The Mechanism of Organic Preservation at Monte Verde, Chile, and One Use of Biomolecules in Archaeological Interpretation

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Monte Verde, a habitation site in southern Chile, is the source of exceptionally well-preserved organic materials. The depositional and chemical circumstances that led to the persistence of this unique assemblage included an anoxic, reducing environment protected by an overlying peat layer and a silica gel-rich substrate.

Utilizing the immunological techniques of ELISA assays and Western blots, a subset of lithic artifacts was tested for blood traces. Geochemical analysis of the soil matrix provided the necessary comparative data for assessing the biological etiology of the residue extracted from these tools. One tool was found to be positive for hemoglobin, and was convincingly above both the chemical and geological background of the immunological assays. These findings complement the archaeological interpretation of the site.

Introduction

Sites with unusual preservation have generated new interest in the field of paleobiology (Briggs 1991), in part because they often possess unique evidence about life in the past. The materials recovered from well-preserved sites are often different in form and substance from those found at more traditional settings where inorganic artifacts may dominate. The methodology utilized in archaeological excavation is constantly evolving with each site discovery, and there is increased evidence that biomolecules are preserved in the fossil record (e.g., Aije et al. 1991; Lawlor et al. 1991; Tuross 1989, 1991).

In order to assess the information potential in an organically-dominated archaeological assemblage, a mechanistic explication is required that details how the site was originally preserved and has been maintained through time. This mechanistic approach allows the formulation of research strategies that maximize the information retrieved at the atomic and molecular levels in archaeological deposits, and encourages the development of experimental approaches that provide meaningful analyses of friable material.

Excavated materials from the site of Monte Verde in southern Chile (FIG. 1) provided the substrate for these studies. Monte Verde is an open-air, wetland occupation site that contains a rich array of economic plants, bones of extinct animals, traces of features, and diverse artifacts of wood and stone (Dillehay et al. 1982; Dillehay 1984, 1986, 1989). A series of radiocarbon dates on stratified noncultural and cultural deposits places one human episode at some 12,500–13,200 years ago. Approximately 1.5 m below these deposits, however, in a different area of the site, are features and worked stones that might belong to an older culture (Dillehay and Collins 1988).

Previous reports of the site have described the geology of the deposits, and the range of materials that were excavated (Dillehay 1989). The purpose of this paper is to explain in geomorphological and geochemical terms *how* the organic archaeological context at Monte Verde was preserved in a near pristine state. Further, by utilizing geochemical and immunological data of the soil in a comparative sense, we performed a residue analysis of some of the lithic artifacts, aimed at determining the presence or absence of blood on these tools.

How Monte Verde Was Preserved: A Depositional and Geochemical Analysis

South-central Chile is defined to the west by a narrow Pacific littoral and low coastal mountain range and to the east by the high Andean mountain range. Several westerly
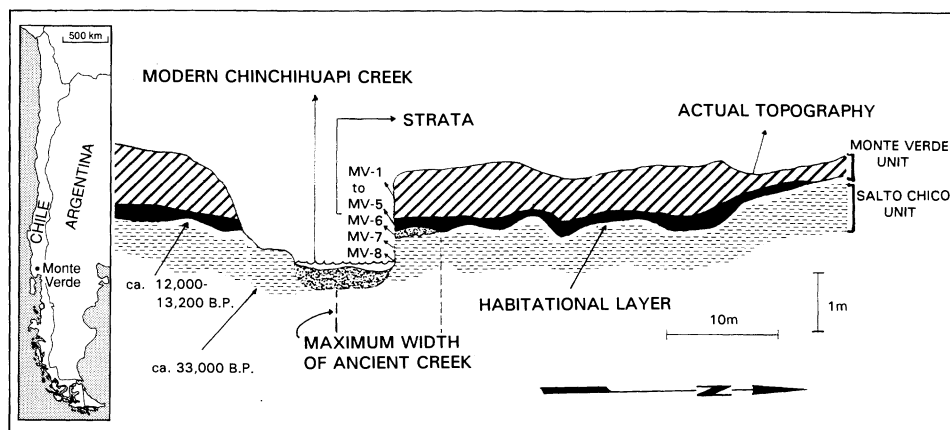
Figure 1. Cross section of the site of Monte Verde illustrating the position of the habitational layer. Note that the overlying MV-5 peat layer is discontinuous on the south side of the deposit. Inset shows location of Monte Verde in southern Chile.

Table 1. Organic content in Monte Verde soils.

Attributes	MV-5 (overlying peat)	MV-6 (cultural context)*	MV-7 (noncultural control)
%N (dry weight)	0.4	1.5–3.5	trace
%C (dry weight)	13.5	22.2	0.6
%Combustible[†]	29	45	3
%Extractable[‡]	14	21	none
Molecular weight range[§]	60–10kD	150–10kD	–

*These samples were recovered within a wishbone-shaped structure that may have served as a site for dressing meat, preparing hides, or making stone tools.

[†]Dry weight loss upon heating at 600°C for 2 hours.

[‡]Dry weight of > 6,000 dalton molecular weight material extracted by 7M Urea for 24 hours.

[§]Range in molecular weights (kD is kilodaltons) as determined by size exclusion chromatography (FPLC-Superose 12), and by SDS gel electrophoresis (see text notes on materials and methods).

flowing rivers descend the Andean slopes and empty into the Pacific Ocean, and several lakes dot both the mountainous and lowland areas. Geological evidence indicates that the Pleistocene glaciers of the region receded out of the lake area into the Andes, probably before 14,000 B.P., and that extensive deglaciation occurred by 13,000 B.P. (Mercer 1984; see Villagran, Silva, and Vera 1983 for nearby Chiloe). Glaciers emanating from the Andes have left extensive, poorly-sorted till deposits and moraines in this part of Chile (Mercer 1972, 1976a, 1976b, 1984; Pino 1989; Porter 1981). In places, these features have been reworked by the rivers and their tributaries, but the lakes, moraines, bogs, and other traces of glaciation dominate much of the landscape (Porter 1981).

Monte Verde is located on the north shore of Chinchihuapi Creek, an ancient tributary located up on a Pleistocene plain and terrace of the Maullin River. Pino (1989: 89–131) has defined two geological formations for this area, the Salto Chico Formation (SCH) comprising lower strata MV-7 and MV-8, and the Monte Verde Unit (MV) defined by upper strata MV-1 to MV-6 in the immediate vicinity of the site (FIG. 1). These deposits were the high terraces of the drainage system of the Maullin River during late Pleistocene times, and consisted of large grain sands, thin lenses of volcanic ash, and igneous and metamorphic rocks. These substrata of the cultural layers of Monte Verde are *not* soil. Deposition of the upper layers (MV-7) of the SCH unit has been dated to between approximately

22,000 and 17,000 years ago (Pino 1989). The deeper, possible cultural component, dated around 33,000 B.P., is buried in a lower layer of this formation, about 1 m below the buried surface of stratum MV-7 and about 1.5–2 m below the present ground surface. This formation is overlain by the Monte Verde Unit.

Interdisciplinary paleoecological research in the site area (cf. Dillehay 1989) shows that a rise in temperature and rainfall between approximately 13,000 and 12,500 B.P. combined with increased vegetation growth choking the stream flow, led to a buildup in a chronological sequence of muddy peat deposits (MV-5) in the old Chinchihuapi channel of the Monte Verde Unit (FIG. 1). The deposition onto the abandoned living surface preserved the cultural materials and provided much ecological information about the past environment and site conditions around the time of human occupations.

Chemical analyses of the lower Salto Chico deposits revealed two important factors that directly affect the archaeological interpretations of Monte Verde. First, organic matter, as measured by carbon and nitrogen content and amino acid analysis, could not be detected at nanomolar levels (10^{-9}) of sensitivity in these strata (TABLE 1). Given the thickness (minimum = 8.2 m) (Quivera and Dillehay 1988) of the Salto Chico formation below the cultural deposits, organic contamination of the culturally-associated materials up through the organic-free substratum is unlikely.

The second notable chemical aspect of the Salto Chico formation (MV-7 and MV-8) is the presence of large amounts of silica gel pebbles (FIG. 2). The identification of silica gel is based on: 1) the hydrophobic properties of the strata; 2) the elemental composition as determined by energy dispersive spectroscopy (EDS) (FIG. 3); 3) comparative Fourier-transformed infrared spectroscopy; and 4) powder pattern x-ray diffraction analysis of selected silica pebbles. Silica gel in the contiguous subcultural strata would act as a desiccant, pulling water from the cultural layer and the associated artifacts. Some of the Monte Verde organic materials, particularly the bone fragments, exhibited an unusual resistance to wetting in aqueous solutions: the objects seem waterproofed.

The weight of the overburden pressed the silica gel pebbles into the interstices of most of the organic materials of the cultural layer (FIG. 4A). In a decalcified sample of mastodon bone, the first signs of silicification were observed in the midst of collagen fibrils preserved in register (FIG. 4B). Silicification of organic materials is a common occurrence in the paleontological record, and has been studied extensively in the formation of petrified woods.

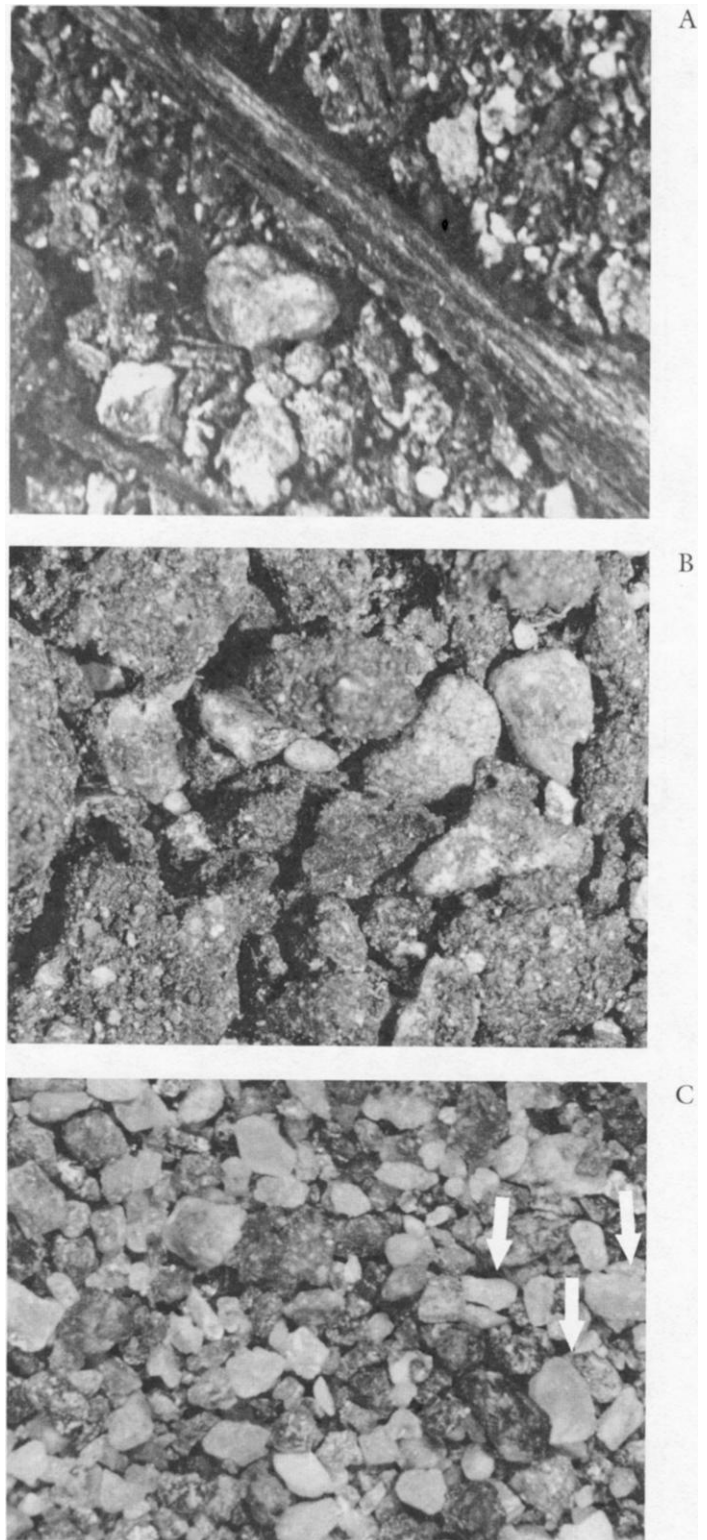Overlying a narrow strip of MV-7 is MV-6, an ancient

Figure 2. Light micrographs of the sediment excavated at Monte Verde: top to bottom: A) MV-5, the peat rich overburden, B) MV-7, culturally associated soil from the wishbone shaped structure, and C) the nonculturally associated gravel from MV-7. Silica gel pebbles are marked with arrows.
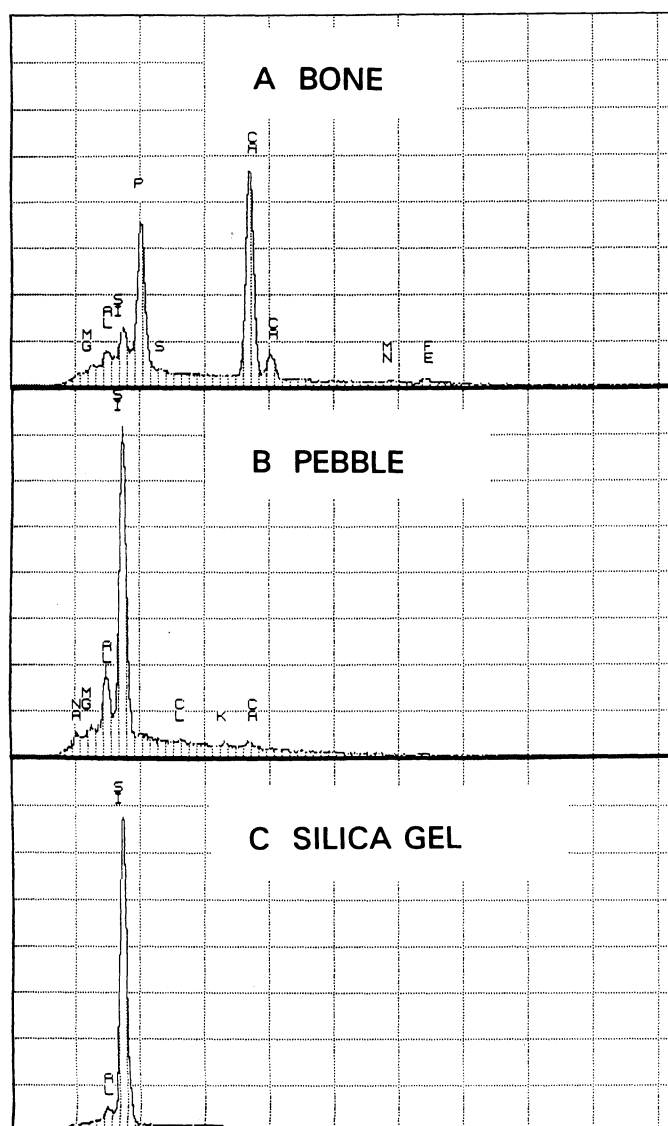
A BONE

B PEBBLE

C SILICA GEL

Figure 3. Energy dispersive analysis by X-ray (EDS) of laboratory silica gel used as a drying agent (C) compared to bone pieces (A) and white pebbles (B) found throughout the cultural and noncultural sediments at Monte Verde.

creek-bed deposit, 4 m wide and 15 cm thick, with associated gravel-strewn, sandy point bars and sandy beaches. The 13,200 to 12,500 year-old habitational layer containing the cultural materials rests on the contemporaneous surfaces of the gently inclined, sandy beaches of the old channel (MV-6) and on the sandy terrace bank (surface of stratum MV-7, in the upper part of the SCH unit at the time of human occupation). The present Chinchihuapi Creek has cut into and partially exposed the earlier, filled channel (MV-6) of the same creek. Only the ancient creek bed (MV-6) and its shoreline (MV-7) are superimposed by

MV-5, a peat-like layer that developed in the narrow stream basin, covering and sealing the cultural materials.

Microscopically, MV-5 is an immature woody peat (FIG. 2) with plant remains, microfauna, insects, and mollusks present (Quivera and Dillehay 1988). These constituents make up what is known as the "cellular" fraction of soil organic matter (SOM) (Swift, Heal, and Anderson 1979). The amorphous organic fraction is referred to as humus. The major constituents of humus are subdivided into three fractions based on solubility: 1) humin, an insoluble fraction in 0.5 M NaOH; 2) humic acid, a fraction that is soluble at basic pH, but precipitates at about pH 1; and 3) fulvic acids, soluble in both acidic and basic solutions.

The current study documented an average amount of combustible material in MV-5 as 29%, in close agreement with the 20% reported by Quivera and Dillehay (1988). The amount of extractable organic matter from MV-5 averaged 14%. The extracted material had a molecular weight range (60–10 kD) by size exclusion chromatography (FPLC, Sepharose 12) that is most consistent with fulvic acids (Brady 1974; Swift, Heal, and Anderson 1979) (TABLE 1). The nitrogen content in MV-5 (average 0.4%, n = 3) is also in line with reported values for fulvic acid, although the total carbon content in the peat layer is lower than expected for either fulvic or humic acids (Paul and Clark 1989). The analyses of the MV-5 peat reveal no cohesive evidence for the presence of measurable amounts of humic acids within the deposit. The lack of humic acids in this deposit could have important implications for the radiocarbon dating of organic material such as bone collagen and in the potential for future DNA studies.

The production of a full humus profile (containing humin, humic acid, and fulvic acid) in soils is thought to involve an extensive reworking of degradation products by microorganisms (Flaig, Beutelspacher, and Reitz 1975). As detailed below, there is scant evidence for microbial activity in any of the deposits at Monte Verde. The lack of microbial activity, the low mean annual temperature (12°C), and the compression of this immature peat layer into the silica gel-invaded cultural layer could well have worked in tandem to minimize the decay of the MV-5 basal peat layer and the intercalated habitational layers.

It is important to understand that the overlying MV-5 peat bed extends only along the channel and lower banks of the ancient creek. No peat existed beyond the creek bed. (Sediments and microorganic content of the ancient bog suggest a maximum length of about 200–300 m; a maximum width of about 25–30 m at the site and con-
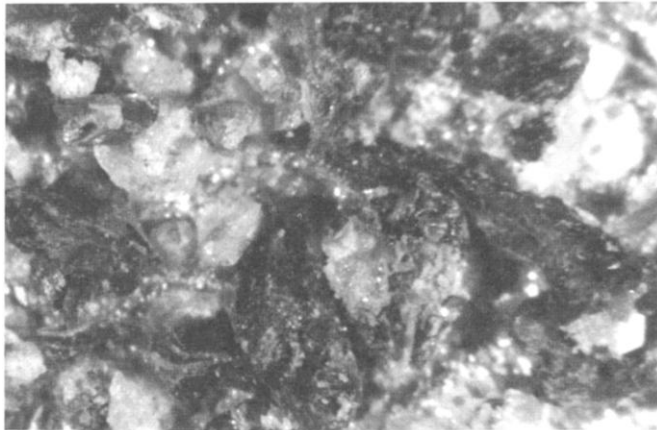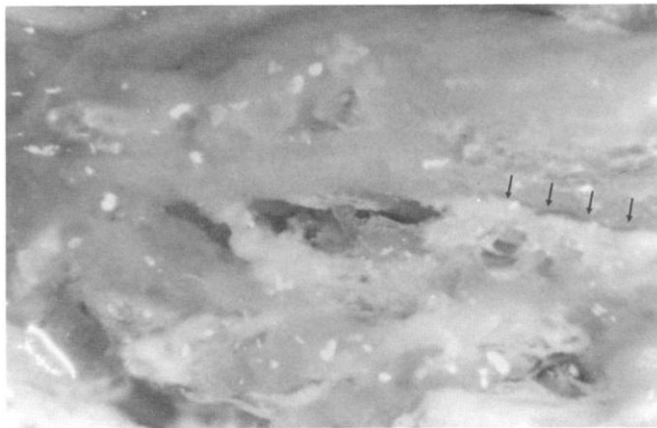
Figure 4. Silica gel pebbles imbedded in the partially decalcified mastodon bone (A). Incipient silicification (arrows) of collagen fibrils from the same piece of fully decalcified mastodon bone (B).

tinuing about 50–70 m downstream; and a maximum depth of 1 m.) The basal deposits of the peat have a floral component indicative of an environment that during and shortly after site occupation was one of reed-bog vegetation.

Above MV-5 are strata MV-1 to MV-4 (FIG. 1), with a combined depth of 85 cm and composed of small clasts and sands. The thickest stratum is MV-3 (20–40 cm): a very hard, tightly compacted layer of fine gravel; lenses of clay, hematite, limonite, and iron oxides; and small pyroclastic rocks. The compactness and sedimentological composition of this layer serve as a sealant to impede the penetration of tree roots and the downward filtration of ground water, thus protecting and preserving the underlying cultural deposits. Strata MV-1 to MV-5 are culturally sterile.

In sum, a unique set of ecological and geological circumstances resulted in the rapid development of a gallery

or fern peat that sealed the habitational layer at Monte Verde in an anoxic, reducing, and slightly acidic environment. The biochemical evidence from the soils and the well-preserved plant and animal remains excavated at the site indicate remarkable molecular preservation. In terms of the organic evidence for human activity at the site, the excavation of Monte Verde is analogous to the opening of a sealed tomb. Our present purpose, however, is to interpret the informational content of molecules rather than artifacts.

The Habitational Surface

The habitational surface is a very rich archaeological layer present over ca. 600 sq m of the ancient creek shoreline (MV-6) and terrace (MV-7) on the north side of Chinchihuapi Creek (FIG. 1). This surface is embedded in the upper 2–5 cm of the stratigraphically-homogeneous stratum MV-7. A single occupation is inferred at the site for several reasons. First, no microstratigraphic breaks in the thin habitational layer and the overlying peat layer were detected during excavation, and no evidence for extensive post-depositional alteration of material positions is present. Second, although refitting of lithic, bone, or wood materials has yielded few links, these are primarily long-distance joins, spanning the entire site and suggesting a moderately high degree of integrity (sensu Binford 1981) of deposits. And third, the hearths, pits, and habitation structures (hide- or brush-covered pole frames) uncovered at the site show no evidence of reuse and spatial overlap. In short, the cultural surface, radiocarbon dated to between 13,200 and 12,500 B.P. at Monte Verde, seems to morphologically represent a single occupational layer.

The cultural (MV-6 and MV-7) deposits at Monte Verde are a diverse collection of organic aggregates (FIG. 5), lithics, wood bits, seeds, and bone chips (FIG. 2). The heterogeneity of this deposit at the macro- and micro-stratigraphic and chemical levels of analysis contrasts sharply with both the underlying sterile gravel and the overlying peat-like cover (FIG. 2). Both the organic elemental composition (carbon and nitrogen, n = 15) (TABLE 1) and total amino-acid analysis of individual soil particles from both MV-6 and MV-7 exhibit a wide range in organic content, both qualitatively and quantitatively. In one case, a small amount of organic matter in a soil aggregate was identified as animal, probably bone, by means of an amino acid analysis that gave a clear collagen pattern qualitatively (FIG. 6). Two of the amino acid analyses of soil aggregates and additional analysis of mastodon bone collagen revealed the presence of methionine. This sulphur-containing amino acid, present in small amounts in these
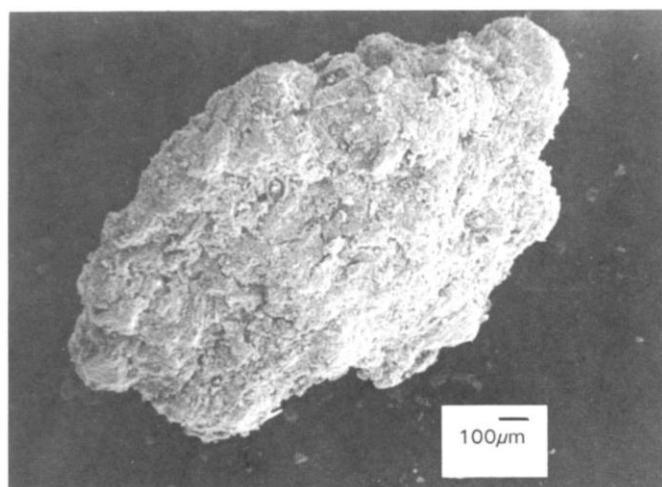
Figure 5. Scanning electron micrograph of a typical soil aggregate found throughout the culturally associated layers. This morphological unit was often found to be rich in nitrogen, and to contain high levels of hydrolyzable amino acids.
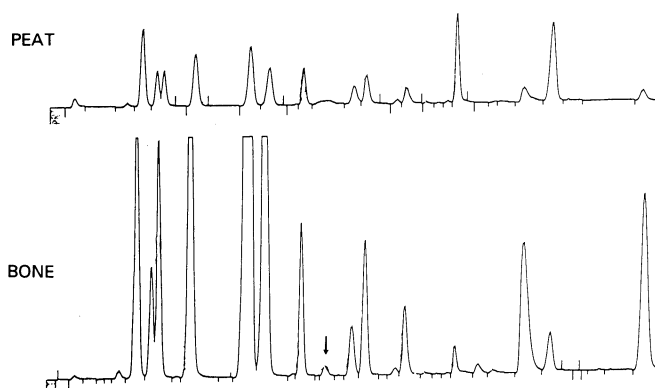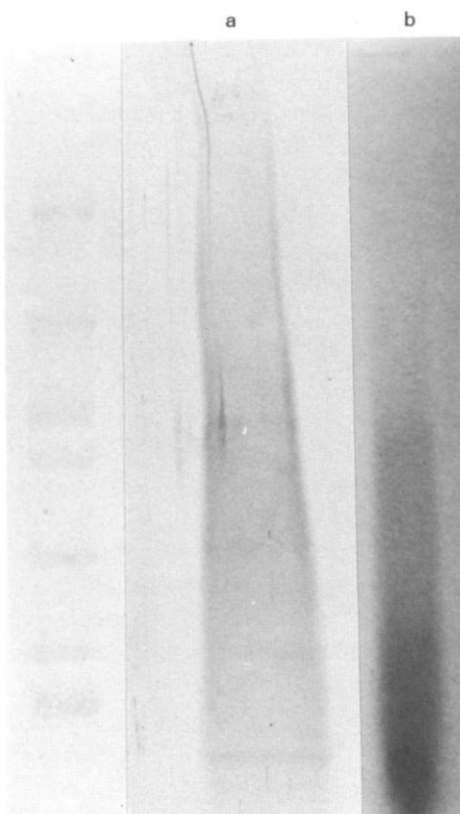

Figure 6. Amino acid analysis of the MV-5 peat and a milligram piece of mastodon bone from the MV-7 strata. The amino acid methionine is marked with the arrow in the collagen-like amino acid pattern of the mastodon bone.

Figure 7. Gel electrophoresis (SDS, 4–20% acrylamide, transfered to nitrocellulose, and gold stained) of the mastodon bone extract (a) showing multiple intact proteins from approximately 100,000 daltons down to less than 14,000 daltons. In lane b, the soil from the culturally associated areas was extracted and electrophoresed without staining, and exhibits mostly low molecular weight products. The lane on the far right is a prestained standard (Amersham) with the highest molecular weight (200,000 daltons) at the top and the lowest molecular weight (14,300 daltons) at the bottom.

samples, is very unusual at this temporal depth due to its extreme susceptibility to oxidation (Tuross, Fogel, and Hare 1988). The preservation of methionine for 12,500–13,000 years suggests rapid burial of the habitation assemblage, and the maintenance of a reducing environment for most, if not all, of the site's geologic history.

Higher order analyses confirm the molecular preservation of animal remains in the habitational layer. By gel electrophoresis, extracts of a 16 g piece of mastodon bone exhibited bandable, presumably intact, protein (FIG. 7A). Three pieces of organic material (mastodon bone, soil aggregates, and one piece of worked wood from a habitational structure) from the habitation layer were examined for the presence of humic acids. The extraction methods used included hot (50°C) basic solutions followed by acid titration to precipitate associated humic acids. There was no precipitate formed in either the mastodon bone or MV-6 soil aggregate extracts. Gel electrophoresis of the soil aggregate extractions exhibited a range on molecular weight products under 40kD (FIG. 7B), consistent with fulvic acid production. Small amounts (5% of the dry weight) of humic acids were extracted from the archaeological woods. The wood-associated humic acids are not necessarily entirely post-habitation developments, as some degradation of organic matter is anticipated during the time the wood was used in structures.

The organic and biochemical data confirm the existence in the habitational area of an organically dominated assemblage that was rapidly buried and that became anoxic very quickly due to the overlying MV-5 peat-like layer. On the
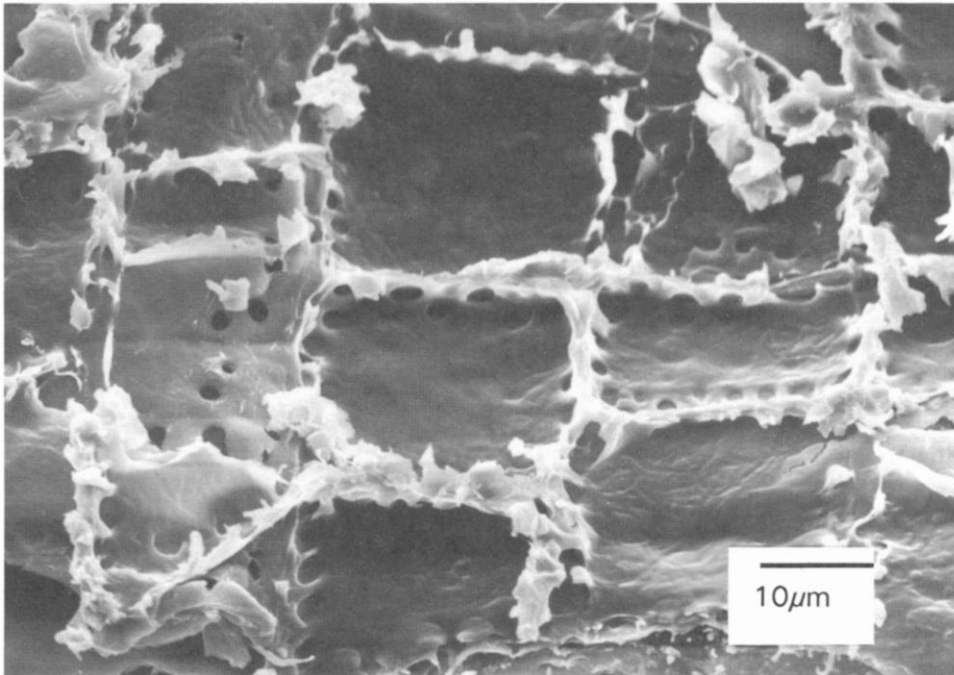
Figure 8. Scanning electron micrograph of a piece of Monte Verde wood found in the cultural layers exhibiting clear cell wall morphology with no obvious degradative changes.

basis of a concentration gradient of organic content and the tendency of substances to move from an area of higher to one of lower concentration, the predicted movement of organics would be *out* of the habitation layer. Given the general sterility of the substrata, and the immature development of the peaty overlayer, substantial organic contamination of the habitational layer from above or below is theoretically unlikely and was not observed in this analysis.

Corroborating evidence of the rapid deposition and burial of archaeological materials is derived from the absence or scant presence of decay and microbial activity in timbers and the soft tissue of animals. Macroscopic and microscopic inspection of archaeological woods reveals few signs of fungal-growth zone lines, insect boring, bacterial decay, or other organic damage in wood (Diaz-Vaz 1989), an observation confirmed by scanning electron microscopy (FIG. 8). A recent pyrolysis gas chromatography/mass spectrometry study of two types of wood excavated at Monte Verde also indicates the lack of microorganism remnants (Diaz-Vaz et al. 1991). Detailed microscopic inspection of numerous thin sections of animal soft tissue (e.g., hide and muscle) from Monte Verde (Cibull 1989) also exhibited very few signs of bacterial decay.

It has been assumed that the microorganisms seen in wood and soft tissue were there when the site was covered by muddy peat since these materials have not been exposed since excavation. Subsequent to excavation the wood and soft tissue were soaked in chemicals to preserve them. The minimal presence of microbial damage must reflect the protection of this organic debris by rapidly rising flood waters and peaty conditions, which would account for the remarkable preservation of all material. Experimental studies of microbial decay by one of us (T.D.) on fresh materials placed on the present ground surface of the site show activity in wood after 4–6 weeks and in meat and hide after 1–2 weeks. If the findings of these studies are acceptable indications of the minimum time necessary to establish microbial activity under the local conditions of the site setting, it can be estimated that the cultural remains at Monte Verde were exposed to decompositional elements no more than a few days or weeks after site abandonment and before permanent burial.

As time passed after site abandonment there was a progressive, rapid build-up of detritus and organic debris, presumably in shallow water that allowed plants such as *Juncus* sp. to invade the flora and become dominant. Indications of this are suggested by the presence of rhizomes of reed (*Juncus procerus*) and sedges (*Carex*), which are intolerant of water deeper than 0.5 m (Rameriz 1989: 154–156) and especially intolerant of widely fluctuating water levels. These are also plants that flourish in nutrient-

rich water. The presence of reeds and sedges suggests a very wet bog with a narrow open-water channel and fringing herbaceous vegetation. This shallow, wet bog seems to have persisted for a considerable period of time to judge from the approximate 1200-year span of radiocarbon dates from the lower and upper levels of the peat layer. Accompanying *Juncus procerus* in the upper levels of the peat are the remains of wood (*Nothofaus* sp). The environment would have been one of shallow water and areas of more stable vegetation, but still marshy until around 11,000 B.P., when the climate became warmer and the bog dried up.

If the ratio of precipitation to evaporation favored precipitation, then there was a long buildup of bog peat which, instead of relying on ground water for its maintenance, depended primarily on rainfall remaining high enough to have exceeded the loss of water by drainage and evaporation. This might tell us that rainfall was sufficiently high at the time of human occupation to allow rapid development of mud and, later, peat. This rainfall figure would have been higher than that at present (Heusser 1989).

In summary, the archaeological assemblage at Monte Verde was buried and persisted through time because of an unusual combination of geomorphological and geochemical circumstances. We have stressed that an interlocking sequence of events contributed to the molecular preservation at Monte Verde. Discussed below is one use of biomolecular analysis that sheds new light on human activity at the site.

An Analysis of Residues on Lithic Tools from Monte Verde

Given the extremely good molecular preservation not only in calcified tissue, but also in amorphous soil aggregates of the habitation layer, we investigated the possibility that preserved biomolecules persisted on the edges of some of the lithic artifacts. A subset of seven lithic artifacts was chosen based on the unambiguous nature of the worked edges, the staining seen in low power microscopy, and the range of raw lithic materials represented. Three of the stones were from the 13,200–12,500 B.P. component, and include two bifacially-chipped tools made of basalt (see Dillehay 1989: 15, fig. 1.9). The remaining tools comprised three flakes and one chipped stone artifact from the deeper, possible cultural layer in the site. One artifact, X1E1, is described in detail because it alone yielded positive traces of blood protein.

Specimen X1E1 is a chipped stone artifact of black, fine-grained basalt with three faces. A remnant of cortex is present on one face. The other two sides are characterized by a single-faceted flake scar that formed a striking platform and a convex face showing at least 10 flake facets. The three largest scars were removed from the platform. The cortex on one side is heavily weathered and moderately battered. This piece was struck from an original cobble (or boulder) that was larger, more fine-grained, and more heavily stream-rolled than any other observed in the Chinchihuapi Creek gravels. Similar basalts have been observed in gravels of deep sand deposits of the Maullin River terrace located several km to the north. This specimen was recovered from the base of the MV-7 stratum, radiocarbon dated ca. 33,000 B.P. The specimen is 96 mm long, 62 mm wide, and 40 mm thick.

Blood protein determination on the edges of stone tools has been reported by a number of investigators using a variety of techniques (Hyland et al. 1990; Loy and Hardy 1992), many of which are qualitative in nature (Loy 1983; Loy and Wood 1989; Newman and Julig 1989). A recent report highlighted the lack of comparability between previously reported immunological approaches used in the study of blood residues on lithics (Downs, Lowenstein, and Newman 1992), and other publications have detailed difficulties in residue analysis (Gurfinkel and Franklin 1988; Smith and Wilson 1992).

For the analysis of putative blood-derived molecules the methodology used on the Monte Verde lithics was tempered by the knowledge that many of the tools had been washed in the laboratory using both dilute acidic and basic solutions that may have removed adhering blood residues prior to these experiments. A denaturing agent (4M guanidine HCl, pH 7.2) was used to extract residual material because we thought it unlikely that the milder extraction techniques used by previous investigators (Loy 1983) would release any of the dried residues. Prior to the extraction in guanidine HCl, however, a careful analysis of stains on the edges of these tools was performed with the use of light microscopy (4–20×), and a photographic record was made. There was no correlation between stains observed and immunological results.

Each tool was partially submerged in 50 ml of the extracting solution, and shaken on a vertical vibrator for 24 hours. After extraction, the solution was concentrated at 4°C to less than 1 ml by Amicon Filtration (Ym 10) over a membrane that will retain molecules above 10,000 daltons. This stirred cell filtration apparatus is dedicated to residue analysis, so never is it exposed to high levels of protein.

The concentrated extracts were used directly in an enzyme-linked immunosorbent assay (ELISA) with controls that consisted of both reagent blanks and the extract of
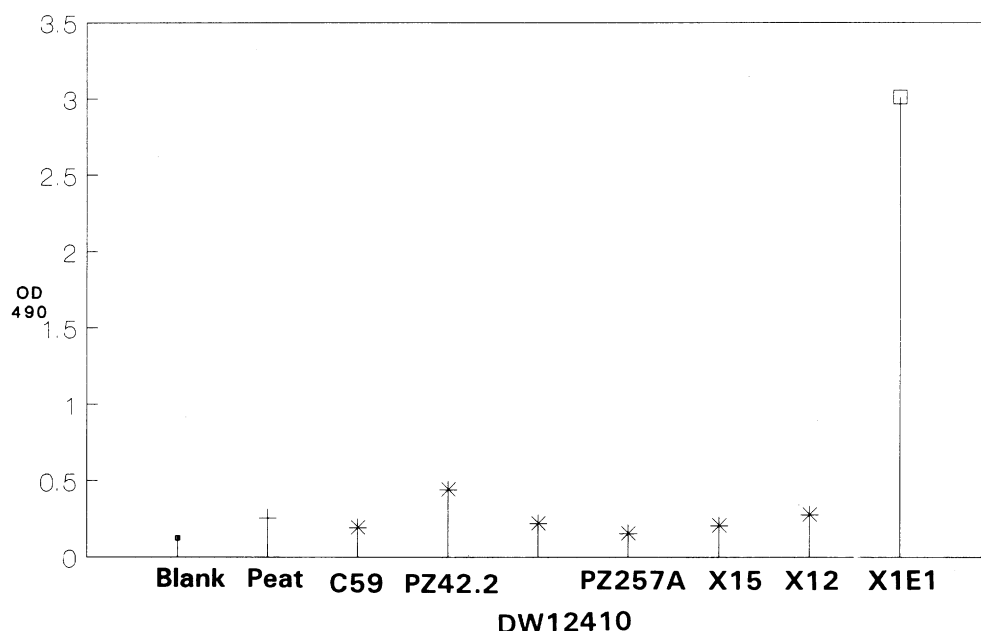
Figure 9. Average optical density (OD) readings (n = 6) of each stone tool extract in an ELISA assay compared to the chemical blank and an extract of the overlying peat. Only X1E1 is considered a positive result.

3 grams of MV-5 peat. Peat from culturally-sterile MV-5 substratum was used as a matrix control because decaying plant material is known to contain complex biomolecules that may produce false positives (Heglar 1972; Hyland et al. 1990; Kooyman, Newman, and Ceri 1992). In addition, we elected not to use the surrounding cultural MV-6 and MV-7 matrix in the experiment because the organic analysis indicated the likelihood of animal remains in these soils. For the direct ELISAs, two separate assays were done, and each tool extract was tested three times in each assay. The combined information from the direct ELISA assays is shown in Figure 9. The absorption at 490 nm is a measure of the amount of antibody bound to the extracts in the ELISA well, and thus is a measure of the amount of hemoglobin in the tool extract.

All of the tool extracts were above the chemical reagent blank, but only one of the extracts (X1E1) was substantially (8 times) reactive above the MV-5 peat extract control (FIG. 9). This experiment highlights the importance of soil matrix controls in residue analyses. Organic soils can be expected to have a significant reactivity, albeit not in a true immunological sense, to many antibodies. In this case the MV-5 peat extract was nearly twice as reactive as the reagent (chemical) blank.

It is important to note that the first antibody used in this reaction was a polyclonal antibody made in a rabbit to *human* hemoglobin. This does *not* mean that the reactive tool extract (X1E1) had remnant *human* blood on it. The rationale behind using a polyclonal antibody is based on the fact that the identification of presence or absence of *hemoglobin* was the first goal of these experiments. A polyclonal antibody, which binds to many places on the target molecule (hemoglobin), will be most sensitive in determining presence of the blood protein.

In order to further test whether the reaction of X1E1 extract in Figure 9 is the result of remnant hemoglobin molecules on the tool, a serial dilution was made of the extract and compared to a human hemoglobin standard and more concentrated peat dilutions (FIG. 10). Two observations are of note. First, the reaction of X1E1 extract "dilutes out"; that is, a less concentrated extract added to the ELISA wells produces a decline in reactivity to the hemoglobin antibody. This is an essential characteristic of the antigen/antibody binding reaction, and the shape of the X1E1 dilution curve suggests that the reactivity of this antibody is to the hemoglobin molecule. A more general discussion on ELISA assays, dilution curve shapes, and hemoglobin identification in skeletal remains can be found in Smith and Wilson (1990). Second, the dilution curve of X1E1 is contrasted with the reactivity seen in the MV-5 peat extract where a fairly consistent and nontitratable reaction is occurring. This reaction is nonimmunologic in
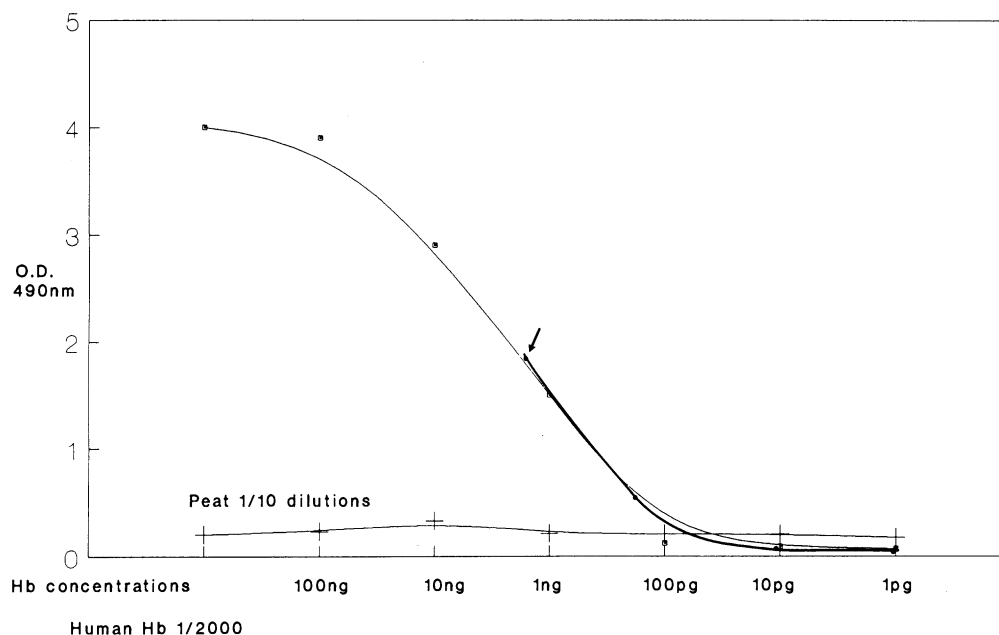
Figure 10. Reactivity of 1/10 serial dilutions of a human hemoglobin standard compared to the peat (MV-5) extract. The dark line (arrow) is a 1/10 serial dilution of the X1E1 tool extract, indicating a maximum concentration of 1 nanogram per 100 microliters of hemoglobin in the tool extract.

nature. This simple dilution experiment can be valuable in distinguishing immunological reactivities from false–positive reactions of a nonimmunological type.

None of the ELISA data address the issue of species origin of the hemoglobin found on X1E1. Attempts at determining species specificity to antibodies made against slowly evolving proteins such as albumin and hemoglobin have presented difficulties to many investigators (e.g., Landsteiner and Prasck 1913; Sensabaugh 1975). Likely blood residue proteins such as hemoglobin and albumin cross-react with other antibodies made to these protein products from other species. Sensabaugh (1976) reported that immunological cross-reactions between species were likely until the amino acid sequence of the target molecule differs by 40%.

Compounding the problem of cross-reactivity of antibodies in attempts at species determination is the serious loss of species specificity of denatured protein first reported by Landsteiner and co-workers in 1913 (Landsteiner and Prasek 1913). Furth (1925) showed that heating various animal sera resulted in a loss in species specificity, and *increased* the inappropriate reactivity in five out of six cases. Whether denaturation and degradation are predictable enough in the geosphere to permit immunological protocols to be developed that would allow for accurate species determination is an open question. A more thorough understanding about the type and extent of the denatura-

tion and degradation of blood proteins on lithic artifacts would permit the informed development of immunological analysis of archaeological artifacts. The use of species-specific monoclonal antibodies (antibodies that target only one specific site on an antigen) may hold some hope of species-specific identification of blood residues, but this hope should be tempered with the knowledge that a large percentage of monoclonal antibodies bind to conformational epitopes (the shape of the molecule, as opposed to the linear string of amino acids) (Benjamin et al. 1984). The preservation of the higher order structure of proteins in the fossil record is unknown, but it is reasonable to assume, based on the relative insolubility of many blood residues, that fundamental degradative and denaturing changes occur in most geologic settings.

In the final stage of this blood residue analysis, a Western blot was done on the remaining extract of X1E1. In a Western blot the reactivity of a sample to the antibody is assessed with the added information of the molecular weight range of the reactive material. From the ELISA dilution experiment (FIG. 10), it was possible to roughly determine how much hemoglobin was present in the extract, and to calculate the theoretical possibility of a reactive Western blot to the remaining material. There was sufficient hemoglobin in the remaining X1E1 extract for one Western blot.

An antibody was chosen based on the distribution of the

faunal assemblage at Monte Verde. Over 90% of the bone excavated at the site was mastodon (Casamiquela and Dillehay 1989), and an affinity-purified elephant anti-hemoglobin antibody (Berkeley) was used. Affinity purification is one method of decreasing species level crossreactivity, albeit at the cost of the intensity of the immunological reaction. The proboscidean antibody reacted at a dilution of 1/1000 to both elephant sera and the MV-5 peat extract, and did not react to the human hemoglobin standard. Preliminary testing of this antiproboscidean affinity-purified hemoglobin antibody in a Western blot was negative to human hemoglobin (no detectable color reaction) (FIG. 11), positive to African and Asian elephant sera, and positive to an extract of MV-5 peat (data not shown). Both elephant sera reacted to the proboscidean hemoglobin antibody at the expected molecular weight (under reducing conditions) in a diffuse band of approximately 16,000 daltons. The MV-5 peat extract showed a positive Western blot reaction to the proboscidean hemoglobin antibody in a light smear of molecular weight from about 40,000 daltons to the end of the electrotransfer. The molecular weight distribution of the MV-5 Western blot results suggests that the nonspecific reactivity to antibodies described previously in the ELISA assays may well be to the similarly-sized fulvic acid component of the peat. The important point to be made is that, although a geosphere interference must be considered in any residue analysis, it is possible to distinguish between nonspecific immunological reactions and preserved proteins based on dilution ELISA assays and Western blot analysis.

The proboscidean antibody reaction to the X1E1 extract (FIG. 11) indicates that the protein in the blood residue consists of degradation products that reaggregate to form stable, high molecular-weight complexes. This phenomenon has been observed previously from other ancient protein sources (Tuross et al. 1980), and is consistent with the relative insolubility of the protein residue on X1E1.

The Western blot data do suggest strongly that the hemoglobin preserved on this tool is not human. These data are consistent with these remnant hemoglobin molecules being proboscidean, presumably mastodon, in origin. The ability to rule out all other possible sources of blood would require samples from all other species found at the site, and additional extract. These were not available. Definitively determining species origin from blood residues by immunological techniques may remain an elusive goal. These data indicate that while identification of a specific protein on tool residues may be done either by ELISA assay (and dilution experiments) or by Western blotting, the strong immunological data that address spe-
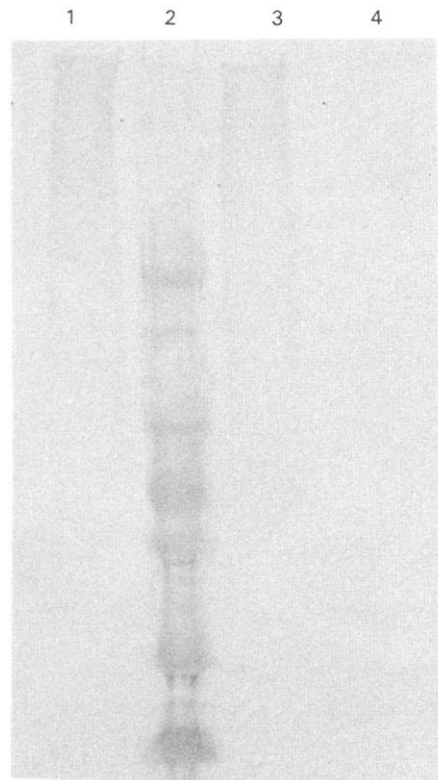

Figure 11. Western blot of the X1E1 extract (lanes 1 and 3), and a human hemoglobin standard. This rabbit antibody was made against proboscidean hemoglobin, and affinity purified to remove cross species reactivity. No crossreactivity is noted in lane 4, the human hemoglobin standard, and the crossreactivity in lanes 1 and 3 is to a range of high molecular weight material. Lane 2 is a prestained standard, and the color in this lane is not associated with antibody reactivity. The standard band closest to the top to the nitrocellulose blot is 200,000 daltons, and the band at the bottom of the blot is 14,300 daltons.

cies origins will tend to be negative, and will allow archaeologists to rule out certain species as blood residue sources.

Summary and Conclusions

The archaeological remains found at Monte Verde persisted for thousands of years because of an unusual set of circumstances, including the rapid anoxic sealing of the site, the relatively low mean annual temperatures, and the leaching of percolating water by silica-gel rich substrata, which all combined to make an environment that is nearly ideal for the long-term preservation of organic materials: cold, dry, and lacking oxygen. By combining the data from the geochemical analysis of the soils at Monte Verde with the immunological experiments of tool extracts we were better able to interpret the tool extract data. One of the tools was positive to antibodies made against hemoglobin, and the protein extracted from this tool had severely

altered and aggregated into a high molecular weight complex.

The rationale for the development of this type of molecular analysis extends beyond an explication of macro-artifactual (e.g., stone tools, modified bone) remains at the site of Monte Verde. The research reported here is the latest in a series of efforts (Dillehay 1989) to design, investigate, and experiment on the atomic and molecular scale in an archaeological site where we know about the content and patterning of macroscale and microscale size data. The site of Monte Verde has afforded the opportunity to test key concepts that may link macro and micro data with molecular and atomic minimum size information. These multiple levels of analysis may yield the same results independently or present conflicting lines of evidence, but, most importantly, minimum size data may reveal more information at sites that are not well preserved.

Notes on Materials and Methods

The soil matrix from the cultural and noncultural deposits of Monte Verde as well as skeletal remains and lithic artifacts were analyzed. The techniques used in these experiments were chosen for their applicability to ancient remains, including sensitivity and lack of interference by degradation products.

Amino Acid Analysis

Microgram to milligram samples are hydrolyzed in 6N HCl under N_2 at 110°C for 20 hours. The samples are dried under a vacuum, and resuspended in pH 2 buffer. The samples can then be directly injected in a postcolumn orthophthaldehyde (OPA) amino acid analyzer that relies upon ion exchange for separation, or reacted with (phenyisothioscyanate-PITC) reagents for separation on the Waters Pico-Tag system. Either analytical method is capable of picomole detection of amino acids.

Carbon and Nitrogen Analysis

Microgram to milligram quantities of samples are weighed and inserted into sample boats. Combustion of the sample in a helium atmosphere is performed at 1800°C. Carbon and nitrogen content is obtained on one set of samples in a Carlo Erba CHNS analyzer and quantitation is referenced to a standard compound (acetaldehyde for C and N).

Protein Extractions

Protein extractions are performed under denaturing conditions in order to solubilize and characterize individual components. Calcified tissue such as bone is subject to dissociative, denaturing extracts (Termine et al. 1985; Tuross 1991) that have successfully removed intact proteins from subfossil bone. For protein analysis the extracts are concentrated by Amicon filtration, desalted (Sepharose 12) in ammonium acetate, and lyophilized or exchanged into 7M urea.

ELISA Assays

Enzyme-linked immunosorbant assays (ELISA) are done either by direct detection (binding the tool extract to the microtiter plates) or by a capture ELISA in which the antibody is first bound to the microtiter wells, and the sample allowed to bind to the antibody (Harlow and Lane 1988). Both direct and capture ELISAs were done on the tool extract from X1E1. General ELISA procedures follow Fisher et al. (1987) with the following modifications: first antibody concentrations (polyclonal rabbit antihuman hemoglobin-Sigma) were 1/1000 in the direct ELISA and 1/2000 in the dilution ELISA; second antibody was horseradish peroxidase conjugated (HRP) goat antirabbit IgG (Kierkegaard and Perry). Color development was achieved with o-phenylenediamine dihydrochloride (Sigma), and all blocking was done in 1% nonfat dry milk.

Western Blot

After gel electrophoresis on a 4–20% gradient polyacrylamide gel, protein is transfered to nitrocellulose (Towbin, Staehilin, and Gordon 1979) at 200mA for 1 hr. The nitrocellulose is blocked with nonfat dry milk, and incubated with first antibody (1/1000) at 4°C overnight. After washing, horseradish peroxidase (HRP) second antibody is added (1/2000) for two hours at room temperature. Visualization is by 4-chloro-1-naphthol. The antibody used on the tool extracts was an affinity purified rabbit antiproboscidean hemoglobin preparation (Berkeley Antibodies) used at a concentration of 1/1000.

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