# INHIBITORY EFFECT OF THE IONOPHORE SALINOMYCIN ON DEAMINATION BY MIXED RUMEN BACTERIA

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## Summary

A series of *in vitro* experiments was conducted to investigate response of rumen bacterial deamination to the ionophore salinomycin. Addition of salinomycin to the inoculum, strained rumen fluid, depressed ammonia production from casein, while increased accumulation of $\alpha$-amino acids. This suggests an inhibitory effect of salinomycin on ruminal deamination. When the effect in washed bacterial suspension was monitored with individual amino acid, aspartic acid degradation was markedly inhibited by salinomycin. This inhibition was not observed when the mixed rumen bacteria were ultrasonically disrupted and used as the enzyme source. Extent of the inhibition tended to be higher in the bacteria source from sheep on a high roughage diet. From these results it was speculated that the inhibition of deamination with salinomycin is caused by a decreased transport of amino acid into the bacterial cells as well as a decreased proportion of deaminating bacteria in the rumen.

(**Key Words** : Deamination, Rumen Bacteria, Ionophore, Salinomycin, Aspartate)

## Introduction

Ammonia produced in the rumen is converted into microbial protein but in most cases the production is much more than the microbial requirement. Most of the excess is considered as loss of nitrogen for the animal, since it is excreted into urine after being converted to urea in the liver. In the case of sheep receiving a lucerne-chaff diet, 36% of ammonia N present in the rumen (3.7 g/d) is estimated to be lost (Nolan, 1975). Therefore, much attention has been paid to the chemicals depressing proteolysis and/or deamination in the rumen that could be advantageous for N economy in ruminants.

Ionophores, some of those candidates, have been known to lower ruminal ammonia level but raise $\alpha$ amino acid level. This suggests a decreased deamination with the chemical, though the mechanism is not documented in full detail. In this report we describe potential of the ionophore salinomycin as a deamination inhibitor in the rumen and how the drug works in degradation of individual amino acid by mixed rumen bacteria.

## Materials and Methods

### Experiment 1

A wether weighing 42 kg and fitting with ruminal fistula was used. The sheep on an Italian ryegrass hay (6.8% of crude protein, 200 g twice daily at 09:00 and 17:00) and a commercial concentrate formula (12.4% and 400 g as above) was fasted for 24 hr to collect ruminl fluid with less feed N sources. The ruminal fluid taken through the fistula was squeezed by 2 layers of surgical gauze and used as an *in vitro* inoculum to assess casein (5 g/l, Van Nevel and Demeyer, 1977) degradation. The bacterial cell suspension for the determination of individual amino aicd degradation was prepared by a differential centrifuging procedure (Baldwin and Palmquist, 1965), i.e. the fluid was spinned (500 xg, 5 min) to remove feed particles and protozoa and the supernatant was spinned again (28,000 xg, 15 min) to collect bacteria. The pellet was washed twice with a buffer (pH 6.8, Maeng et al., 1976) whose volume was same with the discarded supernatant. The washed cells were finally resuspended in the same buffer. Cell free extract was obtained by ultrasonical disruption followed by

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centrifugation (28,000 xg, 15 min) and dialysis (Cellulose dialysis tube 27/32, Sanko Pharmaceutical Co Ltd) against the above buffer over night. Individual amino aicd was added to the suspension or the extract at a level of 3.5 mmol/l (Broderick and Balthrop, 1979). Salinomycin was added to the test tube by dissolving in ether followed by evaporation before inoculum and substrate addition. The final concentration of salinomycin was 2.5 mg/l. Test tube was flushed with $O_2$ free $CO_2$ obtained through a reduced copper colum, capped with a butyl rubber stopper and incubated in triplicate at 39℃. Net changes of ammonia were calculated from differences between ammonia levels in tubes incubated with and without an appropriate amino acid.

### Experiment 2

Three ruminally canulated wether, weighing 37.5 kg in average, were fed on the same hay/concentrate diet as used in the experiment 1. The ratio of hay to concentrate was ranged in 8:2, 5:5 and 2:8 and each diet was offered to sheep for 14d in a 3 × 3 latin square design (500 g twice daily at 09:00 and 17:00). The rumen fluid was taken from sheep on 3 different diets just before the morning feeding and the bacterial suspension was prepared as described above. Incubation with amino acid and ionophore was the same as above.

### Analyses

Ammonia and α-amino acid were determined by the methods of Weatherburn (1967) and Schroeder et al. (1950), respectively. Protein was assayed with Lowry's method. Student's t and F tests were employed for statistical analysis of the results obtained in experiments 1 and 2, respectively.

## Results

### Experiment 1:

When salinomycin was supplemented, ammonia production from casein was drastically decreased, while α-amino acid was accumulated in the *in vitro* culture (figure 1). The extent of the inhibition in deamination was apparently different in individual amino acid, i.e. deamination of aspartate, glutamate, tryptophan and arginine was significantly depressed by salinomycin, while that of others was not (figure 2). Of these amino acids aspartate was the most resistant one to deamination in the presence of salinomycin (approximately 40% inhibition of deamination). This remarkable inhibition was observed when intact bacterial cells were used as an enzyme source, but not when cell free extracts were used (table 1).

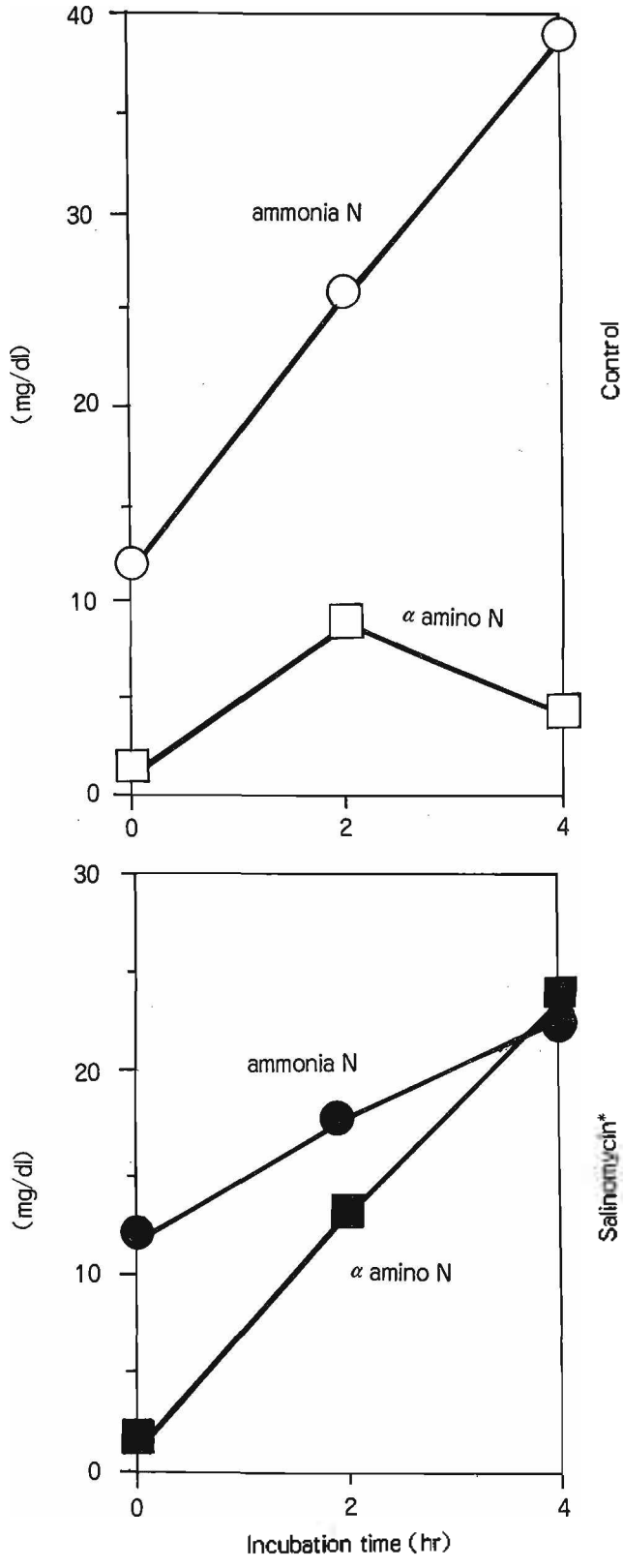


Figure 1. Effect of salinomycin on casein degradation by rumen microbes *in vitro*.
*Salinomycin was added to *in vitro* culture at a level of 2.5 mg/l

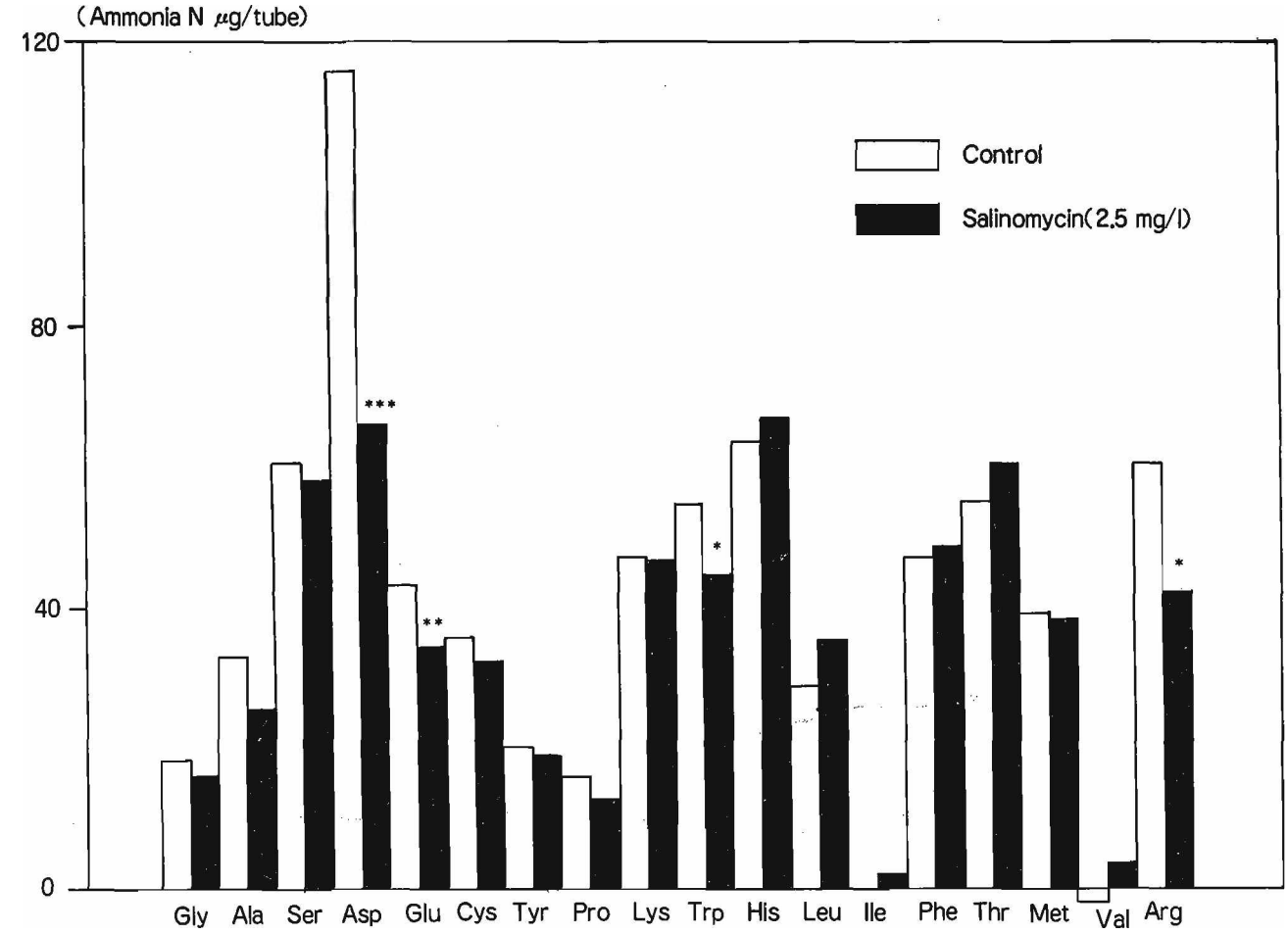


Figure 2. Effect of salinomycin on deamination of individual amino acid by mixed rumen bacteria *in vitro*.
*, **, ***, Significantly different from control at levels of 5, 1 and 0.1%, respectively.

TABLE 1. EFFECT OF SALINOMYCIN AND SUPPLEMENTS ON ASPARTATE DEAMINATION BY INTACT RUMEN BACTERIA AND THEIR EXTRACTS

| Supplements[1] | Washed bacteria | | Cell free extracts | |
|---|---|---|---|---|
| | Control | SL[2] | Control | SL[2] |
| | ··· ammonia released (μg/tube) ··· | | | |
| None | 50.2 | 36.1* | 32.0 | 32.5 |
| NAD | – | – | 30.8 | 33.4 |
| NAD and NADH | – | – | 27.9 | 29.5 |
| NAD, 2OG and B6 | – | – | 32.4 | 31.8 |
| NAD, NADH, 2OG and B6 | – | – | 30.5 | 29.2 |

[1] Supplements were added at levels as follows: NAD, 1 mM; NADH, 2 mM; 2OG (2-oxoglutarate), 0.3 mM; $B_6$ (Vitamin $B_6$), 0.04 mM.
[2] Salinomycin (2.5 mg/l).
* Significantly different from control ($p < 0.05$).

Supplements relating to redox states and transamination did not affect aspartate deaminase activity of the extracts (table 1).

**Experiment 2:**

Aspartate deamination tended to be higher as roughage was given more. Inhibitory effect of salinomycin on aspartate deamination was observed regardless of diet of inoculum donor, though the extent of the inhibition was highest in washed bacterial cells taken from sheep on a high roughage diet (table 2).

## Discussion

This study demonstrates that salinomycin inhibits deamination of particular amino acids as well as ammonia production from casein (figures 1 and 2). Monensin is also known to prevent deamination (Van Nevel and Demeyer, 1977). Some details how monensin depresses degradation of particular amino acids are described by Russell and

TABLE 2. EFFECTS OF DIETS OF INOCULUM DONOR AND SALINOMYCIN ON ASPARTATE DEAMINATION BY MIXED RUMEN BACTERIA

| Treatment* | Diet (Concentrate:hay)* | | | Significance | |
|---|---|---|---|---|---|
| | A(8:2) | B(5:5) | C(2:8) | Diet | Treatmnet |
| | ammonia (mgN/4hr/protein) | | | | |
| Control | 1.90 | 1.99 | 2.49 | NS | $p<0.05$ |
| SL | 1.16 | 1.09 | 1.33 | | |
| | ..... (%) ..... | | | | |
| Inhibition by SL | 39.9 | 45.2 | 46.6 | NS | – |

*Mixed rumen bacteria from sheep receiving 3 different diets are employed in *in vitro* analysis of aspartate deamination with or without salinomycin (2.5 mg/l).

Strobel (1989). They pointed out a prevented transport of some amino acids with $Na^+$ into bacterial cells, an increased intracellular NADH/NAD ratio and a decreased intracellular pH by monensin, all depress the bacterial deaminase activity. This mode of action would be also applicable to that of salinomycin, by which glutamate, one of the highly reduced amino acids, is depressed in its deamination (figure 1). The decreased glutamate deamination with salinomycin has been also reported by Hoshino et al. (1992).

Deamination of other 3 amino acids (aspartate, tryptophan and arginine) was also decreased with salinomycin (figure 2). Of those amino acids catabolism, we focused on attention on the characterization of aspartate deamination, since aspartate is one of the major free amino acids easily degradable in the rumen (Prins et al., 1979) and is strikingly inhibited from its degradation by salinomycin (figure 2). Newbold et al. (1990) have found that tetronasin, another ionophore, decreases amino acid degradation by *Ruminobacter amylophilus* and *Prevotella ruminicola*, both originally ionophore-resistant ruminal species, and speculated that this is caused by the decreased uptake of amino acids. The present results showing a decreased aspartate deamination observed with washed cells but not with cell extracts (table 1) may partly support their speculation.

However, some species of rumen bacteria, sensitive to ionophores such as *Butyrivibrio* sp., are known to be highly responsible for aspartate degradation (Scheifinger et al., 1979).

Aspartate deamination was higher in activity as roughage ratio was increased and its inhibition by salinomycin tended to be greater in bacteria obtained from sheep on a high roughage diet (table 2). Therefore, elimination of aspartate-deaminating bacteria by salinomycin is also an alternative reason. In fact, more ionophore-sensitive and ammonia-producing bacteria have been recently isolated (Chen and Russell, 1990).

To assess the degree of contribution of transamination and glutamate dehydrogenation to ammonia production from aspartate, we tested the effect of co-factor(s) stimulating the transamination. No apparent effects of those on ammonia production from aspartate with cell free extracts (table 1) confirm that the transamination does not have a significant role under the present experimental condition. Accordingly, aspartate is thought to be directly deaminated. The process, probably via aspartate dehydrogenase, may be interfered by salinomycin, though the precise mechanism involved in the decreased aspartate deamination is not clearly understood at the moment.

Uptake modes of aspartate by deaminating rumen bacteria and salinomycin actions on the cellular events should be explored to elucidate why this amino acid is so resistant to deamination with salinomycin. However, simple selection of bacteria or a change in amino acid uptake, or both combination might be responsible for the decreased deamination of particular amino acids. In addition, it is necessary to note a possibility that salinomycin depresses overall deaminase activity in the rumen through the inhibitory effects on rumen protozoa (Kobayashi et al., 1988) that are proteolytic and deaminating (Hino and Russell, 1985).

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