Microstructural, elemental and biomolecular preservation of Tyrannosaurus rex cancellous tissues
by Mary Higby Schweitzer

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences
Montana State University
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Abstract:
A well preserved, articulated Tyrannosaurus rex, recovered from the Hell Creek Formation in Eastern Montana, showed little evidence of diagenetic alteration. The cancellous bone tissues were apparently well protected from water infiltration or mineral deposition, both by the dry climate in the region of burial and by the very dense cortical bone which surrounded them. These tissues provided an opportunity to test the hypothesis that indigenous biomolecules might be preserved over the course of millions of years.

A variety of analytical techniques were used to test this hypothesis. Light microscopy, electron microscopy and confocal microscopy provided structural information, and verified the minimal degree of diagenesis which was apparent upon gross examination. Elemental analysis confirmed that there was no addition or subtraction of elements from the bony matrix through exchange with surrounding sediments.

HPLC analysis of extracts from these bone tissues revealed the presence of molecules with light absorbance maxima consistent with nucleic acids, peptides/proteins, and heme-containing molecules. Extracts of the bone were also analyzed for amino acid content. A high molar glycine ratio and the presence of hydroxylysine peaks in the T. rex samples suggested the presence of collagen type I remnants. In addition, in an attempt to verify that the amino acids were derived from an ancient source, ratios were obtained for the D/L isomers of each amino acid, and the data are consistent with this hypothesis. High resolution NMR spectrometry revealed peaks characteristic of heme containing proteins.

It was found that DNA could be extracted from the T. rex bony tissues, but the extracted DNA is of high molecular weight, making it unlikely to be entirely dinosaurian in nature. However, DNA-specific staining resulted in visualization of DNA within individual osteocyte lacunae, and it is possible that these DNA fragments may be dinosaurian in origin.

Our results indicate that these dinosaur tissues contain numerous biomolecules. While some may be contaminants, the probable presence of collagen type I, visualization of DNA in the osteocyte lacunae, and the potential for hemoglobin-derived molecules provide evidence that some molecules of dinosaurian origin may remain within these tissues.
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Mary Higby Schweitzer

A thesis submitted in partial fulfillment of the requirements for the degree of
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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date

Graduate Dean
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Date 5/12/95
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# Table of Contents

1. Introduction .................................................................1
   References cited .............................................................12

2. Lack of Replacement in *Tyrannosaurus rex* Cancellous bone Provides a Microenvironment favorable to the Preservation of Biomolecules ...........................................19
   Introduction .................................................................19
   Materials and methods ......................................................22
   Results .........................................................................31
   Discussion .....................................................................52
   Conclusion .....................................................................59
   References cited .............................................................62

3. Preservation of Putative Hemoglobin Compounds in Trabecular Tissues of *Tyrannosaurus rex* ...........................................66
   Introduction .................................................................66
   Materials and Methods ......................................................70
   Results .........................................................................76
   Discussion .....................................................................86
   Conclusion .....................................................................88
   References cited .............................................................91

4. Extraction and Analysis of DNA from the Trabecular Tissues of *Tyrannosaurus rex* ......................................................94
   Introduction .................................................................94
   Materials and methods ......................................................99
   Extraction Protocols .......................................................99
   Primer Design ..............................................................104
   PCR Protocols ..............................................................108
   Results and Discussion ...................................................110
   Conclusion ...................................................................117
   References cited ............................................................118

5. Conclusion .................................................................123
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>PITC Derivatized Amino Acid Values in Mole-Percentages</td>
<td>51</td>
</tr>
<tr>
<td>2-1</td>
<td>Racemization State of Selected Amino Acids Found in Cancellous Bone Extracts, Given in Mole Percentages, Derived from Run Totals</td>
<td>80</td>
</tr>
<tr>
<td>3-1</td>
<td>PCR Reaction Mix and Amplification Profile for a 50μl Reaction</td>
<td>109</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 2</td>
<td></td>
</tr>
<tr>
<td>1. Trabecular bone samples from <em>Tyrannosaurus rex</em>, Hadrosaur, and recent horse, and corresponding densities</td>
<td>32</td>
</tr>
<tr>
<td>2. Light micrographs of trabecular bone tissues</td>
<td>34</td>
</tr>
<tr>
<td>3. Confocal laser micrographs of bone tissues</td>
<td>35</td>
</tr>
<tr>
<td>4. Scanning Electron Micrographs of dinosaur tissues</td>
<td>37</td>
</tr>
<tr>
<td>5. Topographic and Compositional studies of dinosaur trabecular tissues</td>
<td>38</td>
</tr>
<tr>
<td>6. Elemental analysis of bone tissues by electron probe</td>
<td>40</td>
</tr>
<tr>
<td>7. Transmission Electron Micrographs of decalcified bony tissues, revealing fibrillar nature of the bone matrix</td>
<td>42</td>
</tr>
<tr>
<td>8. HPLC profiles of extractions of <em>Tyrannosaurus rex</em> tissues and controls, monitored at 214nm and 410 nm to identify absorbance maxima</td>
<td>44</td>
</tr>
<tr>
<td>9. Electron Diffraction analysis</td>
<td>46</td>
</tr>
<tr>
<td>10. Proton NMR spectrum of <em>T. rex</em> extractions</td>
<td>48</td>
</tr>
</tbody>
</table>

| Chapter 3 | |
| 1. Gross and microscopic analysis of trabecular tissues shows lack of diagenetic changes | 77 |
| 2. UV/VIS absorbance spectrum of extracts of dinosaur tissues | 81 |
| 3. Proton NMR spectrum of *T. rex* extracts after filtration to remove impurities | 82 |
| 4. Electron Paramagnetic Resonance profile of *T. rex* extracts compared with known sample | 83 |
5. Resonance Raman Profile of dinosaur extracts, compared with known samples......................85

Chapter 4

1. Alignment of PCR primers against a segment of Ostrich DNA sequence............................108

2. Extractions of T. rex tissues, electrophoresed, stained, and visualized with UV light..............113

3. PCR amplification products, using T. rex extracts as template for reactions..........................114

4. Alignment of sequences obtained from PCR amplification of extracts of T. rex tissues........115
ABSTRACT

A well preserved, articulated *Tyrannosaurus rex*, recovered from the Hell Creek Formation in Eastern Montana, showed little evidence of diagenetic alteration. The cancellous bone tissues were apparently well protected from water infiltration or mineral deposition, both by the dry climate in the region of burial and by the very dense cortical bone which surrounded them. These tissues provided an opportunity to test the hypothesis that indigenous biomolecules might be preserved over the course of millions of years.

A variety of analytical techniques were used to test this hypothesis. Light microscopy, electron microscopy and confocal microscopy provided structural information, and verified the minimal degree of diagenesis which was apparent upon gross examination. Elemental analysis confirmed that there was no addition or subtraction of elements from the bony matrix through exchange with surrounding sediments. HPLC analysis of extracts from these bone tissues revealed the presence of molecules with light absorbance maxima consistent with nucleic acids, peptides/proteins, and heme-containing molecules. Extracts of the bone were also analyzed for amino acid content. A high molar glycine ratio and the presence of hydroxylysine peaks in the *T. rex* samples suggested the presence of collagen type I remnants. In addition, in an attempt to verify that the amino acids were derived from an ancient source, ratios were obtained for the D/L isomers of each amino acid, and the data are consistent with this hypothesis. High resolution NMR spectrometry revealed peaks characteristic of heme containing proteins.

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CHAPTER ONE

INTRODUCTION

Paleontology is a somewhat subjective science, based largely upon assumption and inference. For example, statements that are made about depositional environments or decay processes are based upon the assumption that processes and forces we observe acting upon organisms today are the same as those which acted in the past. When conclusions are drawn about the biomechanical constraints of an animal (Carter, et al., 1987, 1992; Van der Meulen, et al., 1992, 1993), or about the physiology of organisms through what is revealed by histological studies (Reid, 1984a, b, 1985; Chinsamy, 1991, 1995; Chinsamy, et al., 1994; Varrichio, 1993; de Riolles, 1980, de Riolles, et al., 1983, 1991) they are based upon inferences that those characters preserved in bone tissues had the same meaning as similar characters seen in extant organisms today.

One assumption which has been pervasive in paleontological thought ever since the inception of the science is that the organic constituents of an organism, namely soft tissues, the component cells, and the proteins and nucleic acids which were produced by its living cells, were either destroyed in the process of fossilization, or rendered uninformative by the diagenetic changes accumulated during geological time (Curry, 1990). With advances in the
fields of analytical biochemistry, molecular biology, and geochemistry, it is becoming increasingly evident that this is not the case, and that there is a wealth of information to be gained through the study of molecules preserved in the fossil record. Examination of such molecules may provide a means for adding another degree of objectivity to the scientific discipline of paleontology.

Molecular preservation is enhanced by certain conditions, most importantly, rapid burial and desiccation. Rapid burial enhances preservation two ways; by slowing bacterial decay through the creation of anoxic microenvironments, and by protecting the specimen from exposure to destructive environmental conditions (Allison, 1990). Desiccation is a crucial factor in the preservation of these molecules, since hydrolytic damage caused by biomolecules interacting with water is one of the primary causes of degradation (Lindahl, 1993; Tuross, 1994).

The possibility of biomolecular preservation in ancient tissues was suggested as early as the turn of the century (Seitz, 1907), although methods were not yet developed to fully test this hypothesis. Microstructural preservation led others to suggest that organic constituents remained in the bones of extinct animals, and that they may be recognizable and identifiable. (Swinton, 1934; Moody, 1923; Nowicki, et al., 1972). However, until the mid to late 1970's most of these conclusions were based strictly on morphological
evidence, rather than rigorous biochemical tests. The problem of relying on morphological evidence alone was illustrated by the identification of apparently perfectly preserved fibers in 450 million year old graptolites (Towe and Urbanek, 1972) from which few amino acids could be identified.

In 1974, DeJong, et al., demonstrated the retention of the antigenic components of proteins within the shells of mollusks which could be dated to approximately 70 MY BP through precipitation reactions with antisera. This work was continued by others (Weiner, et al., 1976; Westbroek, et al., 1979), again showing that indigenous biomolecules corresponding to structural proteins remained within the matrices of invertebrate shells.

Other work has demonstrated the presence of proteins and/or amino acid protein constituents through the use of sensitive amino acid analyses (Armstrong, et al., 1983; Gurley, et al., 1991). With the realization that amino acids degrade over time to a racemic mixture of their D/L isomers, and that each amino acid had a characteristic rate of degradation, it has been proposed that such amino acid analyses may provide an independent means of verifying the ages of the specimens (Schroeder and Bada 1976; Bada, 1985). In addition, it was felt by some that analysis of the isomeric ratios could be used to determine the degree, if any, of modern biomolecular contamination (Armstrong, et
(Kimber and Griffen, 1987), and a method for verifying the indigeneity of such constituents using both amino acid racemization analyses and stable isotope geochemistry has been proposed (Macko and Engel, 1991).

Lowenstein (1980, 1981, 1985) demonstrated that immunological probes, in the form of solid phase radio-immunoassays, yielded positive results in fossil bone. He was able to extract and identify proteinaceous compounds in a variety of bone materials, including human, which dated to 2 million years BP. He proposed utilizing such immunological methods with fossil bone to elucidate phylogenetic relationships (Lowenstein, 1985, 1988).

The search for proteins in fossil bone has primarily concentrated upon collagen type I. The structural constraints of this protein, as well as the incorporation of the amino acids hydroxyproline and hydroxylysine, which are unique to collagen, give it an easily recognized signature upon amino acid analysis. Since this is a structural protein with a high affinity for hydroxyapatite, the mineral prevalent in bone, it has a high potential for preservation (Tuross, 1994). In addition, it is the most prevalent protein in bone matrix (Van der Rest, 1991), making it a likely candidate for identification. Its unique cross banded pattern and fibrillar nature, when visualized in
electron microscopy, makes its presence easy to verify. Collagen has been identified in several ancient bone samples (Ho, 1965; Baird and Rowley, 1990; Tuross and Stathoplos, 1993; Jope and Jope 1989;), including dinosaurs (Wyckoff and Davidson, 1976).

While most early work focused on proving the presence of amino acids, or identifying structural proteins, it soon became apparent that proteins other than collagen could be identified in appropriate fossil specimens. Proteins such as IgG and albumin (Cattaneo, et al., 1992; Tuross, 1989) have been shown to be preserved in fossil bone. In addition, the vertebrate-specific protein osteocalcin has been identified from bone and tooth samples (Ulrich, et al., 1987) including those of dinosaurs (Muyzer, et al., 1992).

Hemoglobin, the protein involved in oxygen transport, has also shown a surprising potential for preservation in the fossil record, having been identified in association with stone tools (Loy, 1983, 1987; Loy and Wood, 1989), as well as ancient bone samples (Ascenzi, et al., 1985; Smith and Wilson, 1990; Cattaneo, et al., 1990). Hemoglobin is important both as an indicator of certain aspects of the physiology of an organism (Dickerson and Geis, 1983) and as an estimate of phylogenetic divergence (Nikinmaa, 1990; Perutz, 1983). Its presence in dinosaur bone may shed light upon questions of metabolic rates, as well as the relationship of these animals to modern taxa.
However, of all the biomolecules produced by an animal, the most readily obtainable phylogenetic information is contained in the sequences of its DNA, and therefore the ultimate goal of molecular palaeontology has been to recover DNA sequences from extinct taxa. Data bases now exist (Hobish, 1986) which allow comparison of sequences obtained from fossil specimens with those of extant taxa. Computerized algorithms have been established to analyze generated sequence data, not only for the establishment of phylogenetic trees (e.g., Felsenstein, 1981) but also for use in inferring evolutionary distance (Lewontin, 1989). These tools not only enhance the information contained in the base pair sequences of the chains of DNA, but also provide the investigator a means with which to establish the validity of DNA sequences obtained from ancient tissues.

The majority of scientific opinion has long held that DNA is unstable and easily degraded; therefore its presence in tissue samples older than a few thousand years is highly suspect (Lindahl, 1993; Curry, 1990). As our understanding of the chemical nature of this molecule increases, it is becoming evident that under certain conditions DNA may be more stable than previously assumed. Desiccation, protection from oxidative damage through rapid burial, and presence of a mineral substrate to which the molecule may adsorb and thus become stabilized, all enhance the preservation potential of DNA (Eglington and Logan, 1991;
Tuross, 1994). More efficient means of extraction (Hoss and Paabo, 1993), as well as the advent and increasing sophistication of the polymerase chain reaction (PCR) have greatly expanded the range of usefulness of DNA in determining phylogenies (Handt, et al., 1994; Erlich, et al., 1991; Paabo, et al., 1989). However, the sensitivity of PCR, which has made possible the study of rare template molecules such as those from ancient specimens, itself creates problems in the analysis of these same specimens. The most notable of these problems are the ease with which contaminating molecules are amplified and the misleading results due to template damage in ancient samples (Handt, et al., 1994; Paabo, et al., 1990; DeSalle, et al., 1993). Therefore, until these problems can be adequately resolved, the proof of indigeneity of molecules recovered from ancient samples must rest on phylogenetic analysis.

In spite of the assumptions made of the chemical instability of DNA, this molecule has been detected in many fossil specimens from relatively recent extinct animals such as the quagga and mammoth (Higuchi, et al., 1987; Hagelberg, et al., 1994) to much older specimens extending back millions of years (Golenberg, et al., 1991; DeSalle, et al., 1992; Cano, et al., 1992), and these results have been verified through phylogenetic analysis. Recovery of DNA from specimens extending well into the age of dinosaurs has also been reported (Cano, et al., 1993), although
dinosaurian DNA has yet to be shown unambiguously to exist in fossil specimens.

The phylogeny of the Dinosauria has been well elucidated through morphological analyses which align this taxa most closely with Aves among extant groups (Gauthier, 1986). DNA sequences recovered from dinosaur bone, which are purported to be indigenous, must reflect this alignment. Until such alignment can be demonstrated and such data are consistently repeatable, reports of recovery of indigenous "dinosaur" sequences (Woodward, et al., 1994) will remain suspect, regardless of the preservational state of the bones from which these DNA sequences are obtained (Hedges and Schweitzer, 1995). However, evidence for the preservation of indigenous proteins in fossil bone makes the case for authentic DNA preservation much stronger.

Mechanisms for the preservation of protein or other organic compounds over the course of geological time remain to be elucidated. However, it has been proposed that these compounds, in the process of degradation and bond breakage, may react with each other to form complex biopolymers which resist further degradation (Curry, 1990). While these reactions may be a key factor in the preservation of these molecules, analysis becomes very complicated in light of the difficulties involved in separating out the components of such an aggregation.

A second mechanism which has been proposed for
preservation of biomolecular materials is the stabilization of these molecules through complex interactions with organic breakdown products of the surrounding soils, in particular humic or fulvic acids (Tuross, 1994). These associations, while an important factor in the preservation of biomolecules, are also deleterious from an analytical standpoint. Humic acids fluoresce at the wavelengths of some proteins, amino acids, or nucleic acids (Tuross and Stathoplos, 1993), and may therefore interfere with or mask indigenous biomolecular signals. In addition, these compounds inhibit the action of some enzymes which may be used to identify organic remains (Tuross, 1994).

Finally, a primary factor in preserving both proteins and nucleic acids over geological time may be the association of these proteins and/or nucleic acids to a mineral substrate, such as is found in bone (Ambler and Daniel, 1991; Logan, et al., 1991; Tuross, 1989). Adsorbance of biomolecules to minerals may be among the most important of mechanisms involved in biomolecular preservation.

Undoubtedly, the preservation of biomolecules over the course of geological time is enhanced by a combination of the above mechanisms, to varying degrees. There are also, most likely, other interactions involved in such preservation that have yet to be identified. There is little experimental evidence for a temporal limit to
preservation enhanced by such mechanisms.

Specimen Description

In the spring of 1990, a Tyrannosaurus rex skeleton was recovered from the Hell Creek formation in eastern Montana. The articulated condition and state of completeness of the skeleton indicated that burial had been rapid enough to forestall destruction through scavenging, bloat or weathering, although not rapid enough to prevent some minimal displacement. It was found in a stream channel, surrounded by a grey to white, fine grained sandstone which was mostly well cemented. The sediments surrounding the specimen also contained abundant plant material, most of which was coalified. The pelvic region was covered by 1.0 to 1.5 meters of overburden. When the specimen was jacketed, there was still 0.5 to 0.6 meters of sediment between the unexposed hind limb used in this study, and the exposed pelvis.

The bony tissues within the trabecular cavities of the long bones were, upon gross examination, unreplaced and air filled, and showed no evidence of secondary crystallization or deposition as is commonly seen in most dinosaur bone. The trabecular tissues of the long bones were lightweight, brittle, and unreplaced, and had the look and feel of
similar extant tissues which have been naturally desiccated. In addition, these tissues were reddish brown in color. Since it is the percolation of groundwater through the tissues, and the subsequent precipitation of minerals out of this interstitial water that causes secondary crystallization (Richardson and McSween, 1989), the lack of such may be interpreted as evidence of the lack of or minimal of exposure to groundwater in these tissues. Because of the gross appearance of these tissues, as well as the preservational conditions, this seemed to be an ideal specimen with which to test the hypothesis that molecules may be preserved within the tissues of this dinosaur which could be shown to be indigenous.

The analysis of these tissues was carried out as a three-tiered approach. First, microscopic and elemental analyses were conducted to verify minimal diagenetic alteration of the bone. Next, attempts were made to identify proteinaceous components within the tissues consistent with proteins expected to be associated with bone elements. And finally, DNA extractions and analyses were carried out. The following pages delineate methodologies and results of this study.
REFERENCES CITED


CHAPTER TWO

LACK OF REPLACEMENT IN TYRANNOSAURUS REX CANCELLOUS BONE PROVIDES A MICROENVIRONMENT FAVORABLE TO THE PRESERVATION OF BIOMOLECULES

Introduction

Histological studies of fossil bone sections have been conducted since the early days of paleontology (see de Ricqles, 1980), and structures such as Haversian systems, osteocyte lacunae, and vascular canals have been demonstrated to be preserved in ancient bone. Correlations of the characteristics seen in thin sections of cortical bone with the physiology of the animal have become increasingly important, and links between structures present in the cortical bone, such as primary and/or secondary osteons and growth rings, and metabolic rates have been proposed (Reid, 1984a, 1984b, 1985; de Ricqles, 1980, 1983; Bakker, 1986). In addition, as more data become available, estimates of growth rates, onset of maturity, and tentative age of the organism may be made through histological studies of ontogenic series of bones from different species of dinosaurs (Horner and de Ricqles, 1994; Chinsamy, 1991, Chinsamy, et al., 1994; Varrichio, 1993).
The fossil record is capricious in its preservation. Whereas most fossils are well permineralized, individual specimens can show little evidence of permineralization. Surprisingly delicate structures such as feather barbules can sometimes be seen, and, in fossil Lagerstatten such as the Messel Shale (Schaal and Ziegler, 1988), pigment, hair, and individual sarcomeres of muscle fibers have been preserved. Such intricately preserved specimens can reveal additional details which contribute to our understanding of how extinct organisms lived, looked and functioned. For example, the impressions of feathers in the muds surrounding Archaeopteryx led to its placement in the bird lineage, and the recent discovery of oviraptor eggs (Norell, et al., 1994) may help to illuminate the nesting and nurturing behaviors of this little known group of dinosaurs, as did the earlier discoveries of hadrosaur nesting grounds (Horner and Makela, 1979). However, little has been done until lately to examine the possibilities of preservation of the molecules which constituted the fossil organisms. This may be due in part to the rarity of appropriate fossil finds, which precludes destructive analyses, and in part to the fact that adaptations of technologies developed for the field of molecular biology have only recently been developed for examinations of fossil specimens.

DNA and proteins carry much information about an organism, and can provide important clues as to its biology
and evolutionary relationships (Patterson, 1987; Avise, 1994, Hedges, et al., 1990, Hedges, 1994; Hedges and Sibley, 1994). To be able to detect and identify these biomolecules is one of the goals of molecular paleontology. Identifying the type of fossil tissue samples that may best preserve organic remains is the first step in the search for informative biomolecular compounds. Specimens that are well preserved in both morphological and microstructural features may also be those which are most likely to harbor indigenous biomolecules. This report analyzes the physical and molecular state of preservation of one dinosaur specimen which was largely unreplaced with geological materials.

A specimen of Tyrannosaurus rex, Museum of the Rockies (MOR) 555, was recovered from the upper one-third of the Hell Creek Formation in McCone County, Montana in the spring of 1990, and was the source of the material examined in this study. Chapter one contains a description of the depositional environment of this specimen.

Macroscopic examination of the trabecular bony tissues within the marrow cavities showed that sections of the trabeculae were devoid of geological sediment. In histological sections of both the cortical and trabecular tissues, the vascular canals and osteocyte lacunae were free of sedimentary infilling or secondary recrystallization. These conditions suggest little penetration of the fossil by water. This was an important consideration because a
dehydrated state is expected to favor molecular preservation. Based on these preliminary examinations further studies were conducted upon samples of this specimen to determine the extent of its sub-macroscopic preservation.

Materials and Methods

Gross Examination

Trabecular tissues were removed from the right and left tibiae and femora of MOR 555, as well as from metatarsal and phalangeal elements. Cortical bone from the right femur, tibia, and fibula were also examined. As controls, sandstone sediments immediately surrounding the bones were subjected to most of the same conditions of preparation and examination, as the MOR 555, and samples of well permineralized dinosaur bone were visually examined for signs of sedimentary deposition or other diagenetic change. As a further comparison, trabecular tissues from a desiccated horse bone which had weathered on the Montana prairies for an indeterminate amount of time, were also examined. The specimens were grossly examined for the presence of secondary crystallization, sedimentary infilling, or deposition within bone tissue spaces. The bone tissues were weighed as a rough estimate of the extent of geological replacement of the tissues. After sealing the
T. rex and horse tissue samples with a shrink wrap, all three specimens were submerged, and volume displacement measurements were obtained. From these, weight/unit volume values could be calculated as a measurement of the density of each.

Confocal Microscopy

Trabecular tissues from MOR 555 were coarsely crushed, placed on a glass slide, and exposed to the DNA-specific fluorochrome, Hoescht 33258 (10 mg/ml, Sigma) for 60 seconds. As a control, a mounted section of modern ostrich bone was also examined, after similar exposure to the dye. This dye intercalates with double-stranded DNA molecules, and fluoresces when stimulated by appropriate laser light. Different methods were required for ancient and modern bone due to the different physical states of the tissues. Undecalcified modern bone proved to be very difficult to crush to the point where the fragments allowed for transmission of light, and the use of an acid decalcification process would be expected to hydrolyze any DNA which might be present. In addition, the fatty tissues associated with the modern bone prevented full infiltration by the embedding material used. On the other hand, the tissues of the dinosaur were extremely dehydrated, and infiltration by the plastic embedding material was complete.
This effectively blocked access of the Hoescht stain to its target molecules in the tissue. Crushing the bone without any decalcification or embedding process maximized the fluorescent signal which could be observed in the dinosaur tissues. Therefore, for this study, the bony tissues of MOR 555 were coarsely crushed and exposed to the dye, while the undecalcified ostrich tissues were embedded and sectioned as for light microscopy.

Microscopic Examination

The bony tissues and sandstone were embedded in either Silmar™ or a polymethyl methacrylate embedding media. Samples of bony tissues from extant alligator and/or ostrich received identical treatment when possible, and were used as controls. When hardened, these embedded tissues were sectioned using a 320 grit diamond blade on an Isomet static arm saw. The wafers were mounted to glass slides with either a two-ton, two-part epoxy, or a super glue (e.g., HotStart™ or Jet™) and ground to the desired thickness on a series of decreasing grit aluminum oxide papers mounted on a single speed grinder, then polished by hand with 5 and 1 micron aluminum oxide gel.
Scanning Electron Microscope (SEM) Analyses

Samples of well replaced dinosaur bone, the unreplaced MOR 555 trabecular bony tissues, and modern ostrich and alligator bone samples were coated with carbon for examination under scanning electron microscopy (SEM). Embedded sections as well as unembedded bone fragments (not shown) were used. Both secondary electron (SEI) and back-scattered electron (BSE) images were obtained, and topographical and compositional studies were carried out on a JEOL 6100 electron microscope. Elemental analyses were performed as a standardless assay using a Noran Voyager Energy Dispersive X-ray (EDX) system coupled to the SEM.

Transmission Electron Microscope (TEM) Analyses:

Fragments of bone from MOR 555 and from modern ostrich bone were subjected to transmission electron microscopy (TEM). The bone samples were bathed in 44% formic acid overnight at room temperature to remove the mineral phase from the tissues, leaving only organic components. The T. rex tissues were also examined in the undemineralized state. Tissues were fixed in 3% glutaraldehyde in 0.1 M potassium sodium phosphate buffer (PSP), pH 7.2 for 3 hours at room temperature under vacuum, and at 4°C overnight. This was followed by a series of buffer washes in 0.1 M PSP buffer,
then infusion with 2% OsO₄ in 0.1 M PSP buffer for 4 hours at room temperature. Samples were then washed twice in 0.1 M PSP buffer for ten minutes each and were then dehydrated with a series of acetone washes increasing from 25% to 100%. Samples were suspended in a 1:1 solution of propylene oxide (PO) and Spurrs resin overnight at 4°C, then in 2:1 Spurrs:PO for 7 hours on a rotary shaker. The samples were then transferred to 100% Spurrs overnight. Finally, the samples were placed into new solutions of Spurrs in BEEM capsules, and baked at 70°C. Once hardened, the samples were sectioned with a diamond knife in a Reichaert OM-U2 ultramicrotome and placed on a 300 mesh copper grid. The sections were stained with uranyl acetate saturated in 100% methanol for 5 minutes, rinsed in a methanol series, stained for 3 minutes in Reynolds lead citrate, rinsed in 0.2 M CO₂-free NaOH, and finally rinsed in distilled water. Sections were then visualized through a Zeiss EM 10 transmission electron microscope.

**Electron Diffraction**

This examination was done to determine if the crystals identified in the bone were randomly arrayed, as would be expected for secondary deposition through geological processes, or ordered, as occurs in bone, where the hydroxyapatite crystals align along highly oriented collagen
fibers. Bone tissues were prepared by sectioning as for the undecalcified TEM examinations, and then thinning the sections by grinding on SiC paper to approximately 100 microns. Using an ultrasonic drill with a 3 mm bit, the sections were core drilled, then placed in a dimpling machine until they reached a thickness of about 10 μm. Further thinning was accomplished by ion-thinning with argon gas at energies of 6 kV, using a 15 degree grazing angle. This was done at liquid nitrogen temperatures to reduce heat-induced changes. The samples were then subjected to electron diffraction spectroscopy, which uses electrons at very high voltages to determine crystal orientation patterns (see Zocco and Schwartz, 1994, for details). Examination was performed using a JEOL 2000EX Scanning/Transmission Electron Microscope, equipped with a Tracor Northern Energy Dispersive Spectrometry system, at 200kV accelerating voltages. The samples were cooled during data collection by using a liquid nitrogen holder to minimize beam heating effects upon the apatite crystals.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was used to separate organic components present in extracts of the T. rex bone. Samples of trabecular tissues from MOR 555 were crushed in a sterile, baked (180-200°C) mortar and pestle
and extracted with 20 ml of an extraction buffer consisting of 3M NaCl, 5% glycerol, 5mM dithiothreitol, 2 mM EDTA (pH 7.5), 1% CHAPS, 7.5 M Guanidine-HCl, and 0.1 M Tris-HCl (pH 7.6) overnight at room temperature. The suspension was then centrifuged at 5,000 x g for 5 minutes. After extraction, the supernatants were dialyzed across a membrane with a molecular weight cut-off of 3500 KDa against 10 mM Tris (pH 7.9) to remove buffer components. The resulting material was concentrated on a Savant speed-vac, and 20 µl were injected onto either a C18 analytical or a phenyl analytical HPLC column. Extraction buffers alone were similarly analyzed by HPLC. The HPLC elution profiles were monitored with a dual wavelength Spectraphysics UV200 detector. Run parameters were as follows: reservoir A contained HPLC grade water (VWR Scientific, Philadelphia, PA) and reservoir B contained HPLC grade acetonitrile (VWR). Both solvents were buffered with 6mM HCl. The gradient protocol was 1-10 minutes at 100% A, at a flow rate of 0.5 ml/min; 10-45 minute ramp to 40% B at 1 ml/min; and 45-60 minute ramp to 100% B at a rate of 1 ml/min. Elution profiles were monitored at 214 nm and 260 nm, to scan for molecules with the optical absorbance characteristics of peptides and nucleic acids, respectively.

In a second set of experiments, elution profiles were monitored at 410 nanometers using a Spectraflow 757 absorbance detector. This wavelength was chosen because it
is the wavelength at which the heme prosthetic group absorbs strongly. Elution solvents were 400 ml HPLC grade water (VWR) in reservoir A, and HPLC grade acetonitrile in reservoir B. Both solvents were buffered with 400 μl of TFA (0.1%). The gradient protocol was: 100% A for 5 minutes, ramp to 75% B at 55 minutes, remain at 75% B until 60 minutes, then ramp to 100% A at 62 minutes. The flow rate used was 0.3 ml/minute, and sensitivity was 0.05 AuFs.

Amino Acid Analysis

Since a high glycine content and the presence of collagen specific amino acids, such as hydroxyproline, would be expected to be present if the T. rex bone contained significant remains of indigenous proteins, we subjected the T. rex bone to amino acid analysis. Approximately 1.5 g of trabecular bone from MOR 555, along with desiccated bony tissues of horse and fresh ostrich bone as positive controls, were extracted in 1 N HCl overnight with rotation at room temperature. The samples were centrifuged for 5 minutes at 5,000 x G. The supernatants were collected and the horse and T. rex tissues were concentrated on a Sep-Pak™ column, while the ostrich tissues were derivatized without concentration. Samples were derivatized with either PITC or Marfey's reagent before standard amino acid analysis was
conducted at the San Diego State University Peptide Core Facility to obtain an amino acid profile.

High Resolution Nuclear Magnetic Resonance Spectrometry

Because hemoglobin has a readily identifiable proton Nuclear Magnetic Resonance (NMR) spectrum due to the hyperfine shifts of resonances interacting with the unpaired electrons of the iron, we subjected appropriate extracts of T. rex bone to NMR spectroscopy. Ground bone tissues were extracted in guanadinium-thiocyanate (Hoss and Paabo, 1993) and the supernatant fractions were dialyzed (3500 MW cut-off) against 30mM PBS overnight with 2 changes of dialysate. The resulting solution was lyophilized, and 40 mg of the solid was dissolved in 0.5 ml of D$_2$O. A high resolution proton solution-phase NMR spectrum was collected on a Bruker AC300 NMR spectrometer operating at 300 MHz proton frequency using techniques appropriate for studying paramagnetic proteins (La Mar and de Ropp, 1993) such as hemoglobin. The data were collected as 8K complex data points with a sweepwidth of 83333 Hz using a 5µs pulse length and a total repetition rate of 100 ms. A total of 20,000 scans were collected and the data were processed using a 20 Hz exponential line broadening window function.
Preliminary DNA Analysis

T. rex trabecular tissues, which had been removed under aseptic conditions and kept frozen under desiccation, were subjected to extraction with phenol/chloroform and proteinase K as detailed by Sambrook, et al. (1989) The total extract was concentrated in a Savant Speed Vac and subjected to gel electrophoresis on a 1.5% agarose gel. The gel was stained with ethidium bromide, and visualized under UV light. The extracts were also subjected to digestion with DNAse and various restriction endonucleases, and again visualized on agarose gels. Results of the above digestions, as well as a more detailed account of the analysis of this DNA, and the sequences obtained from them, are outlined in chapter four.

Results

Gross Examination

Samples of trabecular bone were gathered from the marrow cavities of the different specimens, and were weighed for comparison. Figure 1 illustrates the samples and corresponding weights and estimated densities for each sample. The tissues of MOR 555 most closely resembled the desiccated horse trabecular tissues in weight, color and texture.
Figure 1. a. MOR 555 (*Tyrannosaurus rex*), weight = 0.72g, density = 0.36g/ml; b. hadrosaur (*Maiasaurus peeblesorum*), weight = 3.69g, density = 1.24g/ml; c. recent horse (*Equus caballus*), weight = 0.34g, density = 0.11g/ml.

The *T. rex* cancellous bone was much too light to accommodate any significant degree of geological replacement. The desiccated horse bone was estimated to be between 10 and 20 years post-mortem, with an uneventful history of being at the surface of the Montana plains for that time period. Lack of protection by quick burial, and consequent exposure to the elements possibly accelerated the loss of organic mass for this sample, and exaggerated its gross similarity to the dinosaur specimen.
Microscopic Examination

Examination by light microscopy revealed little or no evidence of secondary crystallization in the vascular spaces and Haversian systems of the trabecular bone spicules of the *T. rex* specimen. The bone matrix was characteristically anisotropic, but no crystal structure could be detected in cross polarization studies within the vessel channels. The vessel channels did contain small rounded micro-structures that exhibited a translucent outer layer and an opaque inner layer (Figure 2). These structures did not show birefringence under cross polarization, as would be expected for crystalline structures. Other than the microstructures, the vessel channels appeared empty, with no signs of sedimentary deposition. Osteocyte lacunae could be clearly seen, and these, likewise, showed no signs of infilling.
Figure 2. Light micrographs of *Tyrannosaurus rex* bone tissues. a. Trabecular bone from tibia, longitudinal section, X 100, bar = 50 µm. b. Trabecular tissues, including vessel channel and vascular microstructures, X 400, bar = 20 µm.

Confocal Laser Microscopy

When stained with the DNA specific Hoescht 33258 dye and stimulated with laser light at a wavelength of 448 nm, the tissues of both the ostrich and the dinosaur showed positive fluorescence which was highly localized (Figure 3). In the modern tissues, every osteocyte lacuna was seen to fluoresce, and occasionally fluorescence was detected outlining vessel channels as the dye presumably intercalated with the nuclear material of the endothelial cells. In the
Figure 3. Confocal laser micrographs, showing localization of DNA to the osteocyte lacunae, as visualized with Hoescht DNA fluorochrome. a. Ostrich bone, embedded, sectioned and ground. Bar = 50 μm. b. T. rex bone, lightly crushed and attached to slide with double-stick tape. Both samples were exposed to the stain for approximately 1 minute, as indicated in the text. Bar = 10 μm.

T. rex tissues, fluorescence was likewise highly localized to the osteocyte lacunae. However, the regions of fluorescence were smaller (note the greater magnification for the T. rex sample in Figure 3) and fewer osteocyte lacunae exhibited fluorescence compared to the modern tissues. However, it should be pointed out that somewhat different protocols had to be used for the ancient and the modern bone, making direct comparisons difficult.
The lack of infilling or secondary crystallization was verified under electron microscopy. When compared to a typical, well permineralized dinosaur bone, the difference in preservation of MOR 555 is obvious (Figure 4a). The permineralized dinosaur bone has secondary deposition clearly visible in every vessel channel and most osteocyte lacunae, whereas in MOR 555 (Figure 4b, c) these structures are devoid of any infilling or signs of deposition. The microstructures within the vessel channels can again be clearly seen and, while the outer portions of the structures appear amorphous, the central regions possess a regular structure.
Figure 4. Scanning electron micrographs of dinosaur tissues. Tissues were embedded, sectioned and polished as described in the text, then coated with carbon for EM visualization. Magnifications as indicated. a. Well permineralized tyrannosaur bone (RTMP) showing secondary crystallization in vessel channels. Some deposition is also noted in osteocyte lacunae. b. MOR 555 tissues. Vascular channels are devoid of crystallization or infilling. c. Higher magnification of region seen in (b). In this view, the individual osteocyte lacunae can be seen to be devoid of secondary crystallization. d. Longitudinal view of MOR 555 trabecular tissues. In this view, the vascular channels are again seen to be devoid of crystalline deposits, except for the vascular microstructures.
Topographical studies with back-scatter imaging (BSE) (Figure 5a) show that the structures are indeed three-dimensional, and that the central region bulges out beyond the outer portion. Compositional studies show that the central region contains a higher portion of heavy elements (light regions) than does the outer region (Figure 5b).

Figure 5. Computer generated images corresponding to an image obtained with secondary electrons (SEI) and back scatter electrons (BSE). a. Topographical study of *T. rex* tissues, (BSE imaging) showing the three-dimensional aspect of the vascular microstructures. b. Compositional studies showing the localization of heavy elements (light areas) to the central regions of these structures.
The elemental profiles obtained for the bony tissues of MOR 555 are very similar to those of modern alligator and ostrich (Figure 6; a, b, c). There are only minimal traces of elements within the dinosaur bone that are not also found in modern bone. This indicates little diagenetic change in the T. rex specimen. The bony tissues show a calcium to phosphorous ratio of approximately 3:2, which is standard for the mineral phase of modern bone. The profile for the sandstone matrix (not shown) has a high silica peak which is not seen in the bone, whereas the phosphorous peak, dominant in bone, is absent in the sandstone. Line scan analysis through a vessel channel in the T. rex specimen (Figure 6d) verifies the differences in elemental composition between the bone and the observed microstructures within the vascular channels. In this figure, the localization of iron only to the microstructures, and its absence from the bone matrix are evident. Likewise, concentrations of calcium and phosphate are high in the bone matrix and negligible in the microstructures.
Figure 6. Analysis of bone tissues for elemental content. All samples were embedded, sectioned and ground to approximately the same thickness, then polished with a fine aluminum oxide powder. Carbon and oxygen are not figured into the numerical analysis as they are both ubiquitous, and too light for accurate calculations. a. Semi-desiccated alligator bone. b. Fresh ostrich bone. c. T. rex (MOR 555) trabecular tissues. d. Line scan elemental analysis, showing the localization of calcium and phosphorous to the bone matrix, while iron is seen localized to the vascular microstructures. Sulfur can also be seen in association with only the central regions of these structures.
Transmission Electron Microscopy

This study was performed in order to detect the preservation of any fibrillar structures within the bone matrix. The purpose of the formic acid treatment was to remove the mineral phase of the bone, leaving only the organic fibrillar phase. As can be seen from Figure 7a, the extant ostrich bone shows clear evidence of fibers, some of which demonstrate the characteristic cross banding indicative of collagen I fibrillar bundles (arrow). The matrix of the T. rex, treated in an identical manner, also shows evidence of fibers (Figure 7b). However, these fibers are smaller, shorter and more random in orientation. Also, there is no evidence of collagen crossbanding in any of the fibers. At very high magnifications (Figure 7c), the fibers within the matrix of the dinosaur tissues are more clearly defined.
Figure 7. Transmission electron micrographs. Tissues were decalcified, fixed in gluteraldehyde, embedded, microtomed, and stained (see text).  

a. Fresh ostrich bone. Magnification = 26,400X. Note crossbanding in collagen fibril at arrow. 
b. *T. rex* tissues. Magnification = 32,500X. Fibrillar arrangement is present, but crossbanding is not visible. 
c. Higher power (120,000X) of *T. rex* tissues. Again, the fibrillar character of the matrix is apparent, but no crossbanding is evident, and the fibrils are much smaller than in (a).
Elution profiles of concentrated extracts of MOR 555 revealed absorbance peaks at all of the wavelengths at which monitoring was done. When compared against samples of purified rat tail collagen type I or ostrich bone extracts, the _T. rex_ extracts exhibited absorbance peaks that roughly correspond in absorption profile to some of those seen in the known standards (Figure 8a). Also, it is significant that these peaks were not seen in the buffers which were used to extract the bone samples. An exception is the peak for the detergent, CHAPS, which is very difficult to remove through dialysis because of its tendency to form micelles. Instead, this detergent tends to concentrate when associated with proteinaceous compounds. Absorbance at 214nm (absorbance maximum for the peptide bond) supports the hypothesis that biomolecular compounds are preserved in the _T. rex_ specimen, as does the presence of peaks with a strong absorbance at 260 nm, (data not shown) which is the absorbance maximum for nucleic acids. Peaks were also present with absorbance at 410 nm (Figure 8b), which is the value at which hemoglobin and other heme-containing compounds absorb strongly. These tests do not, however, positively identify the compounds that are absorbing at these monitoring wavelengths, nor do they speak to the indigeneity of the compounds.
Figure 8. Reverse phase, high performance liquid chromatography (HPLC) profiles of extracted tissues and/or controls. For each run, 20 µl of concentrated sample was injected. a. From top to bottom the samples are: purified collagen I in extraction buffer, ostrich bone in extraction buffers, *T. rex* tissues in extraction buffers, and the extraction buffers alone. Separation is done on a phenyl analytical column, with monitoring at 214 nm. IP indicates the point of injection, with time running to the right. The small peak (arrow) immediately to the right of the large peak in the *T. rex* sample directly corresponds in elution time to small peaks in both the ostrich and the purified collagen I extracts, and may represent some remnant of this protein. The large peak, likewise, may represent some altered collagen components, eluting earlier than corresponding ones in the modern samples due to increased aqueous solubility of the degraded fragments. Other proteinaceous components may be eluting in the early and poorly separating "shoulders in the first 20 minutes of the run. b. HPLC tracings on a C-18 analytical column, with monitoring done at 410 nm. From top to bottom, samples are: *T. rex* tissues in extraction buffers, sandstone matrix extracted as described, and extraction buffers alone. Again, IP is the point of injection, with time running to the right.
Electron Diffraction

The results of this examination of the *T. rex* tissues are shown in Figure 9b. During the ontogenetic formation of bone tissues, collagen fibers are first laid down in a regular, parallel orientation, and then mineralization occurs as crystals of hydroxyapatite align along the fibers. The presence of partial diffracted rings (arrows) in the dinosaur tissues indicates a preferred orientation of hydroxyapatite crystals consistent with that found by Zocco and Schwartz (1994) in other fossilized and recent bone tissues, and compares favorably with patterns seen in modern crocodile bone (Figure 9a). Electron diffraction pattern analysis of more recent bone differs somewhat in that it also indicates a finer hydroxyapatite crystal size, and a more diffuse series of diffraction rings. This could be due to the presence of a significant organic fraction in the modern bone, as well as the presence of some of the smaller hydroxyapatite crystals that may have been lost from the ancient sample. In any case, the electron diffraction pattern is not consistent with randomly oriented crystals deposited during permineralization, but is consistent with the original hydroxyapatite crystal pattern for the bone.
Figure 9. Electron diffraction analysis. a. Modern crocodile tissues. b. T. rex tissues. The partial rings in these micrographs (arrows) indicate the regular, non-random orientation of the hydroxyapatite crystals of the bone matrix. That the pattern in (a) is more diffuse may be due to the presence of some soft tissues and organic materials not seen in the dinosaur bone.
Nuclear Magnetic Resonance Spectrometry

As can be seen in Figure 10, the proton NMR spectrum shows peaks both upfield and downfield of the standard 0-10 ppm proton spectral window. In the upfield region, three very broad resonances at approximately -9.0, -20.0 and -30.0 ppm are observed, while in the downfield region, four broad resonances are observed at 25.0, 29.0, 45.0 and 72.0 ppm. For a proton to resonate so far upfield and downfield, a paramagnetic ion such as is found in metalloproteins is required (LaMar, 1973; Bertini and Luchinat, 1986). The metalloproteins that are found in the highest concentrations are the heme proteins, hemoglobin and myoglobin. Although the NMR spectrum in Figure 10 does not match a known spectrum for hemoglobin, it is consistent with what could be expected from a highly degraded heme protein such as ancient hemoglobin. While inorganic iron would not be expected to show resonance in solution phase NMR, as most occurs as iron oxide, and there are no free protons in these compounds, the possibility exists that this resonance is due to an undocumented interaction of geologically deposited iron with breakdown products such as humic or fulvic acids.
Figure 10. Proton nuclear magnetic resonance spectrum of T. rex tissues, extracted as described in the text, and lyophilized to concentrate. The arrows mark resonance peaks in the range commonly seen with heme proteins. The reference is D$_2$O.

Amino Acid Analyses

When derivatized with PITC, the extracts of T. rex tissues reveal the presence of amino acids in ratios similar to those reported by Gurley et al. (1991) (Table 1-1). Note
that the numerical values shown indicate molar percentages and are not absolute values. In some cases, the computer identified peaks which are at values small enough to be questionable. When converted to percentages, these peaks (e.g.: His, Met) take on a numerical significance that is not reflected by the original HPLC tracings. In the dinosaur extracts, there was a significant amount of material injected onto the column which was not identified by the computer software as amino acids. This provided a background "noise" and co-elution problems that made positive identification of some peaks difficult. This material may have been humic and/or fulvic acids, common organic breakdown products which are often associated with ancient bone (Tuross and Stathoplos, 1993). Some amino acids, such as hydroxylysine, were better seen with one specific derivatization method (data not shown).

In the T. rex sample, there is a significant decrease of amino acids, such as arginine, which are known to be short lived (Schroeder & Bada, 1975). Longer lived amino acids, such as glutamic acid and valine, are clearly seen in all samples. Although hard to distinguish after PITC derivatization, following derivatization with Marfey's reagent peaks corresponding to the position of hydroxylysine and allohydroxylysine were easily discerned in all samples including the T. rex extracts (data not shown). A peak corresponding to the normal retention time of hydroxyproline
was noted on the PITC derivatized runs, but due to anomalous elution times for the early-eluting amino acids, this peak could not be conclusively identified. The molar ratio of glycine was high in the T. rex sample, and very close to that obtained for modern ostrich bone. Since glycine comprises every third residue in collagen type I, a high glycine ratio was expected. Collagen I is relatively low in tyrosine, and low tyrosine ratios were found in all bone extracts. With the exception of proline and hydroxyproline values, the T. rex analyses showed a good overall similarity to the modern ostrich bone extracts. The T. rex sample also resembled that from the weathered horse bone, but less closely.
TABLE 1-1: PITC DERIVATIZED AMINO ACID VALUES IN MOLE-PERCENTAGES

<table>
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<tr>
<th>SPECIES:</th>
<th>OSTRICH</th>
<th>HORSE</th>
<th>TYRANNOSAURUS REX</th>
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<tr>
<td>Total picomoles:</td>
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<td>650.7</td>
<td>42.9</td>
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<tr>
<td>ASP</td>
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<td>GLU</td>
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<td>10.7</td>
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<tr>
<td>t-PRO-OH</td>
<td>5.9</td>
<td>11.3</td>
<td>---</td>
</tr>
<tr>
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<td>6.8</td>
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<td>14.1</td>
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<tr>
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<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>ARG</td>
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<td>5.5</td>
<td>1.7</td>
</tr>
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<td>3.1</td>
<td>3.0</td>
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</tr>
<tr>
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</tr>
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</table>

*Because these peaks were too small for the computer to accurately calibrate, these numbers represent visual estimates from the HPLC tracings.
** These peaks co-eluted and were difficult to separate from an artifact peak which was not seen in the other samples. See text for discussion.

DNA analysis

DNA extractions from the T. rex bone were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Digestion with restriction enzymes, as well as DNase, demonstrated that the material was indeed
DNA. As all of the extraction controls were negative when analyzed for DNA content, and no PCR amplification steps, which often yield spurious DNA, were used, the DNA we have extracted is unlikely to be a lab contaminant. However, because of its size, it is also unlikely that this DNA consists solely of indigenous material, as molecules from bones much more recent have been found to be highly degraded, if present at all (Meijer, et al., 1992), although large molecular weight DNA has been reported to be associated with fossil material (Hardy, et al., 1994; Tuross, 1994). It is most likely that some, if not all, of this material represents the organic remnants of more recent contamination of the bone by bacterial or fungal species, a phenomenon which has been reported in the literature (Hardy, et al., 1994). For a more detailed treatment of the DNA retrieved from these bone samples, the reader is referred to Chapter 4.

Discussion

Density measurements and microscopic examinations of the trabecular tissues of this dinosaur specimen show no evidence of the permineralization or replacement typically seen in most dinosaur bone, and there is little or no diagenetic addition of elements to the bone matrix. The
ratios of calcium and phosphorous seen in our studies are consistent with those seen in modern bone tissues, and electron diffraction spectroscopy revealed the crystal structure to be well ordered. These data indicate that the inorganic phase of the bone has changed very little from its original state.

Modern bony tissues consist of both an inorganic or mineral (hydroxyapatite) phase, and an organic phase. The organic phase consists mostly of the fibrous protein, collagen type I, in the form of a highly constrained triple helix. There are other, non-collagenous fibrous proteins associated with bone tissues, such as osteocalcin and osteonectin. Removal of the mineral phase of bony tissues of MOR 555 through acid treatment left a somewhat soft, pliable, but still coherent, tissue. The presence of fibers in this tissue, seen under transmission electron microscopy after decalcification, indicates that some of the original organic components of the bone remain. Collagen type I, the primary protein in bone, is characterized by its form as a highly constrained triple helix, and can be recognized by its regular banded pattern on TEM. While fibers can clearly be seen in the dinosaur tissues, this banded pattern, thought to be due to the overlapping stacking of the fibrils which compose the fibers, is not seen in the tissues of the T. rex, implying that if collagen I is present, it has lost sufficient mass to dilute out the banding effect. It is
possible that the fibers which are seen in the T. rex sample are due entirely to the presence of non-collagenous proteins. However, this seems unlikely given their apparent quantity in the sample. Tuross (1994; Tuross and Stathoplos, 1993) has noted that degradation of collagen in ancient samples causes this insoluble molecule to become more soluble. The smaller, degraded, soluble fragments can then be leached away through water infiltration, causing a mass loss for this protein, and this may in turn be reflected in the notable lack of any observable banding pattern. The potential increase in aqueous solubility might also explain the earlier elution time seen for the peak which elutes about halfway through the run in the 214 nm HPLC profile for the dinosaur extracts and which can be compared with similar peaks in the ostrich and collagen samples. This peak cannot be explained by residual buffer components.

The fact that protein components remain associated with the bone is further evidenced by the HPLC elution profiles, which show positive absorbance at both 214 and 410 nm, as well as peaks that correspond in elution times to both purified collagen type I and components of modern ostrich bone extracts. Amino acid analysis lends support to the hypothesis of some protein preservation in these extracts of the dinosaur bone. As would be expected for tissues this ancient, there is lack of the shorter-lived amino acids such
as arginine, and a concentration of longer-lived, more stable amino acids such as aspartic acid and glutamic acid, (Schroeder and Bada, 1975). This is consistent with the inference that these are remnants of original proteins. This conclusion is further supported by the small hydroxylysine and allohydroxylysine peaks seen after derivatization with Marfey's reagent, because these amino acids are unique to the structural collagen molecule, a protein not secreted by microorganisms. Also supporting the presence of collagen I in the T. rex extracts are significant peaks for both proline and glycine, amino acids which are common constituents of this protein. These amino acids are also found in other proteins, therefore, this alone cannot be considered diagnostic evidence for the preservation of collagen type I. The value for proline in the T. rex sample is significantly decreased relative to the other samples. This could be due to several factors, including preferential loss of this amino acid during extraction and/or concentration steps. However, the lack of positive identification of a hydroxyproline peak is troublesome if the claim is to be made for the preservation of collagen I, as this long-lived amino acid is unique to collagen, and not found in any other protein. This needs to be resolved in the future.

In light of the size (25-40 µm) and localization of the vascular microstructures seen within the trabecular
tissues, the presence of absorbance peaks at 410 nm on HPLC and the spectral profiles from the solution-phase NMR studies are intriguing. These data, along with the consistent localization of iron to the microstructures, seem to indicate the presence of blood-derived structures which may still contain identifiable heme groups. These microstructures may not be the original blood cells of this animal, but perhaps conditions were right for some aspects of the blood cell components to be preserved through some as yet undetermined geological process. These cellular derivatives, either singly or as aggregations, could then possibly have formed sites of nucleation for later adsorption of inorganic materials. That blood-derived components may be preserved within vessel channels of dinosaur bone has been noted in the literature by such early workers as Seitz (1907), Swinton, (1934) and Moodie (1923), and more recently, red blood cells have been identified in SEM micrographs of human bone greater than 2000 years old (Maat, 1991; 1993). However, biochemical tests for this hypothesis, while suggested, have not been reported in the literature to date.

Finally, there has been much speculation on the possibility of preservation of indigenous DNA within the tissues of extinct animals, including dinosaurs. While reports of DNA extracted from tissues of various extinct taxa (Higuchi, et al., 1987; Paabo, 1985, 1989; Hagelberg
and Clegg, 1993; DeSalle, et al., 1992; Cano et al., 1992a, b, 1993; Golenberg, et al., 1990) are generally accepted to be valid, and representative of the organisms from which they are derived, DNA isolations from bony tissues as old as MOR 555 have not yet been verified through independent replication. Our studies show that bulk DNA is present within MOR 555 bone, but at sizes and intensities that go against all predictions for highly degraded ancient DNA. However, high molecular weight DNA has been associated with other fossil bones (Doran, et al., 1986; Hardy, et al., 1994, Tuross, 1994). Our most provocative finding was the implication via Hoescht staining in situ, that osteocyte indigenous DNA may still be present in the T. rex bone. We have attempted to amplify the DNA extracted from the dinosaur tissues, but as yet our attempts have been inconclusive.

DNA has a well known affinity for hydroxyapatite, the mineral which predominates in the bone matrix, and this may be both beneficial and detrimental to further molecular studies: beneficial in that adsorbance of DNA to the mineral may in effect remove the molecules from solution and stabilize them against forces that are cited as destructive to the long term preservation of the molecule (Lindahl, 1993); and detrimental in the possibility that the bone matrix may act effectively as a "sink" for DNA molecules from the degradation and infiltration of other organisms.
found within the sediments over the course of geological time. This could explain the presence of the large bulk DNA we repeatedly extracted from this dinosaur and from two other dinosaur specimens (data not shown). The presence of contaminating DNA (if indeed it does concentrate in the ancient bone matrix) from multiple sources may obscure any hope of identifying the rare, degraded template that may be indigenous to the dinosaur tissues. Currently, the best evidence we can present for the indigeneity of the DNA identified in this bone is the localization of Hoescht staining to the osteocyte lacunae, which in life were each inhabited by a single osteocyte. These lacunae, buried deep within the bone and protected by the dense matrix, seem to be ideal sites for the preservation of DNA (Tuross, 1994). In addition, it seems unlikely that the highly localized fluorescence we observed can be explained by invasion of microbial contaminants, as bacterial colonization would be visualized as more than one fluorescent spot per lacuna.

If some of this DNA is indigenous, and truly dinosaurian in origin, it may still be impossible to conclusively demonstrate this via sequence analysis, if the DNA is degraded to a size too small for PCR amplification, or if the bases are so modified by age that they are no longer adequate substrates for the polymerase enzymes (Paabo, et al., 1990; DeSalle, et al., 1993). Until other methods, such as stable isotope analysis, can be developed
and quantified for use in DNA analysis, the case for indigeneity of DNA from ancient tissues must rest on phylogenetic analysis of the sequences obtained from such DNA. Because the phylogenetic position of the Dinosauria, based on morphological characters, is so well known with respect to living and fossil taxa (Gauthier, 1986), and because this phylogeny indicates a shared common ancestor with Aves, we are able to predict where the genetic sequences obtained from these specimens must fall to be considered valid. Without such sequence data, any claims for indigenous "dinosaurian" DNA cannot be made with confidence. Chapter 4 contains a more thorough description of the analysis of the DNA extracted from these dinosaur tissues, and a more detailed treatment of the results obtained from these studies.

Conclusion

Several hypotheses were tested during the course of this study, three of which were: a) that the bone tissues of this dinosaur exhibited no signs of replacement or permineralization, and were very much in their original state; b) that the small microstructures seen within the vascular channels of this bone might be remnants of the original erythrocytes of this animal; and c) that, if these
were cell remnants, perhaps the process which preserved them also preserved biomolecules such as the DNA and proteins associated the dinosaur erythrocytes. While no individual test run on the bone samples stands alone as proof that the last two hypotheses were true, neither did the many tests we conducted falsify either proposition. Indeed, most of the tests supported each hypothesis, and, taken together, are very encouraging for further pursuit of this work.

The excellent preservation seen grossly and microscopically in the tissues of MOR 555 is consistent with a lack of permineralization and diagenetic alteration, and, from our studies, appears to be associated with biomolecular preservation within these tissues. All the tests we have applied to date support this overall hypothesis. As to the second hypothesis concerning the nature of the vascular microstructures, we conclude that these cannot be positively identified at this time. While some of our data, such as the HPLC absorbance peaks at 410 nm and the heme-like NMR resonance spectra support the hypothesis of a biological origin, other data, such as the varying sizes and geometric pattern of the core structures may not. Other tests must be done before a conclusion can be drawn about the source of these structures. Resonance Raman spectroscopy, for instance, should positively identify the heme prosthetic group, and will be attempted as facilities become available to us. Antibody work, both in situ and using Western blots
of tissue extracts, is currently being conducted to identify if antigenic components remain in the T. rex tissues.

While the third hypothesis, that DNA may be preserved in the T. rex tissue, is tentatively supported by the extraction and identification of DNA from these tissues, no claim can yet be made for the extraction of indigenous "dinosaur DNA". The organismal source or sources of the DNA which we have extracted must, for the time being, remain anonymous until further work is done.

There is much to be gained from the study of the molecular components which may remain within the tissues of extinct organisms, including dinosaurs. Application of the latest technologies of molecular biology to the field of paleontology holds great promise for revealing information regarding the physiology and phylogeny of paleontological specimens. Identification of specimens which are most apt to hold such biological information is crucial to the success of such efforts, as time, energy and money will be most productively spent on specimens most likely to contain such a molecular record. The tests done on the tissues of this dinosaur, MOR 555, represent an attempt to identify components which may allow researchers to narrow their search for viable specimens. For example, examination of specimens for elemental composition, followed by preliminary molecular analysis by HPLC, should identify tissues which may be productive for further study.
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CHAPTER THREE

PRESERVATION OF PUTATIVE HEMOGLOBIN COMPOUNDS IN TRABECULAR TISSUES OF TYRANNOSAURUS REX

Introduction

Hemoglobin, because of its vital role in gas exchange, has long been the focus of studies in the field of molecular biology. It is one of the first proteins for which structure and sequence were determined (Dickerson and Geis, 1983), and therefore a protein for which a large body of data exists. The unique characteristics of hemoglobin have allowed the elucidation of its structure using techniques such as nuclear magnetic resonance (NMR: Ho and Russu, 1981; Ho and Perussi, 1994), raman and resonance raman spectroscopy (RR: Asher, 1981; Freidman, 1994; Tu, 1982; Carey, 1982), and electron paramagnetic resonance (EPR: Peisach, et al., 1973; Blumberg, 1981). The amino acid sequence of this protein has been determined for many taxa, making it an important tool in evolutionary and phylogenetic studies. Comparisons of these sequences can reveal distance of relationships across diverse taxa (Dickerson and Geis, 1983). Because the parts of the molecule which interact with the heme prosthetic group at the core are highly
constrained, and therefore conserved across vertebrate taxa (Perutz, 1983), some regions of hemoglobin are appropriate for determining deep phylogenies. Other regions, however, allow for much more variation, and therefore are appropriate for determining closer relationships, even to delineating species (Nikinmaa, 1990). In addition, the association of the hemoglobin molecule and its organophosphate allosteric effectors are useful indicators of metabolic rates (Nikinmaa, 1990; Abbasi, et al., 1987).

Hemoglobin is considered a "protein bound chromophore" because of its association with iron. Iron, with its varying oxidation states and electron shell configurations, affects the characteristics of the protein, and contributes to unique "fingerprint" signals seen using some methods of analysis. At the core of both hemoglobin and the closely related myoglobin molecule is the heme prosthetic group, a porphyrin ring structure with one iron atom at its center. The porphyrin structure is extremely stable, and is found in other proteins, such as the cytochromes and chlorophyll. Porphyrin compounds that can be linked to chlorophyll A have been shown to survive, in a minimally altered state, across geological time (Curry, 1990).

The stability of the porphyrin ring, as well as the ease with which hemoglobin crystallizes, contribute to its longevity; therefore, this protein may be a good candidate for preservation in fossil specimens. Indeed, hemoglobin
residues still containing antigenic components have been identified in fossil bone (Smith and Wilson, 1990; Cattaneo, et al., 1990), even those as old as 4,500 years (Ascenzi, et al, 1985). Blood residues bearing antigenic properties which allow identification of the source species have also been isolated from stone implements as old as 100,000 years (Loy, 1983, 1987). In some cases, even the morphology of the erythrocytes themselves is apparently preserved, to the point that pathological conditions may be identified (Maat, 1991, 1993).

It is commonly recognized that upon the death of an organism, most erythrocytes undergo hemolysis, which releases the hemoglobin protein, and causes a subsequent red discoloration of the surrounding bony tissues (Ascenzi, et al., 1985). It has also been noted that association of proteins with mineral, such as the hydroxyapatite which constitutes the mineral phase of bone matrix, may be a prerequisite for the survival of proteinaceous compounds across geological time frames. This association may retard the decay process of these biomolecules by effectively removing them from potentially aqueous environments, therefore decreasing the potential for hydrolytic damage to the molecule (Ambler and Daniel, 1991).

Traditionally, little hope has been held for the recovery of any biomolecules in fossils older than a few thousand years, and certainly not for dinosaur bone.
However, partial amino acid sequences were identified in the shells of mollusks approximately 80 million years old (Weiner, *et al.*, 1976). In 1991, Gurley, *et al.* reported the isolation and identification of amino acids in the bony tissues of a *Seisomosaurus*, and recently, the small and highly acidic bone protein osteocalcin has been recognized in extracts of dinosaur bone by antibody binding (Muyzer, *et al.*, 1992). These results support the possibility of finding phylogenetically significant protein remnants in fossil bone.

A specimen of *Tyrannosaurus rex* was recovered from the Hell Creek formation in eastern Montana in the spring of 1990. Preliminary examination of trabecular bone elements of this specimen showed little or no evidence of permineralization or replacement (see Chapter 2). This condition may be associated with lack of or minimal exposure to water, and therefore the tissues of this specimen seemed to be possible candidates with which to test the hypothesis of preservation of indigenous biomolecules. For a complete description of the depositional environment of this specimen, the reader is referred to chapter 1. The state of completeness, and minimal displacement is thought to indicate burial rapid enough to prevent extensive scavenging or slow other decay processes (Allison, 1990).

An effort to deduce, from a bio-analytical perspective, the extent of preservation of the unreplaced bony tissues of
this specimen of *Tyrannosaurus rex* has resulted in the identification of apparent hemoglobin compounds in extractions of the trabecular tissues. These results support the hypothesis that microstructures seen localized to the vessel channels preserved within these tissues were derived wholly or partially from blood components of the animal (see chapter 2). A variety of highly sensitive and highly specific analytical techniques were used to obtain these results, all of which are consistent with the interpretation of hemoglobin preservation.

**Materials and Methods**

Trabecular tissues of the *Tyrannosaurus rex* were harvested under aseptic conditions immediately upon removal of the surrounding sediments. These tissues were only handled with sterilized instruments and gloved hands. The bony tissues were wrapped in foil and stored in sealed jars under desiccation at -20° C until used in the following experiments.

Tissues were extracted by grinding the bone to a fine powder in sterile, baked mortar and pestles. This powder was then distributed in amounts of approximately 0.5 g to sterile, silanized 1.5 ml eppendorf tubes. To each tube, 1 ml of a guanidine-thiocyanate extraction buffer (Hoss and Paabo, 1993) was added. The tubes were rocked gently
overnight at 50°- 60°C, then centrifuged at 14,000 x g for five minutes. The supernatant, which was a reddish brown color, was then drawn off. Dialysis tubing (3500 KDa mw cut-off) was prepared by boiling for 5-10 minutes in a solution of sterile, deionized, glass distilled water and sodium bicarbonate, then rinsed several times in sterile, deionized water. The supernatant was placed in the dialysis membrane and dialyzed overnight with two changes of dialysate. For some samples, the dialysate was 0.3X PBS, (pH 7.5). For others, the dialysate was 10 mM TRIS, (pH 7.9). All samples were concentrated to at least half-volumes using a Savant speed vac.

Microstructural analysis

Trabecular tissues from the Tyrannosaurus rex were embedded in a synthetic polymer and sectioned as detailed elsewhere (Chapter 2). Light microscopy was done using a petrographic microscope. Bone sections were also coated with a thin layer of carbon and visualized with a JEOL 6100 electron microscope, which was coupled to a Noran Voyager Energy Dispersive X-ray (EDX) system for use in elemental analyses.
Amino Acid Racemization Analysis

Amino acid constituents of proteins are incorporated almost universally in the L-isomeric form in living systems. Over time, through the processes of degradation, these amino acids are altered to a racemic mixture of L- and D- isomers, and the theoretical rates of racemization for each amino acid have been determined (Schroeder and Bada, 1976). Amino acid analysis of these derivatives was undertaken with extracts of dinosaur tissues in an attempt not only to identify any amino acids present, but also to determine if these amino acids were sufficiently racemized to support the hypothesis of indigeneity (Schroeder and Bada, 1976; Bada, 1985). Tyrannosaurus rex tissues, as well as samples of ostrich and desiccated, naturally weathered horse tissues of indeterminate age were extracted in 1 N HCl, using gentle agitation overnight at room temperature. The supernatant was collected, and sent to the San Diego State University Peptide Core Facility for analysis. The extract was applied to a Sep-Pak® C-18 column for purification, and lyophilized. The resulting pellet was then treated by first adding 100 µl of methyl-t-butyl ether, then 0.5 ml of a solution consisting of 150 µl of 6N HCl to 5 ml of acetone. With a final addition of 5-10 µl of dilute (~ 1M) ammonium hydroxide and subsequent storage at -20° C, the protein fraction precipitated out. The remaining supernatant, which
was a dark-brownish red, was lyophilized and saved for analysis for heme fingerprint signals. Hydrolysis of the protein fraction was carried out in 6 N HCl for 75 minutes at 200° C, and the amino acids were derivatized with the chiral derivative, Marfey's Reagent: FDAA (1-fluoro-2,4-dinitrophenyl-5-L alanine amide), 2 mM in acetone. Derivatized samples were injected automatically onto a C-18 reverse-phase column. Solvent A was 50 mM triethylamine phosphate, pH 2.8 (TEA titrated with phosphoric acid to pH 2.8). Solvent B was 100% acetonitrile. A 35 minute linear gradient from 15% solvent B to 50% solvent B, followed by 15 minutes at 80% solvent B was applied. The column temperature was 45° C and the monitoring wavelength was 340 nm. Control amino acid mixtures were similarly derivatized and separated. In a few cases where trace peaks were recorded by the machine but not verified as identifiable peaks in the profiles, zero levels were recorded.

Spectroscopic analysis

Previous studies, using High Performance Liquid Chromatography (HPLC) separations, monitored at 410 nm, have indicated significant absorbance at this wavelength by extracts of these dinosaur bone tissues (see chapter 2). This strong absorbance peak is known as the Soret band, and
it is characteristic for heme-containing compounds. Extracts were subjected to transmission ultraviolet/visible (UV/vis) spectroscopy to see if such a peak could be detected. Transmission spectra of solid samples of bone were measured through a KBr fused glass. Data were measured at room temperature on either a Hewlett-Packard Diode-array Spectrometer 8452, or a Perkin-Elmer Lambda-9 spectrometer.

Nuclear Magnetic Resonance

Concentrated extracts of T. rex bone, obtained using the guanidinium-thiocyanate method outlined above, as well as the lyophilized supernatant remaining after protein precipitation, were subjected to high resolution, solution phase, proton nuclear magnetic resonance (NMR). This technique recognizes paramagnetic molecules such as the iron in heme compounds, which has a free proton. However, this technique would be insensitive to geological or elemental iron, as these usually occur as iron oxides, which have no free protons to resonate.

40 mg of lyophilized sample were dissolved in 0.5 ml of D$_2$O. NMR spectra were collected on a Bruker AC300 NMR spectrometer operating at 300 MHz proton frequency, using techniques appropriate for studying paramagnetic proteins (La Mar and de Ropp, 1993). The data were collected as 8K
complex data points with a sweepwidth of 83333 Hz, using a 5 µs pulse length and a total repetition rate of 100 ms. A total of 20,000 scans were collected and the data was processed using a 20 Hz exponential line broadening window function.

**Electron Paramagnetic Resonance**

This technique is complementary to NMR, in that it is sensitive to paramagnetic compounds, but instead of looking at the resonance state of the nucleus, it relies on the spin states of the surrounding electrons and the shifts that occur when molecules are exposed to high magnetic fields.

Samples of *T. rex* extracts and/or controls were diluted or dissolved in phosphate buffered saline (pH 7.04), and transferred to a 4 mm quartz tube, which was then rapidly cooled to 77 K. The reported g-values were referenced to DPPH \((g = 2.00232)\). Pigeon met-hemoglobin, used as a positive control, was purchased from Sigma Chemical Co., St. Louis, MO, 63178 (cat #M-3759).

**Raman Spectroscopy**

This technique relies on the unique bond stretching that specifies the heme/porphyrin structure, and allows analysis of the chemical environment of the iron atom.
associated with this structure. This method is commonly used in the study of heme compounds, and the structure of several heme compounds and their interactions with various ligands is well documented (e.g., Asher, 1981; Friedman, 1994).

For this study, spectra were measured on a HR320 spectrograph with a photometrics CCD9000 liquid nitrogen cooled detector. Laser excitation was made using a Spectraphysics 2025 Kr+ laser, operating at 647 nm. The excitation wavelength is longer than is normally used in resonance raman studies, due to a high degree of autofluorescence which interfered with signals when the excitation wavelength was shorter.

Results

Microscopic analysis of the unreplaced trabecular bony tissues (Figure 1a) revealed the presence of rounded microstructures localized to the vessel channels (Figure 1b). They appeared to have two distinct "phases"; an outer, translucent phase with a red tint, and an inner, opaque central core. Scanning microscopy revealed that the inner region possessed a distinct structure, different in character from the outer portion which was amorphous (Figure 1c). Elemental analysis through a vessel channel and the surrounding bone, mapped in several elements (data shown in
chapter 2), revealed the bone to consist primarily of calcium and phosphorous, while the small microstructures are seen to be high in iron (chapter 2).

Figure 1. a. Trabecular tissues extracted from the marrow cavity of the left tibia of *Tyrannosaurus rex* (MOR 555), coated with a polyvinyl acetate preservative. b. Light micrograph of microstructures in trabecular bone vessel channels, X 400, bar = 20 μm. c. Scanning electron micrograph of microstructure. Sample was prepared as detailed in chapter 2. Magnification is as indicated.
The chiral amino acid analysis of extracts of the *Tyrannosaurus rex* bone were complex and difficult to interpret, as there was a significant level of contamination with compounds which co-eluted with the indigenous components of the ancient samples. This caused a high background signal in the *T. rex* samples relative to the modern tissues examined. Most likely, this was the result of humic acid compounds which co-precipitated with components of the dinosaur tissues. In the dinosaur samples, but not in the modern tissues, the co-eluting, non-amino acid components caused a significant shift in retention times of some of the early eluting amino acids. This, in turn, caused the computer to mis-identify the first three or four peaks, and these had to be corrected by hand. Nevertheless, there were enough amino acids which were readily identified and clearly separated to see not only a decrease of those amino acids known to be short lived (Chapter 2) but also to verify a significant degree of amino acid racemization present in the dinosaur sample over the modern samples. Table 2-1 shows some amino acids selected from total runs of extracts of these samples. Aspartic acid, alanine, glutamic acid, and leucine/isoleucine are four amino acids commonly used to determine levels of racemization, and therefore indigeneity in ancient samples (Bada, 1985). In the dinosaur sample, aspartic acid co-eluted with other components of an unidentified background,
and accurate D/L ratios could not be obtained. However, the D-aspartic acid peaks were identified, and in the dinosaur sample, these values averaged 4.6%. For the modern samples, D-aspartic acid was not seen on the elution profiles. The D-values for alanine were not clearly identified in any samples. However, glutamic acid was easily distinguished, and the highest D/L ratio obtained for the three runs of the T. rex samples was 33%, with an average of 24% over three runs. In both modern samples the D-glutamic acid values were not identified as separate peaks. This analysis verifies a significant degree of racemization in the ancient sample, which was not seen in either of the more recent bone samples. Also, in the dinosaur extracts there is an enrichment in glycine over levels of other amino acids, as compared to the modern samples. This may be due to the loss of side chains from other amino acids, causing them to take on the configuration of this simplest of amino acids.
Table 2-1. RACEMIZATION STATE OF SELECTED AMINO ACIDS FOUND IN CANCELLOUS BONE EXTRACTS, GIVEN in MOLE PERCENTAGES DERIVED FROM THE RUN TOTALS

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Ostrich</th>
<th>Horse</th>
<th>T. rex1</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-asparagine/L-threonine/D-serine2</td>
<td>5.06</td>
<td>9.64</td>
<td>11.38</td>
</tr>
<tr>
<td>D-aspartic acid</td>
<td>-0-</td>
<td>-0-</td>
<td>3.28</td>
</tr>
<tr>
<td>L-serine</td>
<td>3.77</td>
<td>6.78</td>
<td>6.45</td>
</tr>
<tr>
<td>D-threonine</td>
<td>-0-</td>
<td>0.05</td>
<td>2.42</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>9.66</td>
<td>13.40</td>
<td>16.99</td>
</tr>
<tr>
<td>D-glutamic acid</td>
<td>-0-</td>
<td>-0-</td>
<td>4.18</td>
</tr>
<tr>
<td>L-leucine</td>
<td>4.89</td>
<td>2.38</td>
<td>2.64</td>
</tr>
<tr>
<td>D-leucine</td>
<td>-0-</td>
<td>-0-</td>
<td>0.29</td>
</tr>
<tr>
<td>Glycine</td>
<td>25.06</td>
<td>14.55</td>
<td>27.66</td>
</tr>
<tr>
<td>L-proline</td>
<td>12.64</td>
<td>-0-</td>
<td>10.74</td>
</tr>
</tbody>
</table>

Values are averages taken over three separate runs.

Peaks could not be separated.

Values are reported for comparison, since they are expected to be high in bone matrix.

Ultraviolet/visible (UV/vis) spectroscopic analysis of extracts of these tissues illustrated that the preservation state of the bone samples varied, with some showing a shift in absorbance from the expected 410 peak to the region of 459 nm. However, in extracts exposed to the HCl/acetone precipitation, a distinct peak in the region of 405 nm is clearly seen (Figure 2). This is consistent with the absorbance characteristic of the Soret band of hemoglobin and other heme proteins (Brown, 1980). One spectrograph of commercially prepared pigeon met-hemoglobin revealed a shift in absorbance to the region of 498-502 nm, while the smaller peak was recorded at 628 nm (data not shown).
Figure 2. Ultraviolet/visible absorbance spectrum of an extract of dinosaur tissues, precipitated in HCl/acetone/ether, as described. The large peak at 405 nm is within the range of variation seen for heme compounds, and is characteristic of these compounds. The smaller peaks at the longer wavelengths (Quasi-allowed, or Q-bands) are also characteristic of these compounds. The inset represents an expansion of the longer wavelengths of the tracing, so that the individual peaks in this region can be visualized and identified.

The proton NMR spectrum seen in Figure 3 shows peaks both upfield and downfield from the standard 0-10 ppm window of other proteins. Four broad resonances at 25.0, 29.0, 45.0 and 72.0 ppm, as well as 3 other peaks at -9.0, -20.0
and -30.0 ppm, require that a paramagnetic atom, such as those seen in various metalloproteins, must be present (La Mar, 1973; Bertini and Luchinat, 1986).

Figure 3. High resolution, solution phase proton NMR profile obtained from extracts of dinosaur tissues, precipitated in HCl/acetone/ether as described. For this spectrum, samples were also filtered through a 5000 MW cut-off filter, and the higher molecular weight fraction was again filtered through a 30,000 molecular weight filter before being subjected to NMR, which gave better resolution. The arrows indicate resonance in regions which are consistent with the presence of a paramagnetic compound such as hemoglobin.

The profile obtained in EPR verifies the presence of a paramagnetic compound in extracts of T. rex bone. While the sharp spike usually seen in hemoglobin and myoglobin proteins at g=6 (Peisach, et al., 1973) is missing from this
profile, it is also missing from the commercially prepared met-hemoglobin (met-Hb) sample (Figure 4). The tracings and g-values for two separate extractions of dinosaur tissues are almost identical with that observed for a dilute sample of the purified met-hemoglobin. This experiment gives additional information as to the oxidation state of the heme in the dinosaur extracts, and indicates that the iron is in the oxidized Fe III state.

![Figure 4. Electron paramagnetic resonance profile of two separate extracts of dinosaur tissues, diluted in PBS as described. Commercially prepared and purified pigeon met-hemoglobin was subjected to the same treatment and used as a comparison. In all cases, the referent is DPPH. The similarity in both profile and g-values of the dinosaur samples to the referent is seen. When monitored at full scale, the g=6 spike was absent from all traces, and this portion of the tracing is not included in this figure.](image)

Resonance Raman spectroscopy adds additional information to the study of the identification and structure of the paramagnetic compound identified in the dinosaur
extracts. This technique elucidates the bonding environment around the iron, the heme group and the ligands bound to these structures (Asher, 1981). There are five peaks generated in resonance raman spectroscopy which are characteristics of heme structure. These are identified as marker Band I, between 1340-1390 wavenumbers; II, between 1470-1505; IV, between 1535-1575; V, between 1605-1645; and VI, between 1560-1600 (Asher, 1981). Marker band III is not identified. The ferric (FeIII) derivatives of heme for marker band I are noted to be shifted to a slightly higher region than are those for the ferrous (FeII) state.

Figure 4 represents the profile of T. rex extracts subjected to resonance raman spectroscopy. The band labeled I is identified at approximately 1373 wavenumbers, consistent with bond stretches involving ferric iron (Asher, 1981). The presence of peaks in the dinosaur extracts consistent with marker bands I, II, IV and V (Figure 5) are strongly suggestive of the hemoglobin protein.
Figure 5. Raman profile of dinosaur extracts, precipitated in HCL/acetone/ether, as described. Human whole blood and commercially prepared pigeon met-hemoglobin are used for comparison. Roman numerals indicate peaks which are mentioned in the literature as marker bands for hemoglobin. Solid lines trace peaks present in either dinosaur and pigeon samples or all three, while broken lines indicate peaks seen in dinosaur and pigeon samples which are not seen in human blood.
Discussion

Because the sensitive techniques outlined above are so specific, they support the hypothesis that hemoglobin is preserved within the trabecular tissues of this specimen of *Tyrannosaurus rex*. However, with the exception of the results obtained from electron paramagnetic resonance, none of the results are identical to those noted experimentally or in the literature for modern hemoglobin, and therefore alternative explanations must be considered for the results shown here. Solution phase NMR requires the presence of free protons within the nucleus of atoms to resonate, a condition which occurs with incorporation of a metal into protein quaternary structure (Cerdonio, et al., 1981) This technique, in most cases, will not measure inorganic or geologically deposited iron, as these generally occur as iron oxides; however, there still remains the chance that the interaction between geologically deposited iron and humic acids or other degraded organic constituents of the soils over the course of millions of years may mimic this state. This has not been investigated, and data in the literature for this type of interaction is negligible.

Another factor which must be considered is that samples which have not undergone precipitation with HCl and acetone do not show a Soret band in the 400-410 region of the UV/visible spectra. It is noted in the literature (Brown,
that the ligands which are bound to the iron in heme may shift the absorbance values somewhat. In addition a shift is also seen depending upon the oxidation state of the iron at the center of the porphyrin unit. It is possible that there is a ligand bound to the porphyrin unit which is responsible for a shift in this absorbance peak to a region of longer wavelengths, and through precipitation, this ligand is lost. Further investigations are being conducted into this possibility.

The presence of additional signals in dinosaur extracts which are not seen in purified or modern samples may indicate additional or unusual ligands bound to the heme compounds in the dinosaur extracts. This would not be unexpected in highly degraded and possibly diagenetically altered biological compounds.

While none of these data will conclusively identify hemoglobin in extracts of these dinosaur systems, taken together the evidence provides very strong support for this hypothesis. In living systems today, the highest concentrations of metalloproteins are the heme proteins, hemoglobin and myoglobin. Indeed, it is estimated that each circulating mammalian red blood cell may contain 300 million molecules or more of this protein, and in birds and other archosaurs, this level is higher yet (Dickerson and Geis, 1983). Myoglobin, a muscle protein closely related to hemoglobin, may be proposed as the source of these heme
signals, and this would seem reasonable, given the sheer mass of muscle surrounding the bones of this animal. However, these studies were conducted only on trabecular bone, which was taken from the endosteal cavities of the bones. While these tissues are rich in blood, and blood forming tissues, they are not in direct contact with muscle tissue.

The cytochrome proteins also contain a heme prosthetic group, and could be offered as a reasonable explanation for the source of these signals. However, this protein is present in all organisms at much lower concentrations than are hemoglobin and myoglobin.

It also must be noted that, even though these tests provide strong evidence for the preservation of hemoglobin protein in extracts of these dinosaur tissues, they do not directly speak to the origin of the vascular microstructures, as the heme signal could be originating only from the tissues, and not the microstructures.

Conclusion

Four independent lines of evidence point to the existence of hemoglobin or hemoglobin breakdown products in extractions of *Tyrannosaurus rex* trabecular tissues, and racemization studies of individual amino acids indicate that the protein components are most likely indigenous to and
produced by the once-living cells of this dinosaur. Absorbance of light in the region of 410 nm is consistent with the Soret band in hemoglobin compounds. Proton NMR studies show resonance both upfield and downfield from the standard "protein window", which is indicative of the presence of a paramagnetic compound in these extracts. The most prevalent paramagnetic compounds in biological systems are the heme proteins, hemoglobin and myoglobin. Cytochromes are also heme proteins, but are found in much lower concentrations than are the hemoglobin proteins in modern taxa. In fact, each mammalian red blood cell contains close to 300 million molecules of hemoglobin (Dickerson and Geis, 1983), each of which is associated with four porphyrin rings and four iron atoms. The blood cells of Aves and Crocodilia may contain even higher amounts (Nikinmaa, 1990). The most parsimonious explanation for the amount of resonance seen in these small samples is that the heme compounds are blood-derived.

This is further supported by EPR, which not only verifies the presence of paramagnetic compounds by looking at the shifts of the electron spin states in microwave-generated magnetic fields, but also gives additional information as to the oxidation state of the heme group. It is found in the more stable met form, which is the ferric or FeIII form, rather than FeII of modern biological systems, and this is to be expected for ancient protein remnants.
The EPR curves are indistinguishable between two separate extractions of the dinosaur tissues and the commercially prepared and purified pigeon met-Hb, and the calculated g-values are almost identical in all of these samples.

Finally, the presence of four marker bands in resonance raman examinations of tissue extracts which are unique characteristics of the hemoglobin protein allow us to conclude that hemoglobin residues remain preserved in the tissues of this Cretaceous dinosaur.

Further work on these tissue extracts may result in the elucidation of the amino acid sequence of this protein. Since a large body of data exists for hemoglobin and the sequences for many taxa are already known, analysis of the amino acid sequence, if obtainable, may resolve some phylogenetic and physiological questions about the nature of this extinct animal.
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CHAPTER FOUR

EXTRACTION AND ANALYSIS OF DNA FROM THE TRABECULAR TISSUES OF TYRANNOSAURUS REX

Introduction

The use of DNA sequence data to establish phylogenetic relationships among taxa is a well known and familiar strategy of evolutionary biologists. DNA sequence data have helped to clarify relationships among different lineages within Animalia (Kocher, et al., 1989), Amniota (Marshall, 1992; Hedges, 1994), Tetrapoda (Hedges, et al., 1990), Reptilia (Hedges, et al., 1991; Hedges and Bezy, 1993; Heise, et al., 1995), and Aves (Hedges and Sibley, 1994). In most cases, phylogenies derived from DNA sequence data support those obtained through morphological analyses (Milinkovitch, 1992). In some cases, however, such as that of the subgroup of Aves dubbed the Pelecaniformes, molecular data revealed that these taxa, which had been grouped according to shared morphological traits, were represented by several different taxa (Hedges and Sibley, 1994).

The phylogeny of the Dinosauria has been delineated through meticulous morphological analyses by Gauthier (1986) and others, and the major divisions of the Dinosauria are well outlined. Based upon such classifications, the dinosaurs share a common ancestor with the Crocodilia, while birds are all the descendants of a line of saurischian
theropods, known as the maniraptors (Holtz, 1994). Because of the speculative and controversial nature of ancient DNA studies, phylogenies based on DNA sequences recovered from fossil specimens must align closely with phylogenies derived from other analyses. In the case of dinosaur DNA, there are certain criteria which must be met in order for the sequences to be judged valid. For example, the phylogeny based upon dinosaur-derived sequences must cluster the specimen with birds, and yet must be basal to all modern birds. If these criteria are not met, then, most likely, the sequences are derived from amplification error or contamination (Hedges and Schweitzer, 1995). The other, but highly unlikely explanation would be that the morphologically derived phylogenies for this group are in error. However, with the field of ancient DNA so new and controversial, such phylogenies cannot be currently challenged by ancient DNA analysis.

The advances in cloning technology, polymerase chain reaction (PCR), and improved DNA extraction techniques over the last decade have provided a means to test the hypothesis that molecules of DNA may be preserved in the tissues of extinct organisms. Recovery of informative DNA sequences from extinct organisms has helped to clarify not only relationships between these taxa and their modern descendants (DeSalle, 1994), but also such questions as rate and direction of evolutionary change at the molecular level.
Although recovery of DNA from ancient preserved tissues has been reported since at least 1984 (Higuchi and Wilson, 1984; Higuchi, et al., 1984; Johnson, et al., 1985), the first detailed published work to use such sequences for phylogenetic analysis was a study based upon DNA extracted from tissues of the quagga (Higuchi, et al., 1987). The quagga is a member of the horse family which became extinct near the turn of the century. This study not only initiated an explosion of research into the recovery of DNA from fossil specimens, but also demonstrated the importance of correlating the morphological characters of all taxa with molecular data to build more accurate phylogenetic trees (Patterson, 1987, 1988).

Since these early analyses of DNA preserved in ancient tissues, much work has been done with fossil specimens of all kinds and ages. DNA has been recovered from ancient human mummified tissues, bones and teeth (Hanni, et al., 1990; Meijer, et al., 1992; Hagelberg and Clegg, 1991, 1993; Merriwether, et al., 1994; Tuross, 1994). Ancient DNA sequences were used to clarify the phylogenies of the extinct marsupial wolf, *Thylacinus cynocephalus* (Thomas, et al., 1989); the saber-toothed cat, *Smilodon fatalis* (Janczewski, et al., 1992); New Zealand wrens of the family *Acanthisittidae* (Cooper, 1994), and the wooly mammoth (Hagelberg, et al., 1994, Hoss et al., 1994).

Studies of ancient DNA have also proven useful in
illuminating dispersions and migratory patterns (Hardy, et al., 1994; Merriwether, et al., 1994; Hagelberg and Clegg, 1993). The value of ancient DNA as a window into host-parasite or symbiotic relationships (Cano, 1994; Cano et al., 1994) and pathogenic states (Spiegelman and Lemma, 1993) is also beginning to be realized.

Recovery of ancient DNA is not limited to Holocene or Pleistocene specimens. Despite theoretical studies which indicated that recovery of DNA was not possible beyond a few thousand years (Lindahl, 1993), DNA has, in fact, been recovered from samples millions of years old. These studies include Miocene plant fossils (Golenberg, et al., 1990), amber entombed insects (Cano, et al., 1992a, b; DeSalle, et al., 1992), and plant tissues preserved in amber (Poinar, et al., 1993). Under certain conditions, it is apparently possible for DNA to have survived in recognizable form from the age of the dinosaurs (Cano, et al., 1993).

Other than amber, or tars such as those found in the Rancho La Brea region, bone may provide the ideal conditions for the preservation of DNA under certain highly constrained conditions. The affinity of DNA for the mineral in bone, hydroxyapatite, is well known (Romanowski, 1991; Jensen, et al., 1992; Tuross, 1994). If the bones of an animal undergo burial rapid enough to protect them from the elements, and if this burial is accomplished in regions of relatively cool and dry climate, then perhaps it may be feasible for the DNA
molecule to be preserved for an indeterminate amount of time by the stability afforded the molecule through its adsorbance to hydroxyapatite. This was the hypothesis that was tested in this phase of the study of a specimen of *Tyrannosaurus rex*, catalogued as MOR 555.

Given the excellent state of preservation exhibited by this specimen, and the preliminary analyses which showed the possibility of DNA preserved within these tissues (Chapter 2), attempts were made to extract this DNA for amplification and sequencing. It must be stressed that the trabecular tissues were handled only when observing stringent aseptic techniques. Tissues were never handled unless instruments were sterilized, and gloves were worn at all times. After the last of the sediment was removed from the bones used in these experiments, the cortical bone of the animal was removed, and the trabecular tissues were extracted from the marrow cavities, using flame sterilized instruments. Specimens were wrapped in foil in small packages, and kept in autoclaved glass jars containing baked desiccation crystals. The jars were kept tightly sealed at -20°C and tissues were removed from them as needed.

Several different methods of extraction were tested, and several different primer pairs were used in polymerase chain reaction (PCR) amplification attempts. The following outline of strategy will be divided into: I. Extraction protocols; II. Primer design; and III. PCR protocols.
I. Extraction protocols.

Protocol 1 (Maniatis)

Attempts to extract DNA from the trabecular tissues of this Tyrannosaurus rex began with a method first outlined by Maniatis, et al. (1989), which is a standard method of extraction of DNA from soft tissues and bone of modern specimens. Dinosaur trabecular tissues were ground in baked mortar and pestles to a fine powder, and aliquoted in amounts of approximately 0.3-0.5 g to autoclaved polypropylene (Eppendorf) microcentrifuge tubes. Approximately 650 μl of extraction buffer containing TE (0.2M Tris: 0.1 M EDTA, pH 8.3), Proteinase K (Sigma, 10 mg/ml) and the detergent, SDS (10%) were added to each tube. After extracting at 60°C for 1-3 hours, the tissues and buffers were incubated overnight at 37°C. 5M potassium acetate (KOAc) was added to the tubes containing the bone and buffers. After centrifugation, the supernatant was transferred to new tubes. A 1:1 mix of phenol and chloroform/isoamyl alcohol was added to the supernatant. This solution was vortexed to mix well, and then re-centrifuged. The resulting supernatant was transferred to new tubes, and the DNA was precipitated by the addition of
an equal volume of isopropanol, then placed in a -20°C freezer for at least 2 hours. After freezing, the tubes were centrifuged at 14,000 x G for 15 minutes to pellet the DNA, and the supernatant was discarded. The pellet was washed with 100% ethyl alcohol and dried in a Savant speed-vac. The DNA was resolubilized in either double-distilled, de-ionized, autoclaved water or autoclaved TE (Tris/EDTA, 10:1) buffer. Concurrent extractions were carried out without adding the dinosaur tissues as environmental controls. Portions of the material obtained from these extractions of dinosaur tissues were subjected to digestion with approximately 1 μg of DNase for 30 minutes at 37°C, then visualized on a 1.5% agarose gel under ultraviolet (UV) light after staining with ethidium bromide. The extracts were also subjected to digestions with the restriction endonucleases, Hind III and Rsa I, and again visualized under UV light.

Protocol 2 (Elu-Kwik)

This was a commercial DNA extraction kit, Elu-Kwik® (Purdue, 1993). To 1.5 μl microcentrifuge tubes containing approximately 0.6 g of ground T. rex trabecular tissues, a solution of 0.5 M EDTA (pH 8.0), 10% SDS, and Proteinase K (14.7 mg/ml) was added. This was rotated for at least 2 hours at 37°C. The tubes were then centrifuged at 3500 x G
for three minutes. The resulting supernatants were removed to new sterile tubes, and lysis buffer and binding buffer, both supplied by the kit manufacturer, were added, as was a glass concentrate. After pipetting to suspend the glass powder the solution was gently rocked for 10 minutes, and centrifuged at 3500 x G for three minutes. The supernatant was discarded, and the pellet was resuspended in a wash buffer. Centrifugation was repeated, the supernatant discarded and this step was repeated. The pellet was then resuspended in a salt reduction buffer, which was provided. This was centrifuged at 7000 x G. The pellet was then washed with 250 ul of 1X TE and incubated at 50°C for 15 minutes to elute the DNA from the glass.

Protocol 3 (Chelex)

To approximately 0.5 g of powdered T. rex bone tissues, 500 μl of 5% Chelex® (Walsh, et al., 1991) was added in 1.5 ml sterile microcentrifuge tubes. The tubes were sealed and incubated overnight at 50°C with shaking. The tubes were then vortexed to mix well, and incubated at 95°C for 15 minutes. Centrifugation at 14,000 x G followed, and the supernatant, containing the template DNA, was removed.
Protocol 4 (RPM)

This extraction method utilized the RPM\textsuperscript{a} (Rapid Pure Mini-prep) kit (Bio 101, La Jolla, CA) as modified by R.J. Cano (Cano and Poinar, 1993). Approximately 1.5 ml of Glassmilk\textsuperscript{b} spin buffer, to which 2 \mu l of 10\% acetic acid had been added, was placed in a siliconized 2.0 ml microcentrifuge tube with approximately 0.5 g of powdered bone. The addition of acetic acid assured that the glass achieved the proper pH for DNA adsorbance. This was vortexed to mix well, and then incubated with slow rocking at 55\° C overnight. The sandstone matrix which surrounded the bone was extracted in an identical manner. After incubation, the samples were transferred to 2.0 ml spin filters provided with the kit. These were centrifuged at 14,000 X G for one minute, and the liquid at the bottom of the tube was discarded. The pellet which remained was washed twice with the "wash buffer" provided in the kit. Subsequently, the DNA which had adhered to the glass beads in the filter was eluted off of the beads with 150 \mu l of sterile, dionized, distilled water. The eluent was lyophilized on a Savant speed-vac, and resolubilized in 30 \mu l of sterile TE buffer (10:1, pH 7.9). This could then be used directly as template for PCR reactions.
This protocol was a modification of the method outlined by Hoss and Paabo (1993). For this method, approximately 0.5 g of T. rex trabecular tissues were ground and placed in a sterile siliconized 1.5 ml microcentrifuge tube. 1.0 ml of extraction buffer (10 M guanidinium thiocyanate (GuSCN); 0.1 M Tris/HCl, pH 6.4; 0.02 M EDTA pH 8.0; and 1.3% Triton X-100) was added to the bone, and the mixture was rocked overnight at 55-60° C. Subsequently, this was centrifuged at 5000 X G. The supernatant was collected and 500 µl was aliquoted to each of two new sterile tubes. To each, another 500 µl of extraction buffer was added, along with 40 µl of specially treated silica matrix, prepared as outlined. The tubes were gently rocked at room temperature for 15 minutes, then centrifuged at 14,000 X G. The supernatant was decanted to new tubes for later use in proteins analysis. The remaining glass pellet was resuspended in wash buffer (10 M GuSCN and 0.1 M Tris/HCl, pH 6.4), vortexed, and centrifuged again. This was repeated a second time, with the supernatant discarded each time. The pellet was then washed twice with 70% ethanol, and once with acetone. The pellets were then dried in a heat block at 55° C for 10 minutes, and then resuspended in sterile, deionized, filtered and irradiated distilled water to elute the DNA. The elution step was repeated a second time, and
the eluents were combined. The resulting eluents were concentrated on a speed vac, and used as template for PCR reactions.

Protocol 6 (Reducing buffer)

This extraction method was outlined in a paper by Merriwether, et al., (1994), and the extraction buffer consisted of the following: 10 mM Tris/HCl, 10 mM EDTA, dithiothrietol, (DTT) 10 mg/ml, Proteinase K, 0.5 mg/ml, and 0.1% SDS. 5 ml of this extraction buffer were added to approximately 0.3-0.5 g of powdered bone. The reason for the addition of the DTT to this buffer was to create a reducing environment that would limit any oxidative damage to the DNA which may occur after it is removed from stabilizing influence of the bone matrix. Again, the resulting solution was either dialyzed against 10 mM Tris, pH 7.9, or filtered using Millipore microcentrifuge filters (30,000 MW) to concentrate and purify the sample. The concentrated sample was used as template for PCR reactions.

II. Primer Design

The current state of technology in ancient DNA research is such that verification of the indigeneity or validity of segments of DNA recovered from ancient tissue sources must
rely on sequence analysis. Since the phylogenetic position of the *Tyrannosaurus rex* is well defined with respect to the most closely related extant taxa of *Archosauria*, namely crocodiles and birds (Gauthier, 1986), it is expected that DNA sequences must reflect this phylogenetic position. As mentioned previously, for any gene region selected for examination, true dinosaurian DNA would cluster with birds by any phylogenetic analysis system, and should be basal to all modern birds, including the ratites, which are the most primitive.

The gene region on which we chose to concentrate our efforts was the 18s ribosomal RNA (rRNA) gene. The rationale for the choice of this gene was fourfold. First, there is quite an extensive database available for comparison with modern taxa. Second, this gene is present in the genome of vertebrates in large copy numbers, and it was reasoned that it was a better target for amplification than a single copy gene (Naito, et al., 1992). Third, it is a highly conserved gene, and as such, primer design could be optimized so that dinosaurian DNA fragments would not be unrecognized because of base-pair mis-matches. However, there exists enough variability within this gene to build primers which would not amplify DNA from microbes, a likely source of contamination in fossil bone (Naito, et al., 1992; Hedges, personal communication). Finally, this is a nuclear gene, and it is believed that the association of DNA with
histone proteins into nucleosomes may afford protection from
degradation to nuclear genetic material. While a case can
be made for using mitochondrial genes for study of ancient
DNA fragments by the first three arguments outlined above,
this DNA is not associated in the same manner with proteins
which may impart added stability and protection to the DNA
over time.

The first primers which were used in attempts to
amplify the DNA extracted from Tyrannosaurus rex bone were
designed by S. Blair Hedges (Pennsylvania State University),
and were provided by Raul J. Cano (California Polytechnic
University). These primers were designed from aligned
sequences of modern taxa to flank a relatively variable
region of the 18s gene. This region contains a 20 base pair
insertion in all mammalian lines, which would allow for easy
screening on an agarose gel against mammalian contaminant
PCR product. These primer sequences (5'-3', IUPAC code)
are: 18s la: ATTC TAGAG C T AAT AC AT GCC GAC GA; and 18s lb
GTGGTCACCATGGT-AGGCACAGAMAG. These primers amplify a region
on the human gene corresponding to positions 185-376, and
are designed to amplify a 201 bp fragment in archosaurs.

Since the DNA fragments which were extracted from the
dinosaur bone were of apparent high molecular weight,
universal primers were also designed by R. Sharrock (Dept.
of Biology, Montana State University) to amplify a segment
of 18s rRNA approximately 500 bp in length. The fragment
amplified by these primers encompassed the above region. From 5' to 3', the sequences of the primers was: 18s #1, CTTGTCTCAAAGATTAAGCCATGC; 18s #2, ATACGCTATTGGAGCTGGGAATTAC.

Several other primers were designed for amplifying varying regions of the 18s gene. Most of these were designed to amplify smaller regions of the gene than either of the two outlined above, and some were nested inside one or the other pair of primers mentioned above. 18s 1c (5'-TTATCAGACCAAAAACCAACCCGGGCT-3') and 18s 1d (5'-GCGTGCGATCG-GCYGAGGTTATCTA-3') were designed either to be used together, or to be paired with 1a or 1b to obtain smaller fragments which would still be informative. 18s 1e (5'-CAGCTAGGAATAATGGGAATAGGACT-3') and 1f (CGGCCGTCCCTCTTAATCATGGCCC-3'); 1g (5'-ATTTGACTCAACACGGGAAACCTCA-3') and 1h (5'-CCACCCACRGAATCGAGAAAGAGCT-3'); 1i (5'-CCATAAACGATGCCGACTAGCGATC-3') and 1j (5'-ATACTCCCCCCGGAACCCAAAGACT-3') were likewise designed to be used in pairs or in conjunction with other primers. The locations of the primers, relative to the ostrich sequence generated by primers 18s #1 and 18s #2, are shown in figure 1. The rest of the primers fell outside of this region, but can be found on any published sequence of the entire 18s gene.

The large subunit of the 28s gene was also examined and regions for suitable primer sites were likewise designed. In addition, primers were designed to amplify conserved regions of the 12s and 16s mitochondrial ribosomal genes.
Results of amplification attempts with these primers were inconclusive, and will not be discussed here.

5'-TTGTCTCAAA GATTAAGCCA TGCATGTCTA AGTACACACG GGTGGTACAG
18s #1 (forward)
TGAAACTGCT AATGGCTCAT TAAATCAGTT ATGGTTCCTT TGGTGCTCC CCTCCGGTTA
CTTGGATAAC TGTGGTAAATT CTAGAGCTAA TACATGCCGG CGAGCCCCGA CCTCCGGGGA
18s 1a (forward)
CGCGTGCATT TATCAGACCA AAACCAACCC GGGCTCGGGC GCGCTTTGGT GACTCTAGAT
18s 1c (forward)
AACCTCGAGC CGATCGCACG CCCCCGTGGC GGCGACGACC CATTCGAATG TCTGCCCTAT
18s 1d (reverse)
CAACTTTGCA TGGTACTGTC TGTGCCTACC ATGGTGACCA CGGGTAACCG GGAATCAGGG
18s 1b (reverse)
TTGATCCGG GAGAGGGAGC CTGAGAAACG GCTACCACAT CCAAGGAAGG CAGCAGGCGC
GCAAATTACC CAATCTCGGC CGGGGAGGT AGTGACGAAA AATAACAATA CAGGACTCTT
TCGAGGCCCCT GTAATTGGAA TGAGTACACT TTAAATCCTT TAAAGGGAT CCATTGGAGG
GCAAGTCTGG TGCCACGACC CGGGTAATT CCAGCTCAA TAGCGTAT-3'
18s #2 (reverse)

Figure 1. Partial sequence of Ostrich 18s rRNA gene, generated from extractions of bone and associated soft tissue, using the method outlined in the text (Protocol I). This sequence was generated with primers 18s #1 and 18s #2. Underlined regions show the placement of other primers used in amplification attempts. Primers which fell outside of this generated sequence are listed in the text.

III. PCR Protocols

Amplification of DNA extracted from the T. rex bones was attempted with the primers outlined above.
Amplification reactions were carried out in a Thermolyne Thermocycler (Barnstead) and the profiles for amplification are seen in Table 3-1.

**TABLE 3-1. PCR REACTION MIX AND AMPLIFICATION PROFILE FOR A 50 μL REACTION**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>template DNA</td>
<td>5-10</td>
<td>μl</td>
</tr>
<tr>
<td>10x PCR buffer&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5</td>
<td>μl</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt; (25 mM)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5</td>
<td>μl</td>
</tr>
<tr>
<td>BSA&lt;sup&gt;3&lt;/sup&gt; (20 μg/ml)</td>
<td>5</td>
<td>μl</td>
</tr>
<tr>
<td>dNTP's (10 mM)</td>
<td>4</td>
<td>μl</td>
</tr>
<tr>
<td>forward primer</td>
<td>2</td>
<td>μl</td>
</tr>
<tr>
<td>reverse primer</td>
<td>2</td>
<td>μl</td>
</tr>
<tr>
<td>water</td>
<td>21.8</td>
<td>μl</td>
</tr>
<tr>
<td>polymerase enzyme&lt;sup&gt;4&lt;/sup&gt; (5U/μl)</td>
<td>0.2</td>
<td>μl</td>
</tr>
</tbody>
</table>

Initial Denaturation: 94°C for five minutes

Cycle: Denaturation: 94°C for 1 minute

Primer Annealing: 50-55°C<sup>5</sup> for 1 minute 40 cycles

Extension: 72°C for 2 minute

<sup>1</sup>Buffer used was dependent on the choice of enzyme

<sup>2</sup>MgCl<sub>2</sub> was only added when the buffer did not include it.

<sup>3</sup>BSA was omitted from some runs

<sup>4</sup>Taq polymerase or Taq Stoffel fragment were used

<sup>5</sup>Annealing temperature was adjusted to the specific primer pairs.

PCR products obtained using 18s #1 and #2 were ligated into the MP 18 and MP 19 strains of the M13 vector. JM 101 strains of *E. coli* were transformed via electroporation, and the transformed cells containing the target DNA sequences were plated onto an agar medium, with added IPTG and X-GAL to allow for blue/white screening of plaques. The plates were incubated overnight at 37°C. The colonies containing
the DNA insertions were purified and used as template for Sanger sequencing. Sequences obtained by this method were aligned to those of the ostrich, using the program ALIGN (Version 1.02, scientific and Educational Software).

Results and Discussion

Every extraction method to which *T. rex* bone tissues were exposed, with the exception of one, yielded a supernatant phase which was a reddish brown in color. The reducing environment created by the addition of dithiothrietol in method 6 resulted in a supernatant which was a deep burgundy. While not significant for the evaluation of DNA, this color change may indicate that any hemoglobin molecules present (see Chapter 3) may still be biologically active in the sense of being able to take up oxygen. Extracts of fresh ostrich bone were clear, with no color phase. However, extractions of desiccated horse tissues, probably 10-20 years post mortem, were indistinguishable from extractions of the *T. rex* in color.

When the supernatants were purified and electrophoresed on agarose gels, then stained with ethidium bromide, in most cases high molecular weight DNA could be visualized with ultraviolet (UV) light. A background smear of degraded material was also usually seen in these lanes (Figure 2a). The methods which utilized a glass matrix to purify the DNA
in solution (methods 2, 4, and 5) did not yield DNA in large enough quantities to be visualized in this manner. However, amplification product was obtained using these extracts as PCR template.

The fact that this material was DNA and not humic acid or other material which also fluoresces under UV light (Tuross, 1994) was evidenced by exposure of the material to the DNA specific enzyme DNase, as well as to restriction enzymes (Figure 2b). The DNA digested to completion with DNase. Of the two restriction enzymes employed, only RsaI, with a four-base pair recognition site, was effective in degrading the DNA.

PCR amplification was attempted on extracts of dinosaur tissues obtained from all extraction methods, with varying degrees of success. The 1a/1b primers did not yield product initially when used with T. rex extractions as template. This may have been due to inadequate removal of co-eluting factors which inhibited the polymerase enzymes (Tuross, 1994). To test this, dinosaur extract and primers were added to a reaction containing extant plant DNA and appropriate primers. After PCR, no product was seen in the reactions containing dinosaur extracts, while controls showed adequate amplification. This allowed us to conclude that the dinosaur extracts were most likely inhibiting the reaction.

Other workers did achieve amplification with the
reptilian/avian specific 1a/1b primers, using T. rex extracts as template, but these results have not been replicated. Of the primers listed, only two yielded amplification products. Primer pairs Ii and Ij yielded product which, upon sequence analysis by S. Blair Hedges proved to be fungal in origin. Primer pairs 18s #1 and #2 yielded amplification product from extractions of both tibia and femur tissues (Figure 3). When clones of these amplification products were sequenced, they showed a high degree of variability.
Figure 2. Extracts of *T. rex* tissues for DNA, as described in the text, and electrophoresed on 1.5% agarose gel, stained for 10 minutes with 10 μg/ml ethidium bromide, and visualized under ultraviolet light. Lanes on the gel are as follows: A: 1. *T. rex* femur extract, 10 μl load. 2: *T. rex* femure extract + DNAse, 10 μl. 3: Lambda HindIII standards, 5 μl, 100 ng/μl. 4: Arabidopsis (sp) DNA untreated, 5 μl (positive control) 5: Arabidopsis (sp) DNA + DNase, 5 μl. 6: *T. rex* tibia extract, untreated, 10 μl. 7: *T. rex* tibia, second extract, untreated, 10 μl. B: 1: *T. rex* femur extract, 5 μl. 2: *T. rex* femur extract + HindIII restriction enzyme, 8 μl. 3: *T. rex* tibia extract, 5 μl. 4: *T. rex* tibia extract + HindIII restriction enzyme, 8 μl. 5: *T. rex* tibia extract + RsaI restriction enzyme, 8 μl. 6: Lambda HindIII standards, 5 μl, 100 ng/μl. 7: *T. rex* tibia extract + DNAse, 5 μl.
Figure 3. PCR amplification products using 18s #1 and #2 primers, visualized on a 1.5% agarose gel with ultraviolet light. Lanes are as follows: 1. Tibia extract, 8 µl load; 2. Femur extract, 8 µl; 3. Alligator, 8 µl; 4. size standards (HaeIII digest, PhiX 174); 5. Ostrich, 8 µl; 6. Wolf, 8 µl; 7. Negative control.

Figure 4 shows the alignment of the sequences obtained from cloned amplification products of 18s #1 and #2. The high degree of variability cannot be accounted for completely by invoking PCR enzyme error or template damage (Paabo, et al., 1990; DeSalle, et al., 1994), and it must be assumed that the clones represent multiple sources of DNA.
Figure 4. Eight sequences obtained from PCR amplification, using extracts of dinosaur tissues as template. Amplicons were cloned into M13 vectors and single stranded DNA was used for sequencing. The sequences are aligned to Ostrich sequence, generated with the same primer pair, with the computer program ALIGN, as indicated in the text. Dots indicate homologies, and dashes indicate gaps inserted into the sequences.
Conclusion

DNA is apparently prevalent in the trabecular tissues of this dinosaur. That the DNA originated from the tissues and not as a lab contaminant is shown by the fact that DNA is visualized before amplification, and that the extraction controls, run concurrently, never yielded detectable DNA. However, the sources of this DNA remain unknown. While some sequences are most likely fungal and/or bacterial in origin, and others show slight homology with arthropods, some sequences do not match any entries in the data bases and therefore, the sources of these DNA fragments remains unknown. It is possible that these DNA fragments are derived from soil contaminant, but another possible explanation is that they were derived from organisms involved in the decay of this specimen.

Since there was no visible evidence of bacterial or fungal contamination even at magnifications of greater than 100K, the possibility remains that at least some, if not all of this DNA, while perhaps not dinosaurian in origin, may still be derived from ancient sources. Future work, perhaps using stable isotope analysis or other methods, may verify this hypothesis. In addition in situ PCR may provide a means for better identification of the sources of this DNA.


DeSalle, R.; M. Barcia; C. Wray. 1993. PCR jumping in clones of 30-million-year-old DNA fragments from amber preserved termites (Mastotermes electrodominicus). Experientia 49:906-909


CHAPTER FIVE

CONCLUSION

It has only recently become feasible to properly test the hypothesis of biomolecular preservation in the fossil record. The increasing sensitivity and sophistication of biomolecular technology applied to fossil specimens is producing results which defy the traditional assumption that indigenous organic molecules are sufficiently degraded so as to be uninformative over the course of geological time. Proteins, the amino acid constituents of proteins and even DNA have been identified in fossil samples over the last decade or two, but with only a few exceptions, these biomolecular markers have not been sought in bone from the age of dinosaurs.

It is noted in the literature (Hagelberg, et al., 1991) that excellent gross and microscopic structural preservation of bone may be a useful indicator of biomolecular preservation. This seems to be consistent with the observations of this study, where minimal diagenetic alteration and excellent preservation on a microscopic level is mirrored by identifiable molecular components. While intensive examinations of the unreplaced and unpermineralized tissues of this dinosaur have shown
apparent biomolecular preservation, there remain several important issues to be resolved. First, little is known of the chemical interactions between biological and geological materials over the course of extensive periods of time. It may be possible that such interactions may either mimic or mask indigenous biomolecular signals.

Second, if analyses of specimens similar in appearance and character of preservation to MOR 555 show similar biomolecular preservation, perhaps certain constraints or characteristics of the depositional environments could be identified which may be used as indicators of such preservation. Microtaphonomic and geochemical analysis of depositional environments may yield clues as to the biological and geological interactions which may enhance molecular preservation.

Also, it is the experience of this investigator that the amount and type of biomolecules recovered from these bony tissues vary greatly, depending upon the particular piece of bone examined. This implies a paradox to future studies similar to the analyses of this T. rex specimen. Demands for independent verification of results are valid, and vital to the usefulness of such studies. However, if these results cannot be repeated, it may not necessarily indicate that the initial results are always suspect. Alterations in the microenvironments of this bone greatly influence the biological materials recovered, both in
quantity and condition. Also, there is very little data in the literature dealing with the profiles which may be expected of highly degraded hemoglobins, collagens, or nucleic acids examined by these methods. Certainly it is not reasonable to hope that such profiles would be identical to modern samples, yet at what point do the differences seen between ancient and modern samples render the ancient ones either unidentifiable or uninformative?

The data presented in this thesis project open the door to a variety of studies in the future, not only for dinosaur tissues, but for any archaeological or palaeontological specimens. The tissues of MOR 555 show little evidence of permineralization or other diagenetic alteration, yet a reasonable theory as to why this should be has yet to be proposed. Elucidating the conditions leading to such preservation would be a valuable study.

Similar preservation, on a gross observational level, has been noted by myself and others for other bones recovered from this same formation. It would be very informative to do a similar set of tests on other bones from this formation, to see if results obtained here could be replicated in other specimens. If so, elucidating the paleoenvironmental conditions which may have made the Hell Creek formation singularly suited to biomolecular preservation would be useful.

Additionally, mechanisms for the preservation of
biomolecules over such vast time periods are unknown. Several theories have been proposed (see Chapter 1), but these theories have not been adequately tested. While it is noted by several investigators in the field that the presence of mineral, particularly hydroxyapatite, seems to enhance organic preservation, the exact role that other entities, such as humic and fulvic acids may play in biomolecular stabilization would make an interesting geochemical investigation.

Finally, this study has concentrated on identifying certain biomolecules, namely proteins or their constituents, and nucleic acids. However, additional studies could be done on the possibility of lipid and/or polysaccharide preservation which may also be informative. In particular, identifying the organophosphate allosteric effectors which bind to the heme units in porphyrins may be particularly informative, both from a physiological and a phylogenetic perspective. This may be possible with some of the techniques outlined here.

It is hoped that this study may contribute to the emerging field of molecular palaeontology through the methodological strategies outlined here. Outlining the most efficient techniques by which to obtain such information may save other workers in this area valuable time, as well as providing them the means to conserve precious sample by avoiding some of the problems encountered in this study.
Also, by making others aware of the information potential of the fossil record, from a biomolecular perspective, perhaps routine collection methods and field techniques can be altered to accommodate similar studies in the future.