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INTERRELATIONSHIP BETWEEN MIXED RUMEN BACTERIA AND PROTOZOA ON THE *IN VITRO* DEGRADATION OF PROTEIN

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ABSTRACT

An *in vitro* study was conducted to examine the degradation of various protein sources by mixed rumen bacteria, mixed rumen protozoa and a combination of bacteria and protozoa. Rumen microorganisms were isolated from the rumen of sheep fed a concentrate mixture and hay. Microbial suspensions were anaerobically incubated at 39°C for 12 h. The results revealed that, mixed rumen bacteria always produced more ammonia than mixed rumen protozoa. Heated soybean meal was deaminated at a faster rate ($P<0.05$) than that of soluble casein, insolube casein and killed bacteria by bacteria. However, protozoa deaminated killed bacteria at a faster rate ($P<0.05$) than that of soluble casein, insolube casein and heated soybean meal. Nonammonia-nonprotein N was greater ($P<0.05$) with protozoa than bacteria. When incubations containing bacteria or protozoa were compared with the combination of bacteria and protozoa, the combination always caused a synergistic increase in ammonia and decrease in nonammonia-nonprotein N ($P<0.05$). It could be concluded that there is synergism between rumen bacteria and protozoa on the degradation of protein.
Keywords: Rumen, bacteria, protozoa, protein degradation, *In vitro*

INTRODUCTION

The degradation of feed proteins in the rumen is an important parameter in determining the protein supply for the rumen microorganisms and ruminant animal with amino acids. However, the role of ruminal protozoa in the degradation of feed proteins is not clear. Abou Akkada and Howard (1962) showed that soluble casein was rapidly degraded by *Entodinium caudatum*, but Onodera and Kandatsu (1970) reported that mixed entodiniomorphid protozoa only digested insoluble casein. In contrast, Shinchi and Abe (1987) and El-Waziry *et al*. (1999) showed that entodiniomorphs were able to decompose a considerable amount of soluble casein dissolved in phosphate buffer solution suggested by Onodera and Kandatsu (1970). Onodera and Yakiyama (1990) examined the coagulation and degradation of dissolved casein by entodiniomorphs plus holotrichs and entodiniomorphs alone, using either phosphate buffer solution (MB9) or Seitz-filtered rumen liquor (SFRL) for the incubation. They suggested that the protozoon has more quickly degraded insoluble casein than soluble casein in all the media except in SFRL with entodiniomorphs alone. Hino and Russell (1987) examined ammonia production from soluble and insoluble proteins by rumen protozoa and bacteria. They suggested that protozoa could contribute to the degradation of insoluble, particulate proteins, though soluble proteins were primarily degraded by bacteria. Therefore, excessive protein degradation in the rumen can be one of the greatest problems affecting

ruminant nutrition (Ørskov, 1982). However, there is little information on the relative contribution of ruminal bacteria and protozoa to the degradation of protein.

The objective of the present study was to examine the interrelationship between mixed rumen bacteria and protozoa on the *in vitro* degradation of various kinds of proteins (soluble casein, insoluble casein, heated soybean meal and killed bacteria, *in vitro*).

MATERIALS AND METHODS

Animals

Four mature rumen fistulated sheep with a mean live weight of 45 kg (S.D. ± 5 kg), fed on a daily ration consisting of 900 g hay and 250 g concentrate mixture given in two equal portions at 0900 and 1700 were used for the experiment. Sheep were housed in individual pens under approximately constant environmental conditions. They had free access to fresh water. Sheep were allowed 21 days for the dietary adaptation before collecting samples.

Preparation of rumen microbial suspensions

Rumen content obtained from the fistulated sheep before morning feed were strained through four layers of surgical gauze into a separatory funnel which was gassed with a mixture of 95% N_2 and 5% CO_2. The contents were incubated at 39□C for up to 60 min. to allow feed debris to float. The suspensions of bacterial (B) and protozoal (P) and their mixture (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 activities of contaminating bacteria (Onodera and Kandatsu, 1974).

Incubation procedure

Tested protein sources (soluble casein, insoluble casein, heated soybean meal and killed bacteria [collected from sheep]) were prepared as described by Hino and Russell (1987). Equal volumes (20 ml) of bacterial or/and protozoal suspensions and MB9 buffer solution (Onodera and Henderson, 1980) were mixed. One gram of protein source was added to the mixtures and incubated at 39°C for up to 12 h.

Sample treatments and analyses

The incubation mixture was centrifuged (10,000 x g, 10 min) and the supernatant was used to assay ammonia and non-ammonia-nonprotein N. Ammonia N (NH_3-N) was measured as described by Chaney and Marbach (1962). Non-ammonia-nonprotein N (NAN-NPN) was determined by micro-kjeldahl procedure as described by Hino and Russell (1987). Microbial N was determined by micro-Kjeldahl methods (AOAC, 1995). Production of NH_3-N and NAN-NPN was expressed as specific activity based on the initial concentration of microbial protein added to the incubation bottles. These compounds were expressed by the differences between the figures of incubations with and without substrates. Data was analysed by the F-test procedure (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

The activity of bacteria was always greater than that of protozoa on the degradation of protein. The rate of ammonia production was also affected by the kind and solubility of protein source (Table 1), since soluble casein deaminated at a faster rate than insoluble casein and killed bacteria as practically insoluble protein (512.12, 445.15 and 135.22 µg NH_3-N/mg protein, respectively, P<0.05) during 12 h incubation. Heated soybean meal was less soluble than casein, but the decrease in solubility was not correlated with a decrease in deamination by bacteria. The heated soybean was deaminated at a faster rate (735.25 µg NH_3N/mg protein) than soluble casein, insoluble casein and killed bacteria during 12 h incubation (Table 1, P<0.05). Hino and Russell (1987) reported that heated casein was deaminated at a rate that was similar to casein, while the heated soybean protein was deaminated at an even faster rate. These results are almost similar to the results of the present study. The present results indicated that the nature of protein, as well as solubility, are important determinants of protein fermentation by bacteria.

Table (1): Ammonia-N (NH_3-N) and Nonammonia-Nonprotein N (NAN-NPN) produced from various protein sources by mixed rumen bacteria[a]

Protein sources[b]	Solubility[c]	NH_3-N (µg/mg protein)		NAN-NPN (µg/mg protein)	
		Incubation time (h)		Incubation time (h)	
		6	12	6	12
Soluble Casein	85	224.15 ±15.24[d]	512.12 ±16.65[d]	20.21 ±3.12[d]	38.42 ±3.25[d]
Insoluble Casein	32	245.12 ±17.15[e]	445.15 ±17.12[e]	14.72 ±1.15[e]	32.52 ±2.21[d]
Heated soybean meal	25	375.35 ±19.23[f]	735.25 ±20.51[f]	15.02 ±1.92[e]	32.98 ±2.25[d]
Killed bacteria	8	72.25 ±16.2[g]	135.22 ±15.12[g]	9.08 ±2.10[f]	20.25 ±3.15[e]

[a]The average of bacterial N was 0.452 mg/ml.
[b]Each protein source was supplied at 1g in incubation medium.
[c]Solubility in MB9 solution after incubation at 39°C for 12h.
[d,e,f,g]Means within a column bearing different superscripts differ (P<0.05).
[h]Means ± SD, n = 4

Protozoa deaminated killed bacteria at a faster rate (P<0.05) than heated soybean meal or insoluble casein (75.65, 60.01 and 48.82 µg NH_3N/mg protein, respectively). Heated soybean meal and insoluble casein were deaminated at a faster rate (P<0.05) than soluble casein (60.01, 48.82 and 32.82 µg NH_3N/mg protein, respectively) during incubation periods (Table 2). These results indicated that the protozoa were generally more important in the fermentation of less soluble and particulate protein. The present results are quite similar to those of Hino and Russell (1987).

Table (2): Ammonia-N (NH_3-N) and Nonammonia-Nonprotein N (NAN-NPN) produced from various protein sources by mixed rumen protozoa[a]

Protein sources[b]	NH_3-N (µg/mg protein)		NAN-NPN (µg/mg protein)	
	Incubation time (h)		Incubation time (h)	
	6	12	6	12
Soluble Casein	15.92 ±2.12[c]	32.82 ±1.25[c]	46.08 ±5.45[c]	90.20 ±5.15[c]
Insoluble Casein	25.45 ±3.25[d]	48.82 ±2.95[d]	65.08 ±6.65[d]	125.25 ±6.25[d]
Heated soybean meal	29.30 ±3.28[e]	60.01 ±3.25[e]	82.25 ±7.25[e]	165.54 ±7.55[e]
Killed bacteria	34.88 ±2.15[f]	75.65 ±3.25[f]	22.75 ±1.19[f]	41.55 ±5.12[f]

[a]The average of protozoal N was 0.742 mg/ml.
[b]Each protein source was supplied at 1g in incubation medium.
[c,d,e,f]Means within a column bearing different superscripts differ (P<0.05).
[g]Means ±SD, n=4

Nonammonia-nonprotein N (NAN-NPN) was higher in incubations containing protozoa than bacteria (Tables 1, 2). As shown in Table (2), protozoa produced the greatest amount of ammonia-N from killed bacteria (75.65 µg/mg protein) and the accumulation of NAN-NPN was less than the other protein sources (41.55 µg/mg protein). High NAN-NPN values were obtained with heated soybean meal, insoluble casein and soluble casein (165.54, 125.25 and 90.20 µg/mg protein, respectively, P<0.05, Table 2). These results indicated that the protozoa digested the protein but released most amino acids or peptides, and are limited in their capacity to up take peptides and amino acids into cytoplasm (Hino and Russell, 1987).

Table (3) shows that interrelationship or potential synergism between protozoa and bacteria in the degradation of protein. Synergisms are normally defined as situations in which the combination elicits greater observed response than the sum of the individual parts (Hino and Russell, 1987). However, a positive synergism occurs if the combination is greater than the average of the parts. An additive response is seen if the combination is equal to the average of the parts, while with a negative synergism the combination is less. According to these criteria, positive synergisms were observed as shown in Table (3). The amount of ammonia-N produced by the combination of bacteria and protozoa more than that would be predicted by the average of bacteria and protozoa, and NAN-NPN was lower (P<0.05). These results indicate that amino acids and peptides produced by protozoa were deaminated and utilized by bacteria (Hino and Russell, 1987).

Early studies indicated that more than one-half of the proteolytic activity of mixed ruminal microorgnisms was due to protozoa (Warner, 1956 and Blackburn and Hobson, 1960). However, it has been reported that bacteria are primarily responsible and that protozoa only play minor role (Nugent an Mangan, 1981). Other researchers have likewise suggested that most of the proteolytic activity in the rumen is associated with bacteria (Brock *et al*., 1982; Kopecny and Wallace, 1982; Wallace and Brammall, 1985).

Table (3): Effect of combination of ruminal bacteria and protozoa[a] on the degradation of protein sources during 12-h incubation.

Protein sources[b]	Measurement	Bacteria (B) [a]	Protozoa (P) [a]	Combination of BP	Mean of BP
Casein	NH_3-N[c]	682.15 ±18.25[d]	52.75 ±3.45[e]	425.25 ±8.65[f]	367.45[g]
	NAN-NPN[c]	70.55 ±2.75[d]	215.75 ±7.25[e]	86.85 ±2.25[f]	143.50 [g]
Killed bacteria	NH_3-N	110.55 ±10.15[d]	22.16 ±1.15[e]	95.75 ±5.16[f]	56.33 [g]
	NAN-NPN	45.35 ±2.42[d]	90.20 ±2.23[e]	58.84 ±3.12[f]	67.78 [g]

[a]The average of microbial N was 1.078 , 0.415 and 0.785 mg/ml for BP, B and P, respectively.
[b]Casein or killed bacteria was supplied at 1g in incubation medium.
[C]µg/mg protein
[d,e,f]Means of bacteria, protozoa and their combination with different superscripts within a row differ (P<0.05).
[g]Mean values of bacteria and protozoa differ from combination of them (P<0.05).
[h]Means ±SD, n=4

The way by which food uptake and digestion is different between bacteria and protozoa (Hino and Russell, 1987), and this difference should be taken into account. Mixed ruminal bacteria adsorbed proteins onto the cell wall and hydrolysis occurred at this site (Nugent an Mangan, 1981; Kopecny and Wallace, 1982; Wallace, 1985). In this process, solubility and the primary sequence of amino acids are the most important determinants of proteolysis. Ammonia is a major product of protein degradation in bacteria. On the other hand, proteolysis by protozoa can occur intracellularly and factors affecting engulfment are important. Shinchi and Abe (1987) have pointed out that the existance of extracelluar proteolytic enzymes may explain why entodiniomorphs can degrade considerable amounts of soluble casein. Entodiniomorphids protozoa only take up particulate protein sources, but the holotrichs protozoa are able to utilize both particulate and soluble proteins (Abou Akkada and Howard, 1962; Onodera and Kandatsu, 1970). Recently, El-Waziry *et al*. (1999) found that the holotrichs can coagulate soluble casein in the rumen fluid and the coagulated casein can be degraded mainly by entodiniomorphs.

Nonammonia-nonprotein N was increased (P<0.05) in incubations containing protozoa than in those that contained bacteria (Tables 1, 2, and 3). These results indicate that the bacteria are more competent at peptide or amino acid uptake. Coleman (1975) showed that protozoa excreted amino acid N after the engulfment of bacteria. Hino and Russell (1985) reported that ruminal protozoa have high specific activities of deaminating enzymes.

Generally, solubility of protein is considered to be a deciding factor in ruminal protein degradation (Hungate, 1966 and Craig and Broderick, 1981). The present results indicated that bacteria play a major role in the degradation of soluble casein than that of insoluble casein and killed bacteria (Tables 1, 3). However, heated soybean was also deaminated at a faster rate than that of soluble casein and insoluble casein, although its solubility was only 25% (Tables 1, 2). Synergism between protozoa and bacteria were

observed (Table 3). Protozoa were able to hydrolyze protein, but their capacity to transport (into cytosol) and deaminate amino acid sources is limited (Forsberg *et al*., 1984). On the other hand, bacteria have high capacity to take up amino acid sources and deaminate them. Synergisms involving proteolysis were evident, but the effect was greatest for insoluble proteins, which would have a greater chance of being degraded by protozoa.

Synergistic effects would tend to support the hypothesis that defaunation might lead to a decrease in protein degradation (Table 3). Indeed, it has been reported that ruminal ammonia concentrations are sometimes lower after defaunation (Abou Akkada and El-Shazly, 1964; Eadie and Gill, 1971). Leng and Nolan (1984) suggested that defaunation enhances the availability of protein in ruminants, however, the present study suggests that bacteria are more active at deaminating protein sources than protozoa (Tables 1, 2 and 3). This apparent contradication may be related to the level of dietary protein, and positive effects of defaunation were only observed in animal given low protein diets (Leng and Nolan (1984). Under such conditions, most of the available dietary protein would have been incorporated into bacterial biomass and little of N would have remained in the ammonia pool (Hino and Russell, 1987). Bacteria are, however, a preferred N source for protozoa (Tables 1, 2 and 3). Onodera *et al*., (1974) found that rumen protozoa may contribute to the nutrition of the host animal by producing lysine from the apparently less digestible, diaminopimelic acid (DAP) containing peptidoglycan. Furthermore, *in vitro* lysine production by faunated goat rumen contents was 23 % higher than that from defaunated animals (Onodera, (1986). El-Waziry and Onodera, (1996) reported that lysine production from DAP by mixed rumen protozoa to be about two-fold higher than that by mixed rumen bacteria. These differences in the relative contribution of bacteria and protozoa to ruminal N metabolism may explain the failure of defaunation to enhance animal productivity under other dietary protocols (Rowe *et al*., 1985).

The present study revealed that bacteria were much more active than protozoa in degrading protein. The rate of ammonia-Nwas greater by bacteria than that of protozoa, but nonammonia-nonprotein N was less. Therefore, it could be concluded that there is synergism between rumen bacteria and protozoa on the degradation of protein.

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العلاقة بين بكتريا و بروتوزوا الكرش علي تحلل البروتين معمليا

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تمت هذه الدراسة المعملية لاختبار تحلل أنواع مختلفة من البروتين بواسطة بكتريا و بروتوزوا الكرش و خليط منهما لمعرفة العلاقة بينهما علي تحلل البروتين. تم عزل البكتريا و البروتوزوا من كرش الأغنام التي غذيت علي خليط من المركزات و دريس البرسيم وتم تحضين الأنواع المختلفة من البر وتينات كلا علي حدة مع المعلقات الميكروبية علي درجة حرارة ٣٩ درجة مئوية لمدة ١٢ ساعة.

وتشير النتائج إلى:-

بكتريا الكرش تنتج عادة امونيا اكثر من بروتوزوا الكرش، حيث وجد أن كسب فول الصويا المعامل حراريا يتحلل بمعدل اكبر من الكازين الذائب و الكازين الغير ذائب و البكتريا المقتولة علي التوالي و كانت الفروق معنوية، أما بروتوزوا الكرش تحلل البكتريا المقتولة بمعدل اكبر من الكازين الذائب و الكازين الغير ذائب و مسحوق فول الصويا المعامل حراريا و كانت الفروق معنوية، وكان معدل إنتاج الأزوت الغير بروتيني الغير امونيومي بواسطة البروتوزوا اكبر من البكتريا. وعندما تم تحضين خليط البكتريا و البروتوزوا معا فان هناك تزامن أو علاقة داخلية بين البكتريا و البروتوزوا علي تحلل البروتين حيث كان هناك زيادة في إنتاج الامونيا وانخفاض في الأزوت الغير بروتيني الغير امونيومي مقارنة بالبكتريا أو البروتوزوا وكانت الفروق معنوية. وتشير هذه الدراسة إلى أن هناك مساهمة أو تزامن أو علاقة داخلية بين البكتريا و البروتوزوا إلي تحلل البروتين.