Scanning Microscopy

Volume 7 | Number 2 Article 29

2-11-1993

Preparation of Dinosaur Fossils for Scanning Electron Microscopic Evaluation of Bone Histology

C. Barreto
University of Wisconsin-Madison

R. M. Albrecht
University of Wisconsin-Madison

Scanning Microscopy, Vol. 7, No. 2, 1993 (Pages 751-754)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/93$5.00+.00

PREPARATION OF DINOSAUR FOSSILS FOR SCANNING ELECTRON MICROSCOPIC EVALUATION OF BONE HISTOLOGY

C. Barreto and R. M. Albrecht*

Dept. of Animal Health and Biomedical Sciences
University of Wisconsin-Madison
Madison, WI 53706

(Received for publication September 21, 1992, and in revised form February 11, 1993)

Abstract

The quality of preservation is a major determinant of the amount and kind of information to be derived from a paleontological specimen. Various fossilization regimes may produce specimens that contain biological information on a microscopic level. Light microscopic inspection of adolescent fossil dinosaur bones from the Two Medicine Formation (northwestern Montana) revealed that precise preservation of tissue may occur at a cellular level. In the following report, we test and compare techniques of specimen preparation for examination by scanning electron microscopy and illustrate an example of faithful preservation of dinosaur bone histology.

Key Words: Calcite, chicken, dinosaur, fossil preparation, histology, long bones, *Maiasaura*, paleontology, scanning electron microscopy.

*Address for correspondence:
Ralph M. Albrecht
Dept. of Animal Health and Biomedical Sciences,
University of Wisconsin-Madison,
1655 Linden Drive,
Madison, WI 53706-1581, U.S.A.
Telephone Number: (608) 263-3952
FAX number: (608) 262-7420

Introduction

The fossils examined for this report were from a duck-billed dinosaur, *Maiasaura*, from the Two Medicine Formation of northwestern Montana. The excellent preservation and abundance of rare material recovered from this formation has contributed greatly to our knowledge of dinosaurs. The specimens we studied were collected from dinosaur nesting sites previously described (Horner and Makela, 1979). *In situ* preservation of an established duck-billed dinosaur rookery has provided insight regarding their ethology, including the complexity of dinosaur social structure (Horner, 1984).

In the present study, we examined bones of adolescent dinosaurs from these nesting sites using light and scanning electron microscopy (SEM). In addition, we collected, prepared and examined normal adolescent leghorn chick bone for comparison. Because the structure of bone reflects diverse biological processes, studies on the comparative histology of fossil and modern bone permit inference on the biology of extinct animals. Of course, cells are no longer present in the fossils. However, the extracellular matrix is often well preserved and from the fossil bone histology, physiological information can be derived (Ricqles, 1976). We concentrated on examining newly forming metaphyseal bone from long bone ends in the area of the growth plate. The overall goal of our research is to gain an understanding of mechanisms of bone elongation in dinosaurs.

The key to the success of the present work was to differentially etch sedimentary matrix versus fossilized bone. Thin sections of the fossils viewed with the light microscope indicated that the bone structure was well preserved. However, intertrabecular spaces were completely filled with calcite crystals. It was necessary to remove the calcite to allow light and scanning electron microscopic viewing of the underlying bone histology. The problem was to find an agent with the capacity to demineralize the sedimentary matrix, but also to prevent demineralization of the fossil bone. We herein report three etching treatments and evaluate the efficacy of each process in specimen preparation. The samples examined were serial longitudinal sections from the same distal femur, and were therefore, equally preserved.

Materials and Methods

It is known that either acids or chelating agents would remove the calcium carbonate crystals which filled the portions of the fossil specimens between biological structures. We chose three different etching solutions to test if they would remove the calcite without adversely affecting the fossilized bone. We were concerned that the fossilized bone, depending on its mineral content, might also be demineralized. Treatments were tested for periods of 5 minutes to 48 hours. Specimens were removed from the etching solution and examined at 5 minute intervals over the first hour and at 6 hour intervals overall, so that optimal timing could be determined. In treatment #1, specimens were etched in a chelating agent of 15% ethylenediamine tetra-acetic acid (EDTA), prepared by a method previously described (Baumgartner and Carson, 1987). A subsequent jet wash procedure removed the loosened particles. A 2 ml Monoject syringe with a 23 gauge needle was filled with a sodium citrate acid solution (see treatment #2 below). The specimen was held in a petri dish and viewed through the dissecting microscope during jet washing. Specimens were then rinsed in distilled water and placed in a desiccator to dry.

Treatment #2 utilized a sodium citrate-formic acid solution prepared as follows: 50.0 grams sodium citrate in 250 ml distilled water is mixed with 125 ml of 88% formic acid in 125 ml distilled H_2O. These specimens were then jet washed and dried in treatment #1.

Treatment #3 entailed etching in a 10% glacial acetic acid solution, followed by jet washing. Subsequent experiments have shown that jet washing with additional 10% acetic acid (instead of solution #2) yields equally good results.

Fossil long bone ends, of approximately 2 cm^3, were longitudinally sectioned on an Isomet (Buehler) saw to 1 mm thickness. Specimens of approximately 8 mm x 8 mm x 1 mm were treated as described above and one control specimen was not etched.

For comparison, adolescent chick bone was prepared. Long bone ends of 2 week old Leghorns were collected and fixed in 0.5 M phosphate buffered saline (pH 7.4) with 2% glutaraldehyde for 24 hours. To digest and dissolve soft tissue, the bone ends were cut in half longitudinally with a razor blade and placed in 3% Biz (proteolytic enzyme-detergent) solution for 96 hours, followed by papain digestion for 48 hours. Both solutions were changed daily and specimens were periodically agitated. The chick bones were subsequently dried in a desiccator.

Both fossil and modern specimens were mounted on Cambridge style stubs and sputter coated with 10 nm of gold. The specimens were viewing using a JEOL JSM 35-C SEM operated at 15-20 kV.

Results

In the untreated specimens, calcite crystals precluded viewing fossil bone (Fig. 1a). However, once etched, the well preserved fossil bone histology is evident (Fig. 1b). Calcite crystals were completely removed from the specimens etched with treatment #1, the 15% EDTA solution. The optimal treatment period was 48 hours. The fossil bone was not completely removed and histological features could be recognized. However, unlike the results of Hirsch and Quinn (1990) on fossil eggshells from the Two Medicine Formation, it was obvious that the bone had been degraded (Fig. 2) and that histological information was lost. The damage may have been compounded by jet washing with the sodium citrate-formic acid solution.

Treatment #2, with sodium citrate-formic acid also completely removed calcite crystals. Optimal calcite removal occurred in 10 minutes. However, the fossilized bone became fractured and flaky (Fig. 3). It was possible to detect that the bone histology had been preserved, but the treatment subsequently rendered the specimens unsuitable for study.

Treatment #3, with 10% glacial acetic acid resulted in the best preparation. The optimal treatment period was 25 minutes. Complete removal of the obscuring calcite crystals was accomplished without any detectable damage to the fossilized bone (Fig. 4). We were able to make detailed histological observations at magnifications greater than 8000x.

Treatment of the modern chick bone with the proteolytic enzyme-detergent solution followed by the papain solution greatly enhanced viewing of the mineralized tissue (Fig. 5). Previously we had attempted to prepare the chick bone by dehydration in a graded series of ethanols and critical point drying (Boyde, 1972; Dillaman *et al.*, 1979). However, with this method of preparation soft tissue remains on the bone and the samples are less suitable for comparison with fossil bone (in which only mineralized extracellular matrix is preserved).

Discussion

The outcome of this project was dependant on our ability to selectively etch the fossil specimens. The mineral content of biological versus non-biological material must be considered when preparing specimens for histological evaluation. During diagenesis, groundwater may introduce various minerals into bones. Often, biological

Figures 1-5: Scanning electron micrographs of:

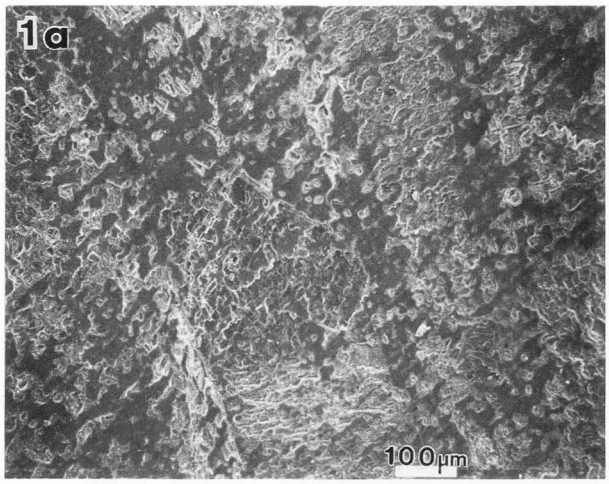
Figure 1. An untreated (**Fig 1a**) and an etched (**Fig. 1b**) dinosaur fossil. In untreated sample (**Fig. 1a**) calcite crystals cover the bone precluding study of bone histology. In etched sample (**Fig. 1b**), after the overlying calcite crystals have been removed, features of the bone histology such as, trabeculae (A), vascular channels (B), and osteocyte lacunae (arrowhead), are evident.

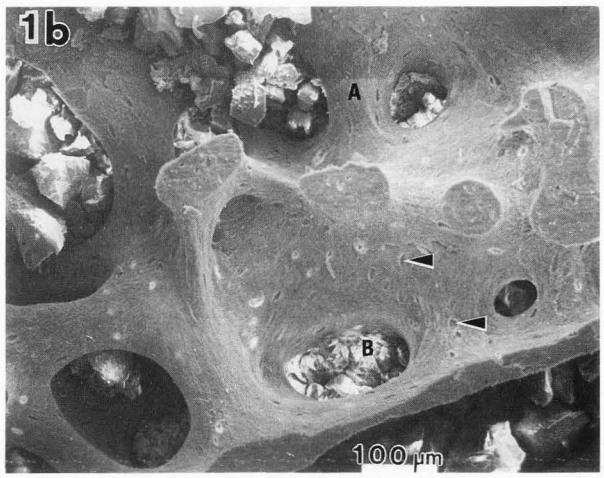
Figure 2. A dinosaur fossil treated with 15% EDTA for 48 hours. An osteocyte lacuna (arrowhead) is recognizable, but the structure has been eroded.

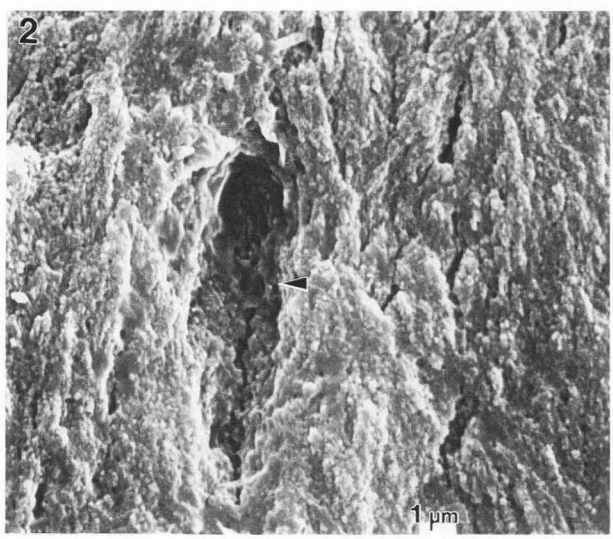
Figure 3. A dinosaur fossil treated with sodium citrate-formic acid solution. Osteocyte lacunae (arrowhead) are recognizable, but the bone has been damaged by the etching treatment.

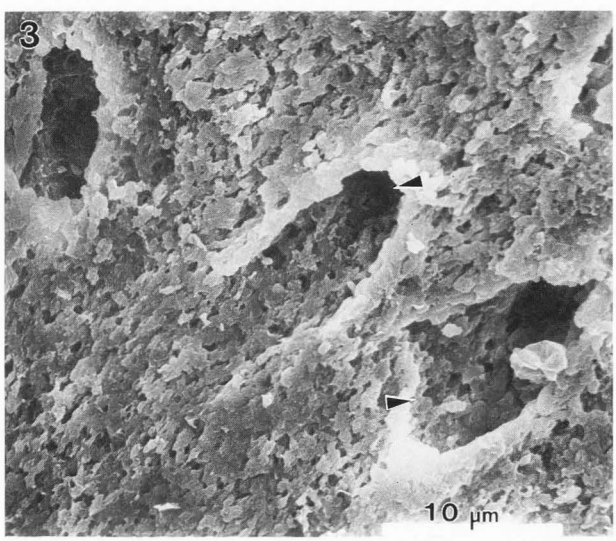
Figure 4. A fossil specimen treated with 10% acetic acid. Osteocyte lacunae (A) and canaliculi (arrowhead) are preserved.

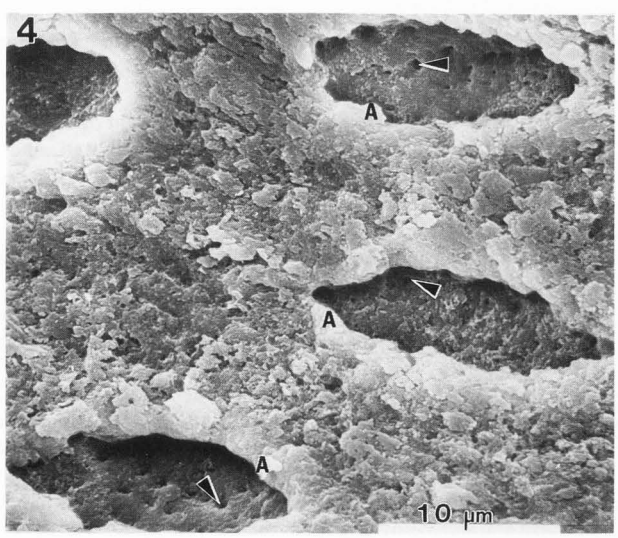
Figure 5. A specimen of modern chick bone. Note the osteocyte lacuna (A) and canaliculi (arrowhead) for comparison with the dinosaur bone.

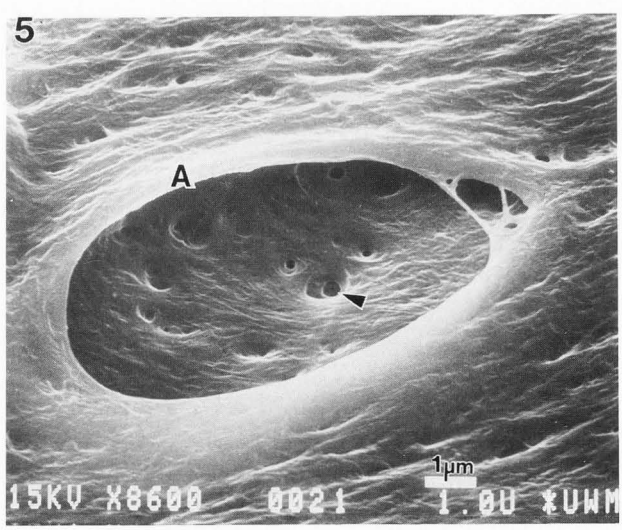

1a
100 µm


1b
A
B
100 µm


2
1 µm


3
10 µm


4
A
A
A
10 µm


5
A
1µm
15KV X8600 0021 1.0U *UWM

materials are removed and replaced by minerals which precipitate from the ground water. The particular sedimentary regime of the Two Medicine Formation afforded a fossilization history that preserved fragile juvenile dinosaur bones and furthermore, preserved their biological ultrastructure. In untreated fossils, however, calcite crystals precluded viewing of the underlying fossilized bone.

Fossilization processes occur variously from formation to formation and even from facies to facies. In addition, a fossil being prepared for microscopic analysis may need to be prepared differently than a gross sample. The sedimentology of a particular geologic formation is important in choosing a technique for preparing fossils for histological examination. In some cases, thin sections need no further treatment (Currey, 1962). The problem encountered with the fossil dinosaur bones from the Two Medicine Formation was that the sedimentary matrix of calcite, which cements the clasts, is present also in the fossil bone interstices. All of the various treatments described above remove calcite; however, treatment with the 10% acetic acid solution etched the samples expediently and without conversion or detectable alteration of fossilized bone. Although the use of acetic acid is not a new technique in paleontological preparation, we now know that it can render samples which are suitable for histological evaluation.

The study of fossil bone histology allows paleontologists to make inferences on the physiology of extinct animals (Bakker, 1972; Bouvier, 1977; Reid, 1984, 1987; Ricqles, 1974, 1980). The ability to study well preserved fossil material with the resolution afforded by SEM broadens the scope of paleontological investigations. For vertebrate animals, bones and teeth are most often preserved in the fossil record. Bone is a connective tissue with diverse biological functions. It can therefore be used as an indicator of metabolism, pathology, nutrition, locomotion and various other life processes. Evaluation of such processes from morphology lends insight into the biology of prehistoric animals. Interpretations on a cellular level define a new degree of understanding of past life. Comparison of the prepared fossilized bone with two week-old chick bone to demonstrates the homology of histological structures observed (Fig. 5). In ongoing studies, we are conducting a comparative histological analysis of adolescent maiasaur and chick long bones.

Acknowledgements

This study was supported by a grant from the Windway Foundation, Sheboygan, WI and by the University of Wisconsin-Madison, Madison, WI. The specimens were viewed at the SEM facility in the University of Wisconsin-Madison, Dept. Engineering and Materials Science.

References

Bakker RT (1972) Anatomical and ecological evidence of endothermy in dinosaurs. Nature **238**: 81-85.

Baumgartner JC, Carson LM (1987) A scanning electron microscopic evaluation of four root canal irrigation regimes. J. Endodontics **13**: 147-157.

Bouvier M (1977) Dinosaur haversian bone and endothermy. Evolution **31**: 449-450.

Boyde A (1972) Scanning electron microscopic studies of bone. In: The Biochemistry and Physiology of Bone. Bourne GH (ed.), Academic Press, pp. 259-309.

Currey JD (1962) The histology of the bone of a prosauropod dinosaur. Paleontology **5**: 238-246.

Dillaman RM, Wilbur KM, Crenshaw MA (1979) Growth of embryonic chick tibiae *in ovo* and *in vitro*: a scanning electron microscopic study. Calcif. Tiss. Intl. **27**: 33-40.

Hirsch KF, Quinn B (1990) Eggs and eggshell fragments from the upper Cretaceous Two Medicine Formation of Montana. J. Vert. Paleo. **10**: 491-511.

Horner JR (1984) The nesting behavior of dinosaurs. Scientific American **250**: 130-137.

Horner JR, Makela R (1979) Nest of juveniles provides evidence of family structure among dinosaurs. Nature **282**: 296-298.

Reid REH (1984) Primary bone and dinosaurian physiology. Geol.Mag. **121**: 589-598.

Reid REH (1987) Bone and dinosaurian "endothermy". Modern Geol. **11**: 133-154.

Ricqles AJ de (1974) Evolution of endothermy: Histological evidence. Evol. Theory **1**: 51-80.

Ricqles AJ de (1976) On bone histology of fossil and living reptiles, with comments on its functional and evolutionary significance. In: Morphology and Biology of Reptiles. Bellairs A, Cox CB (eds.). Linn. Soc., London. pp. 23-150.

Ricqles AJ de (1980) Tissue structures of dinosaur bone. In: Am. Assoc. Advancement of Science Symposium 28. Thomas RDK, Olson EC (eds.). Washington, DC. pp. 103-139.

Discussion with Reviewers

S.H. Ashrafi: The technical tips used by the authors are well known and used to decalcify mineralized tissues!
Reviewer III: The paper describes a method of acid preparation that is not new. The result that acetic acid is to be preferred over other acids is general knowledge!
Authors: The objective of our project was not to demineralize the fossil bone, but rather to differentially etch the minerals deposited by ground water from between the bone structures of interest. At the same time, we wanted the fossil bone to remain unaltered to allow histological evaluation. We do not claim that the use of any of these etching agents is new. We are aware that fossils have been prepared with acetic acid before. What we did not know at the outset was, if we could differentially etch the calcite and leave the fossilized bone histology in tact. To date, we have determined through microprobe analysis that the fossil bone has a Ca:P ratio similar to that of modern bone (and assumedly is the original hydroxyapatite). However, for fossils recovered from other sedimentary regimes, mineral content of the fossil and that of the sedimentary matrix must be evaluated, and in some cases it may not be possible to differentially etch specimens for histological study.
J. Wakely: The similarity of ancient and modern bone is interesting. Could it throw light on the evolutionary relationship between dinosaurs and birds?
Authors: There are two objectives to our research on the comparative histology of dinosaur and modern mammal, reptile and bird bone: 1) to identify shared derived characters in bone that might be applied to phylogenetic analysis; and 2) to use histological characters to make inferences concerning the mode of long bone growth in dinosaurs. To date, we are able to demonstrate shared derived characters in the growth plate of juvenile birds and dinosaurs (in press), which we feel are indicative of common ancestry.