IMMUNOLOGICAL ANALYSIS OF A MUSKET BALL FROM THE FORT CLATSOP SITE, OREGON.

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Scientific research carried out by reputable scientists in North America and Europe in the past 10 years clearly show that organic residues recovered from lithics, ceramics, coprolites and soils, can be identified through he use of chemical and molecular biological techniques. Although these techniques are used with confidence in the 'hard sciences', their application to archaeology is relatively new and, as such, there are still problems areas that need to be resolved (Thomas 1993). However, it is clear that data obtained by the use of these modes of analysis can provide unique insight into the evolution of animals and humans, prehistoric environments, prehistoric diet and subsistence, and tool function, information that cannot be obtained by other means.

Although questions concerning the preservation and viability of ancient protein materials have recently been made (Eisele 1995, Fiedel 1996) evidence shows that proteins are extremely hardy molecules. Proteins have been recovered from shells of planktonic foraminifera dating between 2 and 4Ka BP (Robbins and Brew, 1990), from dinosaur bones (Miller and Wyckoff 1968) and dinosaur eggs (Voss-Foucart, 1968), from frozen mammoth dated ca. 40,000 BP (Prager *et al.* 1980), and in 1500-year-old bones (Cattaneo *et al.* 1992). Although proteins may not be preserved in their tertiary form, linear epitopes are generally preserved which can be identified by Western blot and other immunological methods (Abass *et al.* 1994). Given the viability of proteins under the conditions discussed there is a high probability that artifacts used in hunting, butchering, plant collection and processing will also retain adequate amounts of detectable protein.

In forensic work stains are obtained from a variety of sources - clothing, metal, plaster, cement etc. Moreover, criminals frequently attempt to remove bloodstains by a variety of methods such as laundering, scrubbing with bleach, etc. yet, such degraded samples are still identified by immunological methods (Lee and De Forest 1976; Milgrom and Campbell 1964; Shinomiya *et al.* 1978, among others). It is only in very recent years that immunological analysis has been replaced by DNA testing in crime labs. Forensic wildlife laboratories use immunological techniques in their investigation of hunting violations and illegal trade, often from contaminated evidence (Bartlett and Davidson 1992; Guglich *et al.* 1994; Mardini 1984; McClymont *et al.* 1982; among others). Immunological methods are also used to test the purity of food products such as canned luncheon meat and sausage, products which have also undergone considerable degradation (Ashoor *et al.* 1988; Berger *et al.* 1988; King 1984). The age of stains does not preclude obtaining positive results (Gaensslen 1983:225).

Immunological methods have been used to identify plant and animal residues on flaked and groundstone lithic artifacts (Hyland *et al.* 1990; Kooyman *et al.* 1992; Newman 1990; Yohe *et al.* 1991) and in Chumash paint pigment (Scott *et al.* 1996). Plant and animal residues on ceramic artifacts have been identified by their amino acid sequences (Broderick 1979) and by analysis of lipid and fatty acids (Fredericksen 1988; Heron *et al.* 1991; Bonfield and Heron 1995), while serological methods have been used to determine blood groups in skeletal and soft tissue remains (Heglar 1972; Lee *et al.* 1989) and in the detection of hemoglobin from 4500-year-old bones (Ascenzi *et al.* 1985). Human leukocyte antigen (HLA) and deoxyribonucleic acid (DNA) determinations made on human and animal skeletal and soft tissue

remains have demonstrated genetic relationships and molecular evolutionary distances (Hansen and Gurtler 1983; Lowenstein 1985, 1986; Pääbo 1985, 1986, 1989; Pääbo *et al.* 1989). Recent studies have shown that it is possible to detect DNA in ancient wheat and radish seeds (Brown *et al.* 1995; O'Donoghue *et al.* 1995), providing the potential for evolutionary studies of plant domesticates.

Materials and Methods.

The method of analysis used in this laboratory is cross-over electrophoresis (CIEP). Minor adaptations to the original method were made following procedures used by the Royal Canadian Mounted Police Serology Laboratory, Ottawa (1983) and the Centre of Forensic Sciences (Toronto). Although this test is not as sensitive as RIA, it has a long history of use in forensic laboratories, does not require expensive equipment, is reasonably rapid and lends itself to the processing of multiple samples (Culliford 1964). In this test the antigen and antibody are driven together by an electrophoretic force instead of simple diffusion as in the Ouchterlony test. The test is performed in agarose gels with a pH of 8.6. Paired wells, approximately 1.5 mm. in diameter are punched in the agarose gel 5 mm. apart. The antigen (unknown extract) is placed in the cathodic well of the pair and the antiserum in the anodic one. The gel is placed in an electrophoresis tank containing a barbital buffer, pH 8.6, and triple thicknesses of filter paper are used as wicks to connect the ends of the slides with the buffer. The application of an electrical current, set at a constant 100v, moves the two reactants towards each other. If the unknown sample contains protein corresponding to the species antiserum against which it is being tested, an extended lattice forms as a result of cross-linking, and a precipitate forms where they reach equivalence concentrations between the two wells. Weak positive reactions, common in archaeological samples, are more readily observed if the gel is dried and stained with a protein stain, such as Coomassie Blue. Appropriate positive and negative controls, prepared in 5% ammonia solution, are run with each gel. These are: positive - blood of species being tested for e.g., deer blood for deer antiserum and negative - blood of species in which antiserum is raised e.g., rabbit if raised in that animal. Duplicate testing is carried out on all positive results.

A musket ball recovered from the Fort Clatsop site near Astoria, Oregon, was submitted for potential identification of animal protein residues by immunological analysis. Possible residue was removed from the artifact using a 5% ammonium hydroxide solution. This has been shown to be the most effective extractant for old and denatured bloodstains and does not interfere with subsequent testing (Dorrill and Whitehead 1979; Kind and Cleevely 1969). The artifact was placed in a shallow plastic dish and 4.0 mL of 5% ammonia solution applied directly to it. Initial disaggregation was carried out by floating the dish and contents in an ultrasonic cleaning bath for two to three minutes. Extraction was continued by placing the boat and contents on a rotating mixer for thirty minutes. The resulting ammonia solution was removed with a pipette and placed in a plastic vial. The sample was concentrated by lyophilization then reconstituted by the addition of 200μl of sterile phosphate-buffered-saline (PBS). Initial testing was carried out against pre-immune serum (i.e., serum from a non-immunized animal). A positive result against pre-immune serum could arise from non-specific protein interaction not based on the

immunological specificity of the antibody (i.e., nonspecific precipitation), however, a negative reaction was obtained. Complete testing of the sample was continued against the antisera shown in Table 1.

Antisera obtained from commercial sources are developed specifically for use in forensic medicine and, when necessary, these sera are solid phase absorbed to eliminate species cross-reactivity. However, these antisera recognize epitopes shared by closely related species and will often identify other species within the individual family. The relationship of animal antisera used to potential prey species identified is shown in Table 3.

Table 1: Animal antisera used in analysis.

ANTISERA TO:	SOURCE
bear	Organon Teknika
bovine	"
cat	"
chicken	"
deer	"
dog	"
guinea-pig	"
human	"
rabbit	"
rat	"
sheep	"
elk	University of Calgary

Table 3: Relationship of animals to antisera used in analysis.

ANTISERA	MOST PROBABLE SPECIES
Bear	Black, grizzly.
Bovine	Bison, cow.
Cat	Bobcat, lynx, mountain lion, cat.
Chicken	Chicken, turkey, quail, grouse, pheasant.
Deer	Deer (all species), elk, moose, caribou, pronghorn.
Dog	Coyote, wolf, fox, dog.
Guinea-pig	Porcupine, squirrel, beaver, guinea-pig.
Human	Human, monkey.
Rabbit	Rabbit, hare, pika.
Rat	Mouse (all species), rat (all species).
Sheep	Sheep, goat.

Results

A weak positive reaction to human antiserum was obtained on this artifact. This reaction is probably due to the presence of recent human saliva on the artifact as noted by the excavator. No other positive results were obtained in this analysis. The absence of identifiable proteins on artifact may be due to poor preservation of protein or that it was used on species other than those encompassed by the antisera. It is also possible that the artifact was not utilized.

REFERENCES CITED

Abass, A. K., D. H. Lichtman and J. S. Pober
1994 *Cellular and Molecular Immunology*. W.B. Saunders Co. Philadelphia, PA

Ascenzi, A., M. Brunori, G. Citro and R. Zito
1985 Immunological detection of hemoglobin in bones of ancient Roman times and of Iron and Eneolithic Ages. *Proceedings National Academy of Sciences USA* 82:7170-7172.

Ashoor, S.H., W.C. Monte and P.G. Stiles
1988 Liquid chromatographic identification of meats. *J. Assoc. Off. Anal. Chem.* 71:397-403.

Bartlett, S.E. and W.S. Davidson
1992 FINS (Forensically Informative Nucleotide Sequencing): A procedure for identifying the animal origin of biological specimens. *Biotechniques* 12:408-411.

Berger, R.G., R.P. Mageau, B. Schwab and R.W. Johnston
1988 Detection of poultry and pork in cooked and canned meats by enzyme-linked immunoabsorbent assays. *J. Assoc. Off. Anal. Chem* 71:406-409.

Bonfield, K. and C. Heron
1995 The identification of plant waxes in neolithic pottery: evidence for "invisible" foods. Paper presented at Archaeological Sciences 1995, University of Liverpool, U.K.

Broderick, M.
1979 Ascending Paper Chromatographic Technique in Archaeology. In: *Lithic Use-Wear Analysis*, edited by B. Hayden, pp. 375-383. Academic Press, New York.

Brown, T., K.A. Brown, R.G. Allaby, R. Sallares and M. Banerjee
1995 DNA preserved in wheat seeds. Paper presented at Archaeological Sciences 1995, University of Liverpool, U.K.

Cattaneo, C., K. Gelsthorpe, P. Phillips and R.J. Sokol
1992 Reliable Identification of Human Albumin in Ancient Bone using ELISA and Monoclonal Antibodies. *American Journal of Physical Anthropology* 87:365-372.

Culliford, B.J.
1964 Precipitin Reactions in Forensic Problems. *Nature* 201:1092-1094

Dorrill, M. and P.H. Whitehead
1979 The Species Identification of Very Old Human Bloodstains. *Forensic Science International* 13:111-116.

Eisele, J.A., D.D. Fowler, G. Haynes and R.A. Lewis.
1995. Survival and detection of blood residues on stone tools. *Antiquity* 69:36-46.

Fiedel, S.
1996 Blood from Stones? Some Methodologcal and Interpretive Problems in Blood residue Analysis. *Journal of Archaeological Science* 23(1):139-147.

Frederickson, C.
1988 Gas Chromatography and Prehistoric Tool Use Residues: A Preliminary Study. *Archaeology in New Zealand* 31(1):28-34.

Gaensslen, R.E.
1983 *Sourcebook in Forensic Serology, Immunology, and Biochemistry*. U.S. Department of Justice, Washington, D.C.

Guglich, E.A., P.J.Wilson and B.N. White
1993 Application of DNA Fingerprinting to enforcement of hunting regulations in Ontario. *Journal of Forensic Science* 38:48-59.

Hansen, H.E., and H. Gurtler
1983 HLA Types of Mummified Eskimo Bodies from the 15th Century. *American Journal of Physical Anthropology* 61:447-452.

Heglar, R.
1972 Paleoserology Techniques Applied to Skeletal Identification. *Journal of Forensic Sciences* 16:358-363.

Heron, C.L., R.P. Evershed, L.J. Goad and V. Denham
1991 New Approaches to the Analysis of Organic Residues from Archaeological Remains. In *Archaeological Sciences 1989*, edited by P. Budd, B. Chapman, R. Janaway and B. Ottaway, pp.332-339. Oxbow Monograph 9.

Hyland, D. C., J.M. Tersak, J.M. Adovasio and M.I. Siegel
1990 Identification of the Species of Origin of Residual Blood on Lithic Material. *American Antiquity* 55:104-112.

Kind, S.S. and R.M. Cleevely
1969 The Use of Ammoniacal Bloodstain Extracts in ABO Groupings. *Journal of Forensic Sciences* 15:131-134.

King, N.L.
1984 Species Identification of Cooked Meats by Enzyme-Staining of Isoelectricfocusing Gels. *Meat Science* 11:59-72.

Kooyman, B., M.E. Newman and H. Ceri
1992 Verifying the Reliability of Blood Residue Analysis on Archaeological Tools. *Journal of Archaeological Science* 19 (3):265-269.

Lee, H.C. and P.R. DeForest
1976 A Precipitin-Inhibition Test on Denatured Bloodstains for the Determination of Human Origin. *Journal of Forensic Sciences* 21:804-809.

Lee. H.C., R.E. Gaensslen, H.W. Carver, E.M. Pagliaro and J. Carroll-Reho.
1989 ABH Typing in Bone Tissue. *Journal of Forensic Sciences* 34(1):7-14.

Lowenstein, J.M.
1985 Molecular Approaches to the Identification of Species. *American Scientist* 73:541-547.

1986 Evolutionary applications of radioimmunoassay. *American Biotechnology Laboratory* 4(6):12-15.

Mardini, A.
1984 Species Identification of Selected Mammals by Agarose Gel Electrophoresis. *Wildlife Society Bulletin* 12(3):249-251.

McClymont, R.A., M. Fenton and J.R. Thompson
1982 Identification of Cervid Tissues and Hybridization by Serum Albumin. *Journal of Wildlife Management* 46(2):540-544.

Milgrom, F., Z. M. Tuggac and E. Witebsky
1964 Studies on Species Specificity. *Journal of Immunology* 93: 902-909.

Miller, M.F. II and R.W.G. Wyckoff
1968 Proteins in Dinosaur Bones. *Proceedings of the National Academy of Science U.S.A.* 60:176-178.

Newman, M.E.
1990 The Hidden Evidence From Hidden Cave, Nevada. Ph.D dissertation on file, University of Toronto, Canada.

O'Donoghue, K., T.A. Brown, J.F. Carter and R.P. Evershed
1995 PCR and GC/MS of DNA in 1400-year-old Radish seeds. Poster presented at Archaeological Sciences 1995, University of Liverpool, U.K.

Pääbo, S.
1985 Molecular cloning of Ancient Egyptian mummy DNA. *Nature* 314:644-645.

1986 Molecular Genetic Investigations of Ancient Human Remains. *Cold Spring Harbor Symposia on Quantitative Biology*, 11:441-446.

1989 Ancient DNA: Extraction, characterization, Molecular cloning, and enzymatic amplification. *Proceedings National Academy of Science USA* 86:1939-1943.

Pääbo, S., R. G. Higuchi and A.C. Wilson
1989 Ancient DNA and the Polymerase Chain Reaction. *The Journal of Biological Chemistry* 264:269.

Prager, E.M., A.C. Wilson, J.M. Lowenstein and V.M. Sarich
1980 Mammoth Albumin. *Science* 209:287-289.

Robbins, L.L., and K. Brew
1990 Proteins from the Organic Matrix of Core-top and Fossil Planktonic Foraminefera. *Geochim. cosmochin. Acta* 54:2285-2292.

Royal Canadian Mounted Police
1983 Methods Manual, Serology Section. Ottawa, Ontario.

Scott, D. A., M.E. Newman, M. Schilling, M. Derrick and H.P. Khanjian.
1996 Blood as a binding medium in a Chumash Indian Pigment Cake. *Archaeometry* 38:103-112.

Shinomiya, T., M. Muller, P.H. Muller and R. Lesage
1978 Apport de l'immunoelectrophorese pour l'expertise des taches de sang en medicine legale. *Forensic Science International* 12:157-163.

Thomas, K. D.
1993 Molecular Biology and archaeology: a prospectus for inter-disciplinary Research. *World Archaeology* 25:1-17.

Voss-Foucart, M.F.
1968 Paléoprotéines des coquilles fossiles d'oeufs de dinosauriens du Crétacé Supérior de Provence. *Comp. Biochem. Physiol.* 24:31-36.

Yohe, R., M.E. Newman and J. S. Schneider
1991 Immunological Identification of Small-Mammal Proteins on Aboriginal Milling Equipment. *American Antiquity* 56(4): 659-666.

Zimmerman, M.R.
1973 Blood Cells Preserved in a Mummy 2000 Years Old. *Science* 180:303-304.