

## Effect of a high sulfur diet on rumen microbial activity and rumen thiamine status in sheep receiving a semi-synthetic, thiamine-free diet

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**Summary** — A semi-synthetic thiamine-free diet was used on weaned lambs to test the effect of a high sulfur level on the rumen, microbial activity and on the microbial production of thiamine. In vivo and in vitro kinetic studies, as well as the determination of the thiamine concentrations and thiaminase activity in the rumen, were performed during the 16 week experiment. A high sulfur level (0.6%) in the diet, in comparison with a normal sulfur level (0.2%), did not modify the microbial activity of the rumen with the exception of a slightly retarded decrease in the volatile fatty acid (VFA) rumen concentration. The rumen thiamine level and the thiaminase activity were not modified by the dietary sulfur level. In contrast, the rate of sulfate reduction into sulfide in the rumen increased progressively with the 0.6% sulfur diet. In conclusion, a high sulfur level (0.6%) in the diet of sheep did not modify the thiamine status of the rumen. It strongly increased the production of sulfides but an adaptation period of several weeks was required by the rumen microflora to reduce sulfate at a maximal rate.

**rumen / sulfur / thiamine / synthetic diet**

**Résumé** — Effet d'un régime excédentaire en soufre sur l'activité microbienne du rumen et sur le métabolisme ruminal de la thiamine chez le mouton recevant un régime semi-synthétique dépourvu de thiamine. Un régime semi-synthétique dépourvu de thiamine a été utilisé chez l'agneau sevré, pour étudier l'effet d'un apport excédentaire de soufre sur le métabolisme microbien et la production de thiamine dans le rumen. L'activité microbienne normale du rumen n'est pas modifiée par un régime à 0,6 % de soufre comparé à un régime normal à 0,2 %, à l'exception d'une très légère et tardive diminution de la concentration ruminale en AGV. Des cinétiques effectuées in vivo et in vitro ainsi que des déterminations de la teneur en thiamine et de l'activité thiaminasiq ue dans le rumen ont été effectuées pendant 16 semaines. La concentration en thiamine reste identique dans les deux régimes pendant toute la durée de l'expérience ; il n'y a pas non plus de modification de l'activité thia-

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*minasique. En conclusion un régime à 0,6 % de soufre chez le mouton n'a pas d'effet sur la concentration ruminale de thiamine d'origine microbienne. Il provoque une très forte augmentation de la production de sulfures mais celle-ci n'est maximale qu'après une période d'adaptation de la micropopulation du rumen.*

#### **rumen / soufre / thiamine / régime synthétique**

## **INTRODUCTION**

An adequate concentration of sulfur (about 0.2% of the dry matter (DM)) in the diet of ruminants is required to allow the rumen microflora to synthesize the sulfur amino acids for microbial proteins and sulfur-containing vitamins such as thiamine, at an optimal rate. In contrast, an excess of dietary sulfur (above 0.4% DM) may induce anti-nutritional effects which could decrease the animal's performance and lead to clinical signs of intoxication and eventually death. This aspect has been reviewed by Kandylis (1984). The consequences of a high sulfur diet depend not only on the dietary level of sulfur but also on the type of sulfur compound, on its mode of administration and on the composition of the ration. A sulfur dietary concentration above 0.4% usually decreases both feed intake and animal performance. The effects of an excess of sulfur on the rumen microbial digestion are poorly documented.

The acute toxicity of sulfur is mostly related to a high sulfide production due to the microbial reduction of sulfate in the rumen. It consists of severe diarrhea, respiratory distress and nervous symptoms with muscular tremors, a staggering gait and, finally, coma. More recently, brain lesions identical to those observed in classical thiamine-related cerebrocortical necrosis or polioencephalomalacia (CCN) have been described in ruminants receiving high sulfur diets. Numerous cases of CCN have been identified by veterinarians in France, in beef cattle fattened with sugar beet pulps enriched in aluminium sulfate. Several cases of CCN were observed in the USA in 6–18-

month-old cattle with high sulfate diets (Raisbeck, 1982). In Canada, field studies reported outbreaks of CCN in cattle consuming water with elevated sodium sulfate levels (Harries, 1987; Gooneratne et al, 1989a; Hamlen et al, 1993). Moreover, CCN has been experimentally induced in sheep fed a 0.63% sulfur diet (Gooneratne et al, 1989b; Rousseaux et al, 1991). Experimentally, an intraruminal infusion of sodium sulfide in the rumen can induce brain lesions similar to the classical lesions of CCN within some hours (McAllister et al, 1992). A possible relationship between a high dietary intake of sulfur and the animal's thiamine status have been considered, as the pathogenesis of classical thiamine-dependent CCN, first described in 1961 (Terlecki and Markson, 1961), is not completely understood. Some field studies intended to establish a relation between a high intake of sulfur and a lowering of the blood thiamine content (Olkowski et al, 1991). Further experimental investigations did not show a relation between high sulfur diets and disturbance of the thiamine status (Rousseaux et al, 1991; Olkowski, 1992). However, the diets used in these experiments either had a normal thiamine level or were enriched with large amounts of this vitamin. In other respects, investigations on the effect of high sulfur diets on the microbial metabolism of thiamine in the rumen are limited. In experiments in vitro a low nitrogen/sulfur ratio (Bick et al, 1978) or an addition of sulfate (Olkowski et al, 1993) increases the thiamine-destroying activity of rumen juice or total rumen content cultures.

The aim of the present work was therefore to investigate the effect of a high sulfur diet

on the rumen microbial activity and on its thiamine status in weaned lambs. A semi-synthetic thiamine-free diet was used in order to avoid interference by dietary thiamine.

## MATERIALS AND METHODS

### *Animals and experimental design*

Seven female Texel lambs averaging 30 kg were used in this experiment. Before the experiment they were fed with a classical diet composed of hay and concentrate. After a serological control for brucellosis and an antiparasitic treatment, they were equipped with a permanent rumen canulae. They were adapted over a two-month period to a semi-synthetic diet, whose composition was identical to the normal sulfur diet (NS) used during the experiment, with the exception of the presence of thiamine (2 mg/kg). They were then divided into two groups: a control group (three animals) received the semi-synthetic thiamine-free diet throughout the experiment (16 weeks) with a normal (0.2% sulfur content NS) (table I); and an experimental group (four animals) received a semi-synthetic thiamine free diet with 0.4% sulfur (50% NS + 50% HS) for 4 weeks, and then the HS diet (0.6% sulfur) (table I) for the next 12 weeks.

All animals received their respective experimental diets one week before the beginning of the experiment.

### *Kinetic studies in the rumen*

To compare the effect of the dietary level of sulfur on the rumen microbial activity, the postprandial kinetics of rumen pH, volatile fatty acids (VFA), ammonia (NH<sub>3</sub>), lactate, sulfate and sulfide concentrations were measured once a week in each animal during weeks 2, 4, 6 and 8, to test the eventual adaptation of the rumen micropopulation to the semi-synthetic diet. To allow an acute comparison of the kinetics between both groups, the morning meal was not distributed on the day of the test. As a substitute, 300 g of NS or HS diet were mixed with 1 300 mL artificial saliva preheated at 39 °C and were rapidly introduced into the rumen through the canulae. Hourly samplings of rumen content were undertaken for 7 h after the introduction of the artificial meal.

### *Microbial metabolism of thiamine in the rumen*

In a preliminary assay, no consistent postprandial variation of the rumen thiamine concentration was observed. Subsequently this parameter was determined 5 h after the morning meal, for five consecutive days for each animal at weeks 2, 4, 6, 9 and 16 of the experiment. Rumen samples were collected after withdrawal and homogenization of the total rumen content. This was subsequently reintroduced into the rumen.

**Table I.** Composition of the semi-synthetic thiamine-free diets.

<i>Component (%)</i>	<i>Normal sulfur diet (NS)<sup>a</sup> (0.2% sulfur)</i>	<i>High sulfur diet (HS)<sup>a</sup> (0.6% sulfur)</i>
Corn starch	29	29
Cerelose	24.9	24.9
Cellulose	33	33
Corn oil	3	3
Sodium bicarbonate	1	1
Choline chloride, vitamins A, D <sub>3</sub> , E	0.1	0.1
Urea	4	3.45
Complete mineral mixture	5	5.55

<sup>a</sup> To maintain the same level of N and Na in NS and HS, 0.2% sulfur was added as sodium sulfate in both diets, and 0.4% sulfur was added as ammonium sulfate in the HS diet with a corresponding decrease in N urea.

In vitro incubations at week 8 of the experiment were carried out according to the method of Jouany and Thivend (1986). An inoculum composed of 100 mL of undiluted rumen juice and 100 mL of filtered rumen juice taken from sheep receiving either the 0.2% S diet (NS) or the 0.6% diet (HS) was diluted with artificial saliva up to a volume of 400 mL. Soluble starch, cerelese, cellulose and ammonium chloride were added. Two amounts of sulfate were added: normal (200 mg/L) and high (600 mg/L). The pH variations, VFA and fermented organic matter (FOM) production, gas production, ammonia and sulfate utilization were measured at  $t = 0, 2, 4$  and 6 h. FOM was calculated with the Demeyer and Van Nevel formula (1979). Each incubation was repeated six times.

### **Analytical procedures**

The rumen juice and in vitro incubation fluids were analysed for VFA by gas chromatography (Jouany, 1982), for  $\text{NH}_3$  according to the Weatherburn method (1967), for sulfate and sulfide with the methods of Sorbö (1987) and Cline (1969), respectively, and for lactic acid according to the enzymatic procedure of Gutmann and Wahlefeld (1974).

The total thiamine content of the rumen juice was determined according to the thiochrome method of Strohecker and Henning (1963) after the hydrolysis of the phosphorylated compounds and purification on a ion-exchange column Amberlite CG 50. The potential and actual thiaminase activity was determined according to the method of Thomas (1986) using pyridine as a co-substrate to measure the potential thiaminase I activity.

### **Statistical analysis**

Statistical analysis was carried out using the StatView® program. Repeated measures analysis of variance was used to identify the effects of sulfur level, duration of the experiment and interaction between these factors in the in vivo experiments. The same statistical treatment was used to identify the effect of the origin of inoculum and the level of sulfate in in vitro incubations. The correlation between the potential thiaminase activity and the level of thiamine in the rumen was determined.

## **RESULTS**

### ***In vivo experiment***

#### **Growth, ingestion and pathological status of animals**

The mean weights of animals at the end of the experiment were  $28.55 \pm 1.90$  kg and  $28.24 \pm 1.54$  kg in the NS and the HS groups, respectively. Total gain weight was 0.55 kg in the NS group and negative ( $-0.64$  kg) in the HS group. Both groups lost weight during the first 6 weeks of the experiment because of the poor efficiency of the protein-free purified diet and its poor palatability. Normal growth began on week 11.

Average daily food intakes were respectively  $736 \pm 72$  g and  $701 \pm 54$  g in the NS and HS groups. The two groups were not significantly different.

Predominantly nervous symptoms, consisting of head pressing, blindness, hyper-salivation, polypnea, uncoordination and depression, were observed on week 7 during an approximately 10 h period for one lamb of the HS group after the introduction of the artificial meal into the rumen for kinetic measures. The peak rumen concentration of sulfide measured at this time (80 mg/L) was the highest observed in the animals in the experiment. Gross pathology and histopathology subsequently demonstrated symmetrical necrotic lesions of the grey matter on the anterior cortex.

#### **Kinetic studies in the rumen**

##### ***Effect of dietary sulfur level on microbial activity of the rumen***

Figure 1 indicates the mean kinetics of pH, VFA,  $\text{NH}_3$  and lactate determined on weeks 2, 4, 6 and 8, in the NS group, and on week 4 and 8 in the HS group. Statistical analysis of the NS group showed no significant dif-

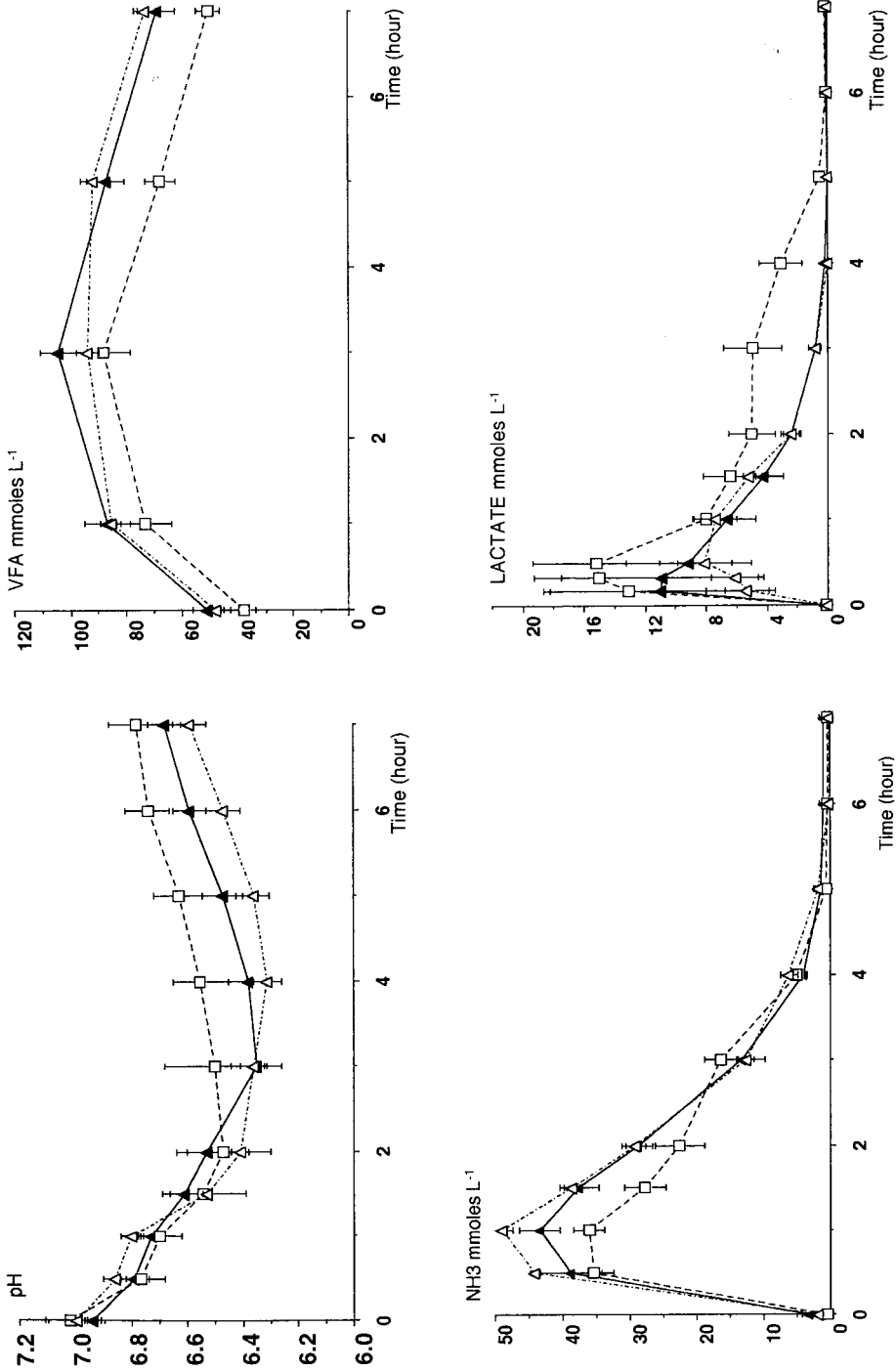


Fig 1. Effect of sulfur level on rumen time curves of pH, VFA, ammonia and lactic acid after an artificial meal daily introduced into the rumen (mean  $\pm$  SEM): — $\blacktriangle$ — control group 0.2% (weeks 2, 4, 6, 8,  $n = 3$ ); - $\square$ - experimental group 0.4% S (week 4,  $n = 4$ ); ---- $\Delta$ ---- experimental group 0.6% S (week 8,  $n = 4$ ).

ference between weeks for all parameters, indicating that the rumen microbial activity was, for this diet, stable throughout the experiment. Consequently, the use of this diet was justified as a basis of comparison for the investigation of the effects of the high sulfur level. No significant difference was observed between the different periods of HS (0.4 vs 0.6% S) or between HS and NS diets for pH and ammonia. A non-significant difference was noted for lactic acid at week 4 (0.4% S) in comparison with week 8 (0.6% S) and the control group NS. VFA kinetics in the HS group were significantly different ( $P = 0.01$ ) from the NS group but only during week 8.

#### *Effect of dietary sulfur level on sulfate uptake and sulfide formation*

The kinetics of the sulfate and sulfide concentrations throughout the experiment in the NS group and for weeks 4 and 8 in the HS group are indicated in figure 2. As sulfate was rapidly converted into sulfide, a peak of sulfide appeared between the first and the third hour of the kinetic. No significant mod-

ification of the sulfate and sulfide kinetics were observed between weeks 2 and 8 of the experiment in the NS group. In contrast the slopes of the sulfate utilization curves in the HS group increased between weeks 2 and 8 and a corresponding increase in the production of sulfide was observed ( $P < 0.001$ ). A noteworthy increase in sulfide production was observed between weeks 6 and 8 in the HS group ( $P < 0.001$ ). The peak of sulfide was about three times higher at week 8 than at week 6 despite the fact that the same concentration of sulfur (0.6%) was present in the diet during those periods.

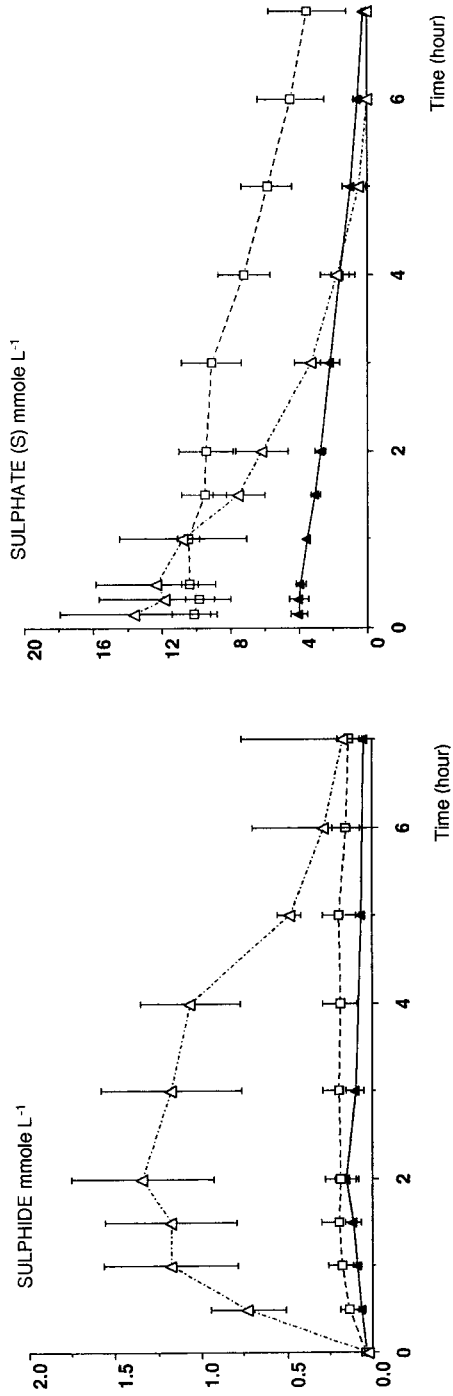
#### **Thiamine status in the rumen**

The thiamine concentrations in the rumen content are indicated in table II. No significant evolution was observed during the 16 weeks of the experiment for both groups. No significant differences were found between the HS and NS groups. The actual thiaminase activity remained very low ( $< 0.01$  mU/mL/min) in all the samples. Large variations in the potential thiaminase I

**Table II.** Effect of the sulfur level on the rumen thiamine concentration of lambs receiving a thiamine-free semi-synthetic diet (mean  $\pm$  SEM) <sup>a</sup>.

Time (weeks)	2	4	6	9	16
<i>Control group * (0.2% sulfur)</i>					
B1 ( $\mu$ g/g total rumen content)	0.60 $\pm$ 0.16	0.71 $\pm$ 0.24	0.69 $\pm$ 0.02	0.73 $\pm$ 0.06	0.86 $\pm$ 0.18
B1 ( $\mu$ g/g DM)	6.62 $\pm$ 2.42	6.86 $\pm$ 3.04	6.22 $\pm$ 0.11	7.13 $\pm$ 0.62	9.65 $\pm$ 2.16
<i>Experimental group **</i>					
B1 ( $\mu$ g/g total rumen content)					
0.4% sulfur	0.76 $\pm$ 0.05	0.63 $\pm$ 0.07			
0.6% sulfur			0.62 $\pm$ 0.08	0.55 $\pm$ 0.02	0.84 $\pm$ 0.25
B1 ( $\mu$ g/g DM)					
0.4% sulfur	7.90 $\pm$ 0.59	5.35 $\pm$ 0.46			
0.6% sulfur			6.38 $\pm$ 1.16	5.80 $\pm$ 0.76	7.19 $\pm$ 1.48

\* Three animals x 4 days; \*\* four animals x 4 days; <sup>a</sup> no significant differences between weeks and groups.



**Fig 2.** Effect of sulfur level on rumen sulfide and sulfate time curves after an artificial meal daily introduced (mean  $\pm$  SEM): —▲— control group 0.2% S (weeks 2, 4, 6, 8,  $n = 3$ ); -◻- experimental group 0.4% S (week 4,  $n = 4$ ); ---◻--- experimental group 0.6% S (week 8,  $n = 4$ ).

**Table III.** Effect of sulfur level on thiaminase activity in the rumen content of lambs receiving a thiamine-free semi-synthetic diet ( $10^{-4}$   $\mu\text{mol}$  of thiamine decomposed  $\text{mL}^{-1} \text{min}^{-1}$ ) (means  $\pm$  SEM) <sup>a</sup>.

<i>Time (weeks)</i>	<i>Control group (n = 3)</i>	<i>Experimental group (n = 4)</i>
1	0.88 $\pm$ 0.75	11.65 $\pm$ 8.37
2	1.05 $\pm$ 0.25	6.75 $\pm$ 3.99
3	1.64 $\pm$ 0.89	9.76 $\pm$ 5.56
5	1.56 $\pm$ 1.12	0.19 $\pm$ 0.09
7	4.94 $\pm$ 4.64	0.92 $\pm$ 0.41
8	0.47 $\pm$ 0.47	1.48 $\pm$ 1.14
10	9.95 $\pm$ 7.70	1.63 $\pm$ 0.70
16	9.14 $\pm$ 3.86	2.46 $\pm$ 1.82

<sup>a</sup> No significant difference between groups.

activity were recorded throughout the experiment (table III) but no significant difference was found between the NS and HS groups. No negative correlation was found between the potential thiaminase I activity and the thiamine concentration in the rumen ( $r = 0.078$ ).

### ***In vitro incubations***

#### **Effect of inoculum origin and sulfate concentration on in vitro microbial activity**

The kinetics of the pH variations, VFA, FOM, gas production and  $\text{NH}_3$  utilization in the NS and HS fermentors with an initial sulfur concentrations of 200 mg/L are indicated in figure 3. The fermentations were not modified by the origin of the inoculum (NS or HS donor sheep). In other respects, these kinetics were not modified by a 600 mg/L sulfur concentration.

#### **Effect of inoculum origin and sulfate concentration on in vitro sulfate utilization**

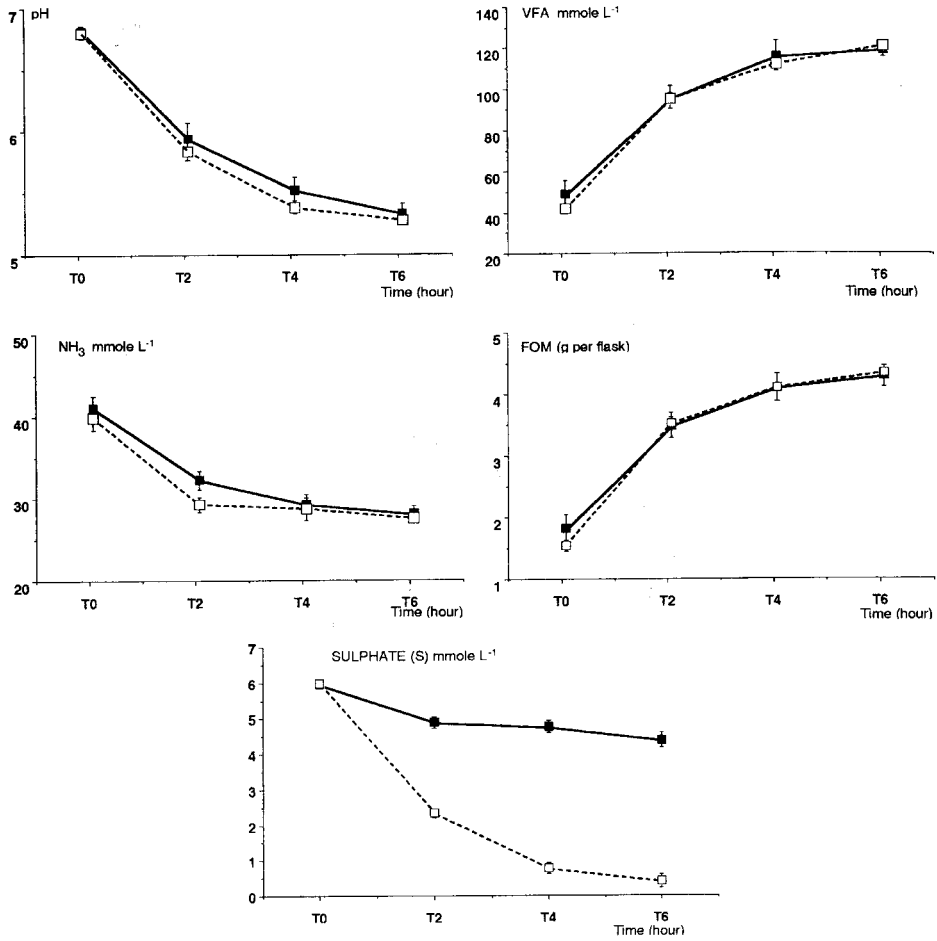
The utilization of sulfate in the flasks containing 200 mg/L sulfur was considerably

enhanced (x 3.5) in the HS donor flasks in comparison with the NS one (fig 3). With the 600 mg/L sulfur concentration, the rate of sulfate utilization was five times higher for the HS donor flasks than the NS. The origin of the inoculum, therefore, had a very strong effect on the utilization of sulfate; inoculum issued from HS sheep adapted to the high sulfur diet utilized sulfate more rapidly than inoculum issued from NS sheep receiving a normal amount of sulfur in their diet. Increasing the concentration of sulfur in the flasks also increased the rate of sulfate uptake.

## **DISCUSSION**

While the toxicological effects of high sulfur diets on animals are on the whole well known, data related to the particular effect of these diets on the rumen microbial digestion are very limited. Kennedy et al (1971) used an in vitro technique with a washed cell suspension and found no deleterious effect on starch digestion with sulfur concentrations as high as 11 mg/mL. Using the same technique, Hubbert et al (1958) observed only a slight effect on cellulose digestion with a sulfur concentration of





**Fig 3.** Effect of inoculum and sulfate concentration on kinetics of pH, NH<sub>3</sub>, VFA, FOM and sulfate in vitro with a synthetic substrate (starch, cellose, ammonium chloride). —■—: inoculum NS, 0.2 g L<sup>-1</sup> S; - -□- -: inoculum HS, 0.2 g L<sup>-1</sup> S.

1 mg/mL and a marked inhibition with 2 mg/mL. No modification in the digestibility of the organic matter, crude protein, ether extract or crude fiber were noted by Johnson et al (1968) in lambs fed a 0.5% sulfur diet. In steers receiving an all-concentrate finishing diet, Rumsey (1978) found non-consistent modification of the ruminal pH, ammonia, VFA and lactic acid concentration with dietary levels of sulfur up to 0.98%.

Our in vivo and in vitro experimental findings are in agreement with these data. In vivo, a 0.6% sulfur diet had roughly no effect on the rumen metabolism. Moreover, our results indicated clearly that a high dietary sulfur level resulted in a progressive adaptation in the rumen microflora's ability to reduce sulfate into sulfide at a faster rate. The resulting increase in sulfide concentration might have been responsible for the

small decrease in the VFA concentrations observed in the HS group on week 8. However, it was not possible to determine whether this was related to a direct inhibiting effect of sulfide on the overall microbial activity or to an increase in the uptake of energetic substrates by the sulfate-reducing bacteria.

In another respect, this progressive adaptation towards reducing sulfate into sulfide could explain the two to three month delay before the onset of nervous symptoms and lesions similar to classical CCN that were observed with high sulfur diets (Gooneratne et al, 1989a). Direct administration of sodium sulfide is capable of inducing the same pathological events within some hours (McAllister et al, 1992). It might also explain why in our experiment, only the sheep that exhibited the greatest efficiency in reducing sulfate into sulfide and consequently had the highest rumen concentration of sulfide, presented the nervous symptoms and lesions similar to CCN.

With natural diets containing various amounts of thiamine, the concentration of thiamine in the rumen content cannot be used as a reliable indicator of microbial thiamine synthesis. In fact, when the dietary amount of thiamine is low, the ruminal synthesis is very active. In contrast, when there is a high input of dietary thiamine, the net microbial synthesis in the rumen (microbial synthesis minus microbial degradation) can be negative (Breves et al, 1981; Miller et al, 1986). These variations are not reflected in the concentration of thiamine in the rumen as there is no differentiation between thiamine from either origin. The use of a semi-synthetic thiamine-free diet eliminated this problem as all the thiamine found in the rumen was of microbial origin. As thiamine is not absorbed through the rumen wall (Höller et al, 1977; Steinberg et al, 1977), its concentration in the rumen can be considered to give good indication of the amount synthesized by the microbes. The thiamine

concentration has often been measured in the rumen. Different authors using different kinds of diets have found average thiamine concentrations within a range of 0.10 to 1.9 mg/g of rumen content. The rumen concentrations found in our experimental animals are in agreement with these data, but if compared with values observed by El Hindi (1977) on sheep fed a similar protein-free, thiamine-free diet, they are roughly three times higher. This apparent discrepancy could be partly explained by methodological differences. In fact, almost all the thiamine is localized in microorganisms. Most of the bacteria are attached to dietary particles. Therefore determination of the thiamine concentration from rumen juice containing only a part of the coarse feed material must give lower results than determinations done on the whole rumen content.

Our results clearly indicated that the microbial synthesis of thiamine remained effective with the semi-synthetic diet during the entire experimental period, either with a normal or a high sulfur level. The actual thiaminolytic activity in the rumen juice remained very low with both levels of sulfur despite that, theoretically, sulfite as an intermediate in the reduction of sulfate into sulfide can readily cleave thiamine. In fact, Bray (1969) indicated that sulfite is a very transient intermediary which does not accumulate in the rumen. Considering the data of Bray as well as the negligible thiaminolytic activity we found in the rumen juice it is unlikely that a high sulfur diet increased the sulfite concentration in the rumen. On the other hand, high values of potential thiaminase-1 activity recorded at weeks 10 and 16 in the control group or at weeks 1, 2 and 3 in the experimental group indicate that a high destruction of thiamine would have occurred if an adequate co-substrate had been present. However, in the absence of a sufficient amount of co-substrate in the rumen juice, no corresponding decrease in thiamine

was noted. In addition, the variations of thiaminase I activity in our experiment were not in relation to the sulfur level. These findings can be compared with the data of Thomas et al (1990) who demonstrated high potential thiaminase I activity in healthy as well as in CCN sheep.

On the whole, our results are in agreement with those of Bick et al (1978) and Olkowski et al (1993). These authors found that high levels of sulfur did not impair thiamine synthesis *in vitro* but slightly increased the level of thiamine destruction.

In our experiment, the net *in vivo* production of thiamine was not measured. It is possible that the rate of thiamine synthesis was high enough to maintain a normal rumen level even with a moderate increase in the thiamine destruction.

In conclusion, a large amount of sulfur in the diet, corresponding roughly to three times the normal level, had little negative effect on the rumen microbial activity and no effect on the level of thiamine in the rumen. It is unlikely that such an amount of sulfur could induce a thiamine deficiency related to a lack of thiamine microbial production in the rumen. In addition, it must be emphasized that the rumen ecosystem seemed much more resistant to a high sulfide levels than the other tissues of the host animal.

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