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TECHNICAL COMMENTS

Identifying Species of Origin from Prehistoric Blood Residues

In the archaeological world, some excitement (*1*) and controversy (*2*, *3*) has been generated by reports (*4*–*6*) that hemoglobin from blood residues could survive intact for tens of thousands of years on stone tools and that the species of origin could be identified. One proposal (*4*) suggested that hemoglobin recovered from blood residues on artifacts could be crystallized by a simple procedure and the species of origin determined by microscopic examination of the crystals. Such a technique would be of great interest in establishing ancient hunting patterns, animal migratory movements and in analysis of human blood residues (*1*–*6*).

The basis for the proposed procedure appears to be an exhaustive study conducted by Reichert and Brown at the turn of the century (*7*), which established that hemoglobin from the blood of many vertebrates can be crystallized. These authors showed that when hemoglobin from a single species was crystallized, often several different crystal forms could be obtained, but within each crystal form the crystals were isomorphous (that is, each example had the same symmetry and unit cell dimensions). Further, it was shown that crystals from two different species were often easy to distinguish from one another, while those from similar species were often similar, sometimes differing only slightly in unit-cell dimensions (*7*, pp. 325–327). These results suggest that animal species might be identified by examination of crystals of their hemoglobin.

The proposed procedure (*4*), however, as applied to material obtained from prehistoric artifacts, has been challenged on two major fronts (*2*, *3*): First, does microscopic examination of crystals permit unambiguous identification of the species of origin? Second, does protein survive in the intact, correctly folded native state in quantities sufficient to provide well-formed crystals that would be large enough to analyze?

The first problem arises from a consideration of allowed crystal morphologies. There are 32 point groups (the symmetry of polyhedra), but protein crystals have only the symmetry of the 11 proper ones, that is, only those lacking operations of inversion and reflection. Likewise, of the 230 crystallographic space groups, only 65 are available for protein crystals. These limitations are severe in the face of the number of extant and extinct species having hemoglobin, and in many instances it may be impossible to distinguish crystals from different species by optical methods. For example, upon examination of the family *Canidae*, crystals of hemoglobin obtained from the blood of 10 distinct species of dogs, wolves, and foxes were studied by Reichert and Brown, who reported (*7*, p. 265) that "[a]ll members of the family furnished oxyhemoglobin crystals which closely resembled each other, so that the differences between species were not readily made out."

In order to identify the species of origin of a hemoglobin crystal unambiguously by the proposed technique (*4*), the minimum requirement is a complete and accurate analysis of the interaxial angles, and the axial length ratios of the unit cell. X-ray diffraction is the method of choice and can yield accurate information about space group and cell dimensions from a single well-formed crystal (*9*). A possible drawback is that the minimum protein crystal size for x-ray analysis is about 100 × 100 × 100 μm (*9*), which contains about 0.5 μg of protein. Optical analysis is much more painstaking, but may be possible with smaller crystals. This requires the use of an optical goniometer, with which one must measure the angles between crystal faces to an accuracy of about 10 arc minutes. One must also determine the relationship of optical axes to crystal faces and edges (*7*, pp. 146–148). From this information, one can often deduce the crystal system and determine the relative unit cell lengths. Usually, this would require several well-formed single crystals and, of course, they must all be of the same material.

Optical examination of crystals cannot, however, identify the space group. At best it can only provide crystal system (and perhaps point group), axial length ratios, interaxial angles, optical axes, and birefringence sign. This compounds the problem raised by the limited number of ways in which proteins can crystallize.

Armed with data obtained by either x-ray or optical analysis, one can then consult a compendium (*7*) of crystal forms obtained from the hemoglobin of known species and attempt to identify the species of origin. For several proteins [citrate synthase, for example (*10*)] crystals from species as divergent as chickens and pigs are isomorphous, and rigorous optical analysis would not distinguish between the two species of origin. The required measurements of crystal system, unit cell axial length ratios, and interaxial angles have not been reported (*4*–*6*), nor has it been demonstrated that the crystals obtained from putative blood residues on stone tools actually contained hemoglobin. One cannot be confident of the correctness of the species identification in these instances.

The other major problem arises from the amount of material required for crystallization and the fact that crystallization of proteins generally requires intact, correctly folded protein. While dry protein samples can retain biological activity for years, a large percentage of hemoglobin will be degraded in samples taken from ancient artifacts (*6*). In controlled experiments simulating burial conditions (*3*), blood protein was usually not detectable by sensitive chemical analysis after several weeks of contact with damp soil.

While the actual degree of protein degradation varies from sample to sample, it is possible to estimate the minimum amount of intact, native protein required for crystallization. Most protein crystals are about

50% solvent (*11*) and thus contain about 400 to 500 mg of protein per milliliter. Some crystals that may be large enough for rigorous optical analysis are shown in figure 3 of reference (*5*, p. 454). A single crystal 15 × 10 × 5 μm (*5*) would contain about 0.4 ng (10^{-9} g) of intact protein, and several would usually be required for rigorous analysis. Therefore, statements that this analysis can be conducted with picograms (10^{-12} g) of protein (*6*, p. 51) appear to be unjustified.

An optical absorption spectrum of a solution made from crystals, or direct microspectrophotometry, could be used to determine whether crystals do in fact contain hemoglobin. Conversely, crystals obtained by the proposed procedure (*4*) from a *clean* archaeological artifact would not be of hemoglobin. Alternatively, one could make at least a partial determination of the amino acid sequence of the protein in a crystal. As this is sufficient to identify hemoglobin and often the species of origin as well, the proposed procedure of microscopic analysis (*4*) is moot.

S. James Remington
Department of Physics,
University of Oregon,
Eugene, OR 97403–1229, USA

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Response: Three lines of evidence indicate that hemoglobin (Hb) may survive in blood residues. First, red blood cells and other structural features consistent with tool use and deposition of blood and other organic materials during killing and butchery have been microscopically documented within residues (*1–3*). Second, with the use of fundamentally different methods (*2–5*), residues have been shown to contain a variety of proteins, including Hb, immunoglobin G, and albumin. Third, anion-exchange, high-pressure liquid chromotography (HPLC) analysis of 90,000-year-old residues (*5*, p. 27) removed in comparable samples from tool surfaces appeared to contain Hb in amounts of between 7.25 and 12.97 μg. A similarly sampled 6-year-old thin smear of control blood contained 19.88 μg of Hb. With the use of solutions of pure Hb and other pure proteins as a reference (*6*), we identified Hb in all samples by the co-occurrence of absorbance of peaks at 280 nm (indicative of proteins) and 410 ± 5 nm (the soret band indicative of heme) at the same elution times. Additionally, the characteristic Hb band position (pH 6.95 to 7.00) on isoelectric focusing gels was observed (*7*).

Do the crystals grown from extracts of prehistoric residues contain hemoglobin? The current method (*8*) was adapted from that of R. K. Washino (*9*) because it used Hb in quantities comparable with prehistoric residues, that is, solutions 10- to 100-fold more dilute than are routinely used in the crystallization of proteins. Crystals grown in solutions that (i) contained Hb from different individuals of the same modern animal species or (ii) were resampled from individual prehistoric residues were repeatedly consistent in form and growth characteristics. In the absence of residues (that is, on blank controls, samples with no residue on the tool surface, or samples from post-use flake scars), no crystals other than buffer salts formed. Because of evaporative concentration, buffer salts precipitated in the later stages of the procedure and were clearly identifiable as such (*2*, *3*, *9*, p. 15). Soil minerals in residues have been readily identified, and crystals were grown from Hb solutions that had been filtered to remove all particles greater than 0.22 μm (*3*). Archaeological and natural soils were tested, and only soil-mineral and buffer crystals were observed (at butchering sites, however, blood might have been preserved in soils).

Microspectrophotometric observation of diagnostic ultraviolet and visible absorption spectra has been unfeasible because of the small size and number of crystals grown by the current method. Hemoglobin has recently been recovered in bones of a *Diprotodon* sp. approximately 100,000 years old. These large marsupial bones permited analysis of gram amounts of cancellous tissue and produced diagnostic crystals (*10*). Anion-exchange HPLC has given the clearest evidence so far that these crystals contain Hb: a diprotodon bone sample (150 mg) was hydrated with ultrapure water and, by using methods described in (*5*) and (*6*), was separated on the column, and fractions were collected. The Hb peak (co-eluting $A_{280\text{ and }410\pm5\text{ nm}}$) was contained in 6 ml of column eluate, reduced to 60 μl by ultrafiltration, and crystallized. Crystals identical to those grown from an aliquot of the original unseparated sample solution were observed. The other concentrated fractions produced only buffer salt crystals.

The anion-HPLC diprotodon Hb fraction was calculated to contain 1.16 ng of Hb in 6 ml of raw elute. After reduction to 60 μl, the concentration was 19.3 pg/μl (if one assumed no major losses or degradation during concentration). Experimental values (*11*) indicated that the smallest optically distinctive crystal (roughly 100 μm^3) would contain about 50 pg of Hb (0.5 pg/μm^3) (*2*). Remington's example of a 750-μm^3 crystal containing about 0.4 ng (again, 0.5 pg/μm^3) approaches the maximum single crystal size observed when we used the current method. A 20-μl volume (containing at most 386.6 pg) of the concentrated HPLC peak solution yielded two clearly identifiable crystals matching the original crystal form, and their size (300 μm^3) was consistent with the calculated density of Hb per cubic micrometer. Other crystals were grown from the HPLC Hb elution peak and washed in phosphate buffer; a second HPLC separation of these redissolved crystals again showed co-occurring $A_{280\text{ and }410\text{ nm}}$ absorption peaks indicative of Hb. Preliminary laser desorption mass spectrometry results showed the washed and redissolved crystals to contain proteins of masses suggestive of globin subunits (15.1 kD) (*10*).

Can the crystals be used for determination of the species of origin? Some proteins have yielded crystals isomorphic between highly divergent species, but the mutationally driven and functionally permissible variety of amino acid residue substitutions in Hb makes it an ideal molecule for species determination. The crystal form is influenced by the concentration of Hb, buffer salts, and solution parameters (*12*, *13*, p. 151); thus any compendium of crystals must be constructed under similar crystallization conditions. Variation in crystal habits has reflected apparently random amino acid substitutions (*14*); therefore the final crystal habit may or may not be consistent with the degree of genetic relatedness. Members of some genera produced Hb crystal forms that precluded differentiation to species (*11*); however, these crystals were diagnostic at higher taxonomic levels (for example, for Canidae or Scuridae). Species of some genera (for example, *Ursus*) produced exactly similar initial crystals, which over time developed into a second crop of diagnostic crystals having different forms (*1*, figure 2; *9*, p. 116; *11*, pp. 259–263). Formation time and shape changes during the formation process reflect the solubility of Hb in the buffer relative to evaporative increases in salt concentration, thereby adding other diagnostic indicators. Effects

of soil pH, soil particles, and humic acid soil constituents have been experimentally determined (*10*) not to influence crystal formation or form. Washino (9, p. 73) observed that samples from only 3 of 100 mammalian species crystallized when distantly related animals had similar crystal morphology, but found these species were still distinguishable by their characteristic growth times. The combination of morphology, length of time to form crystals, and distinctive twinning (*15*, p. 224) appeared to be sufficient to identify species of origin for a wide range of species (*2*; *3*; 8, p. 454, figure 3; 9, pp. 12 and 73). Successful blind and double-blind trials (*1*, *10*) reinforced this view. Using Hb crystallization for prehistoric tool residues appears to reduce the potentially large number of species to a very much smaller set of culturally significant and (often) regionally separate species used by prehistoric and indigenous peoples.

As Remington suggests, amino acid micro-sequencing, x-ray diffraction, or optical methods would be necessary to distinguish between apparently identical crystals. Immunological methods have been useful where highly specific antibodies are available. Analysis of ancient genomic DNA from blood residues appears at present to be a viable alternative (*2*, *4*, *16*), and initial comparisons between DNA and crystallization results have yielded consistent species identifications (*2*, *3*). Obviously, some of the questions raised by Remington remain unresolved and must be clarified by further research. In my view, however, Hb crystallization will prove to be a useful technique in an array of developing methods for the identification of the animal sources of prehistoric blood.

Thomas H. Loy
Division of Archaeology and Natural History, Research School of Pacific and Asian Studies, Institutes for Advanced Study, Australian National University, Canberra, 0200, Australia

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