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The effect of nitrogen source and concentration on in vitro gas production using rumen micro-organisms

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The effect of nitrogen source and concentration on in vitro gas production using rumen micro-organisms

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Abstract

The effects of nitrogen (N) on an in vitro gas production method were investigated by fermenting two N-deficient substrates (glucose or barley straw) in media with different concentrations of N (from 0 to 165 mg l^{-1}) from two N sources (ammonium sulphate or urea). The response of the in vitro gas production system to N supplementation was broadly consistent with earlier in vitro work, indicating that a minimum of about 80 mg l^{-1} N was required to achieve the maximum degradation of carbohydrate by rumen microbes. The effect of N appeared to be mainly, if not entirely, on the rate of degradation rather than its extent. The in vitro gas production method appeared to be suitable for investigating interactions between feeds. © 1998 Elsevier Science B.V.

Keywords: Rumen fermentation; In vitro gas production; Feed interactions; Supplementation; Ruminant nutrition

1. Introduction

Straws, stovers and native hays are a major source of feed for ruminants in less-developed countries (ldcs). These feeds are often high in fibre and deficient in nitrogen (N) resulting in low digestibility and intake by ruminants. Consequently, there

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is considerable interest in the use of supplements to improve digestibility and intake (for example Goodchild and McMeniman, 1994; Bonsi et al., 1994).

A constraint to investigating the supplementation of tropical roughages has been the lack of suitable in vitro methods. The wide range and variability of roughages and potential supplements means that it is impractical to study more than a limited number of combinations in vivo. An in vitro gas production method, developed by Theodorou et al. (1994), appears to be particularly suitable for investigating feed mixtures and interactions between feeds. The purpose of this paper is to describe how this method responded when N-deficient substrates were fermented with different levels and sources of non-protein N and, hence, to assess whether the method appeared suitable for investigating roughage plus N (protein) supplement mixtures.

The substrates, straw and glucose, were selected to represent two extremely different types of carbohydrates in terms of their fermentation characteristics. Neither would contribute much (or any in the case of glucose) fermentable protein, hence reducing this possible complicating factor. Highly fibrous crop residues are often important feeds in less developed countries. Many feeds, such as tree fodders and sugar cane wastes, are rich sources of soluble carbohydrates. Hence the substrates used represent types of carbohydrates which can be particularly important in ldc feeding systems.

2. Materials and methods

2.1. Substrates

Barley straw (crude protein 43 g kg^{-1} DM, of which 28 g kg^{-1} DM was contained in the ADIN–N fraction; ADF 878 g kg^{-1} DM; NDF 532 g kg^{-1} DM; total ash 49 g kg^{-1} DM) was obtained from a commercial supplier. Other substrates and reagents were of analytical grade.

2.2. In vitro gas production

The method of Theodorou et al. (1994), as described by Sampath et al. (1995), was used. This consisted of the anaerobic fermentation of substrates at 39°C in a buffered medium (90 ml, plus 4 ml of a 6.3 g l^{-1} sodium sulphide solution as reducing agent) in stoppered 125 ml serum bottles. The N-free medium described by Menke et al. (1979) was used throughout. Bottles were inoculated with 5 ml of an inoculum prepared in the following way. Rumen fluid was strained through four layers of cheese-cloth, the liquid collected and the solids macerated in an equal volume of medium. This mixture was then strained and added to the strained rumen fluid, stirred and kept under CO_2. Inocula prepared in this way had a total N content of 550 ± 58 mg N l^{-1}, contributing about 28 mg N l^{-1} to the final incubation mixture.

Gas production was monitored using a pressure transducer, with readings of gas volumes produced being taken every 3 h initially, then at increasing intervals as gas production slowed. The incubation period was usually ended after 166 h, although for treatments with 0 and 20 mg N l^{-1} of urea or ammonium sulphate it was continued to

238 h. At each reading time, gas was removed to adjust the pressure in the serum bottle to atmospheric pressure. The gas removed was discarded. At the end of the incubation period the dry matter disappearance (DMD) of the straw was estimated by recovering the solid residues in a pre-weighed scinter crucible (porosity P160), oven drying at 105°C and re-weighing.

2.3. Experimental design

Substrates were either 1 g barley straw or 0.5 g glucose, to give similar amounts of fermentable carbohydrate within the range where gas production is proportional to the amount of substrate (Theodorou et al., 1994). N was added as urea or ammonium sulphate so as to contribute the following concentrations in the final incubated mixture: 0, 20, 40, 80, 120 and 165 mg N l^{-1} (excluding N from the inoculum and substrate). Fermentations were performed simultaneously in triplicate using a single source of inoculum. For incubation with straw as substrate, additional triplicate incubations were set up and terminated after 52 h to estimate DMD at this intermediate time.

2.4. Analysis of data

Cumulative gas productions (ml g^{-1} substrate dry matter) were calculated and the values at 12, 52, 166 and (where appropriate) 238 h, together with DMD (for straw), subjected to analysis of variance. The analysis was carried out on the treatment means for a 2×6 factorial with three replicates per treatment. The null hypothesis was that there were no differences between the treatment means. Prasad et al. (1994) indicated that the extent of in vitro fermentation best matched in vivo digestibility at about 52 h incubation, while 12 h incubation approximately coincided with the end of the lag phase and 166 or 238 h with the end point of fermentation.

The average cumulative gas productions were corrected for the gas produced by a no substrate control and the France model (France et al., 1993) fitted. This model estimates the asymptotic total gas production (gas pool, A) and gives parameters from which two rate constants, b and c, and lag times can be derived.

3. Results

3.1. Gas production characteristics

The gas production characteristics of barley straw fermented without added N or with the highest level of N (165 mg l^{-1} N as urea) are shown in Fig. 1. In the N-rich medium there was a lag phase of about 12 h followed by a period of maximum gas production (about 4 ml h^{-1} g^{-1} DM for straw) and exponential decline. In the N-free medium there was a similar lag phase, followed by a slightly increased rate of gas production of about 2 ml h^{-1} g^{-1} up to about 24 h, then a decline to a lower (but also constant) rate of about 1 ml h^{-1} g^{-1}, which decreased further after 166 h. In general, gas production from straw in media with intermediate N concentrations fell between these two curves.

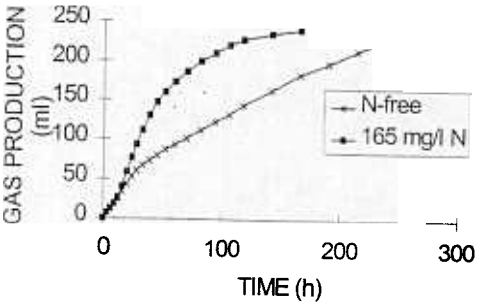

Fig. 1. Cumulative gas production (ml g^{-1} DM) from barley straw incubated in an N-free medium and in a medium containing 165 mg l^{-1} N as urea.

A broadly similar pattern was obtained with ammonium sulphate as the N source and with glucose as the substrate, although the lag phase with glucose lasted for six hours whilst the maximum rate of fermentation was about 23 ml h^{-1} g^{-1} DM. The fermentation of glucose in N-free and in 165 mg l^{-1} N (as urea) is illustrated in Fig. 2.

The gas production characteristics are summarised as the parameters obtained from the fitted France model in Tables 1–4 for the gas pool size (A), rate constants b and c, and lag time T, respectively. Some problems were encountered in fitting the model to the data from the fermentation of mixtures with lower N concentrations. This is reflected in the high asymptotic standard errors in the gas pool size and inability to estimate the lag time for these treatments. Nevertheless the rate constant (b) presented in Table 2 illustrate increased rate of fermentation as N concentration was increased from 0 mg l^{-1} to 80 mg l^{-1}, whereafter responses to higher N concentrations were relatively modest. They also illustrate the relatively rapid fermentation of glucose compared to barley straw at the same N concentration. The France model indicated that lag times for glucose were shorter than those for straw.

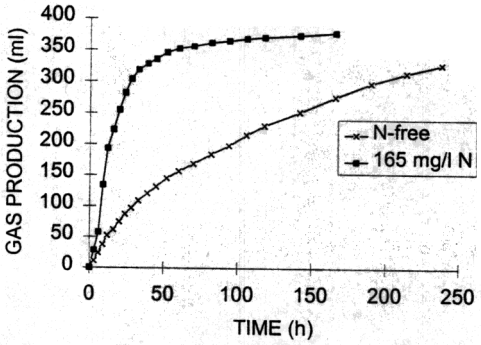

Fig. 2. Cumulative gas production (ml g^{-1} DM) from glucose incubated in an N-free medium and in a medium containing 165 mg l^{-1} N as urea.

Table 1
France model fitted parameters: gas pool size, A (ml), ±asymptotic standard error, for barley straw and glucose with varying levels of ammonium sulphate and urea

Substrate	Barley straw		Glucose	
N concentration (mg l^{-1})	Urea	Ammonium sulphate	Urea	Ammonium sulphate
0				
20				
40				
80				
120				
165				

Table 2
France model fitted parameters: rate constant, b (h^{-1}), for barley straw and glucose with varying levels of ammonium sulphate and urea

Substrate	Barley straw	Glucose
N concentration (mg l^{-1})		Ammonium sulphate
0		0.0035
20		0.0255
40		0.0598
80		0.0849
120		0.0886
165		0.0851

3.2. *Influence of N source and concentration on gas production from straw and glucose*

The average data are presented in tabular form together with a description of the major trends observed. The average (of three replicates) cumulative gas productions from barley straw at different incubation times for each N source and N concentration

Table 3
France model fitted parameters: rate constant, c ($h^{-0.5}$), for barley straw and glucose with varying levels of ammonium sulphate and urea

Substrate	Barley straw	Glucose
N concentration (mg l^{-1})		Ammonium sulphate
0		0.0362
20		−0.0018
40		−0.0659
80		−0.1344
120		−0.1476
165		−0.1452

Table 4
France model fitted parameters: lag time, T (h), for barley straw and glucose with varying levels of ammonium sulphate and urea

Substrate	Barley straw		Glucose	
N concentration (mg l^{-1})	Urea	Ammonium sulphate	Urea	Ammonium sulphate
0	nd	nd	nd	nd
20	nd	nd	nd	nd
40	2.7	2.0	0.9	nd
80	2.9	2.6	1.7	1.5
120	4.0	2.8	1.6	1.7
165	4.0	2.9	1.1	1.7

nd = not derived, values either negative or not obtained.

Table 5
In vitro cumulative gas production from barley straw when incubated for different times with varying levels of ammonium sulphate and urea: individual treatments

N source	N concentration (mg l^{-1})	Gas production (ml g^{-1} DM) at different incubation times			
		12 h	52 h	166 h	238 h
ammonium sulphate	0		86.4		
	20		114.3		
	40		144.8		
	80		154.8		
	120		163.2		
	165		158.5		
urea	20[a]		116.6		
	40		150.3		
	80		157.9		
			138.5		
			141.8		
SED			9.9		
Statistical significance of differences in gas production					
Statistical significance of N source			ns		ns
Statistical significance of N concentration					
Statistical significance for source × concentration interaction				ns	ns

SED = standard error of the difference between means.
[a]For urea-free fermentation data see above (ammonium sulphate 0 concentration).
ns = not significant.
nd = not determined (gas production almost ceased).
$^{***}P < 0.001$.
$^{**}P < 0.01$
$^{*}P < 0.05$

are given in Table 5. Highly significant ($P < 0.001$) differences in gas production were observed at 12, 52 and 166 h incubation between the different treatments. N concentration significantly affected cumulative gas production from straw at all the incubation times selected. N source significantly ($P < 0.001$) affected gas production at 12 h of incubation, but not ($P > 0.05$) at 52, 166 and 238 h, although overall ammonium sulphate tended to give higher gas productions than urea at all selected incubation times. N source × concentration interactions were only significant at 12 and 52 h of incubation.

At 12 h incubation there were no significant increases in gas production with increasing N concentration, indeed 120 and 165 mg l^{-1} urea caused a significant ($P < 0.05$) inhibition of gas production. At 52 h incubation gas production was increased with increasing N ($P < 0.05$), the maximum response apparently being achieved by about 80 mg l^{-1} N for urea and about 120 mg l^{-1} N ammonium sulphate. These trends were still highly significant ($P < 0.001$) at 166 h incubation. At 238 h, the 0 and 20 mg N l^{-1} treatments produced similar total amounts of gas ($P > 0.05$) as the 166 h gas productions achieved by the treatments with higher N concentrations, although overall the effect of N concentration on gas production still achieved statistical significance ($P = 0.0469$) at 238 h (Table 5).

The average DMD data at 52 and 166 or 238 h are given in Table 6. The trends in gas production were paralleled by the trends in DMD, with significant ($P < 0.05$) effects of N concentration on DMD at 52 h, but (unlike gas production) not at 166 or 238 h

Table 6
Dry matter disappearance (DMD) during in vitro gas production from barley straw when incubated for different times with varying levels of ammonium sulphate and urea: effects of nitrogen source and concentration

N source	N concentration (mg l^{-1})	DMD (as a proportion) at different incubation times	
		52 h	166 h or 238 h
ammonium sulphate			
urea			
statistical significance of N source			
	0		
	20		
	40		
	80		
	120		
	165		
SED[a]			
statistical significance of N concentration			
statistical significance for source × concentration interaction			

[a] SED = standard error of the difference between means.
ns = not significant.
*** $P < 0.001$
* $P < 0.05$

($P < 0.05$). Responses at 52 h increased with N concentration of up to 80 mg N l^{-1} for urea (to a proportional DMD of 0.476) before declining at higher N concentrations (0.458 and 0.433 at 120 and 165 mg N l^{-1}, respectively). For ammonium sulphate, the maximum response at 52 h was achieved at 120 mg N l^{-1} (to a proportional DMD of 0.500), the same DMD also being obtained with 165 mg N l^{-1}. Therefore the trends of DMD with N concentration were consistent with those observed in the gas production data.

Table 7 presents the results averaged for each N source and concentration with glucose as the substrate. Although straw and glucose gave broadly the same responses to N, there were some notable differences. The effect on gas production from glucose was similar with either urea or ammonium sulphate as N sources, although the effect of ammonium sulphate tended to peak at 80 mg N l^{-1} and then declined at higher concentrations, while for urea the effect on gas production tended to be lower than for ammonium sulphate up to 80 mg N l^{-1} but continued to increase with increasing N

Table 7
In vitro cumulative gas production from glucose when incubated for different times with varying levels of ammonium sulphate and urea: individual treatments

N source	N concentration (mg l^{-1})	Gas production (ml g^{-1} DM) at different incubation times			
		12 h	52 h	166 h	238 h
ammonium	0	61.4	157.3		
sulphate	20	87.4	270.4		
	40	151.4	303.6		
	80	172.4	335.6		
	120	163.4	320.3		
	165	149.0	305.4		
urea	20[a]	80.7	268.4		
	40	122.0	289.1		
	80	156.1	322.0		
	120	176.1	334.4		
	165	193.1	346.1		
SED[a]		13.1	13.5		
Statistical significance of differences in gas production					
Statistical significance of N source			ns		
Statistical significance of N concentration					
Statistical significance for source × concentration interaction				ns	ns

SED = standard error of the difference between means.
[a]For urea-free fermentation data see above (ammonium sulphate 0 concentration).
ns = not significant.
nd = not determined (gas production almost ceased).
$^{***}P < 0.001$.
$^{**}P < 0.01$
$^{*}P < 0.05$

concentrations. Overall, however, N source effects were not significant ($P > 0.05$). Interactions between N source × concentration were significant ($P < 0.05$) at 12 and 52 h incubation, but not at 166 and 238 h. The addition of N increased gas production for 12 h incubation as well as 52 and 166 h incubation ($P < 0.001$). The increases were proportionately highest at 12 and 52 h incubation.

For glucose fermented with urea as N source, at 12 and 52 h incubation there was a marked initial response which showed little further increase at N concentrations above 80 mg l^{-1}. However, there did appear to be some residual response even at the highest N concentration used (165 mg l^{-1}). At 166 h incubation there was a response to 20 mg l^{-1} N ($P < 0.05$), but no additional significant ($P > 0.05$) response to higher N concentrations. This effect continued to decline with further incubation. At 238 h incubation the response to 20 mg l^{-1} N as ammonium sulphate had ceased to be statistically significant ($P > 0.05$).

4. Discussion

4.1. Gas production characteristics

The gas production curve for barley straw incubated with 165 mg l^{-1} urea (Fig. 1) is similar to that described by Prasad et al. (1994) for finger-millet straw. The lag time is presumably due to adaptation of microbes to the substrate, while the maximum gas production rate is determined by the ability of the microbes to degrade the fibre fraction of the straw. The lag time with glucose was shorter and the maximum rate of gas production higher than found for straw indicating that, as expected, glucose was much more readily fermentable than straw. In contrast, without added N, gas production was apparently determined by the availability of N rather than the degradability of the carbohydrate.

The high gas pool sizes for substrates fermented in N-free medium appeared to be due to problems with the fitting of the model rather than a reflection of a real trend in the data. Increases in rate constant (b) appeared to reliably reflect trends in fermentation rates. The physiological significance of the time dependent rate constant (c) is unclear, but trends were broadly inversely related to those observed for the rate constant (b). Visual inspection of actual and predicted gas productions indicated that the model tended to underestimate the lag time (T). Nevertheless the France model was able to reflect the reduced lag time for glucose compared with barley straw.

4.2. Influence of N source on gas production from straw and glucose

Urea N concentrations of 120 mg l^{-1} or more caused an initial inhibition of gas production from barley straw. This inhibition appeared to account for the generally lower gas productions observed from straw fermentation when urea (at concentrations $\geq$ 120 mg l^{-1}) was the N source. This trend was reflected in the increased lag times for these treatments. These trends were not apparent when glucose was the substrate. The

reasons for this finding are unclear. Possibly higher concentrations of urea inhibited the degradation of the straw fibre fraction by mechanisms which did not affect the fermentation of soluble carbohydrate. Such mechanisms could have included inhibition of the attachment of microbes to fibre and preferential inhibition of microbes most associated with fibre degradation. The fermentation medium contained minerals essential for microbial growth, so mineral deficiencies were unlikely to have occurred.

The apparent inhibitory effect of ammonium sulphate N at concentrations above 80 mg l^{-1}, with glucose as substrate, was not consistent with the trends observed in gas production and DMD for straw. The rate constants for glucose fermented with 165 mg l^{-1} of either N source tended to be below those achieved with 120 mg l^{-1} of N. One possible explanation is that carbon dioxide produced by fermentation reacted with ammonium released by microbial activity to yield ammonium bicarbonate, a reaction which can take place when protein is degraded (Menke and Steinglass, 1988). Thus N not used in microbial fermentation may tend to reduce gas production by preventing the release of some carbon dioxide from the medium. However, the apparent inhibition of gas production by higher concentrations of N were not consistent and could, at least in part, have been due to experimental error.

4.3. Influence of N concentration on gas production from glucose and straw

There have been several earlier studies aimed at defining how much N is required by rumen microbes to facilitate the maximum rate and extent of degradation of feeds. Satter and Slyter (1974), using continuous-culture fermenters charged with rumen microbes, found that rumen microbial protein production was stimulated by adding urea until a concentration of 50 mg N l^{-1} was achieved. No additional stimulation was observed when higher N concentrations were used. It was suggested that the precise limiting N concentration was closer to 20 mg ammonia N l^{-1}, but recommended the higher figure be used as a guideline minimum to give a safety margin. It was also noted that very high ammonia concentrations, up to 800 mg l^{-1}, did not inhibit microbial growth. Oosting et al. (1989), using an in vitro degradation method based on Tilley and Terry (1963), observed that 88 to 100 mg N l^{-1} was required for maximum in vitro degradation of low quality feeds. Erdman et al. (1986), studying in sacco rumen degradation in cows infused with urea, found that N requirement to achieve maximum degradation was dependent on the potential degradability of the feed; the more degradable the feed the higher the N requirement. Rumen N concentrations as high as 250 mg N l^{-1} were required for the maximum degradation of the highly fermentable feeds corn-meal and soybean-meal.

General guidelines on the minimum ammonium N concentrations, at which N supply to rumen microbes is not limiting, range from 60 mg N l^{-1} (Osuji et al., 1995) to 150 to 200 mg N l^{-1} (Preston, 1995). This may well reflect differences in the energy sources being fed; slowly digestible roughage and rapidly digestible by-products of sugarcane, respectively.

In this study some N was supplied by the inoculum and by the substrate in the case of barley straw. The inoculum used here contributed about 28 mg N l^{-1} to the incubation

mixture, but it is unknown how much of this was readily available to the microbes. Similarly, the barley straw could have contributed up to 24 mg N l^{-1} (assuming that ADIN–N was not available to rumen microbes). The fermentation characteristics of barley straw in the N-free medium indicated that any contribution from these sources to the N supply was very limited, so their contribution was assumed to be negligible. However, the N concentrations indicated may under-estimate the true concentrations of N available to the microbes.

In the in vitro gas production system, the expected increase in gas production with increasing N concentration was observed as N deficiencies were alleviated. The near maximum response at about 80 mg l^{-1} N at 52 h incubation, the period most relevant to the in vivo situation (Prasad et al., 1994), for both glucose and straw is broadly consistent with the findings of Satter and Slyter (1974) and Oosting et al. (1989). It was of interest to note that the response was variable with time of incubation and that, for glucose plus urea, there was a response to N at least up to 165 mg l^{-1} at short incubation times. This may indicate some relationship between N requirement and degradability as proposed by Erdman et al. (1986); the presence of a rapidly degradable energy source increasing the initial requirement for N to maintain a balance between the two nutrients.

4.4. Implications for investigating the supplementation of N-deficient feeds

To investigate the supplementation of N-deficient feeds in vitro, a method is required which is sensitive to N deficiencies in the feed, in which roughage plus supplement mixtures can be evaluated, and where the kinetic characteristics can be readily monitored. The in vitro gas production method has these characteristics. This study has demonstrated that the sensitivity of the method to N is consistent with earlier studies. Comparing the fermentation of feed mixtures in N-rich and N-free media could be used to investigate their N status and, thus, identify the level of supplementation required to balance N and fermentable carbohydrate contents in diets. At present, such interactions are usually investigated in vivo in the absence of suitable in vitro methods. The in vitro gas production method promises to fill this important gap.

5. Conclusions

The effect of N concentration appeared to be mainly, if not entirely, on the rate of degradation rather than its extent. As gauged by the France model rate constant *b*, the greatest responses to N supplementation were achieved by increasing N concentration to 80 mg l^{-1}. Cumulative gas productions at 52 h incubation indicated that the maximum of stimulation of gas production from straw was achieved at 80 mg l^{-1} N for urea and 120 mg l^{-1} N for ammonium sulphate. For glucose, 80 mg l^{-1} N ammonium sulphate stimulated the maximum gas production, while for glucose plus urea some residual response was obtained with N concentrations over 80 mg l^{-1} N. The response of the in vitro gas production system to N supplementation was, therefore, broadly consistent

with earlier in vitro work in showing that a minimum of about 80 mg l^{-1} N is required to achieve the maximum degradation of carbohydrate by rumen microbes. Responses did depend to some extent on N source, although differences between sources were not consistent for both substrates. Responses also depended on the period of incubation and largely disappeared as fermentation approached completion. The gas production method can be used for making kinetic measurements of the fermentation of feeds and feed mixtures. The method appears to be particularly suitable for investigating the supplementation of N-deficient feeds with N-rich supplements.

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