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

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.

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Mary Higby Schweitzer

A thesis submitted in partial fulfillment
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Mary Higby Schweitzer

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.

5/12/95
Date

John R Horner
Chairperson, Graduate Committee

Robert S Moore
Co-Chair, Graduate Committee

Approved for the Major Department

5/12/95
Date

Robert S Moore
Head, Major Department

Approved for the College of Graduate Studies

6/27/95
Date

R L Brown
Graduate Dean

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Mary Higby Schweitzer

Advisor: John R. Horner
R. E. Moore, PhD
Montana State University
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Date 5/12/95

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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.

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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 Riqles, 1980, de Riqles, *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 recognizeable 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*

al., 1983) in fossil specimens. This latter proposal has been met with some controversy (Kimber amd 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 phsyiology 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 palaeontolgy 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 algorithims 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 indigeneous "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.

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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% OsO_4 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 CO_2-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_2O. 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.

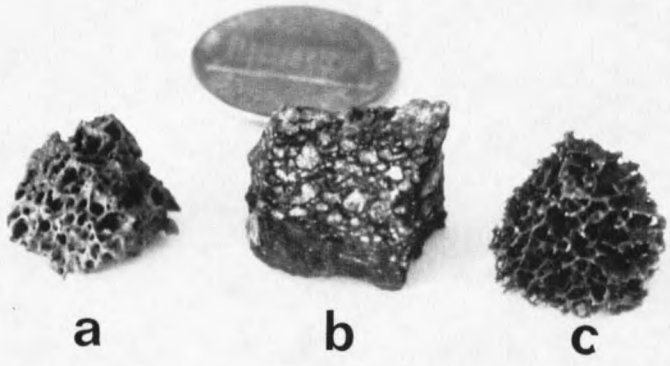

Figure 1. a. MOR 555 (*Tyrannosaurus rex*), weight = 0.72g, density = 0.36g/ml; b. hadrosaur (*Maiasaurus peeblesorum*), weight = 3.69g, density = 1.24g/ml; c. recent horse (*Eqqus caballus*), weight = 0.34g, density = 0.11g/ml.

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.