JOURNAL OF Materials CHEMISTRY

Studies of equid hoof horn material by EPR spectroscopy

Barry C. Cope,[a] Lyn Hopegood,[a] Roger J. Latham,*[a] Roger G. Linford,[a] John D. Reilly,[b] Martyn C. R. Symons[a] and Fatai A. Taiwo[a]

[a]*School of Applied Sciences, De Montfort University, The Gateway, Leicester, UK LE1 9BH*
[b]*Royal Army Veterinary Corps, Defence Animal Centre, Melton Mowbray, UK*

EPR spectroscopy has been used to show that stable free radicals are formed when hoof horn material from equids is cut. The form of the EPR signal suggests that these are stable sulfur-centred radicals. The detection of melanin and thus the distinction between pigmentation levels of hoof horn is noted. Possible implications of these results are discussed.

Hoof horn material is remarkable in that it accommodates major stresses, both chemical and physical, to which the hoof capsule is exposed. Historically, hoof management has been regarded as a skill rather than a science and consequently there is a lack of information concerning structure–property relationships in both the scientific and veterinary literature. It has been suggested recently,[1] however, that multidisciplinary scientific studies of hoof horn involving, for example, biochemists, materials scientists and veterinarians may provide the best way of understanding the structure of this important natural material. The structure of hoof horn is highly complex,[1–3] but it can be described as a composite material based on tubules and intertubular material, whose biomechanical properties are very dependent on its structure. A major structural component of the material is the protein α-keratin, which exists as a triple helix, strengthened greatly by cross-links comprised of sulfur–sulfur bonds.

EPR (electron paramagnetic resonance) spectroscopy has been used to study aspects of other natural materials such as stratum corneum from rats[4] and human fingernail.[5,6] It was reported that when human fingernails are cut at room temperature, remarkably high yields of trapped free radicals were formed that were characterised by EPR spectroscopy.[5] The signals were analysed in terms of a species having highly characteristic features associated with a specific sulfur-centred radical. The structure of these radicals, which is controversial, is discussed below. These radicals are remarkably stable at room temperature, showing no detectable decay over periods of several weeks. It is possible therefore that EPR could provide information on the basic properties of hoof horn together with an understanding of neoplastic hoof horn conditions such as keratoma.

Experimental

Samples

Suitable clippings of hoof horn were obtained by farriers during regular hoof maintenance and sharp hoof cutters were used in order to prevent tearing of the sample. The samples were wrapped immediately in three overlapping layers of Parafilm (Parafilm "M" Laboratory Film, American National Can, CT 06836, USA) to make an airtight seal which moulded to the shape of the sample. The labelled, wrapped samples were then stored at 4 °C prior to examination. The portions removed for testing were from the midline dead centre site (MDC).[7]

EPR spectra

First derivative EPR spectra were recorded at room temperature (*ca.* 298 K) and 77 K (in liquid nitrogen) on an X-band Bruker 6/1 EMX EPR spectrometer operating at a modulation frequency of 100 MHz. Details of instrumental parameters are as stated for the respective spectra.

'Uncut' samples refers to long slivers (usually about $12 \times 2 \times 2$ mm) obtained from original hoof clippings supplied by farriers. 'Cut' samples were obtained by cutting the sliver along its length, into chips (approximately $2 \times 2 \times 2$ mm). Samples from both pigmented and non-pigmented hoof horn were examined.

Results and Discussion

Initial experiments have been carried out on samples from the inner and outer walls of both donkey and, for comparison, horse hoof horn. A typical EPR spectrum for a sample of the inner wall from a donkey hoof is shown in Fig. 1(*a*). Peaks are observed at g values 4.3, 2.003 (line width 9.3 G) and 2.380 (line width 290 G). The spectrum for a sample from the outer wall of the hoof is similar but the peak at $g = 2.380$ is absent, as shown in Fig. 1(*b*). When compared, the spectra for the inner and outer walls of horse hoof horn are similar in appearance, but they differ from those of the donkey hoof horn samples in that the peaks are at g values of 4.3 and 2.2 (broad, line width 1050 G). The EPR parameters of the main features of the spectra of hoof horn are summarised in Table 1.

The signal at $g = 4.3$ is due to Fe^{III} which has the configuration d^5 in the high spin spectroscopic state and high symmetry. This is attributed to haemichrome formation as a result of degradation of haemoproteins, predominantly from blood, whereas no signal is expected from haemoglobin in fresh blood containing Fe^{II} with its d^6 configuration (low spin $S = 0$, high spin $S = 2$ but high transition energy and hence no EPR signal). Its immediate oxidative product Fe^{III} is expected to give a

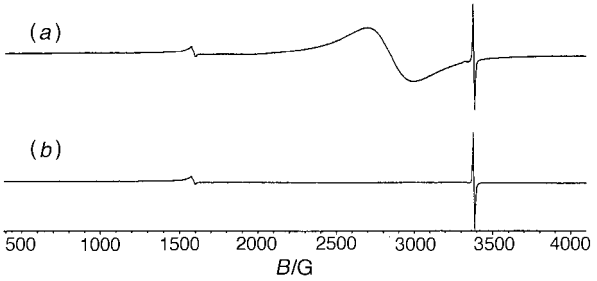

Fig. 1 First derivative X-band spectrum of donkey hoof at 77 K; (*a*) sample obtained from the inner wall section and (*b*) sample obtained from the outer wall section (instrument settings: centre field 2880 G, sweep width 5000 G, microwave frequency 9.480 GHz, microwave power 2 mW, modulation amplitude 4 G, time constant 40 ms, sweep time 84 s)

Table 1 EPR parameters of the main features in the spectra of hoof horn

g value	line width (lw) or A value/G	source
2.382 4.3	lw 290	Fe^{III}—high spin, high symmetry
$g_x = 2.005$ $g_y = 2.004_7$ $g_z = 2.002_5$	lw 5.1 (room temp.) and lw 7.5 (77 K)	melanin[a] (pigmentation element)
$g_{iso} = 2.005$	$A_{iso} = 94.21$	Mn^{II} d^5–low spin (non-protein)
$g_x = 2.061$ $g_y = 2.026$ $g_z = 1.998$	$a^H_x = 8.0$ $a^H_y = 8.0$ $a^H_z = 5.0$	R—S• (on hoof matrix fracture, this work)
$g_x = 2.061$ $g_y = 2.025$ $g_z = 2.000$	$a^H_{x,y,z} = 8.5$	R—S• (human finger nails, ref. 6)

[a]The melanin signal gives different line widths at room temperature and 77 K.

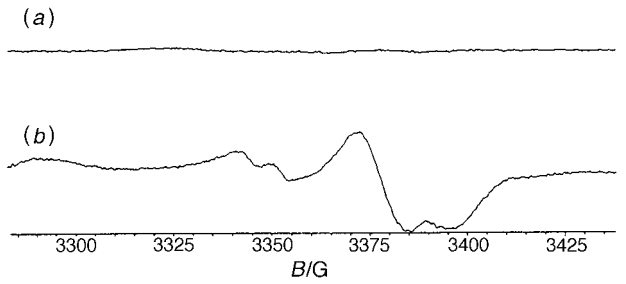

Fig. 2 First derivative X-band spectrum of white (unpigmented) donkey hoof at 77 K; (*a*) uncut sample and (*b*) cut sample (instrument settings: centre field 3381 G, sweep width 200 G, microwave frequency 9.495 GHz, microwave power 2 mW, modulation amplitude 1 G, time constant 40 ms, sweep time 84 s)

signal at $g = 6$ (high spin, low symmetry). The absence of this signal, and the appearance of its decay product at $g = 4.3$, would suggest a history of bleeding into the hoof from the blood supply. Another possible explanation would be the presence in the hoof material of iron species which appear as uptake from soil. This, however, would most likely be present in the form of oxides which are low spin Fe^{III} species, leading to broad lines on the spectrum corresponding to the existence of clusters. Trace amounts of Mn^{II} were also observed. This is attributed to adsorption from the soil as the EPR parameters (see Table 1) are more inorganically derived. Symons *et al.*[5] demonstrated that there is a signal due to the formation of sulfur centred radicals when human fingernail is cut and it is particularly interesting that there are no apparent differences between the EPR spectra of cut and uncut pigmented donkey hoof.† On the other hand, cut non-pigmented samples of donkey hoof do give rise to EPR spectra showing the presence of sulfur centred radicals in Fig. 2. The signals were well defined for white hoof horn material from non-pigmented hooves, but their presence was concealed for strongly pigmented samples because of overlap by very intense signals from the melanin radicals.[8] However, spectral subtraction showed that the sulfur-centred species were still formed after cutting.

There is no obvious explanation for the difference in signals from samples of pigmented and non-pigmented hoof. The role of melanin may be relevant, but the presence of free radicals in hoof material can be detected. These results may prove to be of more than academic interest as hoof horn material is deliberately cut by the farrier, and may undergo microfracture[7] during exercise, on impact with sharp, stony material, for example. EPR spectroscopy also may be useful in the characterisation of neoplastic hoof conditions such as keratoma.

† Although Symons *et al.* did not refer to pigmentation phenotypes in the sources of fingernail clippings, the element of pigmentation and its intensity would be expected to account for spectral differences.

Nature of the free radicals

In terms of chemical expectation, bond-homolysis is likely, such that protein strands are severed. It can be argued that these strands are so long that movement of the sharp blades between the ends of these protein strands is extremely unlikely and thus the only alternative process is bond-homolysis to give radical ends to the strands although some strands may be forced aside by the cutting blade without breaking. In the cutting process it is necessary, however, to break the cross-links and the main protein backbone to sever the strands. The S—S bonds are expected to break, as they are relatively weak, generating RS• radicals which were not detected in these experiments. However, an equal number of carbon-centred radicals, R• are also expected, and are also not detected in hoof horn. This is in contrast with the results for EPR spectra of crushed bone at 77 K.[9,10] Because of the high concentration of RS•S(R)R and RS•S(R′)R Symons[11] has made a strong case that a stable radical centre, frequently encountered in organo-sulfur chemistry, has the general structure RS•S(R′)R. However, at the time this structure was first proposed,[12] Gordy and co-workers[13] proposed that these radicals have the structure RSS•. The former structure is far more probable for the radicals formed herein, mainly because it is difficult to envisage any mechanism for the formation of RSS• centres and this assignment is accepted herein.

$RS•SR_2$ radicals are one of a range of 'three-electron' radicals often described as 'σ^* radicals' because the unpaired electron is in the S—S antibonding orbital. These bonds readily break to give RS• radicals and R_2S molecules. However, if this occurs within the rigid hoof matrix, rapid return to give the σ^* adduct again is to be expected. The results from these experiments indicate that there are significant differences between the EPR spectra of samples from the hooves of donkeys and horses, and also between pigmented and white hoof. The pigment, melanin, contains a relatively high concentration of occluded semiquinone-type radicals, but it is likely that the sulfur radicals are still formed on cutting, even though the EPR spectrum is not detected directly. These radical centres are well separated and cannot react together in the solid hoof horn material. It is not possible to distinguish any spatial differences in the concentration of these radicals. All regions are rich in keratin so this is not surprising.

As stressed above, deliberate cutting or microfracture occurs extensively, so there is probably always a low concentration of these radical centres present. If they can be detected without deliberate cutting, the signals could be of use in providing a measure of the extent of the matrix fracture. It is unlikely that these potentially active radicals present any problem to the hoof in themselves, because they are so firmly occluded within the material.

Although the microstructure of the hoof horn is not yet clearly understood, at the molecular level the predominance of cysteine would suggest a three-dimensional ordered network of –S—S– units such that fracture in the matrix of the fibres by cutting or breaking in any direction would always lead to bond homolysis and formation of an RS•. Depending on the locus of such fractures, RS• formation may be important in the understanding of processes within the hoof horn material. Formation of free radicals initiates a host of biological reactions, often resulting in lipid peroxidation and carcinogenesis.[14] Formation of RS• may be at, or close to, the coronary corium/papillae or the laminar corium, all of which are richly supplied by blood and nerve endings. In these tissues the RS•, though occluded in the rigid fibres, may involve formation of more

reactive species like $O_2^{\bullet -}$ and $RO_2^{\bullet}$, generally referred to as reactive oxygen species (ROS), which are initiators of lipid peroxidation and carcinogenesis, and may be involved in keratoma. Such an incident is a possibility during shoeing when nails are driven into the hoof. A misplacement across such sensitive zones in the laminae would pose potential dangers.

Procedures of hoof resection and other forms of general hoof surgery make significant cuts in the horn and it would be of great importance to note the formation of RS• in the horn.

The line width of the melanin signal varies with temperature. The increase in line width with temperature suggests an increase in the rate of tumbling of the melanin centres in a matrix which undergoes a phase transition. As the matrix becomes more fluid the mobility of the melanin increases and it tumbles faster, leading to a narrower line. Similar observations have been made for lipid–protein interactions in which the otherwise rigid lipid membranes become fluid with increasing temperature, affording increased lipid–protein interaction as reported in spin labelling studies[15] and hydration induced fluidity in stratum corneum,[4] for example.

Conclusions

EPR spectroscopy can provide useful information on a complex natural material such as hoof horn. Cutting or fracture of hoof horn may produce free radicals which are similar to those detected when human fingernail is cut.

The Donkey Sanctuary, Sidmouth, Devon is thanked for the funding of a studentship for L. H.

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Paper 7/06199D; *Received* 26*th August*, 1997