

XVIII. ORGANIC RESIDUE ANALYSIS

Introduction

One hundred lithic artifacts from Lums Pond were examined for the purpose of ascertaining if blood residue was preserved on stone tools. The lithics consisted of bifaces, points, flakes; and hammers fashioned from quartz, jasper, chert, quartzite, argillite, ironstone and andesite. A total of ten soil samples were also examined covering Areas 2 and 3.

Experimental Design

The general approach and specific protocols used in this work derive from previous experience of the Conservation Analytical Laboratory, Smithsonian Institution, with blood residues on stone tools. This includes an appreciation that when protein is preserved on tools, it is in very small quantities and sensitivity in all assay procedures is paramount. Further, it is anticipated that many of the tools will have not adhering residues, and whether this is due to variability in initial use or postburial diagenetic processes is not known. For those tool extracts that do exhibit evidence of protein in the extract, the following protocols allow for not only a sensitive characterization of the material, but also are designed to rule out any interference from soil compounds. Of necessity, the total collection of tool extracts will be subsampled as the range of testing proceeds. The necessity derives almost exclusively from the amount of protein found in association with the archaeological artifact. It makes no sense to do high resolution immunology if the amino acid analysis demonstrates that the sample has no protein on it. This greatest unknown, how much sample is preserved, guides these analyses. The ultimate goal is to answer the question--are the blood residues preserved on the Lums Pond stone tools?

Extraction Protocols

Proteins were extracted from the surface of the archaeological lithics under denaturing, dissociative conditions at room temperature. Lithics were placed individually in sterile polythene bags and a minimum volume (sufficient to cover each lithic, i.e., anything from 2 ml to 40 ml, but most frequently <10ml) of 7M urea (pH 7.2) was added. Extraction was carried out overnight on a vibrating table.

Desalting/Sample Concentration (Dialysis and Freeze-drying)

Sample extract was transferred to Spectra/Por dialysis membrane (pre-soaked to wet the membrane and remove glycerol). The dialysis tubing and sample were then soaked in water at 4 degrees C overnight, for a minimum of three water exchanges to remove salt from the sample extract. Following dialysis, sample extracts were placed in centrifuge tubes (50ml or 15ml), frozen at -20 degrees C and freeze dried. The resulting deposits were resuspended in 1 ml de-ionized water and transferred to microcentrifuge tubes (1.5ml) before they were again freeze dried.

Materials and Methods

Visual inspection of the freeze dried sample residue showed variation in the quantity of extract present. Extract dry weight was selected for amino acid analysis was also selected. The ten largest and ten smallest samples (with two soil controls) were resuspended in 100ul de-ionized water.

Amino Acid Analysis

After centrifugation, 10ul of each sample was placed in a muffled hydrolysis tube, and 200ul of 6N constant boiling HCl was added to each tube and the samples were heated in 150 degrees C for twenty minutes. The samples were placed in a vortex evaporator to remove all remaining acid each sample was taken up in 600 ul pH2 HCl and filtered through 0.45um. Between 0.5 and 1% of the total tool extracts were used in the amino acid analysis. Amino acid analysis was performed by separation over cation exchange resin, and derivatization with the florescent reagent, orthophthaldialdehyde. Appropriate blanks and standards were run.

Gel Electrophoresis

Nine tool extracts with significant dry weight of the residue (L02, L14, L17, L31, L58, L59, L09, L65 and L76) were resuspended in 50ul gel sample buffer, spun and warmed. The extracts (15ul of 50ul) was heated for five minutes at 100 degrees C, and separated on SDS protein gels (4-20% Tris-glycine polyacrylamide gels). Nine samples selected for the largest amount of protein, based on the amino acid analysis (L05, L13, L16, L30, L41, L67, L69, L70 and L85), were analyzed by mixing 15ul of resuspended residue in 15ul of x2 gel sample buffer, and separated as described above. Gel were run at 15mA constant current in Laemmli buffer. Protein was transferred to nitrocellulose membrane by electroblotting at constant voltage (600mA for 45 minutes). The membranes were washed overnight and stained with colloidal gold stain.

A third gel was loaded with sample L64 which had 20ug of protein by amino acid analysis. Three lanes of 30ul of sample each were separated. Protein was transferred (90V, 30mins) to PVDF membrane for protein sequencing. A second blot was done to nitrocellulose for colloidal gold staining. Samples in deionized water were refrozen and lyophilized. The extracts in gel sample buffer were stored at -20 degrees C.

Immunological Assays (Western blots)

Polyacrylamide gels were loaded (30ul per lane) with seven samples that exhibited evidence of protein from colloidal gold staining described above (L05, L13, L16, L14, L17, L59, L09), and the remainder of the Lums Pond extracts. Gels were run at 15mA per gel and were blotted to nitrocellulose for 45 minutes prior to washing. The blots were stored damp at 4 degrees C prior to antibody exposure.

Goat anti-deer serum (Cappel) in phosphate buffered saline/non-fat milk was used as the first antibody. This is a commercially available antibody sold as part of a forensic series (Catalog #56008). The antisera has been adsorbed with a variety of other animal sera, and was found to be negative to a wide range of animal sera including bear, bovine, cat chicken, dog, goat, guinea pig, hamster, horse, human, monkey, mouse, rabbit, rat, sheep, seine and turkey when tested at a concentration of 60mg/ml against sera concentrated in the range of 20-25mg.ml (Cappel product insert). These type of antisera have been used extensively in immunological identification of blood residues on stone tools. The first antibody binding time was two hours. The second antibody, HRP-labeled, affinity purified rabbit anti-goat was used at room temperature for one hour prior to color development using 4-chloro-1-naphthol.

In addition, 14 tool extracts (and two soil controls) were reexamined to Western blot as described above using a goat antihorse antisera (Cappel, Catalog # 56002). The fourteen extracts were chosen on the basis of positive reaction to deer antisera.

Results

Amino Acid Analyses

The results from the twenty-two amino acid analyses show that many samples were totally negative, that is, at the level of detection no amino acids were observed. It should be stressed that only 0.5 to 1% of the dry weight of the residue was used, so that larger injection volumes may have shown some protein. However, the sensitivity of this assay is such that low nanogram amounts of amino acids can be detected, and even two

hundred times more protein (in the case of negative results) would provided barely enough material for one western blot. The results indicate that there is no protein, and thus no blood preserved on many of these tools.

Representative chromatograms of a positive and negative sample are shown in Figure 146. The top trace is a standard amino acid mixture run at a concentration of 225pmole/each, and the elution order is aspartic acid/asparagine, threonine, serine, glutamic acid/glutamine, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and ammonia. The middle trace is sample L67, a quartz flake, which exhibits not evidence of adhering protein. The bottom trace, L30, has significant aspartic/asparagine, glutamic/glutamine, glycine and alanine, as well as trace amount of threonine, serine and valine. Retention times for the amino acids were shifted in the tool extracts, suggesting that other compounds not "seen" in an amino acid analysis were present and caused these chromatographic shifts. All of the amino acid analyses of the tool extracts contained significant levels of ammonia. In general, however, higher levels of ammonia were observed in those extracts with detectable amino acid. These other compounds account for the significant portion of the dry weight that is not accounted for in the amino acid analysis. High ammonia content upon hydrolysis often occurs in the presence of humic and fulvic acids. The potential for interference in immunological assays by these complex geopolymers is a concern, however their presence would not be obvious without this type of analysis or some other chromatographic/spectrometric detection.

Eight of the tool extracts showed clear evidence of protein in the residues, four had trace amounts of amino acids, and eight had not detectable protein. Soil samples had either no detectable amino acids or trace amount. While it is tempting to suggest that the higher levels of protein observed in the tool extracts relative to fairly large soil samples must derive from some non soil source, it must be kept in mind that the analyzed soils have altered over the time course of the deposit. Nonetheless, it is heartening that protein is found on eight of the tools, as this most basic of data is necessary for the demonstration of any blood residues. The pattern of amino acids present were not diagnostic for any particular protein or source (nor is this likely ever to be the case). Rather, this amino acid pattern is observed in many fossil environments, and likely represents the stable protein components that are resistant to diagenesis.

observed: most clearly in L30, L59, L17 and L14. The sharp darkly stained band (and lighter higher molecular weight band) in extracts L30 and L59 is not reminiscent of any blood derived protein product and were not found in any soil extracts. While microbial and fungal sources of the protein are possible, the distribution (two different localities in

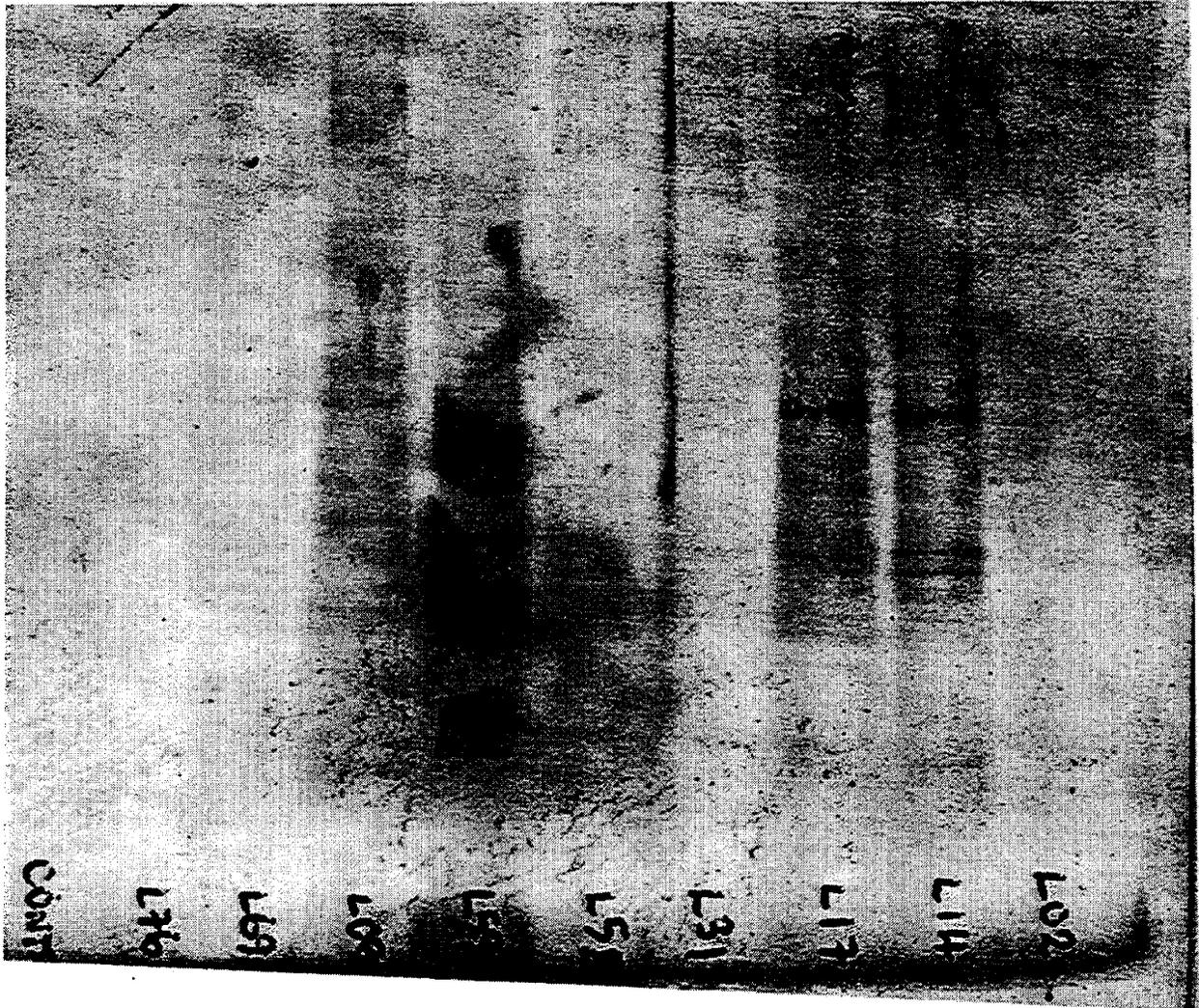


Plate 50. Gold blot of nine tool extracts from the Lums Pond archaeological site. From left to right, gel sample buffer control, L76, L69, L09, L59, L58, L31, L17, L14 and L02. Proteins of discrete molecular weight are observed in L59, L17 and L14. The band at higher molecular weight in L14 and L17 is consistent with that of serum albumin. The bands in L59 are not reminiscent of any blood derived products

the excavation), differing tool types (anvil vs. flake) and different batches of extracts (tools 1-50 and 50-100 were processed separately), and the fact that these products exist only on two tools makes plant sources an intriguing possibility. These extracts can be

assayed immunologically for the presence of the small subunit of rubisco, a major plant protein.

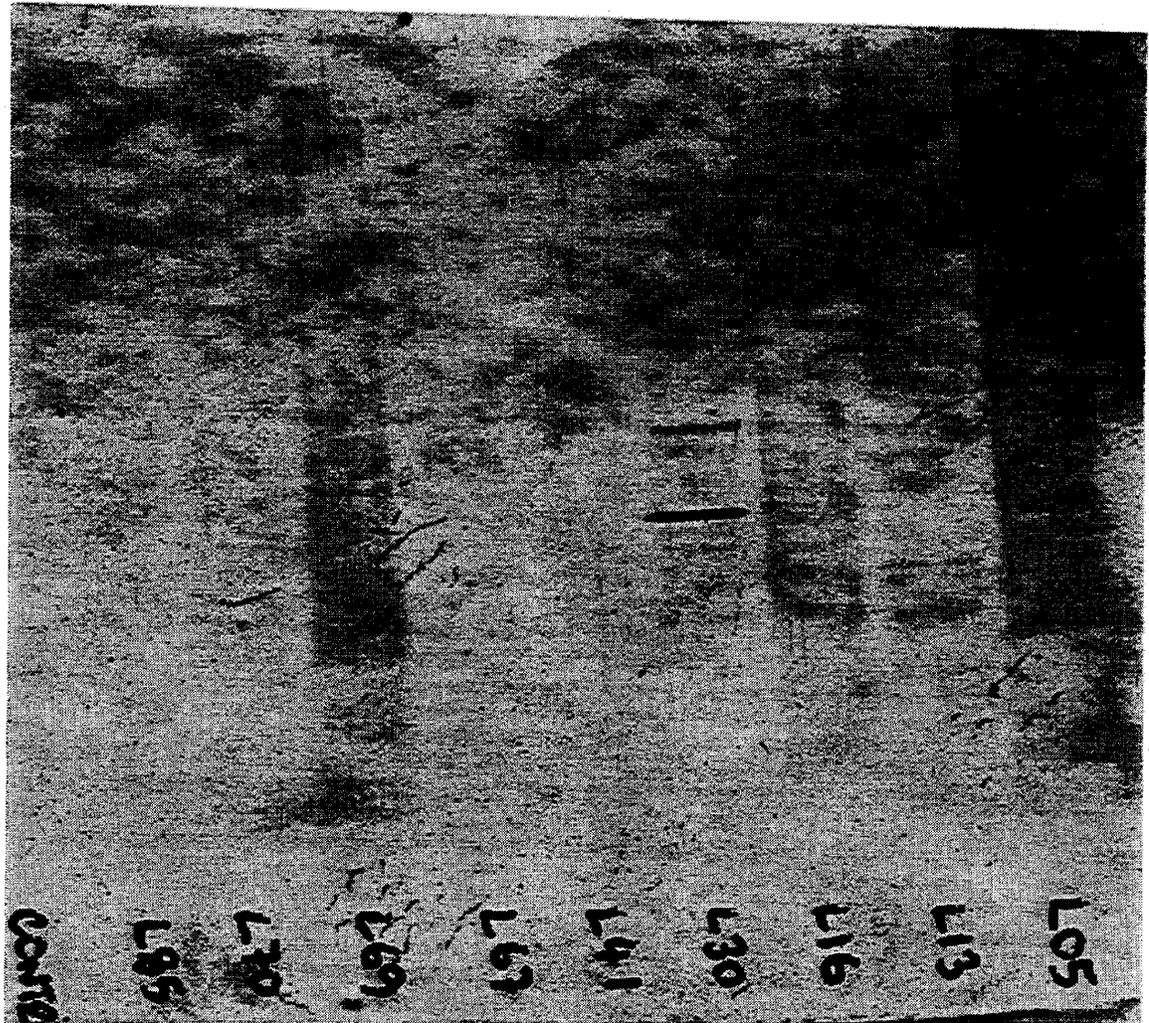


Plate 51. Gold blot of nine tool extracts from the Lums pond archaeological site. From left to right, gel sample buffer control, L85, L70, L69, L67, L41, L30, L16, L13 and L05. Only L30 exhibits evidence of protein at discrete molecular weight, although several other extracts (e.g., L69, L16 and L05) bound colloidal gold through a broad range of molecular weights

Immunological Assays (Western Blots)

The extracts from sixty-seven tools were separated by gel electrophoresis and transferred to nitrocellulose as described above. One sample (L36) showed a clear reactivity to this deer antisera in the molecular weight range of albumin (Plate 52). Sixteen other tool extracts reacted to this antisera as exhibited by a color reaction at high molecular weight ranges in as smear down the nitrocellulose blot (Plate 52, L37, L40, L42). In other cases extracts were negative (Plate 53). Concentrated soil extracts had extremely faint reaction to the antisera, well below the sixteen tool extracts.

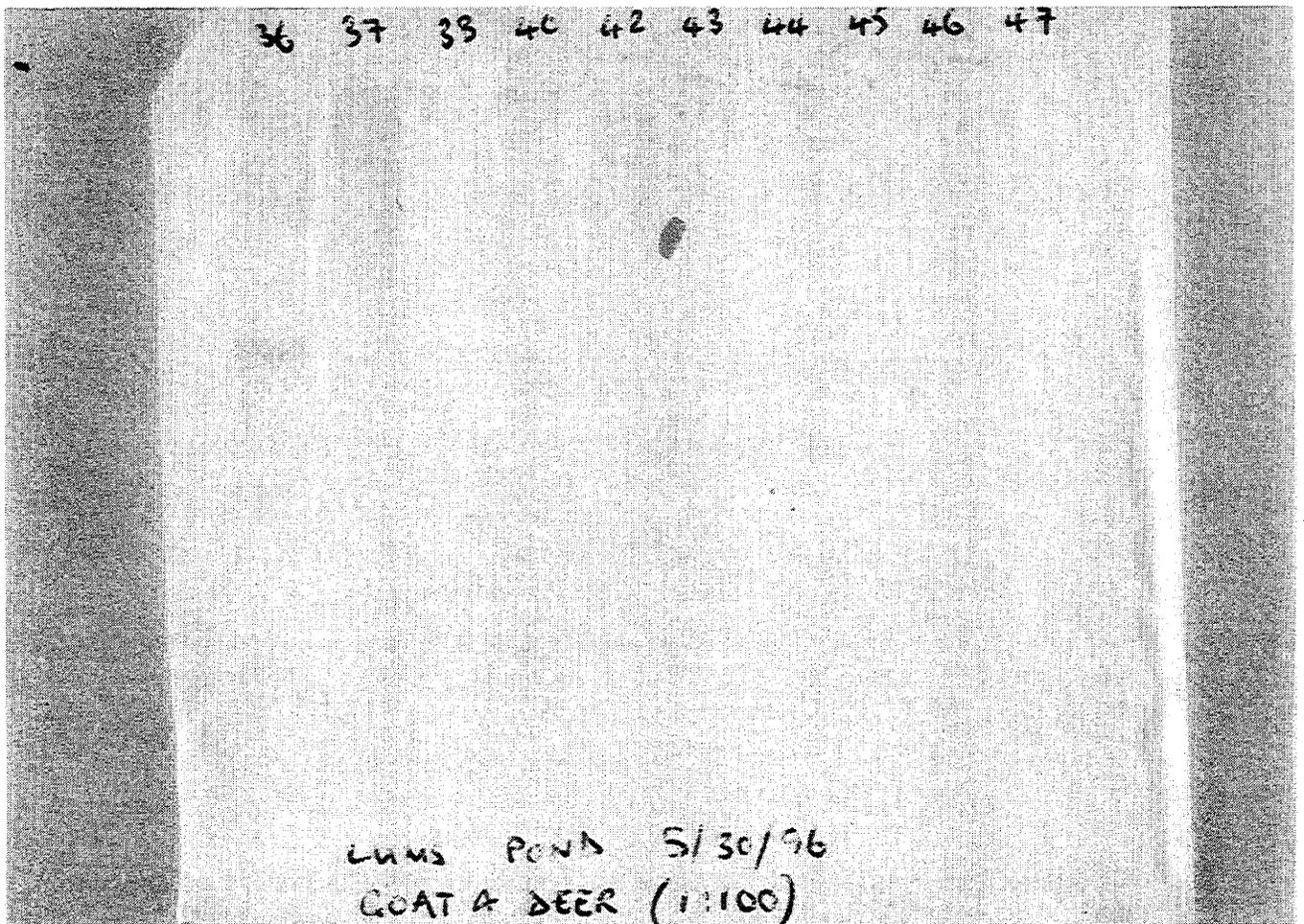


Plate 52. Immunological detection of serum derived projects with an antideer antibody. L36 is strongly positive at approximately 60 kD, and is consistent with albumin. Strong, broad reaction was observed in a smear, especially in L37 and L42

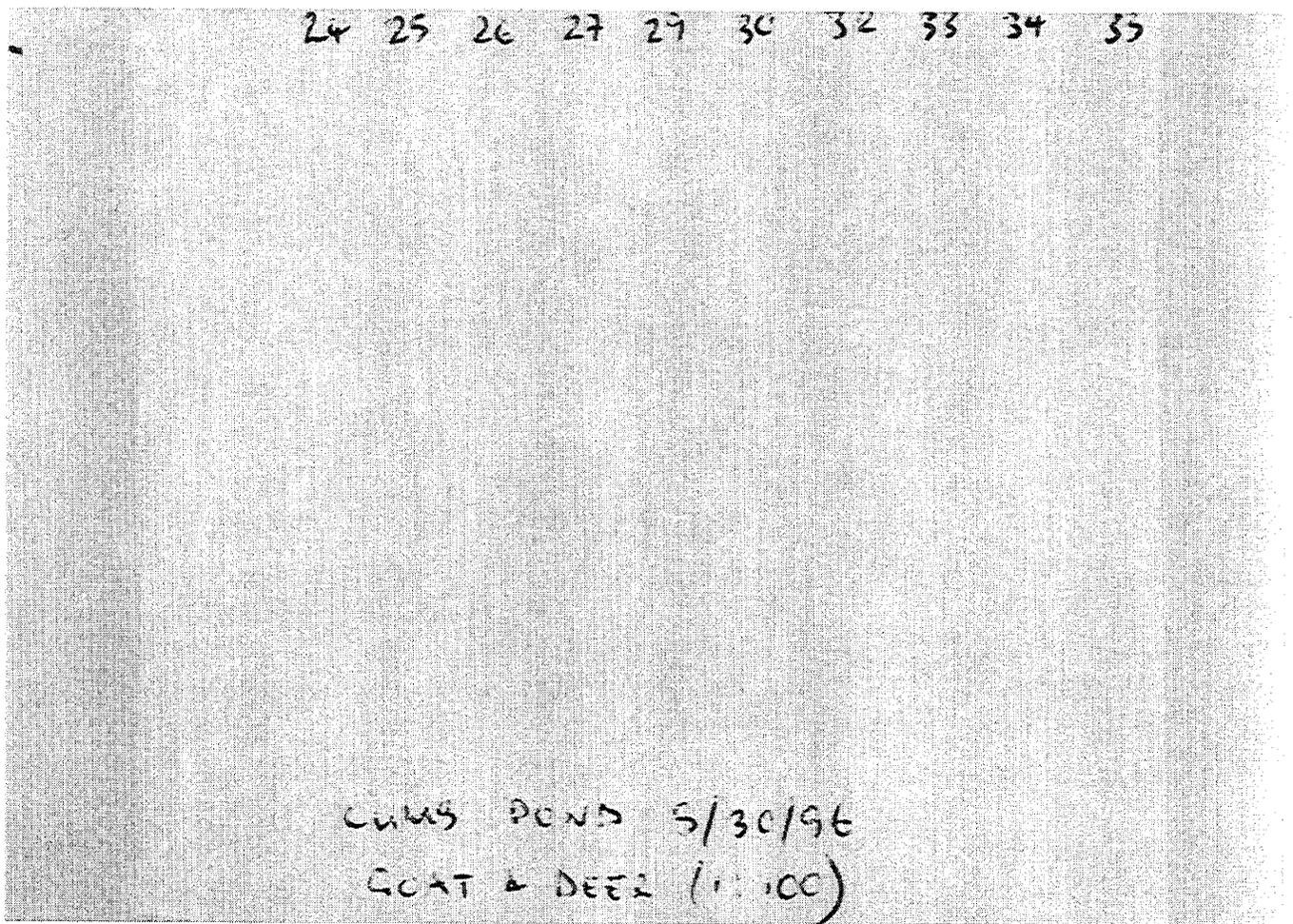


Plate 53. Immunological detection of serum derived products with an antideer antibody. All extracts are negative, and exhibited no reaction to this antibody

Many of the tool extracts were completely negative in their reaction to this antisera, so there was not a uniform background of reactivity. These data are confusing and comforting. It is not known whether this high molecular weight reactivity seen in

sixteen samples represents and "true" immunological reaction, this is whether the reacting proteins are blood. Further it is not known whether any species specificity that has been claimed by the manufacturer of this antisera hold true under these diagenetic conditions. The clear demonstration of one strong positive in the midst of clear negative results is the source of comfort, but the interpretation of the sixteen ambiguous samples is confusing.



Plate 54. Immunological detection of serum derived products with an antihorse antibody. Extracts from L42, L50, L57 and L64 reacted slightly with this antisera at a broad high molecular weight range, the other extracts were negative. As the horse was not introduced into North America at this time, this reactivity must be an inappropriate crossreactivity

In an attempt to resolve this issue, a small subset of samples was reexamined using an antibody that was thought to be specific to horse serum. The Lums Pond site predates the reintroduction of the horse to North America, therefore any reactivity to this antisera is nonspecific and not due to remnant horse blood. In Plate 54, a lightly reactive pattern can be seen in four of the extracts L40, L42, L50, L57, while the other extracts on the blot are nonreactive. It is noteworthy that in the western blot using antisera to deer, L40 and L42 also exhibited the broad high molecular weight reactivity, and the L36, the tool extract with a strong bandable positive in the albumin region was complete negative in the horse antiserum western blot. These comparative immunological assays, then, provide a new approach in determining which reactive tool extracts truly contain remnant blood products.

Conclusions

An examination of one hundred tool extracts from Lums Pond excavation produced evidence of protein on the specimens submitted for analysis. Five of the extracts produced evidence of intact bandable protein, and in two cases the source of the protein remains unknown, and can be tested at a later date with antisera to rubisco. In three cases, the protein albumin was identified, most convincingly in L36 with the reaction to deer antisera. Two other extracts (L14 and L17) with clear evidence for trace albumin did not react to this antisera, suggesting another source of blood. None of the three putative albumin containing extracts reacted with horse antisera, suggesting that when intact albumin is present, some species specificity is obtainable.

The majority of positive immunological reactions and gold stained blots exhibited a high molecular weight smear of unknown origin. The source of these products did not, however, seem to derive from the surrounding soil matrix. The crossreactivity of these high molecular weight products with an "inappropriate" antibody (horse) may go a long way to explaining the fairly high number of positive and odd results in a number of previous blood residue studies. In this study fifteen of sixty extracts (25%) showed the high molecular weight smear reactivity to the antideer antisera. In immunological assays that do not control for molecular weight (e.g., Ochterlony diffusion, CIEP, ELISA), a 25% positive score would have been given by the analyst. These results demonstrate that immunological techniques such as ELISA and CIEP, while useful screening tools, should not be used to identify species of origin from archaeological tool extracts.