Netherlands Journal of Agricultural Science 37 (1989) 157-163

The metabolism of nitrate and proline in the rumen fluid of a cow and its effect on in vivo formation of N-nitrosoproline

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Received 13 October 1988; accepted 6 March 1989

Abstract

In a balance trial, two cows were fed with hay containing nitrate, proline and N-nitrosoproline (NPRO). No in-vivo formation of NPRO was found. The NPRO in the hay was recovered from the urine and the faeces. Nitrite and proline was metabolized for the greater part in the rumen fluid. So, it was estimated that 10 % and 20 %, respectively, will enter the abomasum. In spite of a favourable pH in the abomasum no formation of NPRO was found.

Keywords: nitrate, N-nitroproline, proline, rumen fluid

Introduction

High levels of nitrate in grass and grass products can cause nitrate poisoning in cows (Kemp et al., 1977). The question arose whether the high nitrite concentrations in the rumen fluid of cows after the ingestion of nitrate-rich feed could also be responsible for the formation of N-nitroso compounds (NA). These compounds are well-known mutagenic and/or carcinogenic substances and they may be transferred to meat or milk.

No volatile NA are formed in the rumen fluid after ingestion of nitrate-rich feed (van Broekhoven & Davies, 1981).

Ohshima et al. (1982) have described a method for the estimation of daily human exposure to endogenously formed NA. With this method the excretion of N-nitrosoproline (NPRO) in urine and faeces is measured after the injection of the precursors proline and nitrate in human to estimate daily exposure to endogenously formed NPRO compounds.

This method was used to investigate the possible formation of non-volatile NA in the cow. After feeding cows with hay containing proline and nitrate, no NPRO is found in the milk and all NPRO in the feed is recovered in urine and faeces (van Broekhoven et al., 1984). NPRO is not formed endogenously and it is assumed that both nitrite and proline are rapidly metabolized in the rumen, so that these cannot enter the abomasum.

Here we report the results of experiments on the fate of nitrite, proline and NPRO in the rumen, in order to test this assumption.

Materials and methods

Chemicals

L-proline and DL-pipecolic acid were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA); BF_3-methanol from Chrompack (Middelburg, the Netherlands). Chemicals and standards for analysis of amino acids were obtained from BDH (Chemicals Ltd., Poole, England). All other chemicals (p.a.) were obtained from Merck (Darmstadt, FRG).

Kling gauze (152 cm × 4.6 m) for extraction cartridges was purchased from Johnson and Johnson Products Inc. (New Brunswich, New Jersey, USA).

All chemicals were used without further purification. NPRO and N-nitrosopipecolic acid (NPIC) were synthesized according to the method of Lijinsky et al. (1970).

SEPPAK C-18 cartridges were obtained from Waters Associates (Milford, Massachusetts, USA).

Feeding experiments

The trials were carried out in a cowshed equipment for digestion experiments with two non-lactating Friesian cows fitted with a rumen fistula. One week before the start of the experiments the aimals were fitted with a device for the separate collection of faeces and urine (van Es & Vogt, 1959).

The adaptation period before the first experiment was 10 days. Between the two experiments an adaptation period of three days was used. Earlier experiments (Kemp et al., 1977) show that after these days the cows were adapted to nitrite formation.

After the adaptation period, the collection period of three days followed. The cows were fed in the first experimental period with nitrate-poor hay and in the second period with nitrate-rich hay. The composition of the hay is given in Table 1. The hay was fed in two daily portions (at 8.00 h and 16.00 h).

The consumption of the first portion was interrupted at 8.45 h to measure the disappearance rate of proline and nitrite in the rumen fluid. After the sampling period of the rumen fluid the feeding was continued (11.00 h).

Table 1. Composition of the nitrate-poor and nitrate-rich hay used in the two experimental periods

Period	Total N in dry matter ($g\,kg^{-1}$)	Nitrate-N in dry matter ($g\,kg^{-1}$)	Dry matter content (%)	Proline in fresh matter ($g\,kg^{-1}$)	NPRO in fresh matter ($\mu g\,kg^{-1}$)
1	20,7	0,42	90,8	1,5	168
2	45,7	3,70	90,3	4,2	1673

Sampling

Faeces and urine were sampled and conservated as described in van Broekhoven et al. (1984). For sampling of the rumen fluid a perforated tube was pressed through the fistula almost to the bottom of the ventral sack.

The rumen fluid was divided into three portions. One portion (40 ml) was treated with lead acetate (10 ml) and stored at 4 °C to preserve it for the analysis of nitrate and nitrite (Vertregt, 1977). One portion (10 ml) was mixed (1:4) with ethylalcohol and stored at −20 °C for the analysis of proline. One portion (100 ml) was treated with 0.8 ml 6 mol l^{-1} NaOH and 2 g ammonium sulphamate and stored at 4 °C for analysis of NPRO.

Analysis

NPRO

The extraction and analysis of NPRO in feed, faeces and urine was performed as described in van Broekhoven et al. (1984). Except that an alternative was used for the Preptube extraction cartridges because these were no longer available. Kling gauze (2.3 m) was pressed in an empty cartridge and this cartridge was used in the same way as the Preptube cartridge.

The rumen fluid samples (100 ml) were treated with 3.6 mol l^{-1} H_2SO_4 to pH 1. After centrifugation the precipitate was decanted and 5 g NaCl was added. The solution was extracted with 3 × 50 ml ethylacetate. To each sample, 520 ng NPIC was added as an internal standard. Blanks runs (with water instead of sample) were performed throughout the experiments. The combined ethyl acetate layers were dried over Na_2SO_4 and concentrated in a rotary evaporator. BF_3-methanol was used as described by Sen et al. (1982) to produce the methylesters of NPRO and NPIC.

The analysis was performed with a gaschromatograph (GC)/Thermal Energy Analyser (TEA) combination, under the same conditions as described in van Broekhoven et al. (1984). Recovery experiments were performed and showed recoveries for NPRO and NPIC of 50-70 % (typically 70 % for feed, faeces and urine, and 50 % for rumen fluid). The results were corrected for the recovery of the internal standard (NPIC). No other peaks than these of NPIC and NPRO were found in the chromatogram.

The detection limit of the method is about 2-3 $\mu g\ kg^{-1}$ for feed, rumen fluid and urine, and about 6 $\mu g\ kg^{-1}$ for faeces.

Proline

Proline in the hay was extracted as described by Richter et al. (1975). For the analysis of proline in the rumen fluid, the ethyl alcohol was evaporated from the sample. The residue was filled up to 10 ml. Before analysis, 1 ml of the extracts was cleaned-up by passage over SEPPAK C-18.

After activating with 10 ml methanol, the cartridge was washed with two volumes of 0.1 % trifluoroacetic acid (TFA) in water and 10 ml 0.1 % TFA in water/methanol (80/20). 1 ml of the sample was mixed with 2 ml of 0.1 % TFA in water/metha-

nol (70/30) and passed through the cartridge. The eluent was collected totally and homogenized. The recovery for proline was better than 90 %. An aliquot of the cleaned-up sample was injected into an automated amino acid analysis system with post-column derivatization and fluorescence detection of the orthophtaalaldehyde/ mercaptopropionic-acid derivative after cleavage with hypochlorite (Waters Ass. Milford, Massachusetts, USA).

Other analyses
Nitrate and nitrite were analysed according to Vertregt (1977). Total nitrogen and dry matter were determined by standard methods.

Results and discussion

The experiments were performed with non-lactating cows fed with hay. The conclusions from the present experiments should be limited to those circumstances.

In Table 2 the results are given over the two feeding periods. The NPRO ingested was quantitatively excreted in the urine and faeces and no NPRO was formed endogenously. This is in agreement with the results obtained earlier (van Broekhoven et al., 1984).

In Figure 1 the changes in nitrate and nitrite content and in Figure 2 the changes of the proline content in the rumen fluid of cow 2 on the second day of the experiment are given for the experiments with low-nitrate and high-nitrate hay. No nitrate was measured in the rumen fluid during the experiment with low-nitrate hay. So, as to the endogenous formation of NPRO this experiment has no relevance.

After the feeding of the high-nitrate hay was stopped (8.45 h), the concentrations of nitrite and proline in the rumen fluid decreased rapidly due to microbial reduction of nitrite to ammonia, microbial degradation of proline and spill-over to the omasum and then the abomasum. With the assumption of a spill-over of 10 % of the rumen content per hour a maximum of 10 % of the nitrite and 20 % of the proline could enter the abomasum.

Figure 3 shows the content of NPRO in the rumen fluid of cow 2 on the second day of the experimental periods. Each point is the average of duplo.

Table 2. Average daily intake of feed and excretion of faeces and urine and the N-nitrosoproline (NPRO) content of feed, faeces and urine.

Exp.	Cow	Hay (kg day^{-1})	NPRO intake (μg day^{-1})	Faeces (kg day^{-1})	NPRO in faeces (μg day^{-1})	Urine (l day^{-1})	NPRO in urine (μg day^{-1})	Recovery
1	1	7.4	1243	14.2	706 (57)*	8.2	305 (24)*	(81)*
	2	7.8	1310	16.9	802 (61)	10.1	295 (23)	(84)
2	1	7.9	1321	79.4	6557 (50)	16.4	5336 (40)	(90)
	2	8.0	1338	49.5	7743 (58)	21.8	5690 (43)	(101)

* Figures in parentheses indicate % of intake.

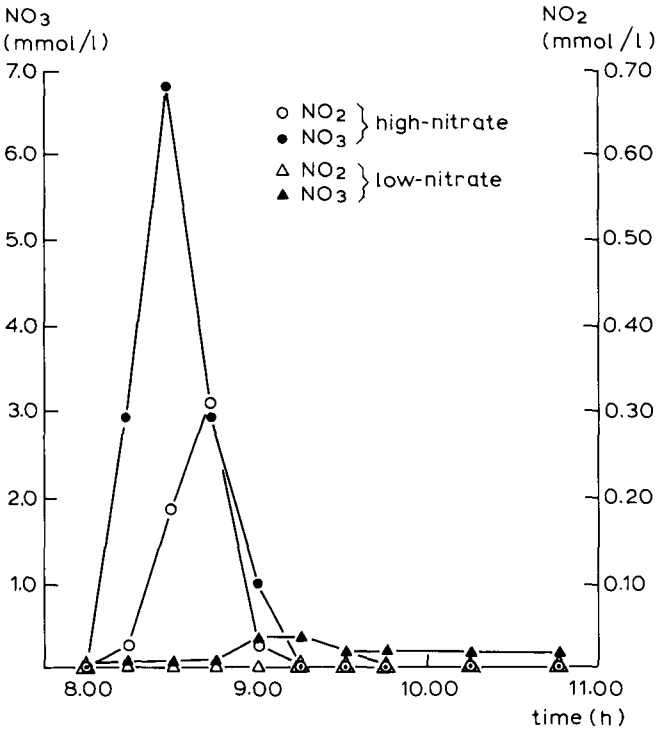

Fig. 1. Nitrite (open symbols) and nitrate (filled symbols) in the rumen fluid of cow 2.

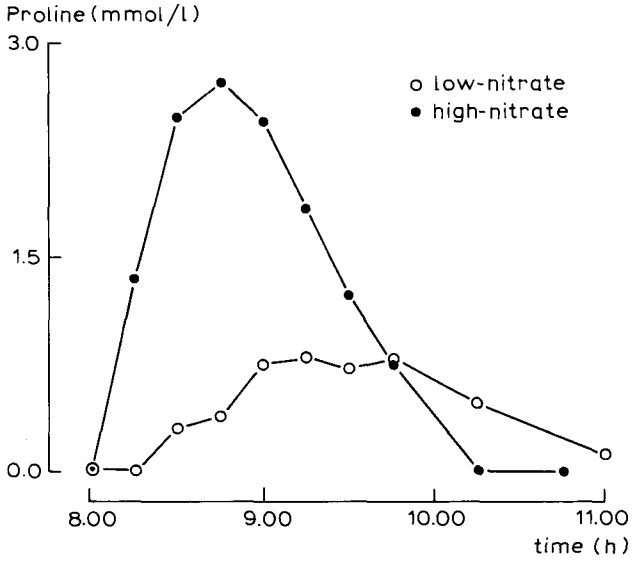

Fig. 2. Proline in the rumen fluid of cow 2 after feeding with high-nitrate (filled symbols) and low-nitrate (open symbols) hay.

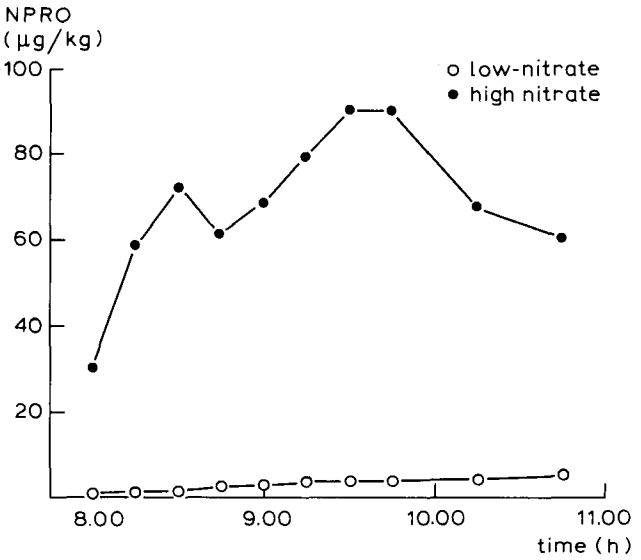

Fig. 3. N-nitrosoproline in the rumen fluid of cow 2 after feeding with high-nitrate (filled symbols) and low-nitrate (open symbols) hay.

Before feeding some NPRO was already present. On the basis of the duplo determination an SD was calculated (±20 µg). Because of the SD no conclusions could be drawn from the NPRO content, other than that no bacterial degradation was shown. The NPRO is probably lost from the rumen by spill-over to the omasum.

Although the results show that the greater part of nitrite and proline is metabolized in the rumen, still an important amount of these products could enter the abomasum. The pH of the abomasum is well-suited for the formation of NA (pH 3). So, the metabolism in the rumen of nitrite and proline explains only partly the fact, that no formation of NPRO could be found. Further investigations are needed to measure more precise the amounts of nitrite and proline that enter the abomasum and to reveal the factors that inhibit the formation of NA in the abomasum.

The results confirm the earlier findings that no formation of NA could be measured in the digestive tract of a row (van Broekhoven & Davies, 1981; van Broekhoven et al., 1984).

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