Copper and manganese in hay samples from scrapie-free, scrapie-prone and scrapie-afflicted farms in Iceland

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SUMMARY

Hay (forage) samples (in total 172) were collected on 47 farms from the summer harvests in 2001, 2002 and 2003 for analysis of manganese and copper concentration. Farms were divided into four Categories (Cat.); Cat. 1: Nine farms in counties where scrapie had never been registered or in areas never affected in otherwise afflicted counties. Cat. 2: Seventeen farms never afflicted by scrapie, or afflicted and restocked prior to 1960, but located amongst scrapie-prone or scrapie-afflicted farms in Cat. 3 or 4. Cat. 3: Twelve farms afflicted by scrapie after 1980 and afterwards restocked with healthy sheep. Cat. 4: Nine farms where scrapie had been diagnosed during the experimental period (April 2002 - March 2004). Farms in Cat. 1 and Cat. 2 are collectively referred to as scrapie-free, farms in Cat. 3 as scrapie-prone and farms in Cat. 4 as scrapie-afflicted. Manganese concentration was significantly higher in hay samples from scrapie-free farms than in samples from scrapie-prone (restocked) or scrapie-afflicted farms. Copper concentration was statistically the same in samples from farms, whether scrapie-free, scrapie-prone or scrapie-afflicted. The Mn/Cu ratio was accordingly significantly higher in samples from scrapie-free than in samples from scrapie-afflicted farms. Nevertheless the amounts of the two metals were positively correlated. When taken separately samples from farms in Cat. 1 had a significantly higher manganese concentration than samples from farms in the other categories. Manganese concentration in samples from farms in Cat. 2 separately was also higher than in samples from scrapie-afflicted farms but not when compared with samples from scrapie-prone farms. It was concluded that high manganese concentration, or a high Mn/Cu ratio, might have a protective effect on the occurrence of clinical scrapie in Iceland. It was furthermore postulated that this effect could basically be connected with the gastrointestinal mucosa of the sheep, which seems to be a major target for prion entry and replication, and where manganese and copper might have an opposite effect on the transport of prion proteins through the cellular border.

Key words: copper, hay, manganese, Mn/Cu ratio, scrapie, sheep

YFIRLIT

Kopar og mangan í heysýnum á ósýktum býlum á ósýktum svæðum, riðulausum býlum á riðusvæðum, fjárskiptabýlum og riðusýktum býlum á Íslandi

Heysýnum úr rúlluböggum, alls 172 sýni frá uppskeru áranna 2001, 2002 og 2003, var safnað á rannsóknartímabilinu (frá vori 2002 – til vors 2004) á 47 býlum og mangan og kopar ákvarðað í sýnunum. Býlum var skipt í fjóra flokka: níu ósýkt býli í ósýktum sýslum eða á ósýktum svæðum í riðusýslum (sýslum, þar sem riðu hefur orðið vart); 17 riðulaus býli (eða riða greind og fjárskipti gerð fyrir 1960) í riðusýslum; 12 fjárskiptabýli (riða komið upp eftir 1980, en síðar skipt um fé) og 9 riðusýkt býli (riða í gangi í hlutaðeigandi hjörðum á rannsóknartímabilinu). Í enska textanum eru býli í tveimur fyrstu flokkunum sameiginlega nefnd „scrapie-free", fjárskiptabýli í þriðja flokki „scrapie-prone" og riðusýktu býlin í fjórða flokki „scrapie-afflicted". Þéttni mangans í heysýnum frá býlum, þar sem riða hefur aldrei komið upp (1. og 2. flokkur samanlagt) var marktækt meiri en í heysýnum frá fjárskiptabýlum og riðusýktum býlum. Þéttni mangans í heysýnum frá ósýktum býlum í fyrsta flokki var sömuleiðis marktækt meiri en í heysýnum frá býlum í hinum flokkunum. Þéttni mangans í heysýnum frá riðulausum býlum í öðrum flokki (staðsett meðal fjárskiptabýla eða riðubýla í riðusýslum) var einnig marktækt meiri en í heysýnum frá riðusýktum býlum (4. flokkur), en var ekki marktækt meiri en í heysýnum frá fjárskiptabýlunum (3. flokkur). Þéttni kopars var staðtölulega hin sama í heysýnum í öllum flokkum. Hlutfallið Mn/Cu var samkvæmt því marktækt hærra í heysýnum frá býlum, þar sem riða hefur aldrei komið fyrir (1. og 2. flokkur samanlagt) en í sýnum frá riðusýktum býlum. Marktækt samband var engu að síður milli þéttni mangans og kopars, ef öll sýni voru metin saman. Ályktað var, að mikil þéttni mangans í heyi, eða hátt Mn/Cu hlutfall, gæti haft varnandi verkun gegn uppkomu klínískra einkenna um riðu. Bent var á þá hugsanlegu staðreynd, að mangan gæti seinkað innferð príonpróteina í frumur í slímhúð í meltingarvegi, en þar á riðusýking að margra áliti einkum upphaf sitt, og kopar gæti haft gagnstæða verkun.

INTRODUCTION

As far as is known scrapie arrived in Iceland in 1878, presumably with an infected, Oxford Down ram imported from Denmark. The disease gradually spread from its origin in Skagafjördur, northern Iceland, to parts of most counties when preventive measures were first systematically enforced in the autumn of 1978 (Sigurdarson 1991; see Figure 1). This system has since been largely effective in curbing the number of scrapie-afflicted farms. Scrapie is, however, found sporadically each year on one or more farms, especially in five localities in southern and northern Iceland (nr. 3-5, 8, 11 in Figure 1). The sporadic recurrence of scrapie thus apparently occurs in a non-random fashion, suggesting a link to some environmental factor (or factors) that either cause or constitute a risk for the development of clinical scrapie. This idea is supported by recent work (Purdey 2000). He showed that a high concentration of manganese and/or low concentration of copper in herbage in an area in northern Iceland apparently favours the occurrence of scrapie in sheep in this area, while a low concentration of manganese in herbage in an adjacent area seemingly had a preventive effect on the occurrence of scrapie in that area.

In the present study hay samples were collected from the summer harvests in 2001, 2002 and 2003 on more than 40 farms in eleven localities in Iceland. The farms were divided into four categories and comprised farms characterised as scrapie-free, scrapie-prone and scrapie-afflicted and concentrations of copper

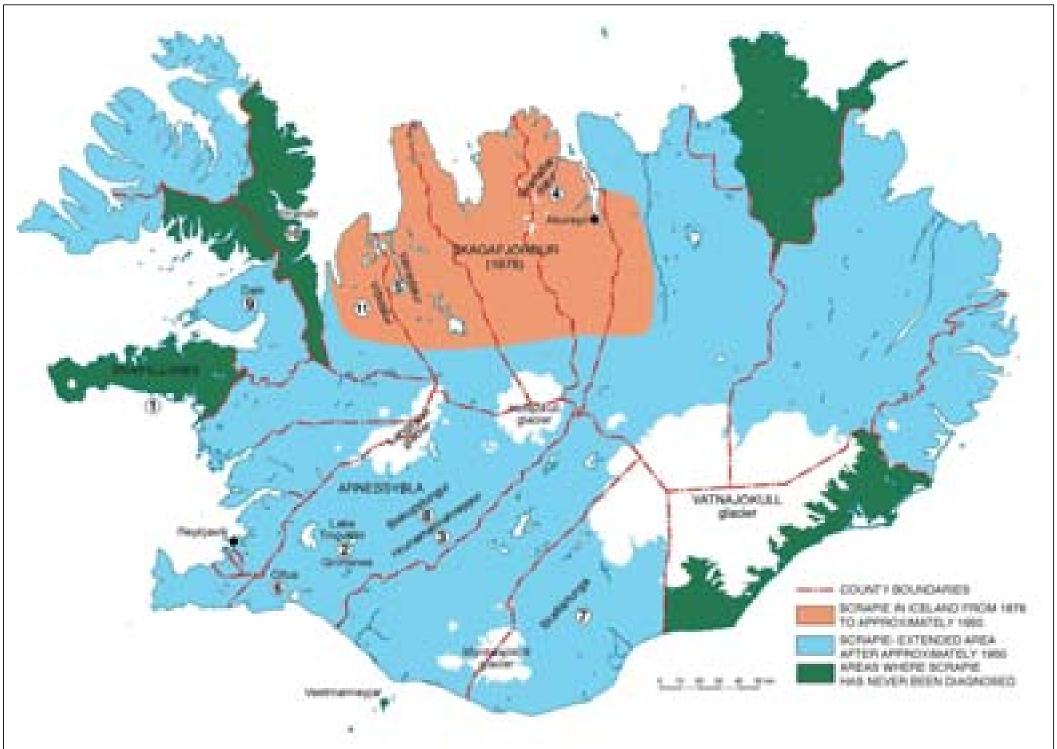

Figure 1. Scrapie in Iceland and localities of farms. Scrapie arrived in Iceland with an infected, imported ram of foreign stock in Skagafjörður in 1878. It was confined to a part of northern Iceland until ca. 1950 (orange). Scrapie afterwards spread patchily to greater or lesser parts of all counties (blue) except for four (green) until 1978 when preventive measures were first systemically enforced. The large green area seen in the north-east indicates one of the main areas in the country used to provide scrapie-free, healthy sheep to restock formerly scrapie-afflicted farms. The numbers 1-11 indicate eleven different localities of the 47 farms where hay samples were collected for Cu and Mn analyses in the present study.

and manganese were determined in the hay samples.

This research is intended to increase our understanding of the possible role of an imbalance in the trace elements Cu and Mn in the onset and mechanism behind the occurrence of scrapie in Iceland.

MATERIALS AND METHODS

Categories of farms and collection of hay samples

Hay samples, mainly from round-bale silage, were collected on 26 farms from the 2001 and 2002 harvests and on 34 farms from the 2003 harvest. On 10 farms samples were collected from the 2001 harvest only and on 21 farms from the 2003 harvest only. On 12 farms hay samples were collected from both the 2002 and 2003 harvests and on 4 farms from both the 2001 and 2003 harvests. The number of farms included in the study was thus 47. The total number of hay samples was 172.

The farms were divided into four categories. *Category 1*: Farms located in counties where scrapie has never been found or (in two instances) located in areas never affected of otherwise afflicted counties. *Category 2*: Farms never afflicted by scrapie, or afflicted and restocked prior to 1960, but located amongst scrapie-prone or scrapie-afflicted farms in scrapie-affected counties (same localities as categories 3 or 4). *Category 3*: Farms afflicted

Table 1. Number of farms in each category, their location and total number of hay samples analysed of the harvests 2001, 2002 and 2003 collected on farms in the respective categories.

Category	No. of farms	Total no. of samples	Location (see Figure 1)
1	9	28	1, 9, 10, 11
2	17	68	2-8, 11
3	12	40	2, 8, 11
4	9	36	3-6, 8, 11; (diagnosed 2002-2004)

by scrapie after 1980 and afterwards restocked with healthy sheep. *Category 4*: All farms (except one) where scrapie had been diagnosed from April 2002 to March 2004, the experimental period. Collectively the farms in Categories 1 and 2 are referred to as *scrapie-free*. Farms in Category 3 are referred to as *scrapie-prone* and farms in Category 4 as *scrapie-afflicted,* respectively. The farms are situated in 11 different localities in the southern, western and northern parts of the country (Figure 1).

Farms in Category 3, where scrapie had been diagnosed after 1980, were kept in quarantine for 2-3 years before being restocked with healthy sheep from scrapie-free areas in the northeastern or western parts of the country (Figure 1). On a few of these farms scrapie was diagnosed twice during that period implying that the herds were slaughtered and the farms restocked twice. Most of the farms in Category 4 were still being kept in quarantine at the end of the experimental period.

A summary of the number of farms in each Category, their location in the country and the number of samples collected is given in Table 1.

The forage samples consisted mostly of grass silage from round-bales with variable dry matter content (from 30-70%). From each bale, samples were taken at 3-4 sites by an experienced person wearing plastic gloves (about 300-400 g from each bale), and placed directly into labeled plastic bags. In some instances it was not feasible to slit the bales open. In these cases the grass silage samples were collected by an electric hay sampler with a 4 cm diameter. Although no strictly controlled comparison has been carried out of the two methods, the results of metal analyses in our study (e.g. on grass silage from farms in the Vatnsdalur valley) were very similar independently of which of the two methods was applied. The bags were then tightly closed and kept in a cool place until analysed. About three different grass silage samples (two to five) were collected on each farm at a time. Samples were taken of grass silage from the cultivated home fields and usually from older and more recently cultivated plots, respectively.

A few farmers still adhere to the older practice of hay making; that is harvesting the forage as fully dry (> 85% dry). In those few (three) instances samples were collected by hand as described above.

For convenience all samples are referred to as *hay* whether fully dry (traditional hay) or only partially dry (grass silage).

Preparation of samples and metal analyses

All samples were dried at 65°C in a forced air oven for 48 hours. After being stabilized at room temperature samples were milled in a hammer mill to pass a 1 mm screen. Milled samples were weighed for metal analyses and at the same time weighed to estimate sample dry matter. Samples were then digested by boiling in concentrated HNO_3 (Merck, Suprapur) overnight and subjected to analysis. Analyses for Cu and Mn were carried out by ICP optical emission spectrometry using Spectroflame D sequential instrument (Spectra, Germany).

Three analytical samples were taken from each hay sample. Individual intra sample results did not differ more than ± 5%. The results were, respectively, the means of measurements of three analytical samples and are expressed as mg kg^{-1} dry matter. External standards containing certified reference material (Leaves of Poplar NCS CC 73350) were taken through the analytical procedure to confirm accuracy of measurements.

Determinations of the metals were performed at the Agricultural Research Institute, Reykjavík, Iceland.

Statistical analyses

One way analysis of variance (ANOVA) was used for comparison of respective metal concentrations in hay samples from farms in different groups. The Student-Newman-Keuls test was used for all pairwise comparisons. Linear regression analysis was used to test the relationship between copper and manganese concentrations.

RESULTS

Manganese concentrations varied more than tenfold and were in the range of 40-550 mg kg^{-1}. The highest concentrations (> 400 mg kg^{-1}) were found in hay from two farms in the western part of the country and the lowest concentrations (< 50 mg kg^{-1}) in samples from a farm in the southern part of the country (nr. 10 and 3, respectively, Figure 1). Copper concentrations varied fourfold from 4 to 16 mg kg^{-1}, the highest amounts were in samples from a farm in the western part of the country (nr. 10, Figure 1).

The results of copper and manganese analyses in samples from scrapie-free, scrapie-prone and scrapie-afflicted farms as well as the calculated Mn/Cu ratios are shown in Figures 2-5.

The concentration of manganese was significantly higher in hay samples from scrapie-free farms than in samples from scrapie-afflicted farms (186 mg kg^{-1} ± 94 and 129 ± 40 mg kg^{-1}, respectively). Manganese levels were also significantly higher in samples from the scrapie-free farms than in samples from scrapie-prone farms (151 mg kg^{-1} ± 63). Manganese concentration was in turn higher in hay from scrapie-prone farms than in hay from scrapie-afflicted farms but the difference was not significant for the number of observations carried out (Figure 2). Manganese levels were highest in hay from farms in Category 1 (scrapie-free farms located in areas never afflicted) and were significantly higher than in samples from farms in the other categories (P = 0.001 or less, not shown in Figure 2). Manganese levels in hay from farms in Category 2 (scrapie-free farms but located amongst scrapie-prone or scrapie-afflicted farms in scrapie-affected counties) were also significantly higher than in hay from scrapie-afflicted farms (P = 0.017) but not from scrapie-prone farms (P > 0.05) (results not shown in Figure 2).

The copper concentration was similar in hay samples from farms in all groups (on average 8-9 mg kg^{-1}) and the difference was not significant (P > 0.05, Figure 3).

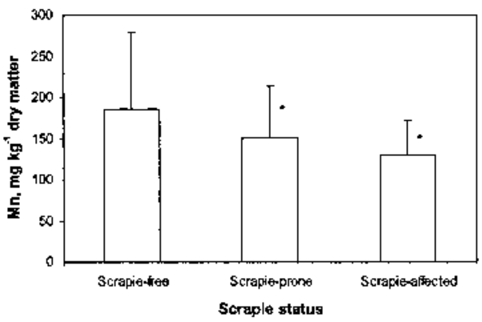

Figure 2. Mean concentrations of manganese (mg kg^{-1} dry matter± S.D.) in hay samples from scrapie-free, scrapie-prone and scrapie-afflicted farms.
* significantly lower concentrations than in samples from scrapie-free farms (P < 0.05).

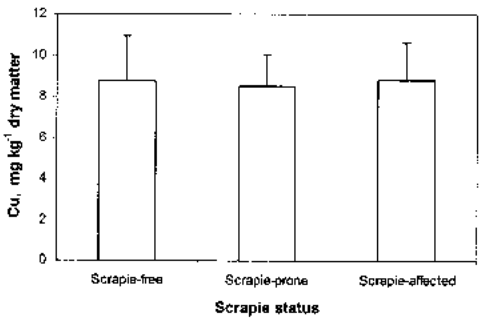

Figure 3. Mean concentrations of copper (mg kg^{-1} dry matter ± S.D.) in hay samples from scrapie-free, scrapie-prone and scrapie-afflicted farms.

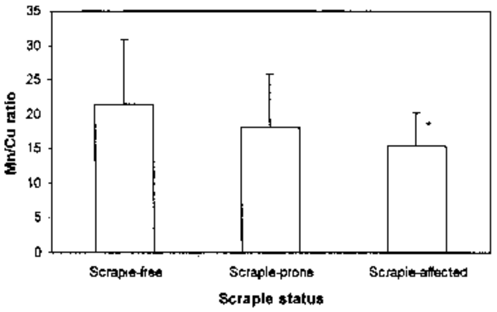

Figure 4. The calculated manganese / copper ratio in hay samples from scrapie-free, scrapie-prone and scrapie-afflicted farms (mean values ± S.D.). * significantly lower ratio than in samples from scrapie-free farms (P < 0.05).

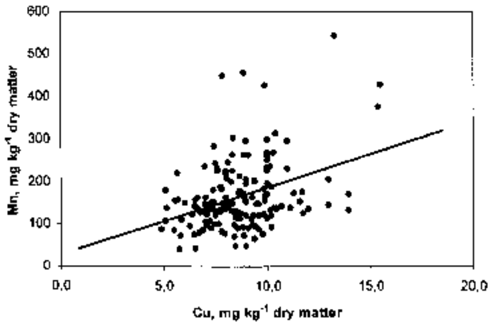

Figure 5. The relationship between manganese and copper concentrations in hay samples from all farms (P < 0.05).

The Mn/Cu ratio was significantly higher in samples from scrapie-free farms than in samples from scrapie-afflicted farms but not significantly higher than in samples from scrapie-prone farms (21.4 ± 9.4, 18.1 ± 7.8 and 15.0 ± 4.8, respectively, Figure 4). The Mn/Cu ratio for farms in Category 1 was significantly higher than for farms in the other categories (P < 0.05) and the Mn/Cu ratio for farms in Category 2 was also significantly higher than for scrapie-afflicted farms (P = 0.009) but not for scrapie-prone farms (P > 0.05) (not shown in Figure 4).

When subjected to regression analysis the copper and manganese concentrations were found to be positively correlated ($R^2 = 0.1288$) (Figure 5).

DISCUSSION

Although manganese concentrations were found to vary more than tenfold in the hay samples they were in general in the same range as is considered to be "normal" for plants (Adriano 2001). Manganese concentrations were also occasionally quite variable in individual samples collected on the same farm. This variability in our study was seemingly haphazard in nature and could not definitely be ascribed to a certain type of soil, but should obviously warrant further study. In this context note should be made of the fact that the availability of manganese in soil is highest at a pH below 6 (Pálmason 1972, Adriano 2001) and availability of the metal is not modified by forage quality but by its content (Lamand *et al*. 1977).

The highest levels of manganese were found in samples from scrapie-free farms located in areas never afflicted (Category 1). They were significantly higher than in samples from farms in the other categories. When manganese concentrations in hay from all farms characterized as *scrapie-free* (Categories 1 and 2) were compared with manganese in hay from *scrapie-afflicted* farms (Category 4) the difference was still significant and also when compared with samples from *scrapie-prone* farms (Category 3) (Figure 2). Taken together these results appear to indicate that high manganese concentrations, or high Mn/Cu ratios (Figure 4), are somehow connected with the absence of clinical scrapie in Icelandic sheep. These results are, however, opposite to the results of Purdey (2000).

Purdey determined manganese and copper in herbage (i.e. leaves, stem and flowerheads from the upper half of the plants that sheep feed on in the free) in Svarfadardalur valley, one of the scrapie-prone areas in northern Iceland (nr. 4, Figure 1), and in an adjacent valley to the southwest that is known to be essentially free of scrapie. In the scrapie-prone valley herbage contained more manganese and less copper than in the scrapie-free valley. He therefore concluded that high amounts of manganese and/or low amounts of copper, i.e. high Mn/Cu ratios, would favour the occurrence of clinical scrapie. Since our study is based on hay samples the two studies are not strictly comparable. It should also be noted that in Svarfadardalur valley, although generally a scrapie-prone area, there are both scrapie-free, scrapie-prone and scrapie-afflicted farms found within the valley. This is of fundamental importance as we have found significantly higher manganese concentrations in hay samples from scrapie-free farms located in scrapie-affected areas (Category 2) as compared with hay samples from scrapie-afflicted farms located amongst the former in the same affected areas.

Copper concentrations varied much less than manganese concentrations or fourfold and were in the same range as is considered to be "normal" for plants (Adriano 2001). There was no significant difference between copper in samples from scrapie-free, scrapie-prone or scrapie-afflicted farms (Figure 3). The fact that the Mn/Cu ratio was significantly lower in samples from scrapie-afflicted farms than from scrapie-free farms may also indicate that there was, in relative terms, abundant copper in hay on the afflicted farms (Figure 4). In summary, our results indicate that it is unrealistic to ascribe the occurrence of clinical scrapie in Iceland explicitly to low levels of copper.

Whether or not low Mn/Cu ratios in hay may be used as an index for the likelihood of occurrence of clinical scrapie, or conversely high Mn/Cu ratios for the absence of scrapie (Figure 4), it was found that the amounts of copper and manganese in the hay samples were significantly correlated (Figure 5). Any disparity in the relative concentrations of the two metals from optimal conditions, although still positively correlated, might then be indicative of proneness to scrapie in the sheep. Indeed, further studies based on a greater number of farms are needed in order to elaborate the predictive value of a high Mn/Cu ratio, or in particular high levels of manganese, in hay samples from scrapie-free farms for the absence of clinical scrapie on these farms.

Manganese is a major element in the Earth's crust whereas copper is rather a trace element. Thus the average concentration of manganese is twentyfold higher in the Earth's crust than that of copper (Ragnarsdóttir & Charlet 2002). In the hay samples from scrapie-free farms the average concentration of manganese was also about twentyfold higher in relation to the average concentration of copper (Figures 2, 3). In the blood of ewes the relationship between the two metals is reversed, i.e. the average concentration of copper is about twentyfold higher than that of manganese (unpublished results). Thus in stoichiometric terms it should be anticipated that manganese has the greatest effect relative to copper in the gastrointestinal tract. The preventive effect of high amounts of manganese (or of high Mn/Cu ratios) on scrapie as indicated by our results could therefore possibly be based in the gastrointestinal mucosa of the sheep, which, with its lymphoreticular tissue, is considered to be a major site of prion entry and replication (Brown 2001, 2003).

Glycosyl transferases are prominent among enzymes that require manganese for activity (Scott & Eagleson 1988). The normal prion protein (PrP) is bound to cell membranes by means of a glycosylphosphatidylinositol anchor and when converted to the pathological form (PrP^{sc}), the protein cannot easily be released from the cells by specific phospholipase cleaving of this anchor (Stahl *et al*. 1990, Brown 2002). If manganese is a necessary cofactor for the enzyme systems efficient in attaching the prion proteins to cell membranes in the gastrointestinal tract, and thus retarding or preventing their entry through the border of the mucosal epithelium, this could perhaps explain the absence of clinical scrapie on scrapie-free farms and especially on scrapie-free farms in scrapie-affected areas (Category 2). In this context it should also be mentioned that copper may facilitate the endocytosis of the prion protein (Pauly & Harris 1998), and administration of a copper chelator significantly delayed the onset of prion disease in experiments with mice (Sigurdsson *et al*. 2003). These results are therefore compatible with our findings in so far as high concentrations of copper in hay, relative to manganese, are seemingly favourable for occurrence of clinical scrapie.

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