Comparison of X-ray absorption spectra from copper-loaded bovine and ovine livers

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ABSTRACT

Copper toxicity and hepatic copper accumulation pose a serious risk to ruminant health and production. Differences resulting in susceptibility of the copper-handling mechanisms of cattle and sheep have been noted, not only in comparison to each other, but also in comparison to 'copper-tolerant' monogastric species. Ruminants appear less able to cope with rising liver copper concentration than their monogastric counterparts, and sheep appear less able to cope with elevated copper intake than cattle. X-ray absorption spectroscopy (XAS) was used to investigate the differences between these species with a high liver copper status. The X-ray absorption fine structure (XAFS) and X-ray absorption near edge structure (XANES) spectra indicated that the hepatic copper is most likely bound to metallothionein; consistent with monogastric species. Although there may be a role for glutathione as a short-term, intermediate copper buffer which may have more relevance to sheep than cattle. The potential that thiomolybdate bound copper can be stored in the liver could not be ruled out.

KEYWORDS

Copper toxicity; ruminant; glutathione; metallothionein; XAS

INTRODUCTION

Hepatic copper accumulation is prevalent in dairy cattle [1–3], with as many as 40% above 'normal' (>5618 μmol/kg DM) [4]. The prevalence of copper toxicity in both cattle and sheep has been reported in the farming press as a health concern [5–10], and is likely linked to the understanding of the complexities of copper interactions [11]. Copper toxicity culminates as a haemolytic crisis; when the hepatocytes, saturated with copper, cause the lysosome to rupture resulting in intravascular haemolysis followed by; haemoglobinuria, jaundice and methaemoglobinaemia [1,12–16], typically followed by the animal's death or euthanasia. Prior to haemolytic crisis there is usually slow hepatic copper accumulation, ranging widely from weeks to years [17–19]. Under practical conditions, hepatic copper loading may continue without showing any clinical signs [4] until a stress on the liver as simple as a dietary change, turning out to pasture, infection, or change in social group, triggers the haemolytic crisis [4,12].

The unique propensity of ruminants to suffer haemolytic crisis in comparison to monogastric species is likely linked to their relative lack of hepatic metallothionein, which is around three times lower [20–22]. Ruminants appear less able to respond to rising copper concentration through a limited metallothionein transcription response in the lysosome, which reaches a

plateau at less than half the concentration of monogastric counterparts [20,22–24]. Sheep appear to be more susceptible than cattle with a limited ability to increase biliary copper excretion in response to copper intake [16] and a more restricted capacity to accumulate copper bound to metallothionein in the liver [20,22–26]. Analysis of copper concentrations for animal status in practice is reported on an elemental basis. As such, it is presently unclear how copper is bound in the ruminant liver at elevated concentrations and if any species difference exists between cattle and sheep.

This work aims to compare the oxidation state and local co-ordination bonding of hepatic copper and to identify how copper is bound in copper-loaded ruminants. A secondary aim was to determine if copper was bound to metallothionein and/ or thiomolybdate.

MATERIALS AND METHODS

Liver selection and preparation

Bovine and ovine livers of high copper status (>8000 µmol Cu/kg DM) were identified through the use of the NUVetNA[1] database and previous trial work [27]. Surplus archived liver, in excess of 300 g which had been continuously stored at -20 ˚C, was prepared through the removal of the outer capsule and slices of ~1 mm thickness were cleaved from the central frozen tissue using a clean scalpel until ~8 g ww had been collected. Resulting cut liver sections were immediately returned to -20 °C prior to being subjected to freeze-drying (Modulyo® M143, Edwards, Leicestershire, UK) until no further weight loss was recorded. Samples were released from vacuum under nitrogen (PRISM, Air products, Surrey, UK) to prevent changes in oxidation state and the sample split into two aliquots (~1 g DM).

Copper concentration analysis

One aliquot (~0.5 g) was subject to wet acid digestion and ICP-MS to reconfirm the previously measured copper concentration. Approximately 0.1-0.2 g of freeze-dried liver was weighed directly into a Teflon microwave digestion tube (HVT50, Anton Paar, St Albans, UK) and incubated for 1 h with 3 ml ≥69% HNO_3 (Fisher Scientific, Loughborough, UK), 3 ml deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm), and 2 ml 30% H_2O_2 (Fisher Scientific, Loughborough, UK >99%), then digested for 45 minutes (10 min ramp to 140 ˚C, 20 min hold, then 15 min cooling at 55 ˚C) in a Multi-wave 3,000 microwave (Anton Paar, St Albans, UK), alongside blanks and reference material (1577c, Bovine Liver, National Institute of Standards and Technology, USA). Digested samples were transferred and then washed into 150 ml polypropylene tubes (Sarstedt Ltd., Leicester, UK) with 7 ml of deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm) and inverted to mix, prior to a 500 µl sample taken for ICP-MS (XSeriesII, Thermo Fisher Scientific, Waltham, USA). Samples and calibration standards were diluted (500 µl sample with 9.5 ml diluent) in a diluent containing 0.1% of a non-ionic surfactant (‘Triton X-100’ and ‘antifoam-B’; Sigma Aldrich, Dorset, UK), 2% methanol and 1% HNO_3 (Fisher Scientific, Loughborough, UK.

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>99%) including the internal standards Ir (5 mg/l), Rh (10 mg/l), Ge (50 mg/l) and Sc (50 mg/l). All calibrations were in the range 0-50 mg/l (Claritas-PPT grade CLMS-2 from Certiprep/Fisher Scientific, Loughborough, UK). Results were then calculated to pre-diluted concentrations and adjusted for background using blank correction.

Liver preparation for XAS

The second aliquot (~0.5 g) was prepared for XAS analysis by grinding in a nitrogen atmosphere into a homogenous powder using a pestle and mortar and sieving to <120 µm. Ground samples were nitrogen flushed, capped and sealed in the nitrogen atmosphere until subsequent analysis. The nitrogen atmosphere was attained through placing a 45 l polypropylene box into a fume hood with a continuous tapped nitrogen supply. The nitrogen supply was allowed to fill the box and a continuous supply of nitrogen flowed into the box during preparation to allow for displacement.

Powdered samples were carefully placed into the centre of a 7 mm square piece of polyamide film (25 µm thickness, DuPont, Stevenage, UK). Samples were compacted with a spatula in the centre to a thickness of 0.5-0.8 mm and covered with a second square of polyamide film secured at all 4 edges with silicone adhesive polyamide tape (Kapton®, DuPont, Stevenage, UK) to create a sealed compartment. The sealed sample compartments were then placed into the centre of a 20 mm aluminium sample holder (Diamond Light Source, Oxfordshire, UK) so that the sample aligned with the central 5 mm diameter window and the back screwed in place to secure the sample.

A standard of copper sulphate ($CuSO_4 \cdot 5H_2O$, Acros Organics, New Jersey, USA. 99-102%) was prepared into a 13 mm diameter pellet using a pelleting press (Atlas 25T, Specac, Kent, UK) containing 120 mg of reference compound (calculated by formula weight) weighed and mixed into homogeneity with dried cellulose binder in an agate mortar and analysed in transmission mode alongside a copper foil (I20, Diamond Light Source, Oxfordshire, UK) which was placed in-line with the incident beam with the standard.

XAS Analysis

XAS data for the samples was collected in fluorescence mode at beamline I20-Scanning at the Diamond Light Source (Oxfordshire, UK). Samples were subject to the X-ray beam monochromated by Si (111) crystals. The XAS spectra was collected at the copper (8979 eV) K-edge. Spectra were recorded in fluorescence mode using the 64-pixel Ge detector (Canberra, Oxfordshire, UK) at 90° to the incident beam. The incident and transmitted X-ray intensities were monitored using ionization chambers. During data collection, samples were maintained at a temperature of -196.15 ˚C using a LN2 cryostat (Oxford instruments, Abingdon, UK) to minimise the effects of radiation damage. For each sample, six to eight 30 minute scans for copper K-edge data were performed due to the dilute nature of the samples. Radiation damage of the sample was visually observed when repeated scans were taken at the same point, therefore the beam position (beam size at sample point HxV = 400 x 300 µm) was adjusted by altering the scan position using the x and y axis between each scan. The K-range of the XAS spectra was limited to 10 Å due to the presence of a zinc absorption edge at 9659 eV, which distorted the XAS signal beyond this point.

Data analysis

All XAS scans, transmission and fluorescence, were calibrated, aligned, background removed, rebinned and normalised using Athena v 0.9.25 (Ravel and Newville, 2005) generating X-ray absorption near edge structure (XANES) spectra. Where appropriate, scans were merged to reduce the signal to noise ratio at further Å. X-ray absorption fine structure (XAFS) spectra were extracted from $\mu(E)$ based on an estimate of the free-atom absorption. Data was extrapolated into K-space where the amplitude and phases were calculated using FEFF to fit $\chi(k)$ to a variation of non-linear least-squares curve fitting. Historically, this data type is difficult to evaluate in K-space thus, the data and the fit were transformed through Fourier transform to a radial structure function (R-space) for analysis. XAFS spectra were then peak fitted in R-space using IFFEFIT of Fourier transformed data analysed through Artemis v 0.9.25 (Ravel and Newville, 2005). Goodness of fit was determined where R values were <0.02 and Debye–Waller factors (σ^2) were ~0.02.

RESULTS

XANES

Analysis of the absorption energy (E^0) indicates that the average oxidation state of copper in both ovine and bovine livers lies between Cu^0 and Cu^+. The E^0 for hepatic copper was reported at 8981 eV specifically, 8980.8 for ovine and 8980.5 eV for bovine (Fig 1.). The spectral shape reported in these samples is consistent with that of three co-ordination. The intra-species variation in XAFS spectra were close to identical in overall shape and magnitude.

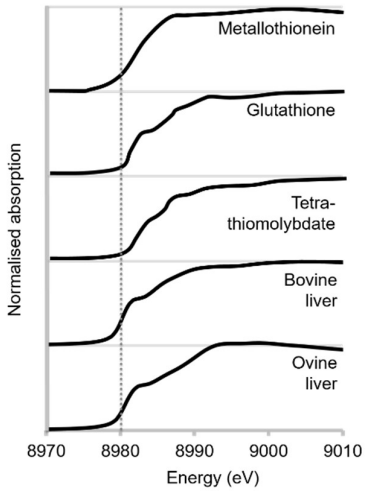

Figure 1: Cu K-edge XANES spectra in energy (μE) for ovine and bovine liver samples versus XANES spectra data reported by Abrahams *et al.,* [28] for metallothionein (6Cu:3ZnMT), Ceko *et al.,* [29] for glutathione and Zhang [30] for tetra-thiomolybdate treated Long-Evans Cinnamon (LEC) rat liver. Dashed line indicates E^0 value for copper K-edge at 8979.3 eV [28,31].

XAFS

Fourier transform magnitude of the sample data plotted in R-space showed some differences between the normalised data for the different species (Fig 2). The difference in peak magnitude and first peak shape between the species indicates that there is only small difference in the local co-ordination bonding which surrounds the copper atom in the livers of these two species. Analysis of the XAFS data plotted in K-space demonstrated the signal to noise ratio present past 8 Å would negatively impact model fitting results beyond this point. K^2 weighted XAFS data was peak fitted between 1-4 Å for each of the data sets across both bovine and ovine livers. The peak spectra differs most in magnitude in the first peaks, with key features appearing at similar distances. Good fits were created across both bovine and ovine liver data using the bonds indicated in Table 1.

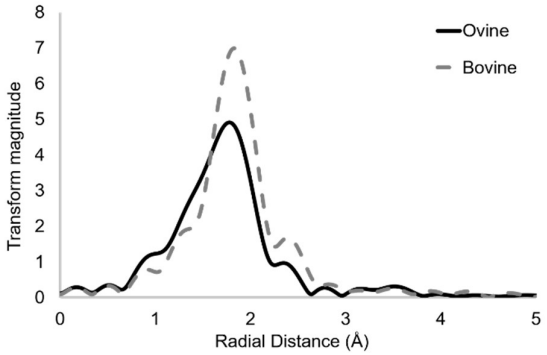

Figure 2: Normalised Fourier transformed data plotted in R-space for bovine and ovine livers.

Table 1: XAFS peak fit n co-ordination number, interatomic distances R(Å) and Debeye-Waller factors σ^2(Å^2) for best fit data for bovine and ovine livers. R-factor indicates goodness of fit (<0.02).

				Co-ordination shell				
Sample	**Path**	**n**	S_0^2	**R(Å)**	**σ^2(Å^2)**	E_0	**Reduced χ^2**	**R-factor**
Ovine Liver	Cu-N	1	1.2	1.972	0.016 (±0.007)	3.98	923	0.004
	Cu-S	3	-	2.248	0.014 (±0.002)	-	-	-
	Cu-C	4	-	2.89	0.015 (±0.003)	-	-	-
	Cu-C	3	-	3.837	0.022 (±0.009)	-	-	-
	Cu-C	8	-	4.257	0.029 (±0.006)	-	-	-
Bovine Liver	Cu-S	3	1.2	2.248	0.009 (±0.0004)	5.19	7496	0.01
	Cu-C	4	-	2.884	0.007 (±0.003)	-	-	-
	Cu-C	4	-	3.483	0.016 (±0.008)	-	-	-
	Cu-C	6	-	4.257	0.02 (±0.008)	-	-	-

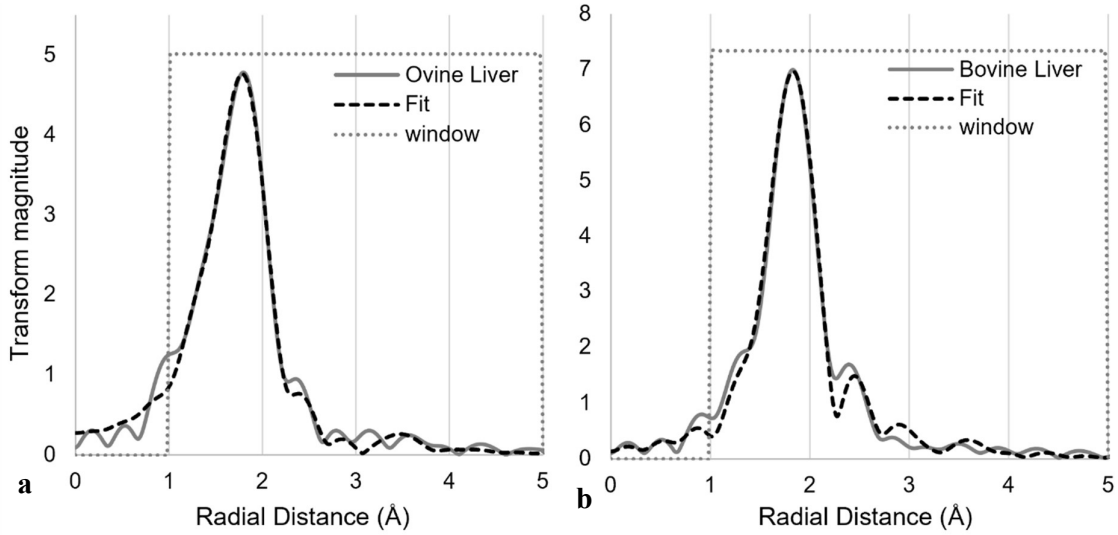

Figure 3: XAFS peak fitting to Fourier transformed data plotted by magnitude in R-space for ovine (a) and bovine (b) livers with high copper status. Solid line denotes sample. Dashed line denotes peak fitting used in the model.

Alternatively, a single scattering molybdenum could be fitted at 2.65 Å in place of the carbon in both the bovine and ovine livers; creating a good superficial peak fit in both species. The fit for the bovine livers (Table 2) needed to be constrained for S_0^2 and E_0 parameters indicating that this fit was not ideal. However, in the ovine livers the fit was statistically meaningful; generating an equally likely fit.

Table 2: XAFS peak fit n co-ordination number, interatomic distances R(Å) and Debeye-Waller factors σ^2(Å^2) for best fit data using molybdenum for bovine and ovine livers. R-factor indicates goodness of fit (<0.02). Asterisk indicates mathematically constrained factor.

				Co-ordination shell				
Sample	**Path**	**n**	S_0^2	**R(Å)**	**σ^2(Å^2)**	E_0	**Reduced χ^2**	**R-factor**
Ovine Liver	Cu-N	1	0.96	1.972	0.009 (±0.004)	3.63	817	0.003
	Cu-S	3	-	2.248	0.01 (±0.002)	-	-	-
	Cu-Mo	1	-	2.653	0.016 (±0.005)	-	-	-
	Cu-C	2	-	2.901	0.012 (±0.006)	-	-	-
	Cu-C	3	-	4.153	0.019 (±0.007)	-	-	-
Bovine Liver	Cu-S	3	1.2*	2.248	0.008 (±0.0003)	10*	3888	0.006
	Cu-Mo	1	-	2.653	0.01 (±0.001)	-	-	-
	Cu-C	2	-	2.901	0.016 (±0.006)	-	-	-
	Cu-C	1	-	4.153	0.038 (±0.062)	-	-	-

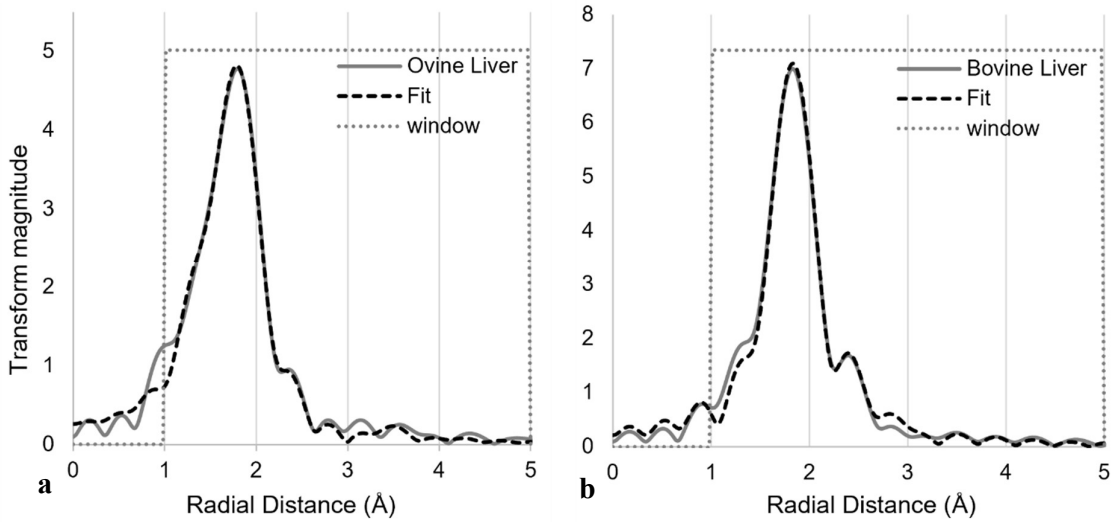

Figure 4: XAFS peak fitting including molybdenum to Fourier transformed data plotted by magnitude in R-space for ovine (a) and bovine (b) livers with high copper status. Solid line denotes sample. Dashed line denotes peak fitting used in the model.

DISCUSSION

The XANES spectra and E^0 values are consistent with copper in its monovalent (Cu^+) state in the hepatic compound of both cattle and sheep. Cu^+ has typical edge positions which range from 8983-8984 eV [29]. The absence of a feature at 8979.5 eV, indicative of the 1s f 3d transition in Cu^{2+}, also supports that copper is present in both species livers as Cu^+ [30]. Monovalent copper is the primary substrate for intercellular transport, despite its divalent (Cu^{2+}) form being more soluble [32]. Within the intestines Cu^{2+} can be reduced into Cu^+ through catalysis using reductases in the plasma membrane [13,33,34] and in most biological compounds Cu^+ is the prevalent form [13,35–39].

The XANES spectra alongside the XAFS peak fitting support three co-ordinated sulphur surrounding the copper atom in both species livers. The spectral shape reported in these samples is consistent with that of three co-ordination [40], and is consistent with the work of Abrahams *et al.*, [28] who found that copper metallothionein from pig liver was likely to be three co-ordinated, and the work of D'Angelo *et al.*, [41] which generated remarkably similar XANES spectra from three co-ordinated sulphur ligands and Cu^+. The fitted XANES spectra from the work of Abrahams *et al.*, [28] (Fig 1) shows remarkable similarity to the shape and structure of the sampled livers, although not identical, it does suggest a strong likelihood that the majority of copper is bound to metallothionein in the liver of copper-loaded ruminants. This is also consistent with the Hard-Soft Acid-Base (HSAB) theory; Cu^+ is a much softer Lewis acid (an ionic compound which can accept an electron pair) than Cu^{2+}, which is considered borderline between hard and soft, Cu^+ has a lower charge with a larger ionic radius [42]. The soft-soft interaction of Cu^+ and sulphur will occur more rapidly and form a stronger bond than Cu^{2+} with sulphur [43]. This is also seen in biological thiol complexes as Cu^{2+} complexes with glutathione and cysteine are much less stable than Cu^+ complexes

relating to the soft Lewis acid character of Cu^{+} [44]. Glutathione is known to reduce Cu^{2+} into Cu^{+} during complex formation [45]. This suggests that in Cu^{+}-thiol compounds, Cu^{2+} is first reduced to Cu^{+} in the presence of the thiol, and then bonds, as a result of the specific complexation of Cu^{+} [45].

The XANES spectra from liver of both cattle and sheep share distinct similarities with glutathione spectra from previous work (Fig 1) [29]. Similarity to copper bound tetra-thiomolybdate is discussed later in this work. Although, this is not an identical spectra it may be indicative of a small proportion of copper binding to glutathione in the copper-loaded ruminant liver. Copper is able to bind to glutathione, a small cysteine containing tripeptide, at a much lower affinity than metallothionein. Within the cell, glutathione appears to shuttle imported copper onto its specific chaperones for production of cellular cuproenzymes and cuproproteins [46,47]. Glutathione is present in almost every cell at concentrations 1-10 mM [48], and it may act as a low-molecular ligand to stabilise Cu^{+} in the intracellular environment [49]. Binding to glutathione could represent a potential buffering role, preventing cellular damage in the absence of sufficient metallothionein transcription [37,47].

The analysis of the XAFS and XANES spectra for the bovine and ovine liver samples was consistent with previous work in suggesting that copper in mammalian livers is predominantly bound to metallothionein. XAFS analysis of rat liver metallothionein has previously provided evidence of three co-ordination to copper in the first shell by single scattering sulphur atoms at copper-sulphur distances of 2.25 ± 0.03 Å with amplitudes and distances consistent with trigonal co-ordination [28,50], which creates a match with the sulphur fitted in this study at 2.248 Å in combination with a low magnitude single scattering nitrogen ligand at 1.97 Å in the ovine samples. Nitrogen is also present in cysteine residues and is consistent with other copper ligands in biological complexes [51]. The similarity between the current XANES spectra and copper bound to metallothionein from previous studies [28] is a close, but imperfect fit; this could potentially indicate a combination of metallothionein and glutathione. Both of which produce similar spectra due to the thiol co-ordination in the first shell surrounding the copper atom.

Cellular studies in other mammals have demonstrated that copper chaperones and copper-binding proteins handle cytosolic copper using cysteine-thiols. These are ideal ligands for intracellular Cu^{+} as they are able to create a stable Cu^{+}-thiol complex which protects the cell from oxidative damage while allowing transfer to copper acceptors for functional use [52]. The binding sites on the copper chaperones are often of a trigonal planar geometry and have co-ordination numbers of 2 or 3 consistent with the findings in the present work [52].

The lysosomal store of metallothionein bound copper is thought to act as a buffer for shifts in cellular copper concentration [13,33,35,53]. The expression of metallothionein, regulated by Metal Transcription Factor (MTF1), is rapidly induced in response to elevated copper in monogastric species [38]. But, the limited metallothionein response of ruminants prevents this rapid binding of copper when copper is elevated and inhibits the mobilisation of stored copper when copper is lacking, especially in sheep [25,26,47]. Despite this, there has been an observed rise in plasma copper where, the known copper antagonist, tetra-thiomolybdate was intravenously infused in sheep suggesting a small labile fraction of hepatic copper

mobilisation which is absent in cattle [54,55]. This response may indicate the potential for a short-term, lower affinity copper buffer in sheep that is not fulfilled by metallothionein.

The data in this work supports metallothionein as the most likely copper binding compound in both bovine and ovine livers of high copper status. However, the presence of a single scattering nitrogen pathway which was absent in the cattle livers could implicate glutathione as a short-term, lower affinity buffer for excess copper beyond the metallothionein carrying capacity in sheep; where their metallothionein synthesis and biliary excretion is unable to keep up with rising copper [16,26]. The subsequent accumulation of copper would cause cellular damage if it is not chaperoned; giving glutathione a potentially vital role in this species.

The data demonstrates that there is a notable difference between copper compounds in the bovine and ovine liver. XAS represents only the average structure surrounding the core atom, and as such, differences between the two species are harder to distinguish where they share a contributing major compound. Both livers carry a large degree of similarity, which suggests their major copper-containing compound is synonymous for both species. Although, biological differences in metallothionein transcription and their ability to cope with an increasing cellular concentration of copper could be responsible for the observed differences [16,20,23]. Previous work has established that in sheep metallothionein transcription declines markedly after birth, as a result of a reduction in metallothionein mRNA production, which also contributes to the observed inhibited response to rising copper.

In some high copper status livers, copper accumulation may not be a simple direct effect of oversupply. If systemic thiomolybdate absorption has taken place [11], the copper accumulating in the liver may be bound to a thiomolybdate complex, which has the potential to interrupt its function and reduce its bioavailability. The livers used in this work were abattoir recovery samples and samples submitted for diagnostic purposes, and therefore had not been previously fed controlled diets. ICP-MS analysis confirmed the livers had molybdenum concentrations (46±5, 42±7 µmol/kg DM bovine and ovine respectively) towards the upper end of normal range (5-52 µmol/kg DM bovine, 11-45 µmol/kg DM ovine[2]). Subsequently, it is not possible to determine if these samples had systemic thiomolybdate or another molybdenum-containing compound present. The XANES spectra obtained showed some similarity to previous data from Long Evans Cinnamon (LEC) rat livers exposed to tetra-thiomolybdate after hepatic copper accumulation, however, this was not enough to conclude its presence [30]. The current data also lacks the peak towards the top of the rising edge (~8986 eV) to be analogous, and the appropriateness of LEC rat data in this case can be debated. A Cu^{+} oxidation state, as observed in the present work, would be conducive to thiomolybdate binding alongside the XAFS peak fitting of three co-ordinated sulphur atoms also found in this work [30]. Thiomolybdate is known to bind to copper in three co-ordination through one thiol group and two sulphur atoms which bridge to molybdenum [56–58]. The molybdenum bond at 2.7 Å from the copper atom in the previous work was consistent with the molybdenum fitted in this trial. However, in this case it is unlikely that the bovine hepatic storage compound had any notable amount of copper-

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thiomolybdate since the second shell did not create a good fit with the molybdenum atom without mathematical constraint. However, the ovine liver did create a good fit for copper-thiomolybdate and despite the normal concentrations of molybdenum in the liver, the differences in XAFS spectra and inhibited metallothionein response in the species mean that the storage of some copper as copper-thiomolybdate in the ovine liver cannot be ruled out. The comparison of the bovine and ovine liver spectra to the tetra-thiomolybdate treated LEC rat spectra remain consistent with the previous conclusions of a three co-ordinated thiolate bond with the copper in its Cu^{+} oxidation state [30,56]. However, the similarity in thiolate bonding between metallothionein and tetra-thiomolybdate means that the contribution of thiomolybdate to hepatic liver accumulation in these species cannot be determined.

CONCLUSION

The present work found that the oxidation state and local co-ordination bonding support the notion that copper is predominantly bound to metallothionein in both ovine and bovine livers of elevated copper status. Although some small differences exist between the two species. The difference in XAFS peak fitting between the hepatic copper compounds in sheep and cattle suggested the presence of glutathione, with a greater potential role for glutathione as a short-term buffer in sheep.

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