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Attempted induction of reductive acetogenesis into the rumen fermentation in vitro

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Summary — Rumen and caecal contents, obtained from slaughterhouse cattle and rumen contents obtained from a fistulated wether were incubated in vitro with ground hay in the presence and absence of, respectively, casein hydrolysate and mucin. Differences in stoichiometry of rumen and caecal fermentations, indicative of reductive acetogenesis in the caecum, were confirmed, except for incubations with free amino acids. Net fermentation end product production was determined after correction for amounts formed in incubations without the substrate. These determined amounts of hay fermentation end products were compared with the amounts calculated from incubations of hay with added casein hydrolysate or mucin, corrected for amounts formed from the latter added substrates incubated alone. With casein hydrolysate, no differences between the determined and calculated amounts were observed, excluding the occurrence of reductive acetogenesis from hay in the presence of free amino acids. With mucin, the calculated amounts indicated an inhibition of methanogenesis, accompanied by increased amounts of proprionate, butyrate and valerate production. This finding was probably related to the greater availability of easily fermented carbohydrates in the presence of mucins. The absence of an increased acetate production in the incubations with added head space hydrogen gas, also indicate the absence of reductive acetogenesis from hay in the presence of mucin. Stoichiometric considerations also indicate that neither free amino acids, nor mucin, induce reductive acetogenesis in short-term in vitro incubations of rumen contents with hay.

reductive acetogenesis / rumen fermentation / amino acids / mucin

Résumé — Essais d'introduction de l'acétogenèse réductrice dans la fermentation de rumen in vitro. *Des contenus de rumen et de cæcum, récoltés à l'abattoir sur des bovins et des contenus du rumen provenant d'un bélier castré et fistulé ont été incubés in vitro avec du foin en présence puis en l'absence de caséine hydrolysée ou de mucine comme substrat. Des différences dans la stœchiométrie de la fermentation du rumen et du cæcum, indicatives de l'occurrence de l'acétogenèse réductrice dans le cæcum, ont été confirmées, sauf en présence d'acides aminés libres. La production de produits de fermentation a été déterminée en corrigeant les quantités produites par celles produites dans des incu-*

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bations sans substrat. Pour le foin, ces quantités ont été comparées avec celles calculées à partir des incubations de foin avec addition de caséine hydrolysée ou de mucine, après correction pour les quantités formées avec les derniers substrats, incubés seuls. Avec la caséine hydrolysée, aucune différence entre les quantités déterminées et calculées n'a été observée, mise à part l'acétogenèse réductrice provenant du foin en présence des acides aminés libres. Avec la mucine, les produits calculés ont mis en évidence une inhibition de la méthanogenèse, accompagnée d'une augmentation de la production de propionate, de butyrate et de valérate. Ces résultats seraient dus à une forte disponibilité en sucres facilement fermentescibles suite à la présence de mucine. Des considérations stœchiométriques indiquent que ni les acides aminés, ni la mucine n'induisent l'acétogenèse réductrice dans les incubations in vitro de courte durée des contenus du rumen en présence de foin.

acétogenèse réductrice / fermentation ruminale / acides aminés / mucine

INTRODUCTION

The plant cell wall polysaccharides cellulose, hemicellulose and pectin can only be utilized in animal metabolism after their fermentation by the indigenous microbial communities found in the digestive tract. Sites of such fermentation in the rumen and hindgut precede and follow the site of acid hydrolytic digestion, respectively. Optimal energy yield of fermentation requires continuous removal of metabolic hydrogen and electrons in 'sinks' by the activities of hydrogen utilising methanogenic, acetogenic and sulphate-reducing bacteria (Gibson et al, 1993).

In the rumen, methanogenesis dominates over H_2 disposal. Bacteria capable of reductive acetogenesis have been isolated from the rumen (Greening and Leedle, 1989) although their numbers remain low in the adult animal (Doré et al, 1995). Although some evidence exists for the initial presence of reductive acetogenesis in the rumen of newborn lambs (Doré et al, 1992), this reaction does not occur in the adult rumen. In addition, assimilatory sulphate reduction only seems to be of quantitative importance in rumen fermentation (Demeyer and Hendrickx, 1967). This is in contrast to the termite gut and the hindgut of a number of mammals including man where sulphate reduction and/or reductive acetogenesis have been identified as major alternative pathways of CO_2 reduction (see eg, reviews in Demeyer and De Graeve, 1991 and Durand and Bernalier, 1995).

Understanding the factors that determine the relative competitiveness of metabolic hydrogen disposal in methanogenesis versus sulphate reduction and acetogenesis is of considerable importance for human as well as for animal nutrition. Indeed, the relative proportions of these different metabolic routes in the human large intestine is related to the incidence of disease (Gibson et al, 1993). Moreover, it is clear that the substitution of acetogenesis for methanogenesis in the rumen would result in considerable improvement of feed energy utilisation in ruminants, as well as in the reduction of overall methane emission in the environment (see eg, Van Nevel and Demeyer, 1995). A greater competitiveness of methanogenic bacteria may be related to more favourable reaction energetics, lower H_2 thresholds, their association with rumen protozoa and the potential of acetogens to shift to organotrophic growth in the presence of organic substrates, in contrast to methanogens. On the other hand, a moderately low rumen pH, induced, for example, by low forage diets may give a competitive advantage to acetogens (Leedle and Greening, 1988). The greater competitiveness of methanogens in the hindgut has recently been demonstrated by the increase in $^{13}CO_2$ incorporation into acetate, following inhibition of methanogenesis with the specific methane inhibitor bromoethane sul-

fonic acid (BES) in the methanogenic hindgut flora of the pig (De Graeve et al, 1994) and in the human faecal flora (Durand et al, 1994). In the rumen, however, methane inhibition by BES increases propionate proportion (De Graeve et al, 1994).

This paper describes two attempts to induce reductive acetogenesis in the rumen in vitro through the addition of free amino acids or mucin to short duration incubations of rumen contents with hay. These attempts were based on the suggestion made in earlier works that the presence of high concentrations of mucin (Demeyer et al, 1989) and of free amino acids (Van Nevel and Demeyer, 1990) in the hindgut, in contrast to the rumen, may be a factor responsible for the induction of reductive acetogenesis in the hindgut. Induction of reductive acetogenesis was assessed from the stoichiometric hydrogen recovery calculated from net amounts of end products formed in the presence and absence of added free amino acids or mucin (Henderson and Demeyer, 1989; Demeyer, 1991) and/or the response of the fermentation pattern to increased head space H_2 (Demeyer and De Graeve, 1991).

MATERIALS AND METHODS

Experiments with free amino acids

The digesta contents were obtained from mature commercial cattle, immediately after slaughtering at our institute. No detailed information on the animals was available. They were Belgian Blue White Beef cattle, probably fed concentrate-rich diets with minimal amounts of maize silage. For each incubation series, three animals were sampled immediately after removal of the intestinal tract. The rumen contents, obtained from the ventral rumen sac, were sieved through a metal sieve (1 mm mesh) and kept bubbling with CO_2. After removal of the visible fat, the caeca were sampled at the distal end. Both the rumen and caecal contents of the three animals were pooled and transported to the laboratory within 15 min of sampling and diluted with four parts of Burrough's solution (Burroughs et al, 1950), followed by sieving of the caecal contents. Both dilutions were decanted once or twice, to eliminate any large particles and 40 ml were incubated for 24 h under CO_2 with or without 500 mg of ground hay. Ten ml of Burrough's solution containing either 40 mg of NH_4HCO_3 (hay and blank incubations) or 138 mg casein hydrolysate (Sigma, MO, USA, 73.2% free amino acids) (amino acid incubations with and without hay) was added to all incubations (Van Nevel and Demeyer, 1990). Incubations were stopped by injecting 1 ml of 10 N H_2SO_4 and the volatile fatty acids, methane, H_2 and ammonia concentrations were determined before and after incubations as described previously (Van Nevel and Demeyer, 1990).

Experiments with mucin

A rumen-fistulated wether (ca 70 kg) was kept in an individual cage and fed 400 g medium quality chopped hay (91.2% DM; 12.6% CP and 66.1% NDF in DM) and 600 g of concentrates (91.3% DM; 17.5% CP and 18.3% NDF in DM) (% w/w: corn 32, oats 37, soja 50% crude protein 14.5, alfalfa pellets 5, lard 1, limestone 5, phosphate 2, NaCl 1, molasses 2, vitamins and minerals 0.5) in two equal parts at 0900 and 1600 hours with water available at all times.

Its rumen contents were obtained before the morning feeding, filtered through a metal sieve (1 mm mesh), diluted five-fold with Burrough's solution and incubated as described earlier. Per flask, 28 mg of NH_4HCO_3 was added. The substrates used were 500 mg of ground hay (91.3% DM; 10.5% CP and 36.0% Cfibre in DM) or 500 mg of mucin (type II: crude from porcine stomach, Sigma M-2378) or a mixture of both. The same substrates were incubated using CO_2/H_2 (50/50 v/v) or CO_2/N_2 (50/50 v/v) as the gas phase. The incubations were stopped and the analyses made as described earlier.

Calculations

For all incubations, the stoichiometric hydrogen and ammonia recoveries were calculated from the volatile fatty acids (VFA) formed as described by Henderson and Demeyer (1989). The significance of differences (at least $P < 0.05$) was tested using the *t*-test on paired observations (Snedecor and Cochran, 1971).

RESULTS

Table I shows the mean values for the production of fermentation end products in incubations with both rumen and caecal contents. Blank incubations without added substrates and incubations with added hay and free amino acids are presented. For the latter, net amounts are shown: the amounts formed in blank incubations are substracted from the amounts formed with added substrates.

The data revealed a large variability in the end products formed in blank incubations (eg, average variation coefficient for total VFA = 26%), whereas the variability was reduced for substrates by correction for blank incubations (eg, variation coefficient for total VFA was reduced to 11%). The results confirmed well known differences between caecal and rumen fermentations: the hydrogen recoveries were significantly higher for the rumen contents than for the caecal contents, in line with the occurrence of reductive acetogenesis in the cecum, as discussed earlier (Demeyer, 1991; Demeyer and De Graeve, 1991). This difference disappeared, however, when free amino acids were used as a substrate, as found earlier (Van Nevel and Demeyer, 1990). The caecal contents formed less methane and butyrate than the rumen contents; however, when free amino acids were used, the former formed more butyrate and propionate, and the methanogenesis became highly variable. Judging from the total VFA productions, the caecal contents were more active towards free amino acids then towards hay.

Eventual induction of reductive acetogenesis through the presence of free amino acids during hay fermentation can now be evaluated through the incubation of hay and free amino acids as a mixture. Assuming that the same amounts of substrates are fermented when incubated alone and as a mixture, the net amounts of fermentation products of hay in the mixture (FP_{hay}) can be calculated as: $FP_{hay} = FP_{mix} - FP_{aa}$ with FP_{mix} = net fermentation products formed in the incubation of mixed substrates and

Table I. Production of fermentation end products (μmol) in rumen and caecal cattle contents, incubated without addition (blank) or with added hay and free amino acids*.

	Blank		*Hay*		*Free amino acids*	
	Rumen	*Caecum*	*Rumen*	*Caecum*	*Rumen*	*Caecum*
Acetate	565 ± 72	533 ± 78	655[b] ± 49	638 ± 34	414 ± 25	515 ± 20
Propionate	184 ± 31	204 ± 26	227[b] ± 9	215 ± 15	105[a] ± 3	192 ± 8
Butyrate	205[a] ± 21	71 ± 16	137[a] ± 10	70 ± 7	151[a] ± 11	230 ± 11
Isovalerate	100[a] ± 16	11 ± 6	10[b] ± 5	3 ± 2	143 ± 5	143 ± 9
Valerate	19 ± 10	5 ± 3	23[b] ± 6	10 ± 7	155 ± 7	154 ± 4
Total VFA	1 083 ± 138	823 ± 120	1 054 ± 55	922 ± 54	962[a] ± 20	1 232 ± 35
Methane	295[a] ± 39	61 ± 18	220[ba] ± 25	41 ± 16	99 ± 21	10 ± 5
Ammonia	761 ± 240	494 ± 228	−114[b] ± 3	−82 ± 60	816 ± 116	854 ± 67
% 2H rec	86.8[a] ± 4.1	53.0 ± 6.6	80.0[a] ± 2.6	47.0 ± 7.5	63.9 ± 9.5	54.9 ± 0.9
% NH_3 rec					145[a] ± 5	189 ± 2

* Mean values ± SE of four replicates; [a] significantly different from the caecal fermentation; [b] significantly different from the amino acid fermentation with rumen contents. VFA: volatile fatty acids.

FP_{aa} = net fermentation products formed in the incubation of free amino acids.

These calculated amounts could then be compared to the actual amounts determined in hay incubations, especially for acetate and methane. Indeed, it could be reasoned that induction of reductive acetogenesis would be reflected in the calculated amounts of acetate and methane that would be, respectively, higher and lower than the amounts determined. Table II shows the result of such an exercise for free amino acids.

An identical approach was followed for mucin and table III illustrates the results obtained.

Table IV shows the effect of head space hydrogen gas on rumen hay incubations in vitro in the presence and absence of mucin. It is clear that the only significant effects observed were increases in the production of methane and propionic acid. The production levels of acetate and butyrate did not change.

DISCUSSION

From the results presented in table II, it is clear that the calculated values for hay fermentation end products matched the determined amounts of the end products in all cases, except for ammonia. Hay fermentation is accompagnied by incorporation of ammonia N, obviously reflecting microbial growth, but such an incorporation is decreased by the presence of free amino acids. It is conceivable that this effect is related to the direct incorporation of amino acids into microbial matter, decreasing the use of ammonia N for microbial growth. It is also clear that both in the presence and absence of hay, more than 95% of the amino acids added (635 µmol α-amino N) are degraded. Ammonia production from the free amino acids accounts for 97% of α-amino N degraded in the presence of hay, but for 132% in the absence of hay. Again, this finding may be related to differences in the amino acid incorporation during microbial

Table II. Effect of free amino acids on net amounts of hay fermentation end products (µmol) using cattle rumen contents in vitro*.

	Hay + amino acids	*Amino acids*	*Hay*	
			Calculated	*Determined*
Acetate	1 062 ± 53	414 ± 25	648 ± 44	655 ± 49
Propionate	337 ± 5	105 ± 3	232 ± 6	227 ± 9
Butyrate	311 ± 13	151 ± 11	160 ± 11	137 ± 10
Isovalerate	148 ± 8	143 ± 5	45 ± 41	10 ± 5
Valerate	177 ± 5	155 ± 7	23 ± 5	23 ± 6
Methane	323 ± 35	99 ± 21	224 ± 28	220 ± 25
Total VFA	2 036 ± 51	962 ± 20	1 069 ± 39	1 054 ± 55
Ammonia	597 ± 225	816 ± 116	−56[a] ± 12	−114 ± 3
α-amino N	−617 ± 6	−615 ± 6	–	–
% 2H rec	76 ± 3	64 ± 10	79 ± 5	80 ± 3
% NH_3 rec	–	145 ± 5	–	–

* Mean values ± SE of four replicates; [a] significantly different from the determined amounts. VFA: volatile fatty acids.

Table III. Effect of mucin on net amounts of hay fermentation end products (μmol) using sheep rumen contents in vitro*.

	Hay + mucin	*Mucin*	*Hay*	
			Calculated	*Determined*
Acetate	3 158 ± 37	1 923 ± 205	1 236 ± 103	1 198 ± 92
Propionate	1 186 ± 14	674 ± 32	513[a] ± 20	391 ± 13
Butyrate	327 ± 6	172 ± 26	155[a] ± 8	113 ± 8
Isovalerate	67 ± 4	57 ± 6	10 ± 2	11 ± 4
Valerate	165 ± 4	135 ± 10	30[a] ± 7	19 ± 7
Methane	727 ± 30	371 ± 69	356[a1] ± 33	464 ± 88
Total VFA	4 902 ± 33	2 960 ± 219	1 943[a] ± 123	1 732 ± 96
% 2H rec	72 ± 1	67 ± 4	78[a] ± 3	89 ± 7

* Mean values ± SE of four replicates; [a] significantly different from the determined amounts; [1] $P < 0.07$. VFA: volatile fatty acids.

growth, as well as to the degradation of non α-amino N. As the presence of free amino acids did not affect the total VFA production or proportion from hay, it can be concluded thay hay fermentation in short-term incubations in vitro was not affected in any way by the presence of free amino acids.

Confirming an earlier report (Van Nevel and Demeyer, 1990), the recovery of ammonia N, calculated from the VFA production in free amino acid incubations, was much higher than 100%, indicating that considerable amounts of VFA were formed following reactions other than the model used

Table IV. Effect of head space hydrogen gas on end products of in vitro rumen fermentations of hay and mucin*.

	VFA (μmol)	*VFA (mmol/mol)*			
		Acetate	*Propionate*	*Butyrate*	*Methane*
Hay					
$-H_2$	3 021 ± 20	655 ± 5	236 ± 3	109 ± 3	267 ± 15
$+H_2$	3 046 ± 11	633 ± 7	255[a] ± 9	112 ± 1	539[a] ± 8
Mucin					
$-H_2$	4 304 ± 34	680 ± 7	234 ± 2	86 ± 2	184 ± 12
$+H_2$	4 349 ± 104	677 ± 16	241 ± 6	81 ± 5	395[a] ± 15
Hay + mucin					
$-H_2$	6 253 ± 69	662 ± 8	252 ± 4	86 ± 2	190 ± 7
$+H_2$	6 279 ± 131	652 ± 15	262[a] ± 6	86 ± 1	342[a] ± 11

* Mean values ± SE of three repetitions; [a] significant effect of head space hydrogen gas. VFA: volatile fatty acids.

(Demeyer, 1991). However, such reactions may involve coupled oxidation–reduction reactions between two amino acids (eg, Stickland reactions), rather than reductive acetogenesis (Nagase and Matsuo, 1982; Russell et al, 1991). The occurrence of either reductive acetogenesis of Stickland reactions would result in an erroneous calculation of low hydrogen recoveries.

When the same approach was used with mucins, however, the calculated amounts of hay fermentation end products in the mixture mucins and hay were significantly different from those determined in incubations with hay alone, except for acetate. Higher amounts of propionate, butyrate and valerate were calculated, and lower amounts of methane. Interpretation of these data is of course very difficult. Indeed, no attempt was made to determine the amounts of mucin and hay substrates fermented in the incubations and each substrate may affect the amount degraded of the other when they are incubated as a mixture, compared to when they are incubated as an isolated substrate. It was tempting to speculate, however, that the presence of mucin resulted in a shift of hydrogen disposal from methane to the reduced VFA propionate, butyrate and valerate in the hay fermentation. Such an effect would have increased the total VFA production from the hay, as calculated. The reason for such a shift may have been a drop in pH, due to the extensive fermentation of the mucin added, as the latter contains linear and branched chain oligosaccharides, constituting up to 85% of the mucin by weight (MacFarlane and Cummings, 1991). Although the hydrogen recovery was lowered significantly, it was still within the values acceptable for rumen fermentation (Demeyer, 1991). The absence of a strong inhibitory effect of mucin on methanogenesis contrasted with the findings obtained with human gut bacteria. There, the inhibitory effect was due to the release of sulphate from the mucin and the change was from methanogenesis to sulphate reduction as major hydrogen sink (Gibson et al, 1988). Rumen methanogenesis is, however, not inhibited by the addition of sulphate (Demeyer and Henderickx, 1967). From end products of mucin incubations, whether incubated alone or as a mixture, hydrogen recoveries are calculated that are significantly lower than those normally found for incubations with rumen contents (Demeyer, 1991). This finding was possibly related to the acetate production from acetylated carbohydrate moieties and resulted in low methane/VFA ratios. Although no change in acetate production was observed, suggestive of reductive acetogenesis, definite conclusions cannot be made. Irrefutable evidence for the induction of reductive acetogenesis in the presence of mucin, would have to come from labelled CO_2 experiments (see eg, De Graeve et al, 1990). Considerable information may be obtained, however, from experiments studying the effects of head space hydrogen gas on fermentation, as shown by Demeyer and De Graeve (1991). These effects involve an increase in acetate and butyrate production in the acetogenic hindgut fermentation, which are absent in rumen fermentation. The presence of head space hydrogen gas in rumen hay incubations in vitro (table IV) in the presence and absence of mucin did not change the production of acetate and butyrate, in line with the absence of active reductive acetogenesis, as shown earlier (Demeyer and De Graeve, 1991).

We concluded that neither mucins nor free amino acids activated reductive acetogenesis in short-term in vitro incubations of rumen contents, as had been suggested in earlier works (Van Nevel and Demeyer, 1990; Demeyer and De Graeve, 1991). As incubations lasted for 24 h, a period largely exceeding the potential doubling time of acetogens, it is unlikely that long-term in vivo addition of these compounds to the rumen would initiate this pathway. On the other hand, a long-term enrichment period

may be necessary at very low initial numbers and low growth rates of acetogens.

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