RUMEN MICROORGANISMS

Effects of Diet and Antibiotics on Utilization of Nonprotein Nitrogen

J. M. PRESCOTT

Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas Agricultural and Mechanical College System, College Station, Tex.

Because rumen microorganisms are known to be responsible for the utilization of nonprotein nitrogen by ruminants, and because of recent interest in the feeding of antibiotics, the effects of several antibiotics on the utilization of urea by rumen bacteria were investigated. Under the conditions of the in vitro technique employed, the addition of aureomycin, terramycin, bacitracin, or penicillin caused definite reduction in the utilization of nonprotein nitrogen, the extent of reduction being a function of antibiotic concentration. Rumen liquid from steers which had received a diet containing green roughages possessed a significantly greater ability to utilize nonprotein nitrogen than that of steers fed on grain. These experiments demonstrate that high levels of antibiotics are capable of reducing the utilization of nonprotein nitrogen by rumen microflora in vitro, and indicate the desirability of in vivo studies before ruminants are fed routinely on a diet containing both urea and antibiotics.

EXPERIMENTAL FEEDING OF ANTIBIOTICS TO RUMINANTS and the increased use of urea as a nitrogen source in certain feeds made desirable an investigation of the possible effects of antibiotics on the utilization of nonprotein nitrogen by rumen microorganisms. Chance, Duncan, Huffman, and Luecke (*8*) have studied the effects of aureomycin on the levels of certain amino acids and vitamins in the rumen, but reports on the effect of antibiotics on utilization of nonprotein nitrogen are lacking.

It was felt that such an investigation could profitably begin with in vitro studies on rumen contents. Although the results of experiments performed in vitro are not directly applicable to living systems, considerable promise is offered by recently developed in vitro techniques for studying the chemical activities of rumen microflora. Smith and coworkers have developed such methods (*15*) and have employed them in determining the effects of nitrogenous compounds, carbohydrates, and metals on the utilization of nonprotein nitrogen by rumen microorganisms (*12*, *17*). Burroughs *et al.* have developed an artificial rumen technique with which they have investigated the digestion of cellulose (*4*, *5*, *7*) and the effects of a number of substances on the utilization of urea (*1*, *3*, *6*). Marston (*13*) and Louw, Williams, and Maynard (*11*) have developed in vitro methods for studying cellulose digestion.

Experimental

The procedures adopted for the preparation and incubation of rumen liquid were very similar to those described by McNaught, Owen, and Smith (*12*). The results from a preliminary experiment indicated that essentially the same amount of nonprotein nitrogen was utilized during an 8-hour period whether the rumen liquid was incubated under a carbon dioxide atmosphere or under air in a tightly stoppered container. This can probably be attributed to the extensive evolution of gases from the rumen liquid which was observed during incubation. Such gases may have produced conditions in the container which approached anaerobiosis. In view of these results it was deemed unnecessary to add a carbon dioxide or nitrogen atmosphere to the flask. Care was taken, however, to keep the rumen liquid in a closed container as much as practicable during the preparation of samples.

Preparation of Rumen Liquid Rumen samples were obtained from steers as soon as possible after slaughter, and always before the rumen contents had cooled to an appreciable extent. The rumen contents were pressed through nylon gauze to remove

solid ingesta, and the resulting liquid was then centrifuged for 5 minutes at 2000 r.p.m. (800 × g). It has been demonstrated (*17*, *19*) that similar treatments are effective in removing protozoa and vegetable matter, leaving in the supernatant liquid those bacteria chiefly responsible for protein synthesis.

To each 100 ml. of supernatant liquid were added 0.15 gram of urea and 1 gram of glucose in order to provide a sufficient supply of nonprotein nitrogen and a readily available energy source. A 10-ml. sample was immediately withdrawn for analysis, while other 10-ml. samples were placed in tightly stoppered 25-ml. flasks or test tubes with various levels of antibiotics. After incubation at 37° for 8 hours, these samples were analyzed for urea plus ammonia nitrogen. In each experiment one or two control samples were incubated without the addition of antibiotic. It was deemed unnecessary to incubate duplicate samples of each level of antibiotic, as enough levels were included to provide a fairly complete curve.

Determination Of Nitrogen In these experiments interest was centered in the combined utilization of urea and ammonia nitrogen rather than in the individual utilization of urea nitrogen and of ammonia nitrogen. Furthermore, it has been demonstrated that urea is readily converted to ammonia by the ureases of rumen bacteria (*16*, *20*), and it has been suggested that such a conversion is the first step in the utilization of urea by the bacteria (*16*). For these reasons, urea nitrogen and ammonia nitrogen were estimated together. Although these analytical values are sometimes referred to as nonprotein nitrogen, they represent only urea plus ammonia nitrogen and not total nonprotein nitrogen.

Table I. Effect of Type of Diet on Ability of Rumen Microorganisms to Utilize Nonprotein Nitrogen Incubated in Vitro

Animal No.	Diet	Change in Urea + Ammonia Nitrogen, Mg./100 Ml. of Rumen Liquid
1	Grass-roughage	− 8.0
2	Grass-roughage	−16.1
3	Grass-roughage	−10.6
4	Grass-roughage	− 9.1
5	Grass-roughage	− 4.0
6	Grass-roughage	−13.2
7	Grass-roughage	− 7.7
Mean		− 9.81
8	Grain	− 1.0
9	Grain	+ 2.8[a]
10	Grain	− 4.2
11	Grain	− 3.2
12	Grain	− 0.6
13	Grain	0.0
Mean		− 1.03

[a] Incubation period, 9 hours. All others, 8 hours.

Table II. Effect of Level of Aureomycin on Utilization of Nonprotein Nitrogen by Rumen Microorganisms Incubated in Vitro

Rumen Liquid from Animal No.	Concn. of Aureomycin Added, γ/Ml. of Rumen Liquid	Change in Urea + Ammonia Nitrogen, Mg./100 Ml. of Rumen Liquid	Utilization of Urea + Ammonia Nitrogen, % (Based on Controls as 100%)
1	0	− 8.0	100
	10	− 2.2	27
	50	− 2.2	27
	100	− 1.9	23
	1000	− 1.9	23
6	0	−13.8	100
	1	−11.8	85
	10	− 6.3	46
	50	− 6.8	49
	100	− 6.3	46
	300	− 4.6	33
7	0	− 7.7	100
	1	− 6.8	88
	10	− 6.0	78
	50	− 5.7	74
	100	− 4.9	64
	300	− 3.0	39
10	0	− 4.2	100
	1	+ 2.8	0
	10	+ 2.8	0
	50	+ 4.2	0
	100	+ 5.0	0
	300	+ 5.0	0

The method employed was essentially that of Pearson and Smith (*15*), with minor modifications. The sample of rumen liquid was transferred quantitatively to a 100-ml. volumetric flask with 0.166 *N* sulfuric acid, 10 ml. of 10% sodium tungstate were added, and the volume was made up to the mark with 0.166 *N* sulfuric acid. After thorough mixing, the samples were allowed to stand 24 to 36 hours before filtering. Portions of the filtrates from the tungstic acid precipitation were neutralized to the methyl red end point with 1 *N* sodium hydroxide, and 10 ml. of 0.6% potassium dihydrogen phosphate were added for a buffer, followed by 1 ml. of a 1 to 10 dilution of the urease solution described by Hawk, Oser, and Summerson (*9*). The samples were incubated for 30 minutes at 37° C., 2 ml. of 2 *N* sodium hydroxide were added, and the mixture was steam-distilled until 15 to 20 ml. of distillate accumulated. The distillate was collected in 3.1% boric acid and titrated with 0.02 *N* hydrochloric acid. A few drops of octyl alcohol controlled the tendency of the mixture to foam during the distillation. Duplicate determinations were made on each sample, and corrections were made for the reagent blank.

Results and Discussion

The original microflora may have changed somewhat during the course of the experiments, as it has been reported (*17*) that changes in rumen population begin soon after rumen liquid is removed from an animal. The incubation was carried out at a slightly lower temperature than that employed by some authors who have reported in vitro experiments with rumen liquid. Certainly no in vitro technique can be said to duplicate exactly the conditions of the rumen, but studies employing such techniques may still be useful.

Effect of Type of Diet During the experiments, it was observed that the rumen liquid of steers fed on pasture and green roughages could utilize urea and ammonia nitrogen to a greater extent than the rumen contents of animals fed largely on grain. In Table I are shown the changes in urea plus ammonia nitrogen occurring after incubation of rumen liquid with urea. A decrease in this nitrogen indicates that it was utilized for bacterial growth, while an increase in nonprotein nitrogen indicates that a decrease in the bacterial protein occurred during the incubation (*19*). The data in Table I show that under the conditions employed in these experiments, there was a pronounced difference in the ability of rumen microflora from animals on different diets to utilize nonprotein nitrogen. Analysis of the data indicated that the results are statistically highly significant ($p < 0.01$).

The difference in the ability of rumen microorganisms to utilize urea plus ammonia nitrogen may reflect less favorable conditions for microbial growth

Table III. Effect of Antibiotics on Utilization of Nonprotein Nitrogen by Rumen Microorganisms in Vitro

Substance Added	Concn., γ/Ml. of Rumen Liquid	Change in Urea + Ammonia Nitrogen, Mg./100 Ml. of Rumen Liquid	Utilization of Urea + Ammonia Nitrogen, % (Based on Controls as 100%)
None	...	−7.7	100
Aureomycin hydrochloride	1	−6.8	88
	10	−6.0	78
	50	−5.7	74
	100	−4.9	64
Terramycin hydrochloride	5	−6.5	84
	10	−6.0	78
	50	−6.3	82
	100	−4.9	64
Bacitracin	1	−7.2	94
	5	−6.0	78
	10	−4.9	64
	100	−0.7	10
L-Ephenamine penicillin[a]	0.5	−2.7	35
	2.5	−2.2	29
	5	−1.0	13
	25	−0.5	6
	250	+0.8	0

[a] 1000 units per mg.

in the rumen of the grain-fed animals. For example, the rumen ingesta of the grain-fed steers generally contained considerably less moisture than those of the grass-fed animals; hence, it is possible that some purely physical factor such as moisture content was responsible for the difference. The better growth of bacteria from the grass-fed steers might also indicate that the required nutritional factors were more readily available in the rumen of these animals than in the rumen of the animals fed on grain. Alternatively, the difference in nonprotein nitrogen utilization may be due to variations in the microbial population of the rumen. The types and numbers of organisms found in the rumen may be expected to vary both seasonally and geographically. Rusoff *et al.* (*18*) found individual variations in the bacterial population of the rumen of the same animal from week to week, even when the same diet was fed. Therefore, if the difference in nonprotein nitrogen utilization is caused by variations in bacterial population, the effect of the diet on nonprotein nitrogen utilization would also be expected to vary considerably.

Effect of Antibiotics

In Table II are shown the results of several experiments in which graded levels of crystalline aureomycin hydrochloride were added to rumen liquid.

In a similar experiment, various levels of aureomycin hydrochloride, terramycin hydrochloride, bacitracin, and L-ephenamine penicillin were added to rumen liquid obtained from a steer which had been on a grass-roughage diet. The abilities of these four antibiotics to reduce the utilization of urea plus ammonia nitrogen by rumen microorganisms are compared in Table III.

In each instance, the addition of antibiotics resulted in reduced utilization of nonprotein nitrogen. As one might expect, the extent of such reduction varied from one individual to another.

Several groups of investigators have failed to observe any change in the total bacterial population of the rumen when animals are fed on aureomycin (*2, 10, 18*). However, Bartley *et al.* (*2*) and Neumann, Snapp, and Gall (*14*) have found evidence that the bacterial flora is altered when animals receive aureomycin in their diet. The latter investigators have suggested that aureomycin destroyed or reduced certain types of organisms under the conditions of their experiments. It is not surprising, then, that the addition of antibiotics to rumen liquid should reduce the number of organisms which utilize simple nitrogenous compounds.

It is impossible to predict, on the basis of these results, any arbitrary level above which antibiotics have an adverse effect on the utilization of nonprotein nitrogen, as the in vitro technique fails to duplicate in vivo conditions accurately enough for such predictions to be made. These experiments do indicate, however, that high levels of antibiotics can reduce the utilization of nonprotein nitrogen by rumen microorganisms, and point out the desirability of in vivo experiments to determine the effect of feeding antibiotics to ruminants which receive urea as a part of their diet, or receive a diet naturally rich in nonprotein nitrogen.

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