Inhibitory effects of sulphur compounds, copper and tungsten on nitrate reduction by mixed rumen micro-organisms

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1. The inhibitory effects of inorganic and organic sulphur-containing compounds, copper and tungsten on nitrate reduction by mixed rumen micro-organisms were investigated in two in vitro studies.

2. Coarsely strained rumen fluid from nitrate-adapted (Expt 1) or non-adapted (Expt 2) Suffolk Down wethers maintained on lucerne (*Medicago sativa*) cubes was used as an inoculum. In Expt 1, anaerobic incubation was carried out for 24 h for each medium supplemented with 10 mM-sodium nitrate and the following chemicals: 0, 1, 2, 3, 5, 8 and 10 mM-sodium sulphide, 1 and 10 mM-sodium sulphite, 1 and 10 mM-sodium sulphate, 1 and 10 mM-L-cysteine, 1 and 10 mM-DL-methionine, 1 mM-sodium tungstate and 1 mM-copper sulphate. In Expt 2, 1 and 10 mM-Na_2S, 1 and 10 mM-L-cysteine, 1 mM-Na_2WO_4, and 1 mM-$CuSO_4$ were added to incubation media to test for chemical inhibition of microbial reduction of nitrate.

3. In Expt 1, the amount of nitrite formed decreased with increasing concentration of sulphide-S added. The additions of L-cysteine, W and Cu suppressed nitrite formation in media from both nitrate-adapted and non-adapted sheep.

4. In contrast to the effects of sulphide, L-cysteine and W counteracted, to some degree, nitrate-induced reduction of volatile fatty acid (VFA) production. Addition of Cu to the media resulted in a further depression of VFA production.

Enzymic reduction of nitrate is classified into two types depending on the metabolic function (Payne, 1973). In nitrate-respirating and denitrifying species of bacteria, classified into genera such as *Escherichia*, *Proteus*, *Paracoccus* and *Pseudomonas*, the reducing pathways of nitrate have been demonstrated as being dissimilar. Rumen bacteria, in contrast, are assumed to reduce nitrate in an assimilative manner, as do plant cells and fungi (Prins *et al.* 1980). In any event nitrate must be reduced primarily to nitrite, and the nitrate reductase (nitrate reductase (NADPH); *EC* 1.6.6.3) involved in the reaction step is known to be a molybdo-enzyme (Nason & Evans, 1953; Hewitt, 1975). The reducing step is of primary importance to the incidence of nitrate–nitrite poisoning in ruminant animals. The nitrate reduction in rumen bacteria has been reported to be inhibited by tungstate and promoted by molybdate (Tillman *et al.* 1965; Korzeniowski *et al.* 1980, 1981; Prins *et al.* 1980; Marais *et al.* 1988), as is nitrate reductase derived from other ecosystems (Seki-Chiba & Ishimoto, 1977; Oltmann *et al.* 1979; Burke *et al.* 1980; Aslam, 1982; Shen *et al.* 1982; Tachiki & Nason, 1983; Deaton *et al.* 1984; Saracino *et al.* 1986). In addition, it has been proposed that an inorganic combination of molybdenum and copper or sulphur, or Mo + Cu + S, results in the formation of an insoluble complex salt (Grace & Suttle, 1979; Ammerman & Goodrich, 1983) and it is, therefore, possible that Cu and S may inhibit nitrate reductase. Cu is an essential trace element for ruminant nutrition, but its maximum requirement is extremely small (National Research Council, 1988). Both Cu and W may be of minor importance for practical applications as inhibitors against nitrate–nitrite poisoning in ruminants due to their toxic properties. However, it seems that Cu, W and S can be useful elements in finding clues to the possible participation of Mo in the activation of nitrate reductase in rumen microbial populations. In establishing a natural prophylactic for nitrate hazards in animal production, application of S-containing compounds is

valuable, because S is a major element present in several essential amino acids and in the biologically active tripeptide, glutathione, which plays an important part in non-enzymic reduction of erythrocyte methaemoglobin (Takahashi, 1983).

The present paper deals with the inhibitory effects of S compounds, Cu and W on in vitro nitrate reduction by rumen micro-organisms.

MATERIALS AND METHODS

Experimental procedures

Eight rumen-fistulated Suffolk Down wethers, maintained on lucerne (*Medicago sativa*) cubes (50 g dry matter/kg body-weight$^{0.75}$ per d) were randomly allocated in equal numbers to two in vitro experiments. To obtain rumen fluid containing nitrate-adapted microbial populations, each animal was given 0·55 g sodium nitrate/kg body-weight$^{0.75}$ twice daily for 35 d via a fistula. Rumen fluid from each nitrate-adapted (for Expt 1) or non-adapted (for Expt 2) sheep was pooled and strained through four layers of muslin cloth. Strained fluid (1 vol.) was then added to 4 vol. preheated (38°), buffered mineral salts solution (McDougall, 1948) containing 10 mM-$NaNO_3$, 10 mM-glucose and 40 mM-lactate, with or without various amounts of the potential chemical inhibitors under investigation. The suspensions were mixed under an atmosphere of carbon dioxide, and 50 ml portions were added to autoclaved incubation vessels stoppered with one-way Bunsen gas-release valves to maintain anaerobic conditions. Five vessels per treatment were assigned randomly and placed in a water-bath at 38°. The incubation was carried out anaerobically at 38° for 24 h.

Expt 1. To test the inhibitory effects of inorganic and organic S compounds, W and Cu on nitrate reduction by the rumen microbial populations adapted to nitrate, the following compounds were put into separate incubation vessels: sulphide (1, 2, 3, 5, 8 and 10 mM-$Na_2S.9H_2O$), sulphite (1 and 10 mM-Na_2SO_3), sulphate (1 and 10 mM-Na_2SO_4), L-cysteine (1 and 10 mM), DL-methionine (1 and 10 mM), tungstate (1 mM-$Na_2WO_4.2H_2O$) or Cu salt (1 mM-$CuSO_4.5H_2O$). After 24 h incubation, portions of culture media were collected for nitrite determination.

Expt 2. To test direct effects of the inhibitors on nitrate reduction in non-adapted microbial populations, the anaerobic incubation was carried out for 24 h with or without administration of sulphide (1 and 10 mM-$Na_2S.9H_2O$), L-cysteine (1 and 10 mM), tungstate (1 mM-$Na_2WO_4.2H_2O$) or copper salt (1 mM-$CuSO_4.5H_2O$). After 24 h incubation, nitrite in each incubation medium was determined as described in Expt 1.

Analytical procedures

According to the method described by Prins *et al.* (1980), 1 ml of each culture medium was deproteinized and diluted by the addition of 3 ml lead acetate (50 g/l) and 1 ml of a saturated $Na_3PO_4.12H_2O$ solution. Nitrite contents were determined colorimetrically by the diazo-coupling method (Horwitz, 1975). The concentrations of volatile fatty acids (VFA) in the media were determined using a gas–liquid chromatograph (GC-3BF; Shimazu) equipped with a flame-ionization detector. The sample mixture was mixed well and centrifuged at 8000 ***g*** for 15 min. After centrifugation, 2 ml 50 mM-crotonic acid was added as internal standard (Stanier & Davies, 1981) to 2 ml of the supernatant fraction followed by acidification with 0·05 ml 6 M-sulphuric acid. The mixture was injected into a glass column (1·7 m × 3·0 mm) packed with 10% polyethylene glycol (molecular weight 6000) coated on TPA (30–60 mesh). The column was operated at 145° with high purity nitrogen as the carrier gas. The values were processed automatically using a Chromatopac data processing system (C–R 3A; Shimazu).

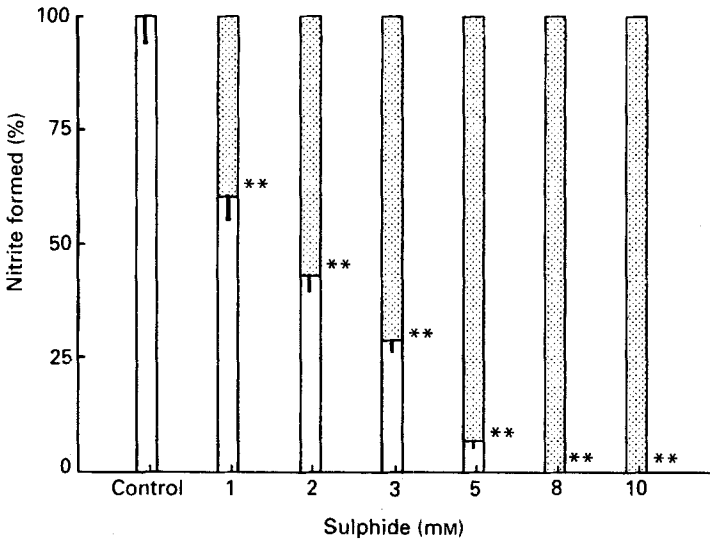

Fig. 1. Effect of sulphide-sulphur on nitrite formation in rumen fluid containing microbial populations adapted to nitrate. Values are means for five determinations with their standard errors represented by vertical bars. (□), Relative amount of nitrite formed (control value indicates 103·20 (SE 5·76) μg nitrogen/ml); (▨), relative amount of nitrite suppressed; ** $P < 0.01$.

Statistical analysis

The statistical significance of the difference between the means of control and treatments was assessed by analysis of variance.

RESULTS

Effects of S compounds, Cu and W on nitrate reduction in vitro

Expt 1. Fig. 1 shows the effect of sulphide-S on nitrite formation in rumen fluid containing microbial populations adapted to nitrate. A marked inhibition of nitrite formation was observed in rumen microbial populations adapted to nitrate as increasing amounts of sulphide-S were added. The addition of 5 mM-sulphide decreased nitrite formation by up to 90%. The addition of more than 8 mM-sulphide completely suppressed nitrite formation by rumen microbes in vitro.

Fig. 2 shows the degree of suppression of nitrite formation from nitrate attributable to inorganic and organic S, and the transition metals tested. Neither sulphate-S nor sulphite-S affected nitrite formation. Of the S-containing amino acids, DL-methionine proved to be inefficient in inhibiting microbial reduction of nitrate. The addition of L-cysteine to the medium, however, significantly ($P < 0.01$) lowered nitrite formation, with an evident dose-dependent effect. The extent of the depression of nitrite formation was 50% at 1 mM-L-cysteine ($P < 0.01$) and 100% at 10 mM-L-cysteine ($P < 0.01$). The metals W and Cu both significantly ($P < 0.01$) reduced nitrite formation after 24 h incubation.

Expt 2. Fig. 3 shows inhibitory effects of sulphide, L-cysteine, tungstate and Cu salts on nitrite formation by rumen microbial populations not adapted to nitrate. All chemicals added, except tungstate, showed approximately the same magnitude of inhibition of nitrite formation for both nitrate-adapted (Expt 1) and non-adapted organisms (Expt 2).

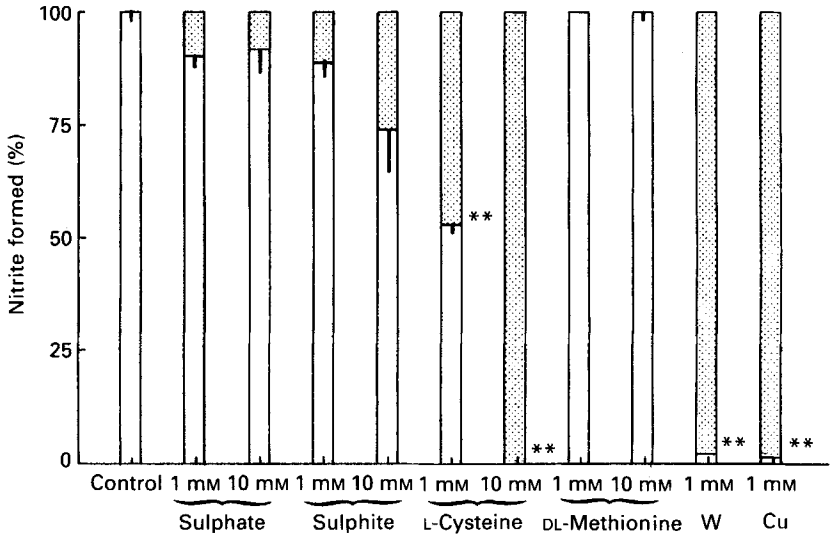

Fig. 2. Effects of inorganic and organic sulphur, tungsten and copper on nitrite formation in rumen fluid containing microbial populations adapted to nitrate. Values are means for five determinations with their standard errors represented by vertical bars. (□), Relative amount of nitrite formed (control value indicates 106·00 (SE 2·12) μg nitrogen/ml); (▨), relative amount of nitrite suppressed; ** $P < 0.01$.

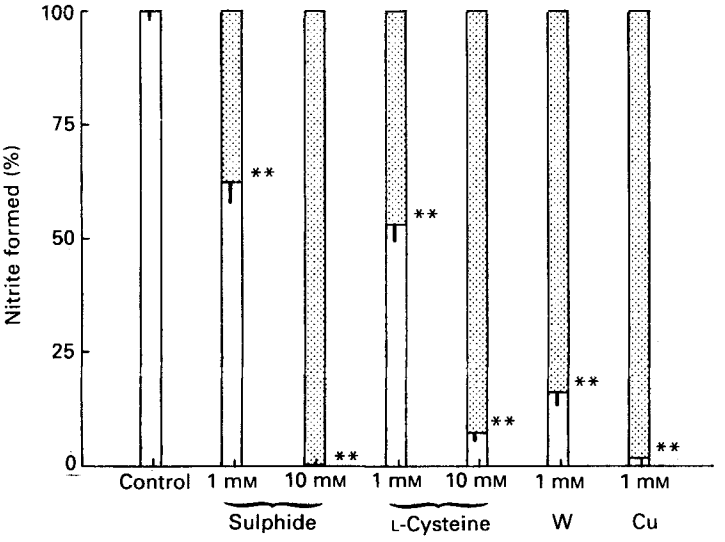

Fig. 3. Effects of sulphide, L-cysteine, tungsten and copper on nitrite formation by rumen microbial populations not adapted to nitrate. Values are means for five determinations with their standard errors represented by vertical bars. (□), Relative amount of nitrite formed (control value indicates 69·81 (SE 1·59) μg nitrogen/ml); (▨), relative amount of nitrite suppressed; ** $P < 0.01$.

Effects of S compounds, Cu and W on VFA production in the presence of nitrate

The effects of S compounds, W and Cu on VFA production by nitrate-adapted (Expt 1) and non-adapted (Expt 2) organisms in the presence of nitrate are summarized in Table 1. Results show that sulphide, L-cysteine and W can be efficient in suppressing nitrite

Table 1. *Effects of sulphur compounds, copper and tungsten on volatile fatty acid production by nitrate-adapted or non-adapted rumen microbial populations in the presence of nitrate (10 mM)*

(Values are expressed as means with their standard errors for five determinations)

	Volatile fatty acids (mM)															
	Nitrate-adapted microbial populations (Expt 1)								Non-adapted microbial populations (Expt 2)							
	Acetic		Propionic		Butyric		Total		Acetic		Propionic		Butyric		Total	
Inhibitors (mM)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
None*	—		—		—		—		20·73	0·42	7·87	0·21	3·26	0·09	31·86	0·60
None	13·63	0·41	2·96	0·30	0·37	0·26	16·96	0·79	12·87	0·23	3·94	0·63	1·88	0·42	18·68	1·28
Sulphide																
1	19·46	0·61	3·78	0·14	1·48	0·10	24·72	0·82	14·51	0·85	4·47	0·20	1·89	0·10	20·87	0·98
2	21·93	0·39	4·51	0·63	2·16	0·15	28·61	0·99	—		—		—		—	
3	21·95	0·56	4·21	0·21	2·69	0·15	28·85	0·82	—		—		—		—	
5	19·19	0·58	4·35	0·31	3·82	0·39	27·36	1·10	—		—		—		—	
8	15·15	0·06	3·67	0·13	3·43	0·17	22·25	0·23	—		—		—		—	
10	13·99	0·50	3·53	0·36	3·55	0·37	21·07	0·90	9·65	0·40	3·01	0·18	2·72	0·13	15·38	0·58
Sulphite																
1	9·93	0·53	2·15	0·13	0·63	0·03	12·27	0·53	—		—		—		—	
10	12·60	0·42	2·44	0·08	1·01	0·07	16·12	0·51	—		—		—		—	
Sulphate																
1	11·10	0·93	3·57	0·26	1·15	0·11	15·82	1·27	—		—		—		—	
10	8·09	0·57	2·14	0·18	0·56	0·06	10·79	0·80	—		—		—		—	
L-Cysteine																
1	15·83	1·44	4·51	0·23	1·67	0·45	22·01	1·22	14·36	0·81	4·05	0·35	1·90	0·11	20·31	1·24
10	16·08	1·15	5·30	0·37	2·85	0·14	24·24	1·62	13·35	0·52	3·21	0·11	1·52	0·08	18·07	0·62
DL-Methionine																
1	18·98	1·11	4·70	0·34	1·42	0·12	25·10	1·51	—		—		—		—	
10	18·87	0·63	4·38	0·30	1·55	0·10	24·80	1·00	—		—		—		—	
Tungstate																
1	15·42	1·69	4·54	0·75	2·55	0·34	22·51	2·77	11·30	1·05	4·37	0·59	2·24	0·26	17·91	1·86
Copper salt																
1	7·27	0·43	1·00	0·35	0·00	0·00	8·26	0·77	4·94	0·28	1·02	0·11	0·15	0·10	6·12	0·41

* Incubated without nitrate.

formation, and also overcome the decrease in total contents and the change in molar ratios of VFA caused by nitrate.

DISCUSSION

Mo is a metal of the second transition series, an essential component of nitrate reductase. Tungsten, an element homologous to Mo, competes with Mo. Thus, nitrate reductases of various origins are inhibited by W, as are other molybdo-enzymes (Johnson *et al.* 1974*a*, *b*). The significant depressing effect of W on nitrite formation by mixed nitrate-adapted rumen microbes (prepared by straining rumen fluid) observed in the present work is in good agreement with other reports of in vitro experiments (Prins *et al.* 1980; Marais *et al.* 1988). However, a smaller suppression of nitrite formation by W was observed with non-adapted microbial populations. In in vitro experiments using inocula from both nitrate-adapted and non-adapted cows, higher sensitivity of nitrite formation to W inhibition has been reported with nitrate-adapted organisms (Prins *et al.* 1980). Additionally, the magnitude of the inhibitory effect of W on rumen formation of nitrite varies with the feeding regimens of the host animals and with substrates used as energy sources for the incubation (Prins *et al.* 1980). The inhibitory effect of W on nitrite formation may be primarily dependent on the ratio, free Mo ions:native molecules of nitrate reductase in rumen fluid.

The interactions between Mo and Cu, and the formation of insoluble copper thiomolybdate, probably inhibit nitrate reductase in rumen micro-organisms. The dietary requirement and physiologically tolerable amount of Cu for ruminants depends on dietary S and Mo (National Research Council, 1988). However, adverse effects of more than 1 μg Cu/ml in the rumen fluid on cellulose digestion by rumen micro-organisms have been reported (Martinez & Church, 1970). The effect of Cu toxicity on VFA production (Table 1) suggests that bacteriostasis caused by this element cannot be denied, due to its inhibitory effect on nitrate reduction (Figs 2 and 3). Apart from the digestive crises in the rumen, possible problems relating to the health of animals and man and environmental contamination remain to be clarified before these transition metals are used as prophylactics against nitrate–nitrite poisoning in ruminants.

In in vivo physiological concentrations (< 1 mM-sulphide), sulphide absorption may be extremely rapid (Bray & Till, 1975). Consequently, undissociated sulphide can be directly absorbed across the rumen epithelium, although the turnover rate is variable depending on the N:S ratio in the rumen. It is probable that the undissociated sulphydryl ions or H_2S absorbed from the rumen can become a metabolic poison for the animal due to sulphide overdose. In terms of partial recovery of VFA production reduced by nitrate (Table 1), it is unlikely that the sulphide added, even at 10 mM, adversely affected microbial fermentation in vitro. Therefore, a marked depression in nitrite formation by the addition of sulphide indicates that elemental S degraded from sulphide inhibits the activity of nitrate reductase as a result of interference with the incorporation of Mo into the enzyme. In an early study of nitrate reductase in wild type *Neurospora crassa* (5297a), Nason & Evans (1953) observed that the –SH group played a significant part in the stability of this enzyme, and demonstrated small inhibitory effects on the enzyme activity at a higher concentration (1 mM) of L-cysteine. Although the *in situ* participation of the –SH group in the activity and *de novo* synthesis of nitrate reductase (Azoulay *et al.* 1969) cannot be ruled out in the present work, it seems reasonable to assume that the sulphide-S generated from L-cysteine by the L-cysteine hydrogen sulphide-lyase (cystathionine γ-lyase; *EC* 4.4.1.1) reaction (Collins & Monty, 1973) of rumen bacterial origin interferes with the nitrate reduction. Currently, interference of undegraded S in nitrate reductase activity from rumen microbial origin has not been established.

Therefore, the significant suppressing effect of L-cysteine on nitrite formation by rumen microbes is of particular interest when considering the dietary control of the hazards of nitrate to livestock.

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