British Journal of Nutrition (1986), **55**, 361–367

The alleviation of chronic copper toxicity in sheep by ciliate protozoa*

BY M. IVAN, D. M. VEIRA AND C. A. KELLEHER

Animal Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada

(*Received 16 April 1985 – Accepted 26 September 1985*)

1. Rams, fauna-free from birth and initially of 48–65 kg live weight, were allocated to two groups of ten each and given a diet containing 14 μg copper/g dry matter; five additional rams were killed and their livers were analysed for Cu.

2. One group (faunated) was inoculated with a mixed population of ciliate protozoa, and contained between 60×10^5 and 195×10^5 protozoa/ml rumen fluid throughout the 184 d experiment. The other group remained fauna-free. Following blood sampling, three rams in each group were killed on day 63, two on day 125 and four on day 184. One sheep in each group died during the experiment.

3. Faunated rams showed higher weight gains and feed consumption than fauna-free rams.

4. Plasma Cu concentration (μg/ml) increased from an initial 0·82 to a final 1·00 in faunated and 1·36 in fauna-free rams. Liver Cu concentration (μg/g dry matter) increased from an initial 745 to a final 962 and 1684 in faunated and in fauna-free sheep respectively, representing a 4·3-fold greater increase in the fauna-free than in the faunated group. The absorption and retention of Cu was 38–50% higher in the fauna-free than in the faunated rams.

5. It was suggested that rumen ciliate protozoa increased rumen production of sulphide (through increased breakdown of soluble proteins) which complexed part of the Cu, making it unavailable for absorption and utilization. Therefore, ciliate protozoa could determine susceptibility to chronic Cu toxicity in sheep.

The Animal Research Centre sheep flock was re-established in 1980 by hysterectomy (Heaney *et al.* 1981) in order to eradicate maedi-visna disease. Although there was no change made in the dietary regimen there was an outbreak of chronic copper toxicity in this flock, 10–12 months later, resulting in a high daily rate of mortality. The toxicity was alleviated by dietary supplements of molybdenum and sulphur and by decreasing dietary Cu from 10–14 to 5–8 μg/g dry matter (Hidiroglou *et al.* 1984). It was later established that the flock remained fauna-free after repopulation by hysterectomy. Therefore, studies were initiated to discover the possible connection between the fauna-free status and the outbreak of chronic Cu toxicity. The present paper provides evidence that the introduction of rumen ciliate protozoa to fauna-free sheep decreased the absorption of Cu and its accumulation in the liver. It was concluded that the outbreak of chronic Cu toxicity in the flock was induced by the absence of rumen ciliate protozoa.

MATERIALS AND METHODS

Animals

Twenty-five cross-bred naturally fauna-free rams were randomly selected from a pool of forty animals. The live weight of the selected rams was between 48 and 65 kg. They were allocated to three mean equal live-weight groups of five, ten and ten animals.

Diet

On a g/kg dry matter basis, the diet comprised: maize silage 824, soya-bean meal 160, vitamin/mineral premix 16. The premix contained (g/kg): 620 cobalt-iodized salt, 186

* Contribution no. 1319 of the Animal Research Centre, Ottawa, Canada.

limestone, 186 dicalcium phosphate, 1·1 copper chloride, 0·037 retinol, 0·005 cholecalciferol and 0·3 vitamin E. Fresh diet was mixed approximately every 2 weeks, using silage from the same silo, and stored in an unheated room (the experiment was carried out during winter). Samples were obtained from each mix and, by analysis, contained (μg/kg dry matter): 12·7–14·6 Cu, 216–255 iron, 53–68 zinc.

Experimental procedure

Each of the three groups of sheep was housed in a separate room and group-fed the diet *ad lib*. To establish a normal rumen population of ciliate protozoa, each animal in one group of ten sheep (faunated) was dosed with 50 ml rumen contents taken from two donor wethers that had a mixed rumen ciliate population. In addition, the two donor sheep were housed with the faunated group during the 2-week adjustment period. A group of ten sheep was kept fauna-free throughout the experiment. The remaining five sheep were killed at the end of the adjustment period and their livers were removed, weighed, sampled and analysed for Cu, Fe and Zn.

Records of total feed intake were kept for each group during the entire experiment which lasted 184 d. The sheep were individually weighed and blood was sampled on days 0, 63, 125 and 184. Three randomly-selected sheep in each group were killed on day 63, two on day 125 and the remaining four sheep on day 184. Livers and rumen contents were removed, weighed and sampled. Ciliate protozoa were counted in samples of rumen fluid. One sheep in each group died during the experiment. Cu toxicity was the cause of death of the sheep from the fauna-free group while that in the faunated group died from injury.

Analytical procedures

Analyses for Cu, Fe and Zn in feed and lyophilized liver samples were performed on a Perkin-Elmer atomic absorption spectrophotometer (model 460) after nitric/perchloric acid digestion. Samples of blood plasma were aspirated directly into the spectrophotometer after appropriate dilution with deionized water. Plasma ferroxidase (caeruloplasmin; *EC* 1.16.3.1) activity (units/ml) was determined by the σ-dianisidine oxidation method described by Mason *et al.* (1980) using a molar extinction coefficient of $1{\cdot}92 \times 10^4$/mol per cm (Schosinsky *et al.* 1974). One unit is 1 μmol of product formed/min. Counting of protozoa has been described previously (Veira *et al.* 1983).

Statistical procedure

Analysis of variance was used to test the statistical significance of differences between means.

RESULTS

Weight gains

Both faunated and fauna-free sheep gained weight during the entire experiment (Fig. 1). However, higher gains and feed consumption were obtained for the faunated than for the fauna-free group. The ratio, mean dry matter intake:weight gain was 13·0 and 10·5 for fauna-free and faunated groups respectively.

Rumen protozoa numbers

Between 60×10^5 and 195×10^5 protozoa/ml rumen fluid were found in the individual faunated sheep. No protozoa were found in the fauna-free sheep.

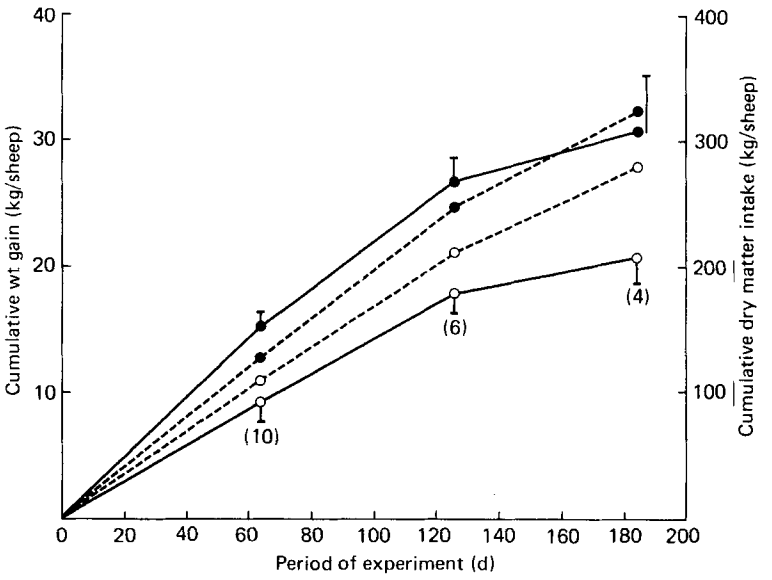

Fig. 1. Cumulative weight gains (——) and dry matter intakes (– – –) of fauna-free (○) and faunated (●) sheep fed on a maize–silage-based diet. Points are mean values, with their standard errors, represented by vertical bars, for the number of sheep shown in parentheses.

Cu concentration in plasma and liver

Mean concentration of Cu (μg/ml) in plasma increased from an initial 0·82 to a final 1·00 and 1·36 in faunated and fauna-free sheep respectively (Fig. 2). The increase was almost linear in fauna-free sheep but fluctuated in faunated sheep. The pattern of ferroxidase activity in faunated sheep was similar to the pattern of plasma Cu concentration. However, the activity in fauna-free sheep was almost constant until day 125 and increased thereafter. Both final plasma Cu concentration and enzyme activity were much higher ($P < 0{\cdot}05$) in fauna-free than in faunated sheep.

Mean concentration of Cu in the liver (μg/g dry matter) increased from an initial 745 to a final 962 and 1684 in faunated and fauna-free sheep respectively (Fig. 3). This represented a 4·3-fold higher increase for the fauna-free sheep compared with the faunated animals. The difference in liver Cu concentration between groups increased with the progression of the experiment. The concentration at the end of the experiment was 1·7-fold higher ($P < 0{\cdot}05$) in the fauna-free than in the faunated sheep, with mean values of 322 (SE 61·0) and 279 (SE 40·6) mg total liver Cu respectively. The ratio, total Cu in the liver (mg): mean daily dry matter intake (kg/sheep) increased in both groups during the experiment but was consistently higher in the fauna-free than in the faunated sheep (Fig. 3). However, the differences between the groups were quite similar (50, 38 and 42% for days 63, 125 and 184 respectively).

Fe and Zn concentration in the liver

Both Fe and Zn concentrations decreased in the faunated sheep with the progression of the experiment (Fig. 4). Although small decreases were apparent after day 63 in the fauna-free sheep, the concentrations remained much lower ($P < 0{\cdot}01$) in the faunated group than in the fauna-free group.

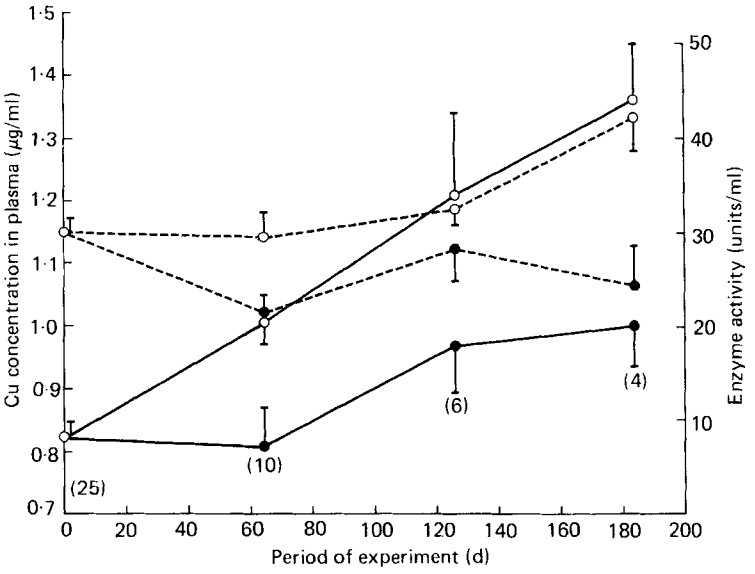

Fig. 2. Mean concentrations of copper (——) and ferroxidase (caeruloplasmin; *EC* 1.16.3.1) activity (---) in plasma of fauna-free (○) and faunated (●) sheep. Points are mean values, with their standard errors represented by vertical bars, for the number of sheep shown in parentheses. One unit of ferroxidase activity is 1 μmol of product formed/min.

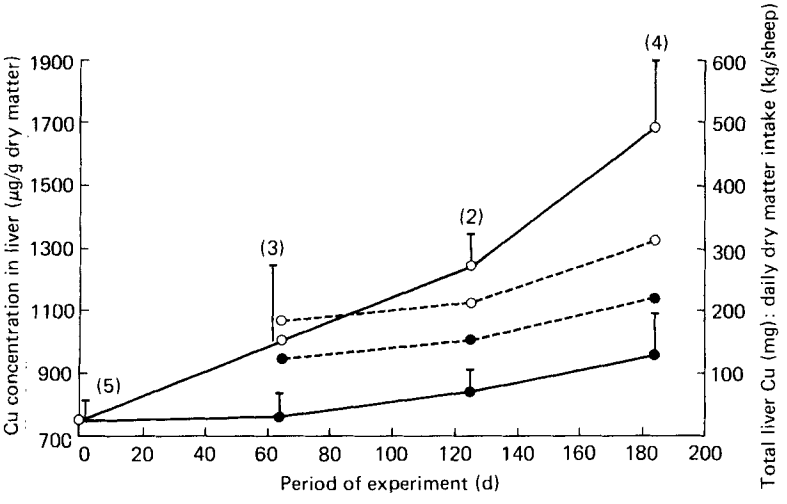

Fig. 3. Mean concentrations of copper in the liver (——) and the ratio, total Cu in the liver:daily dry matter intake (---) for fauna-free (○) and faunated (●) sheep. Points are mean values, with their standard errors represented by vertical bars, for the number of sheep shown in parentheses.

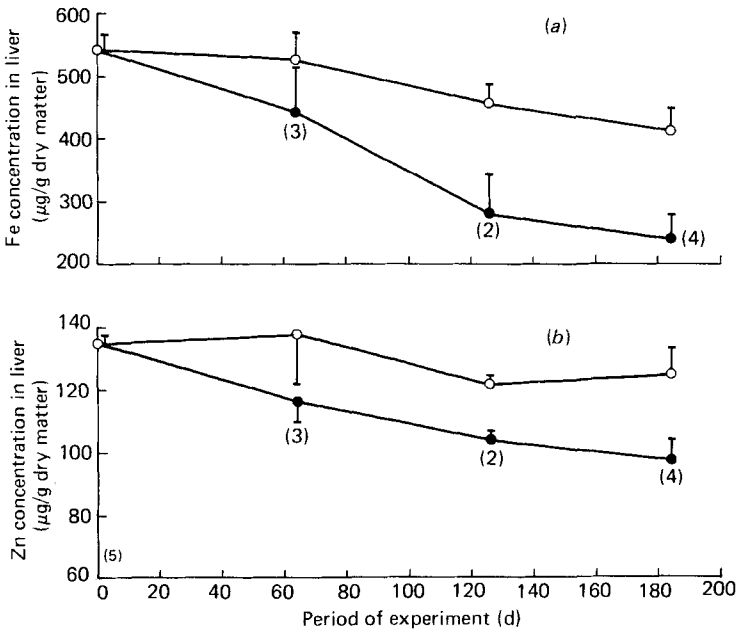

Fig. 4. Mean concentrations of iron (a) and zinc (b) in the liver of fauna-free (○) and faunated (●) sheep. Points are mean values with their standard errors, represented by vertical bars, for the number of sheep shown in parentheses.

DISCUSSION

It is evident from these results that the presence of ciliate protozoa in the rumen markedly decreased the accumulation of Cu in the liver. Although the faunated sheep consumed more feed and therefore more Cu than fauna-free sheep, their increase in the liver Cu concentration over the duration of the present experiment was only one-quarter that observed with the fauna-free animals. When total Cu in the liver (calculated from the liver weight and Cu concentration) was divided by the mean daily dry matter intake, the ratio throughout the experiment was from 38 to 50% higher in the fauna-free than in the faunated sheep. This strongly suggests that there was a higher rate of absorption of Cu in the fauna-free sheep. This is further supported by higher and almost linear increases in the plasma Cu concentration and by higher ferroxidase activity in the fauna-free than in the faunated sheep. All the sheep came from a naturally fauna-free flock and, at the beginning of the experiment, the control sheep had an average liver Cu concentration of 745 μg/g dry matter even though they were given a silage-based diet containing only 5–8 μg Cu/g dry matter. Our records show that before the flock became fauna-free in 1980 it was fed on a similar diet with a higher concentration of Cu (10–14 μg/g dry matter), the liver Cu concentration was only 300–400 μg/g dry matter.

Although both groups of sheep received identical diets, growth rate, feed intake and feed intake:weight gain were more favourable in the faunated sheep. This agrees with previous reports (Abou Akkada & el-Shazly, 1964; Christiansen *et al.* 1965) and is readily explained by the fact that the presence of rumen ciliate protozoa is beneficial to digestion (Conrad *et al.* 1950; Klopfenstein *et al.* 1966; Jouany *et al.* 1981) and to maintenance of more stable rumen pH (Veira *et al.* 1983), although protozoa increase the energy lost as methane (Whitelaw *et al.* 1984) and contribute to inefficient utilization of nitrogen in the rumen (Veira

et al. 1983; Ushida *et al.* 1984). Increased growth rate due to the absence of rumen ciliate protozoa was obtained only when diets high in energy and low in rumen undegradable protein were given to ruminants with a large amino acid requirement (Bird & Leng, 1978; Bird *et al.* 1979).

Previous results (Ivan & Veira, 1981) showed that the solubility of Cu in the rumen and abomasal digesta decreased with increasing dietary supplements of casein, a protein almost completely degradable in the rumen (Hume, 1974). Hartmans & Bosman (1970) reported lower liver Cu and higher rumen sulphide concentrations in grazing cattle than in those given hay which had been harvested at the same growth stage and which had a similar mineral content. Rumen degradability of protein is lower in dry forage compared with fresh forage (Beever *et al.* 1976) and it has been postulated (Ward, 1978) that the high level of very soluble (degradable) protein of fresh pasture results in production of sulphide from the S-containing amino acids during rumen fermentation; this sulphide probably complexes Cu to form insoluble copper sulphide which is largely unabsorbed. Recent results (Veira *et al.* 1984) showed a decreased flow of amino acids into the duodenum with increasing population of ciliate protozoa. Therefore, the evidence leads us to suggest that rumen ciliate protozoa increase the breakdown of dietary proteins in the rumen thereby increase production of sulphide through the metabolism of S-containing amino acids. Cu is bound to the sulphide and becomes unavailable for absorption and utilization. Such binding could also involve Fe and Zn, and possibly other divalent cationic mineral elements. It would appear that the protozoa-associated decrease in Cu utilization amounted to 38–50% in the present experiment.

Liver Cu levels up to 500 μg/g dry matter in sheep are considered to be normal and, in most haemolytic crises associated with chronic Cu toxicity, the levels reach 1000–3000 μg/g dry matter (Bostwick, 1982). Hepatic Cu levels in all fauna-free sheep in this experiment exceeded 1000 μg/g dry matter, rendering these animals much more susceptible to a stress-induced haemolytic crisis. Indeed, one sheep of this group died of Cu toxicity 128 d after the initiation of the experiment. None of the sheep in the faunated group showed any sign of chronic Cu toxicity. It was therefore concluded that rumen ciliate protozoa play an important role in the prevention of chronic Cu toxicity in sheep through a reduction in hepatic accumulation of dietary Cu. More Cu was probably partitioned into extra hepatic tissue in faunated sheep, though too small to explain hepatic Cu differences. It was also concluded that the 1981 outbreak of chronic Cu toxicity in the sheep flock which had been re-established by hysterectomy was induced by the absence of rumen ciliate protozoa. This has another practical implication as excessive levels of some dietary components such as Zn (Durand & Kawashima, 1979) and oils (Ikwuegbu & Sutton, 1982) may significantly decrease the protozoa population in the rumen and thus decrease the formation of CuS.

The authors are indebted to Mr M. K. Bryan for skilled technical assistance. C.A.K. thanks the National Science and Engineering Research Council Canada (NSERC) for financial support.

REFERENCES

Abou Akkada, A. R. & el-Shazly, K. (1964). *Applied Microbiology* **12**, 384–390.
Beever, D. E., Thompson, D. J. & Camell, S. B. (1976). *Journal of Agricultural Science, Cambridge* **86**, 443–452.
Bird, S. H., Hill, M. K. & Leng, R. A. (1979). *British Journal of Nutrition* **42**, 81–87.
Bird, S. H. & Leng, R. A. (1978). *British Journal of Nutrition* **40**, 163–167.
Bostwick, J. L. (1982). *Journal of American Veterinary Medical Association* **180**, 386–387.
Christiansen, W. C., Kawashima, R. & Burroughs, W. (1965). *Journal of Animal Science* **24**, 730–734.
Conrad, H. R., Hibbs, J. W., Pounden, W. D. & Sutton, T. S. (1950). *Journal of Dairy Science* **33**, 585–592.
Durand, M. & Kawashima, R. (1979). In *Digestive Physiology and Metabolism in Ruminants*, pp. 375–408 [Y. Ruckebusch and P. Thivend, editors]. Lancaster: MTP Press Ltd.

Hartmans, J. & Bosman, M. S. M. (1970). In *Proceedings of WAAP/IBP International Symposium on Trace Element Metabolism in Animals*, pp. 362–366 [C. F. Mills, editor]. Edinburgh: E. & S. Livingstone.
Heaney, D. P., Hartin, K., Shrestha, J. N. B., Ainsworth, L. & Langford, G. A. (1981). *Journal of Animal Science* **53**, Suppl. 1, 181 Abstr.
Hidiroglou, M., Heaney, D. P. & Hartin, K. E. (1984). *Canadian Veterinary Journal* **25**, 377–382.
Hume, I. (1974). *Australian Journal of Agricultural Research* **25**, 155–165.
Ikwuegbu, O. A. & Sutton, J. D. (1982). *British Journal of Nutrition* **48**, 365–375.
Ivan, M. & Veira, D. M. (1981). *Canadian Journal of Animal Science* **61**, 955–959.
Jouany, J. P., Zinab, B., Senaud, J., Grolière, C. A., Grain, J. & Thivend, P. (1981). *Reproduction, Nutrition, Développement* **21**, 871–884.
Klopfenstein, T. J., Purser, D. B. & Tyznik, W. J. (1966). *Journal of Animal Science* **25**, 765–773.
Mason, J., Lamand, M. & Kelleher, C. A. (1980). *British Journal of Nutrition* **43**, 515–523.
Schosinsky, K. H., Lehmann, H. P. & Beller, M. F (1974). *Clinical Chemistry* **20** 1556–1563.
Ushida, K., Jouany, J. P., Lassalas, B. & Thivend, P. (1984). *Canadian Journal of Animal Science* **64**, Suppl., 20–21.
Veira, D. M., Ivan, M. & Jui, P. Y. (1983). *Journal of Dairy Science* **66**, 1015–1022.
Veira, D. M., Ivan, M. & Jui, P. Y. (1984). *Canadian Journal of Animal Science* **64**, Suppl., 22–23.
Ward, G. M. (1978). *Journal of Animal Science* **46**, 1078–1085.
Whitelaw, F. G., Eadie, J. M., Bruce, L. A. & Shand, W. J. (1984). *British Journal of Nutrition* **52**, 261–275.

Printed in Great Britain