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## Microscopic, chemical and molecular methods for examining fossil preservation

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### Abstract

Advances in technology over the past two decades have resulted in unprecedented access to data from biological specimens. These data have expanded our understanding of physical characteristics, physiological, cellular and subcellular processes, and evolutionary relationships at the molecular level and beyond. Paleontological and archaeological sciences have recently begun to apply these technologies to fossil and subfossil representatives of extinct organisms. Data derived from multidisciplinary, non-traditional techniques can be difficult to decipher, and without a basic understanding of the type of information provided by these methods, their usefulness for fossil studies may be overlooked. This review describes some of these powerful new analytical tools, the data that may be accessible through their use, advantages and limitations, and how they can be applied to fossil material to elucidate characteristics of extinct organisms and their paleoecological environments. **To cite this article:** *M.H. Schweitzer et al., C. R. Palevol 7 (2008)*. © 2008 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

### Résumé

**Méthodes d'études microscopiques, chimiques et moléculaires pour l'examen de la conservation des fossiles.** Des avancées technologiques intervenues au cours des deux dernières décades ont permis d'accéder à des données sans précédent sur des échantillons biologiques. Ces données ont élargi notre compréhension des caractéristiques physiques, des processus physiologiques cellulaires et subcellulaires, ainsi que des relations évolutives au niveau de la molécule et au-delà. Les sciences paléontologiques et archéologiques se sont récemment orientées vers l'application de ces technologies à des représentants fossiles ou subfossiles d'organismes disparus. Les bénéfices de ces techniques, qui sont du domaine de multiples disciplines, sont difficiles à déchiffrer et, sans une connaissance de base du type d'information fourni par ces méthodes, leur utilité pour l'étude des fossiles peut ne pas être bien mesurée. Cet article décrit certains de ces nouveaux outils analytiques puissants, les données qu'ils permettent d'obtenir, leurs avantages et leurs limites, et comment ils peuvent être appliqués au matériel fossile pour élucider les caractéristiques d'organismes disparus et leurs environnements paléoécologiques. **Pour citer cet article :** *M.H. Schweitzer et al., C. R. Palevol 7 (2008)*. © 2008 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

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## 1. Introduction

The geological record holds the only evidence of life that once existed but is now extinct. This record may consist of fossilized remnants of microbial life (e.g., [25,26,128,155,156,171]), chemical signals such as isotope shifts [11,19,44,104,106], tracks and trails made by both invertebrates [31] and vertebrates [56,92] and physical remnants of eukaryotic organisms, both from plants (e.g., [98]) and from animals (e.g., [118]). Extinct vertebrates, the focus of this paper, are best represented in the geological record by mineralized remains of skeletal elements, although in rare instances soft tissues may be preserved [1,24,32,33,80,94–96,130,137,138,170,174]. In cases of exceptional preservation, fragment molecules may be retained in mineralized matrices of vertebrate remains [2,3,39,40,101,132–135]. Therefore, a combination of morphological and chemical or molecular data recovered from fossils have the potential to contribute to a greater understanding of both extinct organisms and evolutionary and/or geological processes acting upon them.

The recovery of DNA sequences from fossil tissues has the greatest potential for elucidating evolutionary relationships, and hence, has been the focus of many molecular efforts with fossils and subfossil material. But it has been hypothesized that DNA analyses are useless for extremely ancient specimens (i.e. greater than  $\sim 1$  Ma; [71,91,172]). Additionally, ancient DNA studies are subject to contamination and artifact (e.g., [64,68,108,114]). Because of contamination issues and extremely low preservation potential, recovery of DNA from very old specimens is fraught with controversy ([108,114] and references therein, [64]). However, significant chemical and/or molecular information may be retrievable from fossil material from sources other than DNA.

Analytical techniques that are both informative and sensitive can be applied to fossil tissues to provide independent tests of morphologically based phylogenies and evolutionary histories, as well as insight into physiologies, paleoecology and habitats, and chemical environments to which the fossil has been exposed. Thus, analytical approaches to fossil specimens contribute to our understanding of the evolution of life on this planet.

It is highly unlikely that a fossil will preserve DNA without also preserving evidence of other organic components such as proteins [116]. Because of the controversies surround recovery of ancient DNA, authenticity should be independently supported by the identification of other remnants, and more durable molecules. For example, supporting evidence for ancient

DNA may include enzymatic degradation of DNA from tissue extracts, positive anti-DNA antibody binding, immunohistochemical localization of DNA to structures such as osteocyte lacunae within bone tissues, or identification of other biomolecular fragments that are known to be resistant to degradation. It has been hypothesized that one way to do this is to tie DNA degradation to the degree of racemization of amino acids [9,115,116]. However, the conditions that both preserve and degrade proteins may be different than those involved in the preservation of DNA. Indeed, protein fragments have been identified in tissues where no endogenous DNA can be amplified [115]. Therefore, this relationship needs to be evaluated further.

The methods described in this paper not only provide means of independently verifying the authenticity of ancient DNA (aDNA) sequences, but may also provide valuable information not obtainable from DNA analyses alone, and may be effective in retrieving molecular information from fossils that do not preserve DNA. Because methods for aDNA analyses have been addressed in depth elsewhere (e.g., [108,172] and references therein; see also papers by Bollongino, Geigl and Hofreiter in the present issue), we do not address these here, except to mention that some of the methods we describe are as applicable to DNA as to other molecular remains. This paper reviews complementary and supportive methods for molecular and chemical analyses of fossils.

This is not meant to be a complete description of all methods available for the study of fossil tissues. There are many techniques and combinations of techniques that can be applied to analyses of fossil material that are not considered in this discussion. Likewise, comparison of cladistic methodologies and tree building algorithms based upon sequence data are beyond the scope of this paper.

## 2. Fossilization

Scientists have been studying fossilized remains for centuries, but processes acting to preserve these remains are still unresolved. Generally, it is hypothesized that when organic remains are buried, minerals in overlying sediments are solubilized and then redeposited in pore spaces within the fossil as the saturated water moves through them (e.g., [142]), while at the same time removing any endogenous organic constituents.

With new technologies developed over the last few decades, it is recognized that ‘fossilization’ may actually occur through multiple means (e.g., [23,24,127,171]), and regions of a single bone can vary greatly in preservation [59,67]. Some regions may be only minimally

altered, while others, separated by only millimeters, may show more extreme diagenetic alteration. The degree to which a fossil has been altered through diagenesis is linked to molecular preservation, and as such, histological index (microscopic integrity) is viewed as a proxy for possible molecular preservation [67]. The less alteration that has occurred at the gross, microscopic and elemental levels from the living state, the more likely it is that fragments of original biomolecules may be retained [63,66].

### 3. Molecular preservation

Molecular preservation in geologically ancient fossils is thought to be extremely unlikely, or impossible. Controversy surrounds claims for recovery of DNA from Cretaceous-aged fossils, and most are viewed with considerable skepticism [5,64,68,91,114]. These arguments are based upon theoretical kinetics and bench-top experiments that use extremes of heat and/or pH to induce degradative changes as a proxy for geological time. These extreme conditions do not exist in nature [39,91] and may be inappropriate models for molecular diagenesis or longevity estimates. Indeed, several studies have shown that absolute age of a fossil is not the primary determinant of preservation of molecular signal [63,74,162]. Resolution of this issue may be best addressed through the study of exceptionally preserved fossils in which degradation has been arrested early in diagenesis.

### 4. Diagenesis

In order to detect and decode molecular information remaining within fossilized vertebrate tissues, it is important to understand taphonomic and diagenetic processes operating at the molecular level.

Taphonomy describes alterations from the living state that occur at any point between death and discovery to organism remains [63]. Microbial decay, biogeochemical interactions, and autolytic breakdown of cell and tissue components are just a few of the processes contributing to taphonomic loss. Intra- and/or intermolecular bond disruption, denaturation of 3D molecular structure, crosslinking of proteinaceous fragments with soil organic components resulting in formation of melanoidins or humic acids [120], deamidation, depurination, racemization of amino acids or methylation of organic compounds are examples of diagenetic alteration at the molecular level. The removal of endogenous components (e.g., proteins, DNA, lipids, carbohydrates), addition of exogenous organics from invasive microbes, addition of

minerals and/or trace elements from the surrounding environment, or contamination by adsorption of exogenous DNA to vertebrate biomineralized tissues at any point in burial history would also be included in diagenesis.

In the vast majority of cases, taphonomic factors combine to completely degrade tissues until no trace of the organism remains. The presence of fossil remains indicates that at least some of these processes were arrested before complete degradation occurred. In cases of truly exceptional preservation, including preservation of original articulation of skeletal elements, preservation of original, unaltered mineral composition and orientation, labile soft tissues, or cellular structure, indicate that diagenetic processes were halted very early, and/or that mineralization outpaced degradation to preserve components normally lost.

### 5. Retrieving data from fossils

The depth and types of analyses conducted upon fossil bone depend to a large degree upon study goals. Gross observations such as density determination, degree of deformation, and presence or absence of exogenous elements or mineral may be sufficient if morphological description, biomechanics or issues of specimen transport or reburial are the focus of the study. Information about the physiology of the organism, chemical processes that have acted upon the bone, depositional environments, paleoecology, or alterations to the molecular make-up of the specimen require more detailed analyses.

Fossils can be analyzed on at least three levels, and techniques should be chosen according to the type of information sought. Physical characteristics of the specimen, including gross (e.g., deformation, pre- or postmortem fracture) and microscopic (dissolution, recrystallization, permineralization) observations can be contrasted with comparable modern specimens to estimate type and amount of post mortem processes and diagenetic alteration, thus shedding light on burial environments and chemical change through time.

Chemical and/or molecular analyses identify alterations occurring in fossils at higher resolution. They can also be employed to identify the types of molecules or molecular fragments present in fossil material, to elucidate depositional/chemical environments most likely to preserve endogenous molecules, or to show whether retained organic material is original, or if it has accrued in fossil matrices through diagenesis. Observed chemical alterations, such as bond breakage, exogenous ligands, and functional group modifications can

also shed light upon mechanisms of molecular diagenesis. In addition, DNA or protein sequence data derived from fossil specimens may provide independent tests of evolutionary relationships proposed by morphological studies of extinct organisms. Rates and types of evolutionary change in DNA and/or protein constituents over the course of evolutionary history could be elucidated by molecular comparisons, and molecular clocks could be more accurately calibrated.

In this review, we discuss various methods useful for determining the state of preservation of fossil bone at these three levels. This is a general overview of selected analytical procedures that may be applied to studies of fossil tissues, adapted from those commonly applied in analytical chemistry, molecular biology, immunology, and other fields. Analyses of specific proteins will not be discussed, nor will specific protocols; rather we describe the methods and the types of questions each method can address.

### 5.1. Chemical nature of vertebrate material

In order to understand and characterize diagenetic change in vertebrate remains, a thorough understanding of the nature, characteristics, components and processes involved in the biogenesis of original tissue is required. Without knowledge of the chemistry of original components present in bone or teeth, it is impossible to quantify changes from that state.

The structure, formation and composition of vertebrate mineralized tissues are thoroughly discussed in the literature (e.g. [54,169]) and in-depth treatment of this subject need not be repeated here. However, in brief, bones (and other biomineralized elements such as teeth) are a composite material, consisting of an intimate interaction between mineral and organic components. Mineralized tissues are formed by differentiated cells (e.g. osteoblasts, odontoblasts) that secrete an organic matrix consisting primarily (~90%) of collagen protein, with some other non-collagenous proteins such as osteocalcin, osteonectin, and serum proteins such as albumin [65,124,176]). Understanding the molecular characteristics of extant bone yields a starting point for identification of similar compounds in fossil bone.

## 6. Collagen I

Collagen type I is the dominant protein making up the organic phase of bone. This fibrillar structural protein has a strong affinity for the bone mineral hydroxyapatite. Type I collagen consists of three helical peptide chains

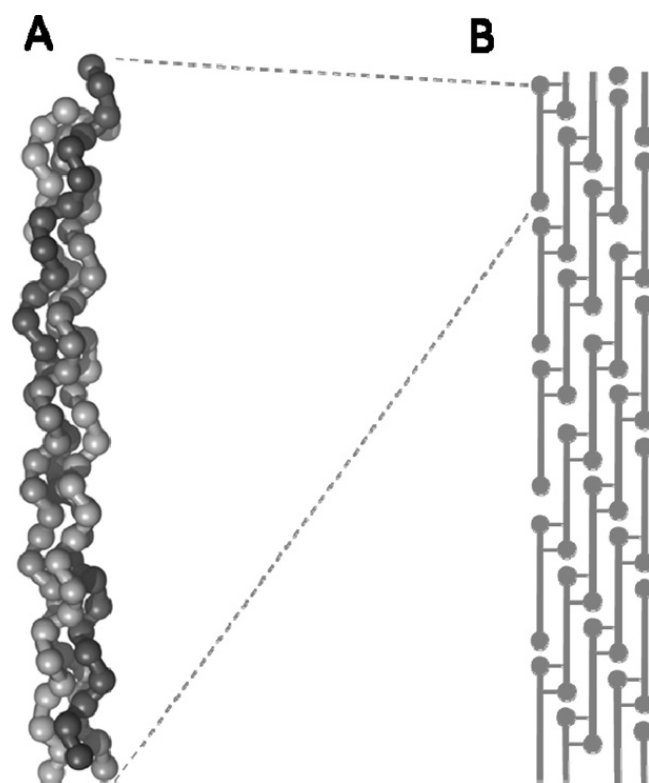


Fig. 1. Schematic view of a single collagen molecule (A) and a collagen fibril (B), showing the arrangement of overlapping molecules and crosslinks.

Fig. 1. Vue schématique d'une molécule unique de collagène (A) et d'une fibrille de collagène (B), montrant l'arrangement de molécules et de réticulations se recouvrant.

that turn about each other at regular intervals (Fig. 1A). To achieve this orientation, the amino acid composition is highly constrained, where every third residue is glycine, the smallest amino acid. Amino acid analyses indicative of collagen will therefore yield a 30% glycine ratio. Collagen also incorporates two uniquely modified amino acids; hydroxy-proline (pro-OH) and hydroxy-lysine (lys-OH). Although highly conserved, the sequence of amino acids making up collagen can be used to determine higher level phylogenetic relationships (e.g., [23,105]).

The tripeptide collagen molecules align with and overlap one another at regular intervals, forming intermolecular crosslinks that make the mature protein relatively insoluble in water (Fig. 1B). This intermolecular overlap gives collagen fibers a diagnostic banded appearance in electron microscopy; but this pattern disappears as the intermolecular cross links are broken during degradation. The collagen fibrils secreted by bone-forming cells are usually laid down in a highly regulated pattern in bone, with the fibrils of each successive layer oriented at right angles to one another. This plywood-like arrangement gives bone great ten-

sile strength and flexibility, and provides resistance to external stresses.

## 7. Bioapatite

Once the primarily collagenous organic matrix is secreted, bioapatite mineral ( $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ ) solubilized in the blood is deposited in the 'gaps' between overlapping collagen molecules (Fig. 1B). The orientation of mineral crystals is dictated by the arrangement of the underlying collagen fibrils [169,170]. In some cases, mineral crystals may form around the protein fibril, creating a closed system [57,125,150,167,168]. The insolubility, helical arrangement, intermolecular crosslinks, and intimate association with mineral make mineralized collagen resistant to degradation, increasing chances of survival during fossilization [57,125,149,160,161].

Both the organic (proteinaceous) and mineral phases of bone and teeth are subject to chemical alteration during the fossilization process, and these diagenetic effects must be accounted for when analyzing fossil material. It is generally accepted that the older the specimen, the more alteration will have occurred.

## 8. Microscopic analyses of fossil vertebrate remains

Because the interaction between the geochemical environment and the organic components of fossils is unpredictable, analyses of bone or teeth should begin with characterization of gross alterations relative to modern counterparts. The less alteration at both the macroscopic and microscopic level, the more likely that some endogenous molecular signal may be preserved [63], although excellent microscopic preservation does not guarantee molecular preservation [67,158].

It stands to reason that if the specimen is articulated, if gross evaluation of the bones does not reveal distortion or crushing, and if density measurements demonstrate that little exogenous mineral has been added, processes of decay and degeneration that normally act on bone have been arrested or retarded to some degree. This can be confirmed at the microscopic level.

### 8.1. Microscopic methods

#### 8.1.1. Light microscopy (LM)

Microscopic preservation (histological integrity) has been strongly correlated with the presence of endogenous molecules in archaeological and fossil bone [63,67], providing a useful first screening to assess the appropriateness of further analytical studies.

To produce ground sections, embedded tissues are sectioned with a diamond encrusted blade (e.g., [36,164,173,175]), and the resultant wafers are mounted to glass slides with epoxy, ground to desired thickness on a series of decreasing grit aluminum oxide papers, then polished to remove fine scratches. Ground sections are visualized with a standard light microscope or a petrographic scope using polarized light. Mineral composition of tissues, infilling, recrystallization or lack thereof can be revealed through standard light microscopy [132]. Polarized light reveals crystal orientation, and can demonstrate alteration from the biologically dictated 'plywood' like orientation. Paleohistology is also used to determine growth rates [37,49,50,73,109], estimates of relative metabolic rates [35,43,121] and biologic age ([50,51,73] and references therein). Microscopic inspection can also address extent of microbial invasion or other alterations to original cellular structure.

#### 8.1.2. Scanning electron microscopy

Scanning electron microscopy (SEM) rasters a beam of tightly focused electrons (diameter of beam  $\sim 1$  nm) across the surface of the specimen for topographical imaging. The specimen (or specimen casts) is usually coated with a conductive substance such as carbon, gold-palladium, or iridium deposited as a fine film (usually only nanometers thick) on the surface of the specimen under vacuum, but newer technologies such as environmental (low vacuum) SEM or field emission SEM (FESEM) do not require coating. Metal coating may yield better images, but carbon coating provides better accuracy for elemental analyses, such as EDX (see below). High vacuum (usually  $10^{-5}$  torr or less) allows the unscattered flow of electrons from the source to the surface of the sample. The high-energy beam generates secondary electrons that are transmitted from the surface at intensities dictated by inherent sample properties to a detector capable of translating electron counts into a three-dimensional topographic image. The shorter wavelength and tighter focusing of electrons increase magnification and resolution by orders of magnitude over images obtained by the most powerful light microscopy, where resolution is limited to about 1.0–0.5 microns. In addition to secondary electrons used in imaging, backscattered electrons and X-rays [58] are also generated, and these can produce additional data, such as the relative distribution of heavy and light elements, elemental identification and mapping, or crystal phase identification.

Specimen chambers for some SEMs have the capacity to study relatively large samples (e.g. teeth, [48]), thus requiring less specimen preparation and

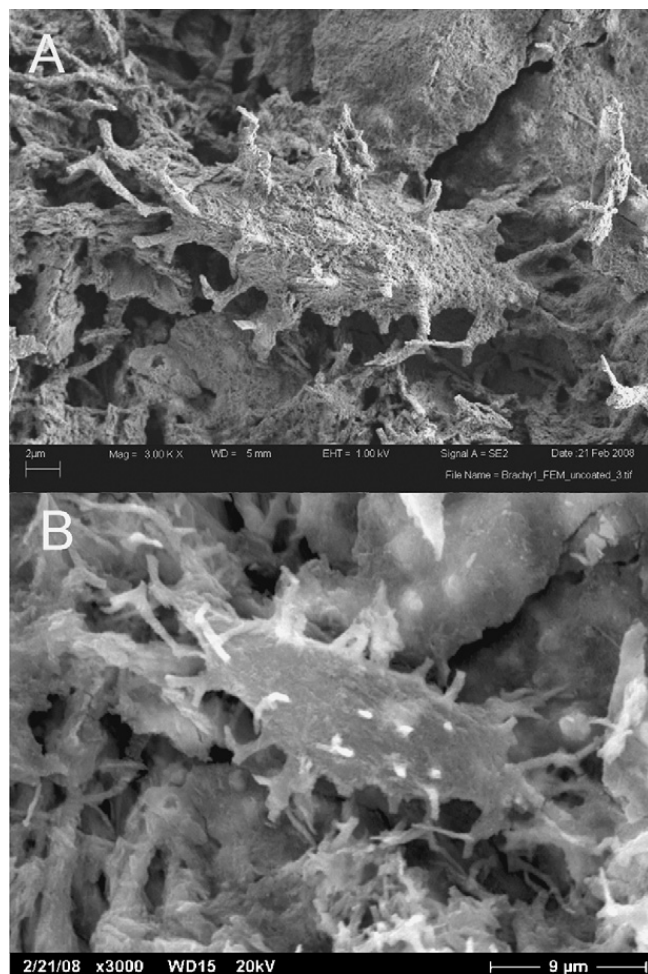


Fig. 2. Comparative SEM images of a dinosaur osteocyte *in situ* on demineralized bone. (A) Image is taken without conductive coating, using a thermal field emission SEM (FESEM) with 1-keV primary electrons. (B) The same sample as in (A) but coated with ~20-nm AuPd film and imaged by a conventional SEM using a lanthanum hexaboride filament with primary-beam energy of ~20 keV. The resolution provided by FESEM is substantially superior to that obtained by the conventional SEM.

Fig. 2. Comparaison d'images (MEB) d'un ostéocyte de dinosaure *in situ* sur un os déminéralisé. (A) Image (MEB) sans recouvrement conducteur, prise sous émission de champ thermique (MEBEC), avec faisceau d'électrons primaires d'énergie 1 keV. (B) Même échantillon que (A), mais recouvert d'un film au Pd d'environ 20 nm, photographié par un MEB standard utilisant un filament d'hexaborure de lanthane avec un faisceau primaire d'énergie 20 keV environ. La résolution fournie par MEBEC est substantiellement supérieure à celle obtenue avec un MEB standard.

less risk of inducing artifact. Low-vacuum methods do not require coating, so specimens can be used in other studies; however resolution may be poorer than with coated samples [15]. Recent advances in thermal field emission SEM (FESEM) technology, coupled with improved electron optics and use of variable pressure environments (e.g., Zeiss Supra55-VP FESEM, Fig. 2), allow imaging and analyses of uncoated samples using

a wider range of primary beam energies (between 0.1 and 30 keV). These newer systems offer ideal platforms for high-resolution imaging of samples sensitive to high energy electron beam damage. Fig. 2 shows a comparison of the same sample (a dinosaur osteocyte) imaged uncoated by FESEM (A) and metal coated and imaged by SEM technologies.

### 8.1.3. Transmission electron microscopy

Fragments of bone or tooth may be treated with a demineralizing agent such as ethylenediaminetetraacetic acid (EDTA) or a dilute solution of hydrochloric or formic acid to remove excess mineral deposit, facilitating thin or ultrathin sectioning for electron microscopy. If no organic matrix remains, complete demineralization will result in total loss of specimen, therefore partial demineralization is recommended. Demineralized or partially demineralized fossil tissue is then embedded in a polymer resin and ultrathin (30–90 nm) sections are taken using a glass or diamond microtome knife [134,135,137,138,140], and collected on special conductive grids for transmission electron microscopy (TEM). To put this in perspective, red visible light has a wavelength of approximately 400 nm, meaning that histological sections are thinner than the wavelength of visible light. Taking sections this thin is difficult for highly mineralized fossil bone without treatment. An alternative to demineralization is ion milling [132,177], where tissues are thinned with a high-energy ion beam (e.g., Ar<sup>+</sup> or Ga<sup>+</sup>) to obtain optimal thickness for TEM. Rather than passing over the surface of a sample, as in SEM, in TEM electrons must pass through the specimen to be detected, requiring very small and extremely thin samples. Extant or unaltered material must be stained with electron dense stains, such as uranyl acetate/lead citrate to visualize fine structures, but this may not be necessary for fossil material. This method is commonly used to detect structural patterns such as the 67–70-nm cross banding commonly seen in collagen fiber bundles [123], ultrastructural features such as interconnecting osteocyte canaliculi [112] or intracellular structures. Fig. 3 shows a dinosaur osteocyte imaged by TEM.

### 8.1.4. Confocal microscopy

Confocal microscopy is similar to standard light and/or fluorescence microscopy, particularly with respect to resolution. However, instead of a standard light source that passes through a thin section of embedded tissues, a laser of a specific excitation wavelength is used as the illumination source. This is useful for localizing immunological probes (to be discussed later) or certain histological stains that bind to molecular components

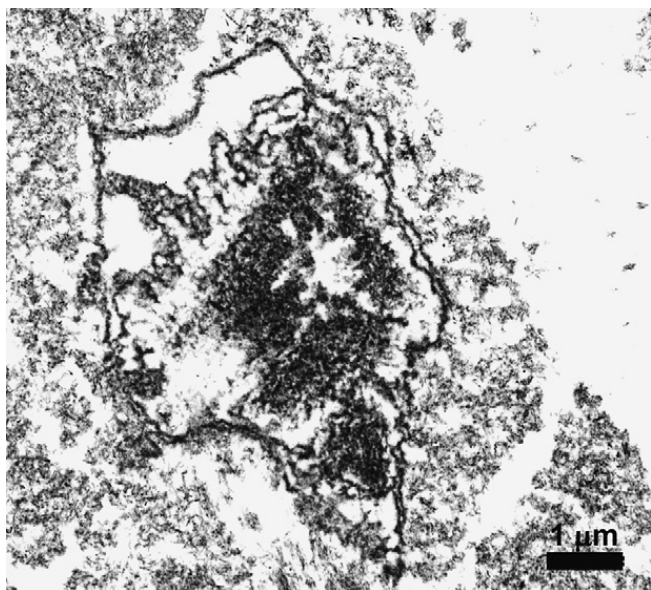


Fig. 3. Transmission electron micrograph of dinosaur osteocyte *in situ* in demineralized bone, sectioned to 70 nm, stained with uranyl acetate/lead citrate and imaged using a LEO 912 AB Omega TEM (G34–651e).

Fig. 3. Micrographie d'un ostéocyte de dinosaure *in situ* sur un os déminéralisé, obtenue au microscope électronique à transmission. Section jusqu'à 70 nm, coloration à l'acétate d'uramyle/citrate de cuivre, photographie utilisant un MET LEO 912 AB Omega (G34-651e).

within a tissue. For example, Hoescht stain (#33258, Sigma) is a fluorochrome designed to intercalate between the bases of double stranded DNA. After timed incubation (~60 min) excess stain is removed with rinsing, and a laser tuned to 480 nm is then used to illuminate the section. If the stain has bound to DNA in the tissues, it will fluoresce, and can be detected at a longer wavelength (approximately 530 nm) using optical filters. The principle is the same when proteins are detected with antibodies labeled with fluorescent markers. Confocal microscopes scan samples multiple times at different focal planes, and signal is integrated using custom software. Integration intensifies the signal, and is useful for fossil specimens where the organic components may be low in concentration and sparsely distributed relative to modern samples. Histochemical staining has been used to demonstrate the presence of DNA in occasional osteocyte lacunae in dinosaur tissues [132]. However, it does not discriminate between endogenous DNA and that arising from a contaminant source.

## 9. Chemical analysis of fossils

### 9.1. Stable isotope analyses

The isotopic composition of the mineral incorporated into bone during the life of an organism can reflect the

ecology [81,151], diet [83,106] and possibly, metabolic rates [10–12] of the organisms. Isotopes of oxygen and carbon incorporated into bones and teeth during formation and remodeling are affected by the chemistry and temperature of pore waters and diet of the organism. Thus, isotopic analysis holds potential for shedding light on many aspects of the biology of extinct organisms. However, possible modification of these values over time from the living state [18,87] requires a detailed understanding of the effect of the burial environment on the original isotopic composition of bioapatite before these data can be accepted as reflecting *in vivo* values.

Postmortem diagenesis results in a range of chemical and mineral changes to the organic and inorganic constituents of fossil bone and teeth. These processes cause trace-element enrichment, dissolution, precipitation, and recrystallization of the original crystal structure of bioapatite, resulting in modifications to fossil bones and teeth from different burial sites and ages [110,154,174]. The resolution and precision of analytical methods for documenting these primary mechanisms of diagenesis in fossil bone have improved since the 1960s and early 1970s with systematic studies of chemical and physical changes of fossilization [16,20,34,47,59,69,76,84,85,90,111,113,159]. It is well established that different diagenetic factors act in different burial environments, even if it is difficult to link a specific process to a specific diagenetic change [126]. A well-preserved 'pristine' fossil with excellent texture and micron-scale preservation of primary structures does *not* guarantee the preservation of original isotopic values [87,93], nor, for that matter, preservation of original molecular signature [157,158].

The widely used proxy for evaluating the robustness of isotopic values derived from fossil bioapatite is more difficult if a modern analog is unknown, or if diet and environmental conditions are not well documented, or have changed through time. This presents a problem when assessing preservation of original carbon isotopic values using the pre-Quaternary fossil record or in deep time before the evolution and global spread in the Miocene (7–10 Ma) of C<sub>4</sub> grasses. To further complicate matters, dietary components that are isotopically distinct may be obscured by secondary fractionation and variable carbon isotope turnover rates in paleoecological studies [152,168].

Oxygen isotope variation within apatite carbonate is more difficult to predict than carbon isotopes derived from the same mineral [21]. The  $\delta^{18}\text{O}$  of biogenic apatite is strongly correlated with that of body water in extant mammals. A linear correlation between  $\delta^{18}\text{O}$  of bone phosphate and  $\delta^{18}\text{O}$  of bone carbonate from the same

bioapatite mineral would support the preservation of original isotope values in extant mammals in most cases [77a].

Plugging calculated  $\delta^{18}\text{O}$  values into the respective temperature equations for the same phase carbonate and phosphate is also used to evaluate isotopic values from fossils [141]. The primary basis for evaluating oxygen stable isotope composition in fossil bones and teeth relies on the comparison of  $\delta^{18}\text{O}$  values between bone, tooth enamel, and dentine. Ranges of these values are compared with known extant systems to test whether fossil isotope values are well supported [7]. Wang and Cerling [166] concluded that  $\delta^{18}\text{O}$  values of structural bone carbonate are vulnerable to diagenesis and unlikely to record original isotopic signature, based on comparison of Tertiary fossil teeth, bone, and sediments from Badlands National Park, South Dakota. Furthermore, efforts to correlate microscopic integrity of dinosaur bone with pristine geochemical integrity [87] were not supported, but instead demonstrated the importance of burial environments in identifying diagenetic alteration. Kolodny et al. [87] calculated  $\delta^{18}\text{O}$  from bioapatite in Cretaceous teleost fish, dinosaurs and reptiles from the same localities across a latitudinal gradient (Alaska, Wyoming, and Texas) and found values determined more by locality, than by expected physiological or life history signal, suggesting environmental overprinting of original endogenous values. No systematic isotopic differences between these diverse taxa were preserved. The similarity of  $\delta^{18}\text{O}$  phosphate in modern fish, turtle, and crocodylians in their data set did not support the preservation of original isotopic composition of drinking water or environmental waters they lived in, but rather indicated isotopic equilibration occurred between fossils and groundwater from the burial environment. They concluded that differences in dinosaur  $\delta^{18}\text{O}$  phosphate composition did not reflect in vivo body temperature variability when the bones in the skeleton were formed, but more likely the chemistry of the burial environment (contra [10–14,55]). Chenery et al. [30] argued that early diagenetic recrystallization resulted in overprinting the  $\delta^{18}\text{O}$  signature of hadrosaurid dinosaurs buried in Late Cretaceous marine sediments in Dinosaur Provincial Park, Alberta. And, while Trueman et al. [160] reached the same conclusions from bones buried in terrestrial and marine sediments from Campanian Bearpaw and Dinosaur Park Formations, Thomas and Carlson [151] concluded that, while absolute oxygen isotopic values may be altered, a seasonal signal may be preserved in their ontogenetic series of the hadrosaurid, *Edmontosaurus*.

Preservation of original isotope values in fossil bioapatite may also be affected by the enzymatic microbial

degradation of phosphate. Microorganisms (e.g., bacteria, algae), through enzyme mediated nutrient cycling of organic constituents of skeletons may be responsible for anomalous phosphate oxygen isotope compositions and trace element concentrations in fossil biogenic apatite [16,61]. Through biogeochemical cycling of phosphate in aquatic environments, sediments, and soil, isotopic fractionation may occur during early stages of diagenesis. Complete re-equilibration of oxygen isotope values or re-setting phosphate oxygen composition from this secondary organic phosphate source may be responsible for inconsistent oxygen isotope values in fossil bioapatite [16,87].

Biogeochemists who use fossil bone and tooth bioapatite as source material for stable isotopes have utilized a variety of methods to determine the effects of burial environments on preservation of in vivo isotope values. This is necessary if these data are used to support hypotheses on climate change, animal and plant migration, evolution, and life histories of extinct animals. For the most part, divergence of fossil isotope values from modern counterparts is attributed to diagenesis, while similarities between the two are evidence of diagenetic resistance [86]. In any case, these techniques alone are insufficient to demonstrate preservation of original isotope values. This has resulted in conflicting conclusions in determining if isotopic values are original or altered in prehistoric and fossil hydroxylapatite.

Even though  $\delta^{13}\text{C}$  values in modern mammals are a result of diet only [84], both  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  isotopic compositions in fossils are potentially exposed to postmortem diagenetic overprinting in the burial environment [89]. Deviations from predicted isotopic values may reflect either diagenesis or changes in diet and physiological adaptations [83]. Despite the variety and complexity of factors acting on a bone or tooth when it leaves the biosphere and enters the geosphere [111], many paleobiological and paleoclimatological isotope studies still do not consider the potential modifying effects of diagenesis from the burial environment on the original isotopic composition of their sample source materials when faced with anomalous results [93].

The above concerns dictate that pretreatment of modern and fossil bone and tooth bioapatite is absolutely necessary to remove exogenous organic matter and diagenetic minerals. Standard protocols recommend the pretreatment of powdered samples with a 2–3% reagent grade sodium hypochlorite ( $\text{NaOCl}$ ) to remove organics (e.g., [82]). Approximately 1 ml of solution is required for each 25 mg of apatite (0.04 ml/mg). Samples are centrifuged and rinsed five times in distilled or megapure water, then soaked in 1 N calcium acetate

buffered acetic acid overnight. Alternate rinsing and centrifugation cycles are followed by lyophilization, or oven drying prior to stable isotopic analysis by mass spectrometry.

Using fossil apatite to infer aspects of paleoecology or paleophysiology when derived from specimens across a broad geological time frame, without a stable support for the preservation of its original isotope values remains a much debated issue in biogeochemistry [18,85,119,141,160], particularly because the mechanisms of alteration of carbon isotope signals derived from fossil bone carbonate have not been fully elucidated [89].

### 9.2. Other chemical analyses of fossil material

Several methods may be applied to analyze specimens for chemical/molecular content. These will be divided into those that require minimal sample preparation (i.e., bulk sample analyses), and those that require some form of pretreatment.

## 10. Bulk analyses

Elemental composition of fossil specimens can demonstrate diagenetic alteration by identifying exogenous minerals incorporated into spaces in bone matrices, and this can be accomplished using different techniques. Lower resolution methods include Energy Dispersive X-ray (EDX or EDS) and electron microprobe, which are similar in outcome. EDX is combined with SEM analyses in some electron microscopes. As with imaging, EDX coupled to a standard SEM requires sample coating with a conducting material, but some environmental SEMs, including FESEM, can obtain elemental analyses without coating. Choice of coating material is dependent upon the goal of the study. For imaging and elemental analyses combined, metal coating is preferred, but for more accurate elemental analyses, carbon coating introduces less risk of masking minute amounts of lighter elements retained in study material. Electron bombardment from the primary beam causes surface emission of characteristic high energy X-rays that allow unique identification of component elements. EDX delivers high accuracy for detection of heavier elements (atomic mass > 11), and is adequate for estimating proportions of lower masses as well, although difficulty in accurately detecting and quantifying low energy X-rays produced by low mass elements introduces more uncertainty in these values [58]. Elemental ratios (both mass percentages and elemental percentages) can be calculated based upon areas under the peaks, and allow identification of specific minerals, but some leeway in

interpretation must be made due to the lack of internal standards. Because carbon and oxygen are ubiquitous as well as falling within the range of uncertainty by these detection methods, there is a risk that their calculated numerical values may be artificially high [132].

EDX can also map element concentrations and distribution in fossil material. When an electron beam is rastered across the sample, subsequent detection of elemental X-rays emitted from each region [58,131] results in a visual image of element distribution. Several elements can be mapped in composite images.

While EDX can detect carbon-containing compounds, it is insufficient to identify either molecular composition or source of those compounds. It does not detect nitrogen with certainty, as N falls between peaks of oxygen and carbon, and is not usually resolvable; therefore, this method is not appropriate for identifying biomolecules that may be present. Both the sensitivity and the accuracy of quantitative analyses increase substantially if an appropriate crystal is used for resolving X-rays, rather than a solid-state detector.

### 10.1. Electron diffraction pattern analysis (EDPA)

This method is coupled to TEM systems, and transmits a very high energy electron beam (200 kV) through the sample. The beam is diffracted at angles determined by Bragg's law, applied to a set of reflecting crystal planes separated by the d-spacings of the crystals. In extant, or relatively unaltered fossil bone, the resultant diffraction pattern occurs as partial rings [140]. In replaced or recrystallized bone, the pattern is diffuse or geometric, reflecting the arrangement of crystals when no pattern is imposed from collagen fibrils [177].

Diffraction patterns can allow identification of *in situ* mineral phases with high accuracy, and can differentiate between bioapatite and other forms by D-ring spacing [140], or between different forms of iron-bearing minerals. It is useful in quantifying degree of diagenetic alteration in fossil samples, or in identifying remnants of original biominerals.

### 10.2. Pyrolysis gas chromatography–mass spectrometry (PY-GC-MS)

Like other methods of mass spectrometry, PY-GC-MS capitalizes upon the fact that every molecule consists of a unique combination of atoms that can be identified with high resolution by their atomic masses [77b]. This method has been used to identify the remnants of original molecular components, including protein frag-

ments, lignins and chitin, of fossils dating back to 25MY BP [144–147,163], and has been applied to systematic differentiation of plant materials [145].

Py-GC-Mass spectroscopy uses intense heat ( $\sim 600^\circ\text{C}$ ) to split and volatilize molecular components, which are then separated according to fragment mass by gas chromatography. Each separated peak separated is analyzed by a quadrupole mass spectrometer to identify the number and mass of each volatilized fragment, producing a spectrogram. Because this is a bulk sample analysis, it is not possible to localize the source of these molecular components within a heterogeneous sample. However, because this method does not require chemical extraction, it reduces the chances of inducing artifact into the analyses. It is not ideal for identification of proteins and DNA, but is highly sensitive to other biomolecular fragments.

### 10.3. Time-of-Flight secondary ion mass spectrometry (TOF-SIMS)

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) rasters a high-energy (e.g., gallium ions, typical energy: 15 keV) ionizing beam with a pulse width of  $\sim 1$  ns and a repetition rate of 10 kHz over a predetermined area to release molecular fragments from a sample surface. Only a small fraction ( $10^{-6}$  to  $10^{-1}$ ) of the total released fragments are measured, because only charged particles (ions) are detectable [165]. Mass of each fragment is then calculated based upon the time it takes for the molecule to move from the source to the detector. This ultra high-resolution detector is capable of differentiating fragments that differ in mass by as little as 0.001 atomic mass unit (amu; 12 amu is, by definition, the mass of a carbon atom). Mass resolution (mass/mass uncertainty,  $m/\Delta m$ ) in practice varies from 2000–5000, and is affected by primary pulse width, sample type and surface flatness, and charge compensation. In addition, whether or not detectable secondary ions are generated is highly dependent on characteristics of the sample; therefore, peak intensities (i.e., peak areas) may differ between samples [77b], but mass resolution is not affected. Other mass spectrometry methods use spectral patterns in addition to absolute mass values to identify fragments, but in ToF-SIMS, the spectral patterns will vary because of inherent differences in the surface composition. For example, fresh bone may yield a spectrum with a pattern that differs from fossil bone, even though they may contain some of the same molecules. However, absolute masses (or peak location) will be the same, regardless of surface, if the source fragments are the same.

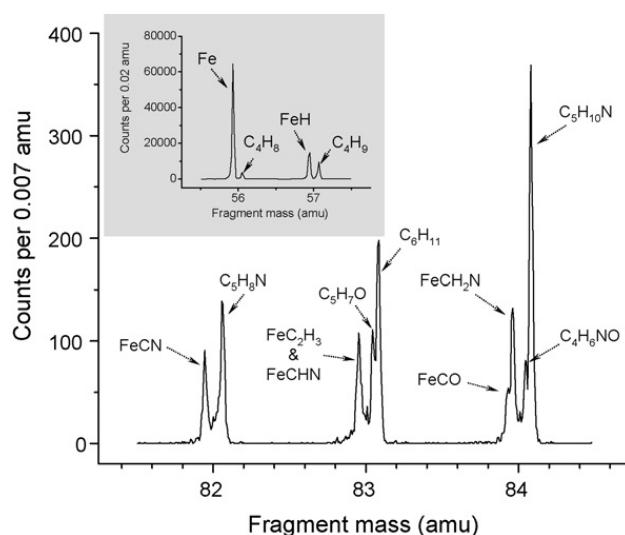


Fig. 4. High-resolution positive-ion ToF-SIMS spectra obtained from demineralized dinosaur vessels. Inset amplifies the mass region around 56–57 amu, showing the identification of iron in the vessel wall, while the main spectra shows the expanded region around 82–84 amu. This allows resolution of organometallics fragments and demonstrates the unique association/complexation of Fe with organic-derived C, N, and H, as well as purely organic fragments containing C, N, and H. Imaging capability of ToF-SIMS demonstrates conclusively that these fragments are associated with the vessels but not with the background (a polished and cleaned Ge disk on which the vessels were mounted). Fig. 4. Spectres haute résolution à ions positifs TOF-SIMS, obtenus sur des vaisseaux déminéralisés de dinosaures. L'encart agrandit la zone autour de 56–57 amu, montrant du fer identifié dans la paroi des vaisseaux, tandis que le spectre principal montre la zone agrandie aux alentours de 82–84 amu. Ceci permet la résolution de fragments organométalliques et démontre l'association/complexation unique de Fe avec C, N et H. Les possibilités d'imagerie offertes par l'appareillage TOF-SIMS démontrent de manière décisive que ces fragments sont associés aux vaisseaux et non au soubassement (disque Ge poli et nettoyé sur lequel les vaisseaux sont montés).

ToF-SIMS provides at least three avenues of information not available by other mass spectroscopy methods. Because only a small fraction (0.1%) of a small area ( $<200 \mu\text{m}^2$ ) of the outermost surfaces is ionized, this method is less destructive, allowing the same sample to be used for other analyses, once mass data is obtained. This can be important with rare, minute, or fragmentary fossil material. Fig. 4 shows a ToF-SIMS profile obtained on dinosaur vessels recovered from demineralized bone. In addition to several peaks that can be associated with amino acids, we have used this method to detect novel compounds involving organics and iron, supporting our hypothesis of a possible chemical pathway for preservation [139,140].

Second, ToF-SIMS measures both positive and negative ion spectra, thereby providing complementary information about fragmentation patterns of sample-derived molecules [2,3,140]. Third, unlike other mass

spectrometric methods, ToF-SIMS allows the localization of molecular sources to specific regions of the sample. Therefore, in fragile specimens where it is desirable to limit handling or sample preparation, the study specimen can be left within the encasing matrix. Comparative mass data can be obtained from both fossil tissues and the surrounding matrix, demonstrating that molecular signal originates with the specimen, and not from exogenous environmental contaminants. Like all mass spectrometric methods, it is imperative that ultraclean methods be used in handling the specimens, and particular to ToF-SIMS, because only surface layers are analyzed, it is important that all samples are handled using aseptic techniques. Additionally, freshly fragmented specimens that expose internal surfaces for analysis, minimize chances of detecting exogenous contaminants. The advantages and disadvantages of other mass spectrometry methods that require chemical extraction of samples will be discussed below.

#### 10.4. X-ray photoelectric spectroscopy (XPS)

X-ray photoelectron spectroscopy focuses monochromatized X-rays on study specimens under ultra high vacuum (UHV), to produce photoelectrons varying in intensity and kinetic energy. Each element within the sample produces characteristic XPS lines, the intensity and position of which determine the concentration and local chemistry or bonding environments of the atom detected. For example, the sulfur 2p line can show as much as 7 eV chemical shift between sulfur in a disulfide environment ( $S^{2+}$ ) and one in a sulfate ( $SO_4$ ) environment ( $S^{6+}$ ). With sub-eV resolution, such shifts can easily be monitored. This method is an excellent complement to both TOF-SIMS and EDX, confirming the presence of elements identified in EDX, and, since it is slightly more sensitive and discrete, identifying light elements not detectable or quantifiable by EDX, or which are masked by other elements, as is nitrogen. XPS can identify the presence of nitrogen unambiguously, as well as identify its bonding environment. Nitrogen is an essential component of both DNA and proteins, and while its identification in fossil samples does not guarantee the presence of these molecular compounds, it is certain that neither DNA nor proteins will be found in fossil specimens if nitrogen is not identified.

Finally, XPS complements both EDX and mass spectrometry (MS) methods, in that the oxidation state of the elements identified by EDX, or molecular fragments identified by MS, can be linked to local bonding environments by XPS [22]. For example, if TOF-SIMS suggests the presence of sulfur-containing amino acids such as

cysteine in a sample, XPS will verify that sulfur is present in the sample, and that it exists in a state consistent with sulfur which has been oxidized either to  $S^{2+}$  for disulfide bonds, or  $S^{4+}$ , consistent with cysteic acids [134]. This method differentiates sulfurs in bonding environments consistent with organics, as opposed to those derived from inorganic sedimentary sulfur.

#### 10.5. Analyses of chemical extracts of fossil material

Many analytical methods besides mass spectrometry require fossil material to be chemically extracted, and several methods can be applied, depending on the downstream analytical technique employed. Organic extractions (i.e., using organic reagents such as benzene) are useful for isolating particular compounds, such as kerogens, lipids and other geochemical components (e.g., [38]). However, for isolation of water-soluble molecular components such as protein fragments, other methods are more appropriate and/or more efficient.

Where possible, all surfaces should be cleaned by abrasion to remove external contaminants. Fossil material is then crushed to a fine powder and washed 2–3 times in water or dilute salt solution to remove any loosely bound contaminants. Wash buffers are removed using centrifugation, and an extraction buffer is applied to the bone powder and allowed to incubate for several hours to overnight, depending on individual protocols. Extracting reagents must be removed before further analyses, usually by ion-exchange column chromatography or dialysis. This removes low molecular weight salts and other buffer components, while retaining the larger organic components of fossil tissues. Fossil extracts should then be dialyzed against sterile distilled water or a neutral physiological buffer such as phosphate-buffered saline (PBS), using low molecular weight cut off (MWCO) membrane, assuming any endogenous organic components are fragmented and therefore smaller than comparable molecules derived from fresh tissues. After dialysis, ultrafiltration or lyophilization can be applied to concentrate the sample, thus optimizing chances of identification of endogenous molecules in low concentration.

#### 10.6. Extraction procedures

Various extraction buffers have been used to isolate organic material in fossils. We briefly review several here that have been used with success with fossil and sub-fossil material. The first three protocols below have in common a demineralizing agent to remove the mine-

ral phase of bone, and one or more protein denaturants. Others contain a reducing agent, such as dithiothreitol (DTT), included for breaking intra- or intermolecular disulfide bonds or other cross-linking bonds.

- (1) Gurley et al. [62] used a reducing buffer consisting of 0.3 M NaCl, 5% glycerol, 5 mM DTT (dithiothreitol), 2 mM EDTA, 1% CHAPS (3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate), 6 M guanidine-HCl, and 100 mM Tris-HCl, to extract dinosaur bone for identification of amino acids.
- (2) A buffer designed to extract fossils for aDNA [75] is also an excellent choice for recovery of other biomolecules, and can be used for modern material as well. This buffer consists of ~10 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 6.4, 0.02 M EDTA, pH 8.0, and 1.3% Triton-X 100. While passing the extract over glass beads at low pH retains relatively pure DNA, dialyzing the whole extract will retain all organic molecules for analyses.
- (3) Schweitzer et al. [137,138] used a modification of Tuross and Stahoplos' method [162], where after surface cleaning, samples were further treated by incubation at 60 °C overnight with gentle agitation in a buffer consisting of 4 ml/g of 6 M GuHCl in 0.1 M Tris pH 7.4. Supernatant was reserved for dialysis, and this step repeated for another 12 h. Both supernatants were pooled, dialyzed, lyophilized and kept in reserve, but not used in primary analyses. Following this initial phase, the pellet was demineralized in 0.5 M EDTA pH 8.0 for five days at 23 °C with daily changes of EDTA. Supernatants were pooled and stored at 4 °C. After demineralization, the remaining pellet was again incubated with guanidine buffer at 60 °C, as described above.
- (4) Reducing buffers [99] denature intramolecular cross-links or molecular bonds with geochemical substances (e.g., humics) that may have formed during diagenesis of organic material in fossils. Extracting under reducing conditions may break apart molecular aggregates so that the components can be analyzed. One reducing buffer, modified, consists of 10 mM Tris-HCl, 10 mM EDTA, 10 mg/ml DTT, 0.1% sodium dodecyl sulfate (SDS).
- (5) For some procedures, it may be adequate to treat the fossil material with a dilute acid (~1 N HCl) or EDTA to remove the excess mineral. This has been used successfully to remove some organic components from relatively unaltered dinosaur tissues [133] and other fossil specimens [159,160]. Prolonged soaking in 0.5 M EDTA, pH 8, with frequent

changes over several weeks is sufficient to remove all soluble mineral, leaving behind what appears to be organic matrix [137–139].

Once the fossil tissues have been extracted, purified and concentrated, there are many analytical techniques that may be performed. The following are some of the analytical techniques that may be performed on extracts of fossil specimens.

#### 10.7. UV/VIS spectroscopy

Organic molecules absorb light at specific wavelengths in the electromagnetic spectrum, and may be identified by the specific absorbance pattern they exhibit. Lyophilized extracts are solubilized in PBS or sterile distilled water, placed in a special ultraclean quartz cuvet, and continuous wavelength light (~190–800 nm) is transmitted through the sample. When light interacts with a molecule, it is absorbed in quantum packets, the characteristics of which are dependent upon the atomic make-up and overall molecular structure of the sample, and the absorbance can be measured quantitatively. Most proteins demonstrate an absorbance maximum at 280 nm that can be attributed to the presence of aromatic amino acid residues (i.e., tryptophan, tyrosine, phenylalanine). Some proteins, such as hemoglobin, have shifted absorbances (~410 nm, [29]), due to their association with pigment molecules. Maximum absorbance characteristic for nucleic acids is seen at 260 nm. Ratios of absorbance measured at 260 nm: 280 nm is often used to determine the purity of a DNA preparation. For absorbance values of commonly occurring amino acids, see Fasman [52].

#### 10.8. Chromatography

Heterogeneous mixtures resulting from bulk extraction of fossil materials present analytical challenges. Chromatographic separation enhances the sensitivity and efficiency of other analytical techniques. In addition, because some components in bulk extractions of fossils may inhibit both polymerases and proteases in amplification of DNA [125,159], chromatographic methods can be used to purify samples.

Column chromatography separates individual components from a complex mix using inherent differences in molecular structure of the constituent compounds. Components may be separated by mass, charge, or hydrophobicity, depending upon the column packing chosen. Differences in migration rates that allow for separation are the result of selective retention on the column packing (referred to as the stationary phase). The mobile phase, in which the analyte is suspended,

can be gas or liquid. In gas chromatography, the sample is vaporized and the vaporized molecules are carried in an inert gas through a fine capillary tube, lined with a high molecular weight liquid. Separation is monitored by a detector that measures the exit of the molecules from the capillary tube. This, in turn, is determined by the interaction of the vaporized molecules in the mobile phase with the liquid of the stationary phase [77b].

In high-performance liquid chromatography (HPLC, [27]), a heterogeneous sample is dissolved in an inert liquid and applied to a column under high pressure. Components of the mixture will adsorb to the column material according to the characteristics of hydrophobicity or charge, and are then eluted individually from the column as the mobile phase changes across a gradient. In reverse phase HPLC, the water-soluble compounds adhere to the non-polar (hydrophobic) material of the column packing. A hydrophilic or polar buffer is then washed over the column, and will remove the most polar components of the mixture as these are least likely to stick to the hydrophobic column. Less polar substances elute from the column later, as the mobile phase changes from polar to relatively non-polar [100].

Chromatographic separations are achieved by varying the characteristics of the column (stationary phase). For organic compounds more likely to be soluble in lipids than water (such as kerogens) normal phase chromatography is often used, where the stationary phase is polar and the mobile phase is non-polar. Separation by charge is accomplished using a stationary phase containing charged groups (e.g.,  $\text{NO}_3^+$ , or  $\text{SO}_3^-$ ) that interact with charged regions of the molecules carried in the mobile phase. Size exclusion packing is used to separate molecular components by molecular weight. Larger molecules elute earlier, while smaller molecules are retained as they pass through small pores in the packing material.

Ionic exchange is another means of chemical separation, whereby the constituent molecules are separated by charge. This method is excellent for removing buffer salts, detergents, or other remnants of the extraction procedure.

Finally, DNA may be selectively removed from bone extracts using apatite columns. Once separated, individual components can be characterized by mass spectroscopy, UV/VIS, or molecular analytical methods such as PCR amplification or antibody testing (see below).

### 10.9. Raman and resonance Raman spectroscopy

Raman spectroscopy uses a laser tuned to a single wavelength to elucidate information about molecular structure [153]. When stimulated, a photon is scatter-

red inelastically, transferring energy and momentum to molecules in the sample. Monitoring light wavelengths that are scattered inelastically yields information on how individual bonds stretch or vibrate as energy is absorbed. Intramolecular bonds such as C=C or C=O can be detected and differentiated, thus yielding information on molecular structure. Resonance Raman (RR) is particularly useful in the study of biomolecules. As the wavelength of the stimulating laser approaches the same frequency as electronic transitions of certain component molecules, Raman scattering is greatly enhanced. Resonance Raman will preferentially enhance certain aspects, at the expense of others, thus identifying unique ligands of known molecules that may arise through chemical cross linking in the fossilization process.

Raman spectroscopy requires only minute samples (e.g., single crystal grains or microliters or less of solubilized material), making it an ideal method for analyzing rare fossil material [60]. In addition, by carefully selecting the excitation wavelength of the stimulating laser, the characteristics of a single molecular class in a complicated mixture [153] can be analyzed. Because organic compounds are often irreversibly crosslinked with other molecules in fossil tissues, purification and/or separation of proteinaceous material is difficult. Raman spectroscopy is particularly well suited for detecting fossil specific molecular modifications. Finally, Raman spectroscopy is a highly accurate and sensitive means of identifying specific molecular compounds such as porphyrins or heme [133,149].

Porphyrins are a class of small biomolecules found in many fundamental molecules such as chlorophyll, hemoglobin, myoglobin, and cytochromes. The function, identification and stability of these small molecules depend upon the central atom. In hemes (which form the reactive portion of hemo- and myoglobin and cytochromes, this atom is iron. In chlorophylls, it is magnesium. The metal atom at the center of the ring imparts unique characteristics and vibrational energies to porphyrins, determining function in life and allowing their detection and unambiguous identification. Porphyrin-based molecules are extremely stable, and have been identified, almost unaltered, in organic kerogens from Carboniferous sediments [41]. This stability makes them an ideal target for molecular studies of fossil bone. Cytochrome porphyrins are also found in aerobic microbes, making this molecule a possible indicator of extraterrestrial life as well [149].

In addition to being a valuable tool for the identification and characterization of biogenic material including proteins, Raman spectroscopy is frequently used to study heme and its ligands [4,17,28], such as oxygen, carbon

dioxide, or carbon monoxide. Hemoglobin is a prominent component of extant bone, as bone in most taxa is highly vascular. For a listing of Raman characterizations of different groups common to organic compounds, the reader is referred to Grasselli and Bulkin [60].

#### 10.10. Solution-phase Proton Nuclear Magnetic Resonance (NMR)

NMR is routinely performed as a means of determining overall structure of biomolecules. The nuclei of atoms in certain molecules possess an inherent magnetism, or magnetic moment, which is recognized by NMR. Atomic nuclei with an even number of protons and neutrons have no magnetic moment, and are not responsive to NMR analysis [153]. This excludes naturally occurring carbon (6 protons, 6 neutrons,  $m = 12$ ) and oxygen ( $m = 16$ ), but isotopes of these elements can be studied by NMR. A pulsed magnetic field is applied to molecules in solution, which in turn interacts with the magnetic moment of the appropriate atomic nuclei. This causes energy splitting, which can be measured by absorbance of an appropriate light (probes and detectors operate in the radio frequency range of the EM spectrum). The chemical bonding environment of the proton can then be positively defined by the variation in positions of absorbance peaks. For example, a proton ( $H^+$ ) bound to carbon will be located in a slightly different region of the spectrum than one bound to oxygen. Additionally, NMR can be used to track changes in conformations of biologically active molecules as their chemical environments change [143]. NMR imparts detailed information about the chemical structure of molecules in an extract, but is not highly sensitive and requires relatively high concentrations of material for detection, a potential problem for analyzing fossil material. Even in low concentrations, however, NMR is adequate for detecting certain characteristics of molecules. Proteins or protein fragments that occur as complexes with metal ions (metalloproteins) are particularly sensitive to changes in magnetic field, because their electrons at the lowest energy state are unpaired, making them paramagnetic. Molecules containing paramagnetic atoms produce a large induced magnetic field easily detected by NMR. In addition, a paramagnetic compound can affect the NMR spectra of other molecules within a sample. Because the core heme of hemoglobin and cytochromes is paramagnetic, NMR is particularly useful in the study of hemoglobin and its related muscle protein, myoglobin (e.g., [72]), and it has been used in conjunction with other evidence to identify the occurrence of heme groups in extracts of fossil bone [133]. Like Raman spectroscopy, NMR

has been widely used in the study of heme-containing biomolecules ([143] and references therein).

The size and complexity of molecules influence the degree to which their structure can or cannot be elucidated from an NMR spectrum. For example, the complexity of chemical interactions in an average protein consisting of 500 or more carbons would be difficult to structurally characterize via NMR alone. However, the presence of characteristic peaks can be used to identify the presence or absence of biomolecules in bulk solutions or in eluents from chromatographic separations.

#### 10.11. Electron paramagnetic resonance (EPR)

Electron Paramagnetic Resonance (also Electron Spin Resonance, or ESR) is an excellent complement to NMR of paramagnetic molecules. Some atoms in molecular complexes contain electrons that exist in a state of unequal spin, detectable by EPR. The range of applications for EPR is more limited than Raman or NMR; however, it is more sensitive than NMR, and provides slightly different information, such as characteristics of electron transitions and molecular interactions [17]. EPR is well suited for verifying the presence of paramagnetic compounds in extracts of fossil bone, and can provide another line of evidence for preservation of molecular fragments [133].

#### 10.12. Solution Phase Mass Spectrometry

Some mass spectrometry ionization methods require extracted samples in solution, rather than bulk sample as described previously for PY-GC-MS or ToF-SIMS. These are usually more sensitive and can provide more information about source molecules, provided that these fragments are not lost or modified beyond recognition in the process of extraction. These include electrospray ionization mass spectrometry (ESI-MS) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) [102,107].

Electrospray ionization (ESI) is favored in the mass spectrometry of large biomolecules (i.e. peptides and proteins) because of its 'softness' (the ability to charge molecules for analysis without fragmenting them). Electrospray is also capable of generating multiple charges on some analytes via surface charge interactions in the ionization process, allowing more accurate mass measurement and as a result, a better chance in identifying a molecule's chemical makeup [53]. A drawback to ESI-MS is that a larger quantity of analyte may be required, because most of the components of the analyte spray do not reach the spectrometer and hence, are not measured.

Larger amounts of sample diluted in solution yields better detection. The development of more advanced mass analyzers, however, makes this problem less prohibitive.

Matrix assisted laser desorption ionization, on the other hand, is a pulsed laser ionization technique where analyte molecules in solution are applied to a solid matrix and are subsequently liberated from a matrix surface for mass analysis via a rapid pulse from a UV or IR laser. This ionization technique yields singly charged, whole analyte molecules, because the process involves rapid neutralization of multiply charged analytes [79].

Although these techniques yield mainly intact charged molecules rather than fragments, it is common to couple these techniques in tandem experiments such as MS/MS where the analyte can be measured intact via ESI or MALDI and then fragmented via collision induced dissociation (CID). The fragmented analyte is then mass-analyzed by another detector. These types of experiments are most commonly performed using Triple Quadrupole Mass Spectrometry or hybrid instruments coupling multiple detectors in series (i.e., LTQ-FT-ICR, LTQ-Orbitrap and 2D-LTQ).

These techniques have in common the ionization, fragmentation and detection of molecular compounds in solution, with the intent of measuring fragment masses. These methods vary in sensitivity; however if sufficient fossil material is available for extractions and concentration, they are very informative. Mass spectrometry methods can also be used to obtain amino acid sequences if samples are otherwise purified [2,3,29,136], and can also be coupled with separation techniques to yield even more accurate molecular information.

### 10.13. Sequencing by Mass spectrometry

The identification and quantification of proteins from an organism (proteomics), spans a continuum of technologies and techniques. The vast majority of these experiments employ a separation technique such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or liquid chromatography (LC) to separate components from complex mixtures. Each component is then subjected to digestion by proteases that cleave proteins into peptides of manageable length. The use of trypsin, for example, predictably results in peptides with a C-terminal arginine or lysine residue. These peptide mixtures are submitted to mass spectrometric analysis via either direct infusion, using an LC coupled directly to a mass spectrometer for detection (LC-MS), or by in-line tandem mass spectrometry (MS-MS). Inside the mass spectrometer, molecules can be fragmented using collision induced dissociation

with an inert gas, resulting in ion spectra from which the amino acid sequence of the parent peptide can be obtained. The sequence information, coupled with the initial mass of the parent peptide, can be used to search proteome databases to identify the original protein.

Recently, a ~300,000-year-old mammoth skull (MOR 604) and a 68-million-year-old *Tyrannosaurus rex* (MOR 1125) were chemically extracted and purified via dialysis, and the resulting product was digested with trypsin and sequenced using solid phase extraction [3,137,141]. Mass spectrometer analyses were performed on peaks separated by elution from a reversed phase microcapillary column coupled to a two-dimensional linear ion trap mass spectrometer. Because no genomic sequences or protein databases exist for these organisms, they were identified by searching sequence data against closely related extant species for which a database existed [2,3,105]. The amino acid sequences can then be used to generate phylogenetic hypotheses, or to predict three-dimensional protein structure.

## 11. Biomolecular analytical techniques

If morphological, microstructural and chemical analyses show potential for preservation of biomolecular fragments within fossil bone, molecular analyses can be undertaken. These experiments are often time intensive and require larger samples, as multiple repetitions are needed to rule out the possibility that positive results may be due to technical artifact. Of the four classes of biomolecules associated with living organisms (nucleic acids, proteins, lipids and polysaccharides), proteins and DNA have the best potential for addressing issues of phylogenetic relationships, evolutionary processes, and physiological strategies of extinct organisms. This section will focus on methods for analyzing protein-derived constituents in fossil specimens.

Protein function is determined by its three dimensional structure. However, the structure of a protein is ultimately determined by the sequences of bases in the DNA molecule that codes for that protein. Protein structure can be described on four levels. The sequence of amino acids as it is read from DNA is the primary structure, where three bases on the DNA helix code for one amino acid in the protein chain. Secondary structure describes moieties within a peptide chain, such as helices or beta-pleated sheet regions. Tertiary structure refers to the overall three-dimensional structure of a protein chain, combining all motifs within the chain. The quaternary structure of a protein is the total sum of

the above, and refers to an assembled functional protein, made up of two or more individual protein chains. Most proteins have a highly constrained quaternary structure upon which proper functioning of the protein depends.

### 11.1. Amino acid analyses

The amino acid is the basic unit of a protein. Although over 60 amino acids have been identified, virtually all naturally occurring proteins from living organisms consist of only 20 of these.

Amino acid analysis is conducted using either specialized column chromatography or mass spectroscopy. Analysis requires solubilized and relatively pure sample extracts which are hydrolyzed with strong acids or enzymes to break apart the bonds holding the constituent amino acids within the peptide chains. The amino acids are identified by a combination of elution/retention time and UV/VIS absorbance of the eluted peaks when compared to known standards.

Estimations of source proteins for identified amino acids may be based upon ratios of particular amino acids. The sequence of collagen, with its highly constrained tertiary structure, requires every third amino acid to be glycine for proper folding. Therefore, amino acid analysis of pure collagen yields approximately 33% glycine. Additionally, collagen incorporates amino acids which are unique to this protein. Proline and lysine are post-translationally modified by the addition of hydroxyl (OH) groups in the finished protein, and presence of these amino acids confirms the identity of collagen.

In addition, most amino acids possess “handedness”, either dextro (D-) or levo (L-) rotatory, because they have 4 unequal groups about the central, or *chiral*, carbon (Fig. 5; [8,130]). While all proteins in living organisms consist of only the L-form of amino acids, post mortem conversion from L- to D- forms (racemization) progresses to equilibrium (i.e., D/L = 1). The degradation of proteins over time as seen in fossil extracts yields both D- and L-amino acids. Using either a special (chiral) column or a post-column derivatizing agent allows separation of amino acids according to chirality. Many factors affect rates of racemization, but general estimates of racemization rates have been calculated for each amino acid [129], based on reaction kinetics of free amino acids in solution. These figures represent a *minimum* time for each amino acid to reach equilibrium, and under ideal conditions this rate may be extended by one or more orders of magnitude [46]. Chiral amino acid analyses of fossil specimens provide an independent control for identifica-

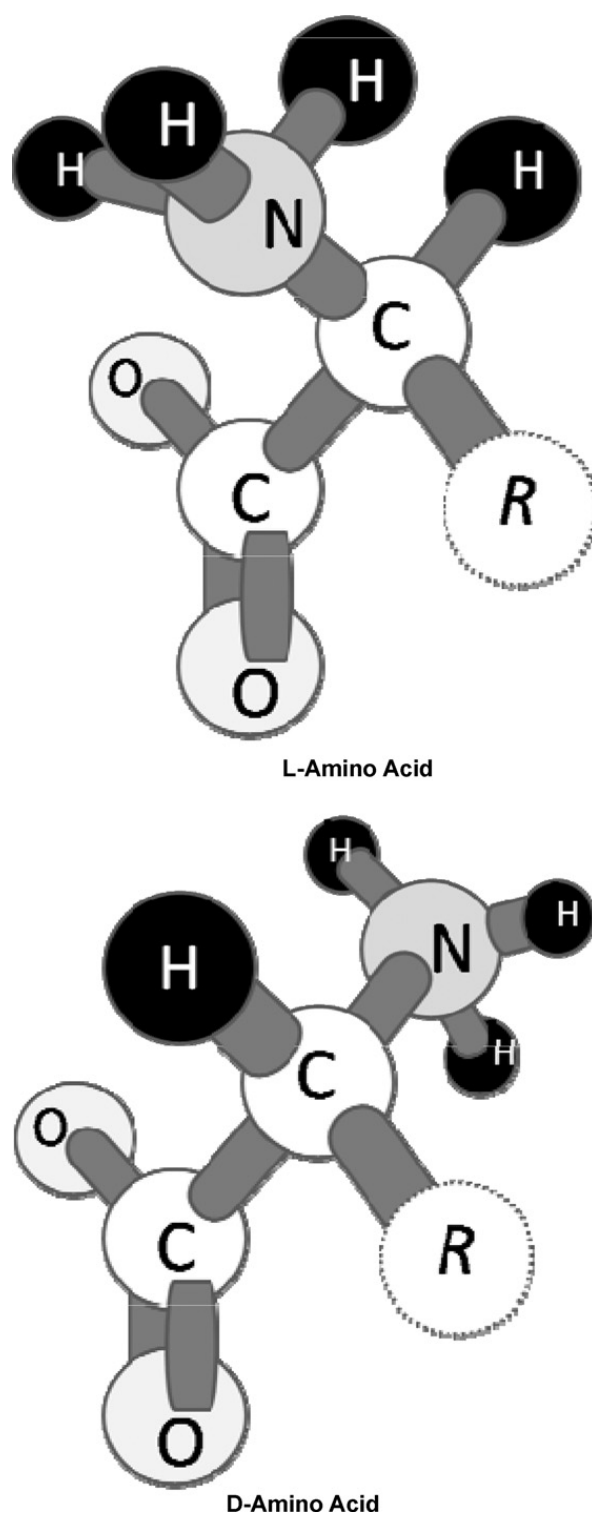


Fig. 5. Schematic of a generic chiral amino acid, showing L- (left) and D- (right) isomers.

Fig. 5. Schéma d'un aminoacide chiral générique, montrant l'isomère L (gauche) et l'isomère D (droit).

tion of contaminating compounds by more recent amino acids. At least some proportion of the D-isomer of more labile amino acids should be detected in specimens older than a few hundred years. Some investigators have pro-

posed a relationship between degree of racemization and the persistence of endogenous DNA from fossil material [116]. While a causal relationship between amino acid racemization and DNA degradation has not been shown, and protein fragments may persist in samples in which DNA has been degraded [115], chiral analyses may be another way of judging the appropriateness of a fossil for DNA analyses.

### 11.2. Electrophoresis

Extracts of fossil material may be separated into constituent fragments using polyacrylamide gel electrophoresis (PAGE). Samples are mixed with a special buffer that imparts a uniform negative charge to component molecules, then loaded onto a gel to which an electric field is applied. The migration rate for negatively charged fragments is determined by the molecular size of the fragment. The gel may then be stained by a variety of agents to visualize fragments for size estimates relative to known standards loaded concurrently. Extracts of extant tissues, loaded to the gel simultaneously with fossil material, provides a positive control, as well as verification of fragment size seen in fossil lanes.

### 11.3. Specific enzyme degradation

Enzymes are proteins that can act with varying degrees of specificity to cut or degrade either proteins or DNA. While enzymes such as proteases (which digest proteins) are commonly used to purify DNA in a mixture by degrading protein fragments, DNAses can also be used to identify DNA in a mixture because of their specific action. Likewise, some collagenases are specific enough that degradation by the enzymes is sufficient to prove the presence of collagen in an extract. If collagenase-treated fossil extracts show smaller fragments of material on PAGE separation relative to untreated material, it provides strong evidence that collagen is present in bone extracts. However, crosslinked material is relatively protected from enzyme digestion, and this may be problematic for protein identification by this method alone. In addition, digestion of fossil extracts by various enzymes may actually have the opposite effect than that seen in modern extracts. While crosslinking is a critical factor in the preservation and retention of original organic molecules over time, enzymatic degradation may partially break these links, thus exposing more antibody binding sites in fossil bone after digestion than is seen in undigested material.

### 11.4. Antibody–antigen interactions

Like protein sequence data, antibody–antigen interactions have potential to address evolutionary relationships, though indirectly, and with less resolution. An *antigen* (ag) is any substance capable of being recognized by an antibody (ab). An *antibody* is a protein produced by the immune cells of host organisms in response to stimulation by a foreign substance. An *immunogen* is a substance capable of invoking an antibody response when introduced to a host. An antigen may not always be immunogenic, but an immunogen is almost always antigenic as well. An *epitope* is that specific region or three-dimensional conformation that an antibody recognizes, and is sometimes used interchangeably with the term antigen. A microbe or a single protein may have hundreds or thousands of epitopes on its surface, each of which is capable of being recognized by a different antibody. Each antibody, in turn, is produced by a different clonal population of cells.

Antibodies are produced by host B-cells in response to exposure to an immunogen. Each B cell recognizes and responds to only one epitope. Upon exposure to an immunogen, the responsive B-cell divides rapidly to produce a population of identical cells secreting identical antibodies, each of which bind to only the one epitope that stimulated its production. Antibodies are secreted into the serum of the host organism, and collected serum will contain all antibodies produced by the different clonal populations, each recognizing a different epitope of the immunogen. This is called a *polyclonal* serum (Fig. 6).

*Monoclonal* antibodies are produced by only one B-cell clonal population; therefore, a monoclonal antibody will only recognize and bind to a single epitope of a foreign molecule (Fig. 6). Monoclonal antibodies are highly specific, and if the test antigen is lacking that one epitope, for example one particular 5-amino acid turn or shape, the monoclonal ab may not bind, while the polyclonal serum from which it is derived will show reactivity. A monoclonal antibody specific to a single epitope of a protein used to immunize, but not present on the same protein from the test taxon will not demonstrate reactivity, even though the protein may be present. Therefore, monoclonal antibodies are not ideal for analyses of proteinaceous material from extinct organisms.

Proteins may be extracted from a tissue, then purified to homogeneity, dissolved in a physiological buffer (saline) and used to immunize a host animal, usually a mouse or rabbit. The host immune system responds by producing antibodies; therefore the injected protein is the *immunogen*. The antibodies produced in response

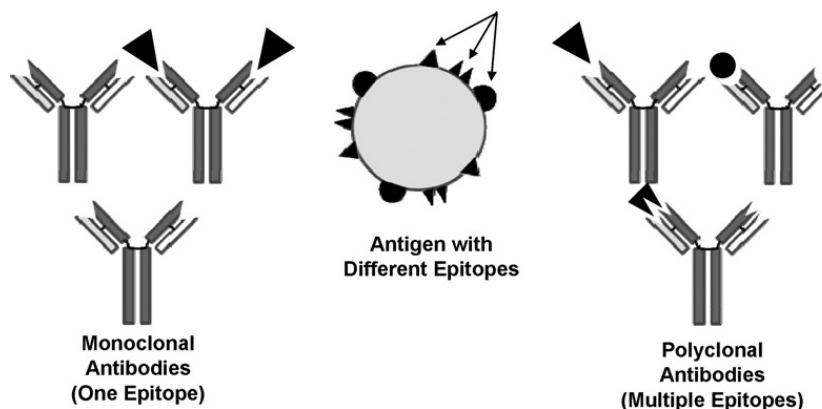


Fig. 6. Schematic differentiating monoclonal (left) and polyclonal (right) antibodies. Central image is an antigen (possibly a protein or virus) exposing multiple epitopes on the surface to which antibodies may bind. Monoclonals will bind only one type of epitope (black triangle), while polyclonal antibodies will bind multiple epitopes (black triangle, circle, or double triangle).

Fig. 6. Schémas d'anticorps monoclonal (gauche) et polyclonal (droit) se différenciant. L'image centrale correspond à un antigène (probablement un virus ou une bactérie), montrant, en surface, de multiples épitopes auxquels les anticorps peuvent s'attacher. Les anticorps monoclonaux ne s'attacheront qu'à un type d'épitope (triangle noir), tandis que les polyclonaux s'attacheront à de multiples épitopes (triangle noir, cercle ou double triangle).

to the collagen remain in the circulating blood and may be collected. Incubation of the antibody containing serum (antiserum) with extracts or tissues derived from either the original source or from a second source may demonstrate varying degrees of reactivity, depending upon the phylogenetic proximity of the animal providing the immunogen with the animals providing the antigen. Antibodies demonstrate greatest reactivity with molecules most similar to those used to immunize, and therefore binding patterns may reveal evolutionary distances. Thus, antibody reactivity provides a means of confirming phylogenetic hypotheses in addition to providing the means to identify the source of protein fragments preserved in fossil tissues. Antibodies can also be raised against proteins that are taxon specific, thus reducing the chances that reactivity is due to contamination.

Antibody–antigen interactions provide a robust identification of protein survival in fossil specimens when a two-fold approach is employed. Fossil extracts that react with antibodies raised against extant proteins can be used to stimulate antibody production in new host animals. If the resultant fossil antiserum binds purified proteins from extant sources, not only does it confirm the presence of protein, but identifies the source proteins in fossil material. However, producing antibodies to fossil material may require the destruction of more fossil material than is practical, as antigenic protein in fossils is extremely low in concentration, and usually degraded, modified or fragmented, compromising immunogenicity of the fossil material. Thus, the proteinaceous material in fossils may be *antigenic*, but too small to be *immunoge-*

*nic*. If fossil tissues contain material that is antigenic but not immunogenic, they can be coupled to a larger carrier molecule called a hapten, making the complex large enough to be recognized by the host immune system.

Antibodies can recognize molecular fragments only 3–5 amino acid residues in length [8], and do not require that a whole molecule be present for recognition, making this a powerful method for studying degraded fossil specimens. Antibodies can be used to distinguish proteins that differ by only a few amino acids, if those differing amino acids result in changes in three-dimensional structure.

Antibodies can also be used in ancient DNA studies, both to confirm and/or localize DNA to regions of fossil tissues, such as osteocyte lacunae within bone fragments, or they can also be used to purify DNA or proteins from heterogeneous extracts, and the purified molecules can then be used in additional studies such as genome sequencing (e.g., [117]). Antibodies raised against DNA molecules are not dependent on sequence, so no *a priori* assumptions regarding DNA sequence are required. While antibodies are not specific enough to distinguish DNA for phylogenetic studies, they can visually confirm the presence of the molecule in fossil tissues.

To purify proteinaceous components from fossil extracts for further and more detailed analyses, antibodies showing reactivity to fossil material may be coupled to a column, binding reactive fragments contained in fossil extracts that are passed over it. Most fossil material contains contaminants or degraded complexes of organics in addition to small amounts of endogenous material, some of which inhibit enzymes or confound

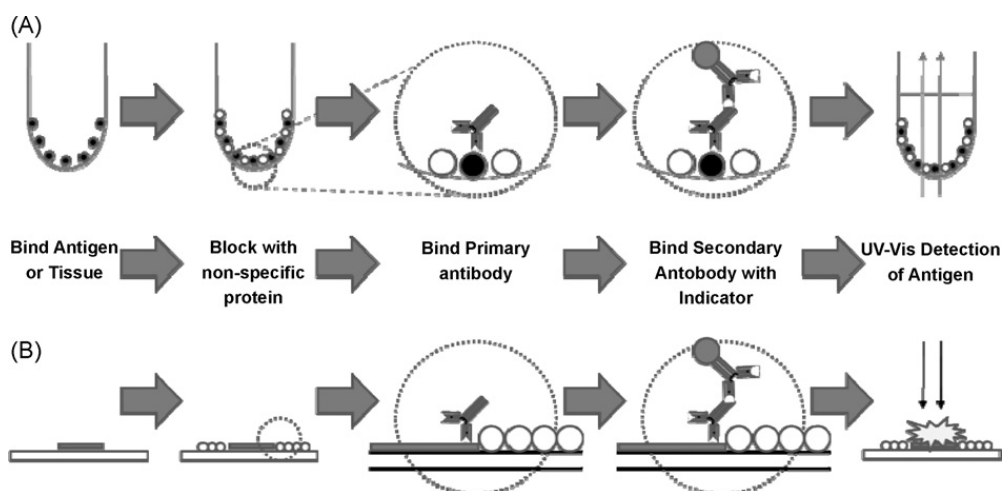


Fig. 7. Schematic drawing illustrating two different methods of monitoring antibody–antigen interactions. **(A)** Enzyme-linked immunosorbent assay (Elisa), with extract in solution applied to coated wells, and then incubated with primary and secondary antibodies with detector. **(B)** *In situ* immunohistochemistry, showing localization of antibody to regions on prepared tissue to which it binds. Tissue is sectioned and mounted to a microscope slide, blocked, and primary and secondary antibodies applied. Secondary antibodies are linked to a fluorescent label, and stimulated with a tuned laser specific to the label used to cause fluorescence.

Fig. 7. Schéma illustrant deux différentes méthodes de contrôle des interactions anticorps–antigènes. **(A)** Essai immunoabsorbant lié à une enzyme (Elisa) avec extrait en solution appliqué en revêtement de tubes, ensuite incubés avec des anticorps primaires et secondaires avec détecteur. **(B)** Immunohistochimie *in situ*, montrant la localisation d'anticorps aux régions des tissus préparés, auxquelles ils se sont attachés. On utilise une section de tissu, montée et bloquée sur une lame de microscope, à laquelle sont appliqués des anticorps primaires et secondaires. Les anticorps secondaires sont reliés à une plaque fluorescente et stimulés par un laser accordé, spécifique pour provoquer la fluorescence de la plaque.

other methods of analyses, and affinity purification using antibodies avoids these problems [63].

### 11.5. Monitoring antibody antigen interactions

Antibody–antigen interactions are measured using various, usually complementary means. Each method provides slightly different information, and particularly when dealing with degraded fossil material, some may work, whereas others may be less effective. Several of these methods are described below.

#### 11.6. Enzyme-linked immunosorbent assay (ELISA)

These assays are extremely sensitive, capable of detecting nanograms to picograms of antigenic material. Solubilized antigen is applied to wells of specially designed microtiter plates. Antigen binds to the charged plastic after incubation for several hours to overnight (Fig. 7A). After removal of antigen, a blocking buffer containing a high concentration of non-relevant protein is applied to block reactive sites on the well not coated with the antigen, preventing non-specific binding of antibody. Specific antibody in solution (primary antibody) is allowed to incubate with the bound antigen, and any unbound antibodies are removed by multiple washings. A detector antibody is then applied, which recognizes

a region on the primary antibody, now bound to the test (fossil) antigen. This secondary antibody is coupled to an enzyme such as alkaline phosphatase or horseradish peroxidase, and after removing excess or unbound secondary antibody a substrate is applied that causes a color change, which is monitored by a special detector that allows the degree of reactivity to be quantified. If the primary antibody does not recognize the antigen, there will be nothing to bind the secondary antibody–enzyme complex, and no color change will be detected (Fig. 7A). ELISA is relatively simple to perform, and allows reactions with non-denatured proteins or protein fragments. However, the ability of the wells to bind fossil antigen may be compromised by the presence of degraded organics such as humic acids, in extracts of the fossil tissues.

#### 11.7. Western blots (also Immunoblots)

Antigen is applied to a polyacrylamide gel and separated by electrophoresis, as previously described. However, instead of applying special stains to the gel to detect the presence of protein, DNA or other organic compounds, the gel is covered with a special membrane (generally nitrocellulose), placed in a chamber filled with electrolysis buffer, and an electric field is again applied. Instead of moving the protein compo-

nents downward for separation, as in electrophoresis, the field moves the components out of the gel, and onto the membrane, where they bind irreversibly. After the proteinaceous components are membrane-bound, the membrane is incubated with a blocking solution as above, then incubated in a solution of diluted antibodies several hours to overnight, allowing antibodies to bind to the separated components immobilized on the membrane. Unbound antibodies are removed, and secondary antibody, labeled with fluorescent chemical, enzyme, or other detection chemical is incubated with the membrane where, similar to ELISA, it binds

the primary antibody. The membrane is then developed, and reactive fragments of fossil material are identified. Assuming that the concentration of antigenic material preserved in fossils is greatly reduced relative both to modern samples and to other co-extracting contaminants, sensitive chemiluminescent techniques are preferred over other detection systems. These are detected using radiographic film, and provide a permanent record of reactivity that will not fade, a problem with other detection systems.

Immunoblots provide information not only regarding overall antibody reactivity, but also molecular

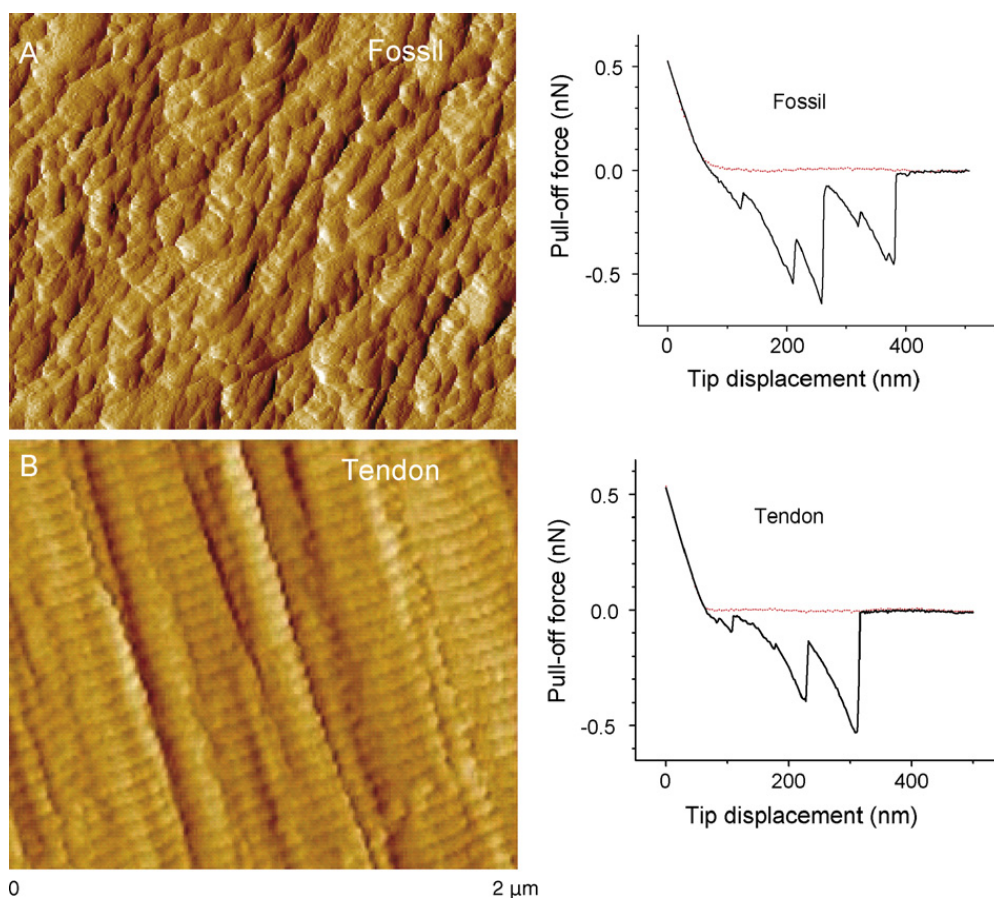


Fig. 8. Examples of AFM imaging and spectroscopy. (A) High-resolution AFM image of a collagenous section of demineralized bone from an embryonic fossil bird. The panel to the right of the image shows collagen pull-off curves associated with fossil collagen. The dotted red (gray) lines correspond to tip approach, while saw-tooth patterned black curves correspond to the pull-off curves associated with the unfolding and breaking (sharp transitions) of individual collagen fibrils. (B) High-resolution AFM image of a tendon from an extant bird. The 67 nm banding of collagen I is clearly visible. The pull-off curves associated with the tendon collagen are shown in the spectrum to the right of the image. Statistical analysis of collagen pull-off curves shows no discernable differences between the tendon and fossil collagen, supporting the hypothesis that the integrity and functionality of the remaining collagen in fossil bone are preserved (for details, see [6]).

Fig. 8. Exemples d'imagerie et de spectroscopie « AFM ». (A) Image « AFM » haute résolution d'une section de collagène d'os déminéralisé d'embryon d'oiseau fossile. La partie droite de l'image montre des courbes d'arrachement de collagène associé au collagène fossile. Les lignes rouges (grises) en pointillé correspondent à l'approche de l'extrémité, tandis que les courbes noires en dents de scie correspondent aux courbes d'arrachement, associées à des transitions brutales (cassantes et brusques), dues à des fibrilles individuelles de collagène. (B) Image « AFM » haute résolution obtenue à partir d'un tendon d'oiseau encore existant. La zonation en bandes de 67 nm du collagène est clairement visible. Les courbes d'arrachement associées au collagène s'observent dans le spectre, à droite de l'image. L'analyse statistique des courbes d'arrachement du collagène ne montre aucune différence discernable entre le collagène du tendon et celui du fossile, ce qui était l'hypothèse selon laquelle l'intégrité et la fonctionnalité du collagène restant dans l'os fossile sont conservées (pour plus de détails, voir [6]).

size. Antigenic components within extracts of fossil bone can be compared with electrophoresed products of known and/or purified proteins to gain insight into identity of source proteins and patterns of molecular diagenesis. Usually, however, this method relies on denaturation of antigenic material. If antibodies used to detect reactivity in fossil material are made against native (non-denatured) proteins, the probability of binding is reduced, even if protein fragments are present in the fossil extracts.

### 11.8. Immunohistochemistry (IHC)

This method is an invaluable complement to the above described techniques. IHC relies upon *in situ* localization of antibody binding to antigen, providing a check for contamination and verifying the presence of reactive compounds in fossil matrices. The procedure is similar to that described for light microscopy, in that fossil tissues are embedded in a polymer, but one specifically designed for antibody studies. The tissues are then microsectioned to thicknesses of 1.0 or 0.5  $\mu\text{m}$ , and adhered to coated glass slides. Sections are then blocked with non-relevant proteins as described above to reduce non-specific binding, followed by incubation with primary and secondary antibodies, similar to the above procedures. For IHC, an additional detector coupled to a fluorescent label is applied, greatly amplifying signal from antibody binding (Fig. 7B). Confocal microscopy or fluorescent microscopy can then be used to visualize signal arising from primary antibodies binding to regions of the sectioned tissue. IHC demonstrates that antigenic components reside within the fossil tissues and are not the result of extraction procedures or contamination. This method has been used successfully to identify proteinaceous components in various fossil tissues (e.g., [101,103,134,135,140]).

### 11.9. Atomic force microscopy (AFM)

In addition to providing the ability to visualize samples at nanoscale levels, this technique enables scientists to probe molecular ligand-receptor interactions with pico-Newton resolution. Increasing sensitivity and resolution of computer, laser and material sciences makes it possible to detect sub-nanometer movements of a cantilever tip that transverses the surface of a specimen [88]. The ability to sense interactive forces between the tip and the surface with pN sensitivity allows quantitative determination of elastic and adhesive properties of materials at nanoscale levels [78]. This is particularly useful when the probe is coupled to a specific antibody.

When the probe is allowed to interact across the surface of the sample, the strength of antibody binding can be quantified [6,42,70,97]. In addition, because AFM microscopes can image at the level of a single molecule, force maps obtained from ab-ag interactions can pinpoint the location of epitopes on the molecule [45,122,148]. The technique is used often to study protein unfolding and its functionality. We have applied this technique to image morphology of and to test the unfolding of collagen obtained from fossils dating to the Late Cretaceous, and compared their morphological and physical properties with those obtained from extant samples [6,138,140]. Fig. 8 shows demineralized Cretaceous bone (A) imaged by AFM, and compared with tendon (B) from an extant bird. In addition to visually observing whole fragments and demineralized tissues, this method may also be used to determine elastic and mechanical properties of components within tissues (Fig. 8C), and to quantify the force of ab-ag binding at nanoscale levels [42,70,97].

In summary, the increasing sensitivity and resolution of chemical and molecular analytical techniques in the last decade or so has increased our understanding of biological and physiological processes in extant animals. The application of these methods to fossil specimens can likewise increase our understanding of the physiology and evolution of extinct vertebrates as well. Complete chemical characterization of cells, tissues and molecules preserved in fossil bone may also provide the means to elucidate the chemical pathways of diagenesis and degradation, as well as preservation, at the molecular level. Finally, by understanding the interactions between biological and geological components of the fossil record, we may gain additional information about the sedimentary and geochemical conditions contributing to preservation. Vertebrate paleontology is, by its very nature, a cross-disciplinary endeavor. We predict that the application of these analytical methodologies in the future will continue this trend, and that an understanding of the methods, data and interpretations will be crucial to vertebrate paleontology in the future.

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