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Analytical Methods

Determination of Arsenic Poisoning and Metabolism in Hair by Synchrotron Radiation: The Case of Phar Lap**

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Phar Lap is a sporting icon and Australia's most famous racehorse. After his triumph in the Agua Caliente Handicap in Mexico, the world's richest race at the time, he died in agonizing pain under suspicious circumstances in 1932 while touring the USA. His legendary status has been associated with one of sport's most intriguing mysteries.

Phar Lap was born in New Zealand and was sold and transported to Australia, where he became a highly successful racehorse. Wins in 32 of his last 35 starts brought fame, but his popularity was not universal, and he was the target of a drive-by shooting from which he luckily emerged unscathed. In 1932 he competed in the Agua Caliente Handicap, which he claimed in record time. Following this great success he died a rapid, excruciatingly painful, and suspicious death in California. Speculative headlines, which reported murder or the misfortune of succumbing to acute colic, raced around the world. Necropsies were inconclusive, and cause-of-death theories included murder by poisoning, overdoses of tonics, colic, and more recently a newly identified form of bacterial gastroenteritis.[1] Evidence for the cause of death has remained elusive to date. Phar Lap's imposing stature was preserved by the exquisite taxidermy of his hide (Figure 1). His heart is preserved in the National Museum of Australia and his skeleton in New Zealand.

Herein, we explore the possibility that a chronological record of any poisonous substances ingested by Phar Lap could be locked in strands of his hair, which incorporates xenobiotics from the blood supply during its growth through the dermal papilla and can reflect a retrospective time span along the hair length. Analysis of hair provides results that are indicative of environmental exposure and/or ingestion of specific substances,[2] though the results may be compromised by exogenous contamination.[3] Fortunately, acute exposure may be identified along the length of a single hair since a longitudinal time-resolved signal is provided.[4] It should be noted that in Phar Lap's case, his hair was suspected of being further compromised by the use of arsenic during the preservation of his remains.[5]

Figure 1. Phar Lap's preserved hide, which is on display at Museum Victoria, Melbourne, Australia.

A region of hide was excised from Phar Lap's mane, and individual hairs, which had an intact bulb and root sheath were dissected from this sample. Only hairs identified as being in an anagen growth phase were used, in order to ensure that these hairs were actively growing at the time of death. Four whole hairs (Figure 2a) were mapped longitudinally with a synchrotron X-ray fluorescence microprobe (XRF) for excellent elemental sensitivity with high resolution and nondestructive analysis (ca. 10×10 micrometers, 12 keV beam at PNC-CAT, Advanced Photon Source, in ambient conditions).[3c] The arsenic map indicated low arsenic concentrations throughout the hair. In the subcutaneous region, an intense band existed in all hairs at the same location (Figure 2b). The longitudinal distribution (Figure 2c) related to this band exhibited a profile strongly indicative of an increase and subsequent decay following administration and

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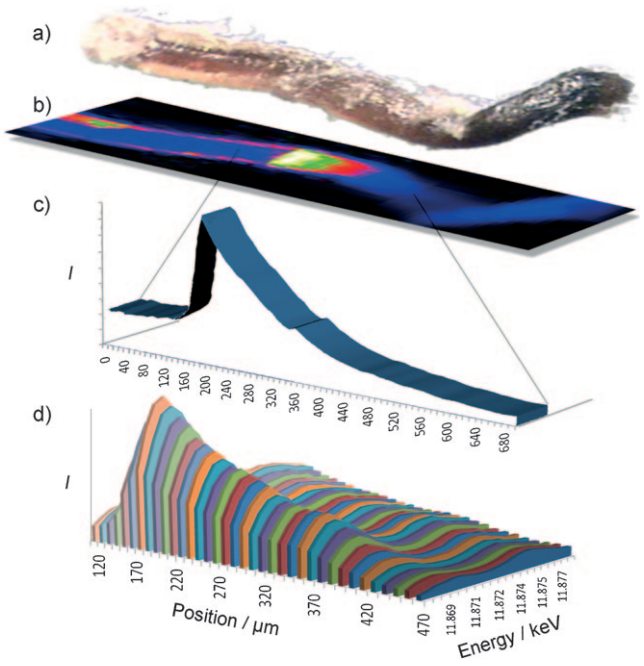

Figure 2. Analysis of Phar Lap's hair. An optical image shows the root end of one hair with the root sheath intact (a). The hair was analyzed with an X-ray microprobe that imaged the internal arsenic distribution (b). The longitudinal profile reflects the hair growing outwards as the arsenic is metabolized (c), while 2D XANES mapping reveals the variation in arsenic speciation ratios (d).

metabolism/excretion of arsenic.[6] Maps over portions of hide and distal hair regions (data not shown) revealed arsenic in all tissues, but at much lower concentrations than in this subcutaneous region. The wide extent of the arsenic distribution shows that Phar Lap's hide had been treated with an arsenic-based preservative.

Horse tail hair has been measured to grow between 390 and 1260 micrometers per day (compared to ca. 330 micrometers for human head hair).[7] The growth of mane hair may be within this range, in which case a 500 micrometer segment would correspond to between approximately 10–30 hours of growth. In swine tests, arsenic in blood plasma has a half-life of less than approximately 5 hours after ingestion and can be entirely removed within around 24 hours.[6a] In the treatment of horses with disodium monomethylarsonate, a half-life in plasma was found to be 44 hours after five consecutive days of intramuscular dosing.[8] This value is considerably longer than that from the swine tests, but reflects the method of administration. The longitudinal chronological profile in Figure 2 reflects a time line consistent with ingestion and metabolism of arsenic.

X-ray absorption near edge spectroscopy (XANES) is a powerful tool that easily discriminates between the chemical environments in which arsenic might exist within a biological context.[9] Arsenic XANES was performed on the same experimental apparatus as the mapping. Spectra were acquired following the protocol utilized previously to assess photodamage during analysis.[10] In brief, scans spanned an energy from 11.866 keV to 11.877 keV in 0.25 eV increments requiring 45 s, comprising 1 s per point plus overheads. This procedure was performed nine times before acquisition over an energy range of 11.717 keV to 12.110 keV, which required 326 s to include an in-line gold standard for energy calibration. A series of XANES spectra were acquired in a sequential manner along the same arsenic-rich region with an energy range confined to the white-line region, thus minimizing beam-induced alteration of the chemical state of arsenic (Figure 2d). The 3D plot shows the relative absorption of the X-rays as a function of incident energy and longitudinal position. The two peaks across the energy span indicate the presence of two arsenic species; in this case, the results are consistent with an As^{III} thiol complex (at the lower energy) and arsenate. On the longitudinal spatial scale, both peaks have a profile similar to the relative arsenic concentration in Figure 2c. However it is interesting to observe that the relative concentrations of the arsenic species vary, with the arsenate peak eventually dominating at the proximal end of the distribution.

The nature of hair growth results in incorporation and subsequent immobilization of blood constituents into the metabolically inert structure, thus the length of the hair can indicate a time course. Analysis of a portion of hair can reflect blood concentrations at the time when that segment of hair was formed, thus longitudinal analysis is effectively interchangeable with a retrospective time line. Assuming that the arsenic in Phar Lap's hair is from ingestion, the results suggest the majority of arsenic is contained in a compound similar to arsenic glutathione. The smaller and longer-lived arsenate peak may reflect a metabolite that has a greater half-life and becomes dominant at the tail-end of the decay profile (that is, higher concentrations are maintained over a greater longitudinal distance).

In chromatographic analysis of digests coupled with mass spectroscopy, arsenic glutathione will typically elute as As^{III} unless special care is taken.[11] Such methods have measured that up to approximately 98% of arsenic in human hair can be inorganic, with around 1.66–8.3 times as much As^{III} (which can include arsenic glutathione) as As^{V}.[12] These data provide evidence that the arsenic in Phar Lap's hair is the result of ingestion. The dominant species in the hide, distal regions, and the taxidermy samples was As^{V} (Figure 3).

It was also interesting to note that the arsenic speciation in the root region of Phar Lap's hair did not suffer from beam-induced alteration. In contrast, seven different hair samples that were used for comparison from various taxidermy specimens, including two horses from the early 20th century held at the National Museum of Scotland, did show beam-induced photoreduction.[10] The hide and all taxidermy specimens studied were very rapidly photoreduced from arsenate to arsenite. The distal hair segments were slightly different, and were rapidly reduced from arsenate to a glutathione-like state with a minor arsenite component. The results suggest that the ingested arsenic does not undergo significant photoreduction while that associated with taxidermy does. While the reasons for this difference are unclear, the effect may be used to discriminate between the two modes of arsenic incorporation.

Hair cross-sections microtomed to 1 µm thick from the arsenic-rich region were prepared and analyzed with a

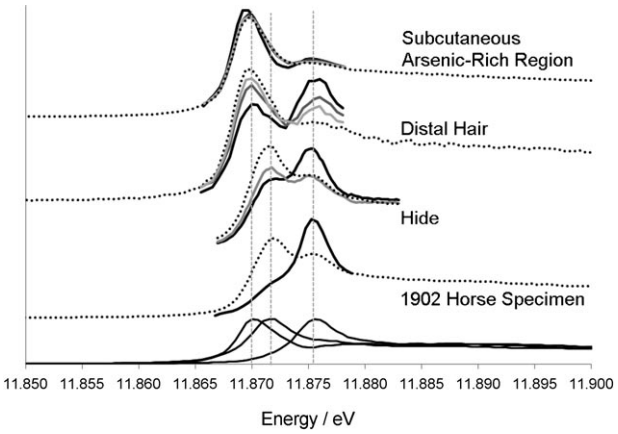

Figure 3. Arsenic K-edge XANES spectra. The solid black lines represent the first scan, with subsequent scans indicated in gray and finally as dotted lines. The vertical lines represent the white line energies of standards (following the procedure of Smith et al.[9a]) arsenic glutathione (11 870.0 eV), arsenite (11 871.7 eV), and arsenate (11 875.3 eV), which were ground and diluted with boron nitride. Arsenic in the hair in the subcutaneous region did not undergo photoinduced changes and indicates that an As^{III} thiol complex dominates the speciation. The distal hair, hide, and all taxidermy samples experienced very rapid photoreduction from an initial arsenate state. The observation that different samples resulted in different photoreduction products is noteworthy. This result is possibly due to the sulfur content of pheomelanin in Phar Lap's hair as opposed to the other samples.

400 nm, 12 keV beam (2ID-E, Advanced Photon Source) in a He atmosphere at room temperature as previously described for taxidermy hair specimens.[10] These images (Figure 4) show a strong physical correlation with sulfur in the cysteine-rich cuticle; this result is consistent with other observations.[6b] Arsenic is also distributed within the cortex and in the outer root sheath. Arsenic enrichment in the cuticle after consumption, as well as at other cortical localizations and within the medulla, has been confirmed by imaging mass spectrometry.[13] A section from above skin level reveals significantly less arsenic (Figure 1 in the Supporting Information).

The longitudinal XANES variation shows differences in arsenic speciation along the hair. Figure 4 shows that arsenic is associated with different parts of the hair composition, with the glutathione-like structure presumably correlated with

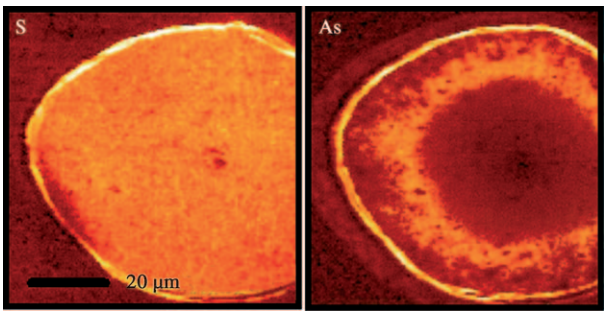

Figure 4. Micro X-ray fluorescence maps of sulfur and arsenic. Arsenic has a strong affinity for the sulfur-rich cuticle layer as well as within the cortex, and to a lesser extent the root sheath.

sulfur-rich proteins. However, there is also arsenic that is not correlated with sulfur, particularly in the outer root sheath. The longitudinal analysis of arsenic speciation may reveal different incorporation processes. The transverse arsenic distribution changes longitudinally; based on the arsenic map in Figure 2b, the cuticular association diminishes towards the bulb. This result is important for understanding longitudinal profiles as it reflects the fact that there are different mechanisms for the preferential incorporation of metabolites into different hair regions. These results could reflect two possible processes: Firstly, metabolites with a greater blood-concentration half-life preferentially interact and become incorporated within cortical structures, that is, a speciation dependant interaction. Secondly, different incorporation mechanisms deposit arsenic in different locations over the same time period, that is, physically distinct deposition and association.

At the time of Phar Lap's demise, he vomited blood and suffered from abdominal pain, high temperature, gastrointestinal inflammation, and ulcers, which are consistent with all cause-of-death theories proposed. A point of contention has been that he did not have diarrhea, however, this symptom is not universally encountered in humans, horses, and cattle.[14] Even in modern veterinary practice, the diagnosis of arsenic poisoning is not easy.[15] There are several documented accounts of horses dying from arsenic poisoning;[16] in one instance, if arsenic had not been identified in the animal's feed, then poisoning would have been indistinguishable from colitis.[16a]

Arsenic concentrations in the organs of horses are typically only around 10 ppm in fatal cases, that is, if they can be detected at all.[15] Small quantities of arsenic were measured in Phar Lap's vital organs. This result supports the poisoning argument, but was dismissed at the time as the concentrations were too low. The suggestion of a fatal dose of arsenic has been highly provocative, and has led to conspiracy theories that raise questions of foul play and involvement of gangsters and illegal racing syndicates. Other theories include a bad "green feed", that is, consumption of monosodium methanearsonate, sodium and potassium arsenite, or thioarsenites that were used as herbicides before 1947. However, other horses had access to the same foliage but did not fall ill. An unfortunate case of misadventure or a simple dosing error provides another explanation. Arsenic-based tonics were common in the racing industry for boosting the oxygen-carrying capacity of the blood, improving stamina, and stimulating appetite to improve an animal's ability. The tonic book of Harry Telford (Phar Lap's trainer), held by Museum Victoria, lists an arsenicalis-based tonic that is "a great tonic for all horses" (Figure 2 in the Supporting Information). Extended XRF mapping of Phar Lap's hair was performed to investigate any regular dosing of arsenic. Faint bands moving distally along his hair were observed, but the bands were not as intense as the band studied in detail here.

Many complexities in the analysis (and interpretation of results) of arsenic in hair exist,[17] yet the results presented here show arsenic distribution and chemistry consistent with ingestion of a large dose of arsenic just prior to death; a

finding that is consistent with his symptoms and autopsy results. Many questions, such as the source of the arsenic, are beyond the scope of this work; it is likely that the events of Phar Lap's last days will remain entrenched in mystery.

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