SI) after 12 h incubation when neither SL nor B₆ were added to the incubation mixture.

The percentage of lysine production to missing DAP-SI in the PB suspensions (58.7%) was higher than that in the B suspensions (40.0%), although it was lower than that in the P suspensions (79.3%) after 12 h incubation.

With the single addition of SL or B₆, the reduction of DAP-SI was 0.939 mm (21.9% higher than that without SL, $p < 0.001$) and 0.860 mm (11.7% higher than that without B₆, $p < 0.001$), and the increase in lysine was 0.820 mm (81.4% higher than that without SL, $p < 0.001$) and 0.630 mm (39.4% higher than that without B₆, $p < 0.001$) after 12 h incubation. The average total microbial nitrogen in the PB suspensions in the experiment was 1.078 ± 0.008 mg/ml, and the amount of lysine produced during 12 h per milligram of microbial nitrogen was 419.3 nmol without the addition of either SL or B₆, while that with the addition of SL or B₆ was 760.7 and 584.4 nmol, respectively.

Thus, the present results clearly show that SL enhances lysine production more than the control by mixed rumen protozoa, bacteria, and their mixture. This suggests that SL stimulated the transport of DAP-SI into the microbial cells. In this connection, Okuuchi et al. (1993) reported that tryptophan production from indolepyruvate by mixed rumen protozoa, bacteria, and their mixture was increased when SL was added to the media. However, they did not check

Table 3. Effects of salinomycin (SL) and vitamin B₆ (B₆) on the breakdown of the three stereoisomers of 2,6-diaminopimelic acid (DAP) and lysine production by mixed suspensions of rumen protozoa and bacteria during 12 h incubation.^a

Substrate	DAP only	DAP+SL	DAP+B ₆
Decrease in substrate			
meso-DAP (mm)	0.483 $\pm$ 0.028 ^b	0.694 $\pm$ 0.051	0.490 $\pm$ 0.062
LL-DAP (mm)	0.177 $\pm$ 0.018	0.147 $\pm$ 0.021	0.140 $\pm$ 0.021
DD-DAP (mm)	0.110 $\pm$ 0.010	0.105 $\pm$ 0.011	0.230 $\pm$ 0.011
Total DAP (mm)	0.770 $\pm$ 0.120	0.939 $\pm$ 0.092*	0.860 $\pm$ 0.112*
Increase in lysine			
Supernatant (S)	0.160 $\pm$ 0.001	0.284 $\pm$ 0.022	0.282 $\pm$ 0.021
Hydrolysates (H)	0.292 $\pm$ 0.041	0.536 $\pm$ 0.001	0.348 $\pm$ 0.012
Lysine in S+H (mm)	0.452 $\pm$ 0.010	0.820 $\pm$ 0.001*	0.630 $\pm$ 0.031*
Lysine (nmol/mg microbial N)	419.3 $\pm$ 9.3	760.7 $\pm$ 0.9*	584.4 $\pm$ 28.8*

^a Mixture of three stereoisomers of 2,6-diaminopimelate (DAP) was added to the mixed suspensions of protozoa and bacteria as substrate (5 mm), with and without SL (5 μ g/ml) or B₆ (10 μ g/ml). Values are shown as the differences between those with and without substrate after incubation at 39°C.

^b Mean $\pm$ SD ($n=3$). *, significantly different from DAP only at $p < 0.001$.

Table 4. Effects of salinomycin (SL) with vitamin B₆ (B₆) on the production of lysine by mixed rumen protozoa, bacteria, and their mixture during 12 h incubation.^a

	DAP only	DAP+SL	DAP+SL+B ₆
Increase in lysine by protozoa (P)			
Lysine (mM)	0.411±0.044 ^b	0.982±0.028*	1.103±0.034*
Lysine (nmol/mg protozoal N)	522.2±55.9	1247.8±35.6*	1401.5±43.2*
Increase in lysine by bacteria (B)			
Lysine (mM)	0.234±0.033	0.361±0.015***	0.438±0.014**
Lysine (nmol/mg bacterial N)	488.5±68.9	753.7±31.3***	914.4±29.2**
Increase in lysine by mixture of PB			
Lysine (mM)	0.452±0.010	0.820±0.001***	0.922±0.066***
Lysine (nmol/mg microbial N)	419.3±9.3	760.7±0.9***	855.3±61.2***

^a Mixture of three stereoisomers of 2,6-diaminopimelate (DAP) was added to the protozoal and bacterial suspensions and their mixture as substrate (5 mM) with and without SL+B₆ or SL. Values are shown as the differences between those with and without substrate after incubation at 39°C.

^b Mean±SD (*n*=3). *, ***, significantly different from DAP only at *p*<0.001; **, significantly different from DAP+SL at *p*<0.05.

the bacterial hydrolysate in their study.

On the other hand, B₆ also enhanced lysine production by 26.8, 32.9, and 39.4% in P, B, and PB suspensions, respectively, where B₆ might act as a coenzyme to increase the production of lysine. In the experiments of this study, the increases in lysine with the addition of SL to the incubation mixture were 88.5, 16.1, and 30.2% higher than that with the addition of B₆ to P, B, and PB suspensions, respectively. These results indicate that the transport of the substrate through the cell membrane may be a major factor in lysine production in rumen protozoa, if the substrate exists as a solution in the medium. In this connection, Hoshino et al. (1992) reported that amino acid increased in mixed rumen microbes when SL was added to the incubation media, and this may be due to the inhibitory effect of deamination (Newbold et al., 1990). The effect of SL on the production of lysine by rumen protozoa from DAP-SI given as particles like rumen bacterial cells was not examined in this study.

The effect of SL together with B₆ on lysine production by mixed rumen protozoa, bacteria, and their mixture

Finally, the combined effect of the addition of both SL and B₆ on lysine production by rumen protozoa, bacteria, and their mixture was compared with the effect of the addition of SL alone. As the result, in P suspensions, lysine production with the addition of SL plus B₆ or SL alone, on average, was 1.103 mM (1,401.5 nmol/mg protozoal N) and 0.982 mM (1,247.8 nmol/mg protozoal N), respectively, while lysine production without additives as a control was 0.411 mM (522.2 nmol/mg protozoal N). Thus, the production of lysine in the media including SL plus B₆ was shown to be 12.3% higher than that with SL alone during 12 h incubation (Table 4). In B suspensions, lysine production with the addition of SL plus B₆ or SL alone, was 0.438 mM (914.4 nmol/mg bacterial N) and

0.361 mM (753.7 nmol/mg bacterial N), respectively, while lysine production without additives as a control was 0.234 mM (488.5 nmol/mg bacterial N). Lysine production in the media including SL plus B₆ was 21.3% higher (*p*<0.05) than that with SL alone during 12 h incubation (Table 4). In PB suspensions, lysine production with the addition of SL plus B₆ or SL alone was 0.922 mM (855.3 nmol/mg microbial N) and 0.820 mM (760.7 nmol/mg microbial N), respectively, while lysine production without additives as a control was 0.452 mM (419.3 nmol/mg microbial N). Lysine production in the media containing SL plus B₆ was 12.4% higher than that with SL alone during 12 h incubation (Table 4).

Generally, the concept that the benefits of ionophores on ruminant production are thought to be at least partially due to their positive influence on ruminal nitrogen metabolism (McAllister et al., 1994) was demonstrated in terms of lysine production by rumen microorganisms in this study.

The study revealed that SL extremely enhances lysine production from the three DAP-SI by rumen microorganisms. On the other hand, although vitamin B₆ has been thought to be produced in the rumen of ruminants and unnecessary to be given in ruminant nutrition, the observations of this study demonstrate that the addition of vitamin B₆ to the suspensions of rumen protozoa, bacteria, and their mixture also increases the production of lysine from the three DAP-SI. Even if vitamin B₆ was used together with SL, it enhances lysine production to a higher level than that with only SL. This may be due to the fact that the use of B₆ as a supplement to diets, especially for high-yield cattle, is suggested to be examined at the practical level.

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