# Letters to the Editors

*Nitrate reduction by rumen micro-organisms and nitrite accumulation* in vitro

Statements in the recent paper of Takahashi *et al.* (1989) have led me to write to you with the following comments.

The title of the report is misleading because the authors have not measured nitrate reduction at all. They have determined nitrite concentration after 24 h incubation of rumen fluid in vitro under different conditions, and the results are given as values relative to a control with added nitrate (100%). No negative controls or blanks with killed or fixed rumen fluid seem to have been included and no initial or final concentrations are given. Therefore, conclusions about formation, production or even accumulation of nitrite due to microbial action may be spurious. The authors should have included analyses for nitrate disappearance or ammonia accumulation, preferably both, in order to make any inferences concerning nitrate reduction as in the excellent paper by Marais *et al.* (1988) which they cite.

Sentence 2 in the introduction is not clear. Perhaps the second part should read 'the pathways of nitrate reduction have been demonstrated as being dissimilative'.

Why were such large unphysiological amounts of sulphide and copper examined? The authors admit that they would be of little practical use in vivo. Normal values for sulphide in rumen contents are around 0·06 mM, but the smallest concentration tested here was 1 mM. Copper concentrations in rumen contents are generally about 0·02 mM. A concentration of 0·17 mM Cu drastically reduced sulphide concentrations in vitro (Nikolić *et al.* 1980).

While it is clear from Table 1 that sulphide reversed the negative influence of nitrate on volatile fatty acid (VFA) production, the authors have not shown that sulphide has any effect on nitrate reductase (sentence 5, paragraph 3 of the discussion). They have demonstrated only that nitrite concentration at 24 h is lower in the presence of added sulphide. It would be interesting to examine the chemical composition of a sterile solution of sodium sulphide with sodium nitrate or nitrite at similar concentrations after 24 h incubation at 38°.

The authors have not stated whether their rumen fluid inocula contained protozoa or not. The motility of these organisms at the end of incubation periods in vitro provides evidence that the conditions have been favourable.

Besides containing molybdenum, nitrate reductase is an iron–sulphur protein (Vincent, 1979), which may be relevant in connection with possible effects of the examined chemicals. However, bacteriostasis caused by high concentrations of copper sulphate is not necessarily due to an inhibitory effect of Cu on nitrate reduction (sentence 4, paragraph 2 of the discussion). Although the high concentration of copper sulphate added to the incubation system decreased nitrite and VFA concentrations after 24 h compared with controls, it has not been shown that this was caused by an effect of Cu on nitrate reductase. Since nitrate and sulphate may compete for reduction by certain rumen bacteria (Bennink & Bryant, 1973), it would have been more appropriate if the authors had used copper chloride or acetate to examine the effects of Cu. Nikolić *et al.* (1984) demonstrated that nitrate added in similar amounts to that examined by Takahashi *et al.* (1989) considerably decreased the rate of sulphate reduction by rumen micro-organisms in vitro.

Finally it should be added that paragraph 4 in the abstract does not agree with the results given in Table 1. Surely the first six words should be removed because all three substances

mentioned, and also methionine, appeared to have counteracted the decrease of VFA 'production'.

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## *Suppression of nitrate reduction by sulphur*

Dr Nikolić raised several points on our recent paper 'Inhibitory effects of sulphur compounds, copper and tungsten on nitrate reduction by mixed rumen micro-organisms' (Takahashi *et al.* 1989).

First, dealing with the most important point, Dr Nikolić claimed that there was a lack of evidence concerning nitrate disappearance. The inhibitory effects of the chemicals studied on nitrate reduction in vitro were shown by the suppression of nitrite accumulation, as was also reported by Prins *et al.* (1980), since it is clear that nitrite detected in the media after 24 h incubation is the product reduced from the nitrate added. The suppression of rumen microbial reduction of nitrate by sulphide and L-cysteine has also been reported in detail by Takahashi (1989) at the VIIth International Symposium on Ruminant Physiology. The validity of the results in our paper has been confirmed in vivo using sheep, and at the enzyme level (J. Takahashi, N. Johchi and H. Fujita, unpublished results). Therefore, we are confident that sulphur could be a specific inhibitor for nitrate reduction in vitro.

In our experiment in vitro, copper and sulphur were used at concentrations higher than those in vivo. As mentioned in the introduction, these chemicals were used to study the possible participation of molybdenum in the activation of nitrate reductase on rumen microbial populations. The results in our experiment showed that Mo is an essential element for promoting nitrate reduction by rumen microbial populations, although the participation of elements present in the rumen fluid other than Mo in nitrate reductase cannot be ruled out. Also, it appeared that nitrate reduction in the rumen can be regulated using a method that interferes with the function of Mo. The possible use of the essential amino acid L-cysteine as a natural prophylactic for nitrate–nitrite poisoning should be stressed, as discussed in the paper.

Dr Nikolić pointed out that in her own investigations in vitro, nitrate decreased the reduction rate of sulphate by rumen micro-organisms. In our experiment, however, we observed that sulphate had no notable effect on nitrate reduction. Consequently, the marked depletion in nitrite formation by the addition of copper sulphate was attributable to the effect of Cu itself on nitrate reduction by rumen microbial populations.

With regard to the possibility of the existence of protozoa under our experimental conditions, ciliate protozoa do not appear to produce either assimilatory or dissimilatory

reductase as reported by Alaboudi & Jones (1985), and direct contribution of protozoa to rumen nitrate reduction was very unlikely in our experiment. The motility of small protozoa at the end of incubation was confirmed by light microscopy.

Finally, two typographical errors in our paper should be corrected. In the synopsis, paragraph 4 should read 'In contrast to the effects of sulphate and sulphite, sulphide, L-cysteine and W counteracted, to some degree, nitrate-induced reduction in volatile fatty acid (VFA) production'. In the introduction, sentence 2 should read 'In nitrate respiring and denitrifying species of bacteria classified into genera such as *Escherichia*, *Proteus*, *Paracoccus* and *Pseudomonas*, the reducing pathway of nitrate occurs by a route which does not involve the assimilation or incorporation of the products into the bacterial cells.'

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