Review

Proteomes of the Past: the pursuit of proteins in paleontology

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Abstract

Despite an extensive published literature, skepticism over the claim of original biochemicals including proteins preserved in the fossil record persists and the issue remains controversial. Workers using many different techniques including mass spectrometry, X-ray, electron microscopy and optical spectroscopic techniques, have attempted to verify proteinaceous or other biochemicals that appear endogenous to fossils found throughout the geologic column. This paper presents a review of the relevant literature published over the last 50 years. A comparative survey of the reported techniques used is also given. Morphological and molecular investigations show that original biochemistry is geologically extensive, geographically global, and taxonomically wide-ranging. The fossil limits of endogenous organics remain the subject of widespread and increasing research investigation.

Keywords: fossils, paleontology, proteins, collagen, biochemistry, immunohistochemistry, mass spectrometry, X-ray, FTIR, optical spectroscopy.

Article highlights

- Over 85 papers containing reports of original biochemistry including proteins in fossils are reviewed in a comprehensive survey of the published scientific literature going back over the last 53 years.
- The number of publications has increased rapidly in the last 10 years indicating a surge in interest in this research area.
- Although reports of original (endogenous) biochemistry from the Cenozoic period are considered the focus of the review is on claims of original biochemistry from the Mesozoic, Paleozoic and lower levels which are more difficult to explain.
- Preservational ideas are considered and reviewed.
- This review of the published scientific literature reveals that apparently original biochemistry in fossils is geologically extensive, geographically global and taxonomically wide ranging.

1. Introduction

In vertebrate hard tissues bone and teeth, precisely arranged proteins combine with calcium hydroxyapatite ("bioapatite") to account for the rigidity and flexibility of bone. Overall, about 40% of the dry weight of bone is organic and 60% inorganic [1], as illustrated in Fig. 1. The collagen family of proteins comprises 90% of the total organics of bone. It contains a high content of glycine, proline, and hydroxyproline, usually in a repeated -gly-pro-hypro-gly- pattern [2]. This arrangement of relatively small residues permits the coiled architecture of each subunit. The flexibility of this fibrous biomolecule provides bone its resilience, while bone rigid structure of the biomineral components provides compressive resistance [3]. Less abundant bone matrix proteins include osteocalcin, a protein hormone involved in signaling bone tissue growth via osteoblast activity.

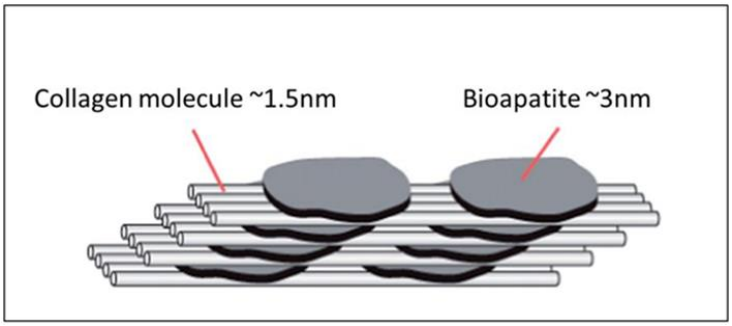

Figure 1 | Illustration of bone biomineral and collagenous microstructure

Collagen's abundance, insolubility in water and hence longevity, and ease of separation from bone by acid dissolution of biominerals have made it a preferred bone component for radiocarbon and stable isotope analysis [4] as well as protein sequencing for species identification in archaeological settings [5].

2. Bone diagenesis

Models of bone decay *post mortem* that assume an absence of microbial biodegradation suggest that tropocollagen helices undergo chemical reactions including glycation, oxidation and hydrolysis as they decay into smaller components (e.g., amino acids, carbon dioxide, ammonia, etc.) that then disperse. Collagen fibres fray from either end, where exposure favors reactivity. In principle, the tight packing of adjacent apatite crystals hinders reactivity and preserves bone collagen [6]. Collagen fibers can interact with surrounding material, but do so under different conditions than bioapatite. Under conditions where collagen decays first, bioapatite crystals can quickly disorganize and bone soon turns to dust. Similarly, if bioapatite undergoes dissolution before collagen decays, then its dispersal exposes collagen to chemical reactants, again hastening whole bone decay. Ideal conditions for bone

preservation thus dampen both the organic and inorganic decay processes. These conditions include a constant low temperature, a uniform, low hydration level with restricted percolation, and near-neutral pH. Extremes in pH react with, help solubilize, and thus hasten collagen decay [7]. Therefore, each broad setting, and even microsite variations that can occur within feet of one another in a single setting, brings its unique set of chemical, thermal, mineral, biological, pH and other conditions to bear on the complicated array of possible diagenetic fates for bone and bone collagen.

Field and lab observations suggest that upon deposition, any bone collagen that escapes scavenging and biodegradation decays rapidly at first, then slows to a more linear decay regime [8]. Such studies model bone collagen decay using ideal conditions, such as near-neutral pH, near sterility, and constant hydration. Even under less than ideal conditions, archaeological bone can still retain sufficient collagen for radiocarbon dating, stable isotope analysis, thermal (collagen) dating [7], and even species identification via collagen sequencing. However, paleontological (fossil) bone does not often retain sufficient collagen to relay any of those types of biological information. Recent research reviewed in detail below shows a surging interest in bone and protein longevity, how long it has actually lasted, and what conditions might promote protein preservation in fossils.

3. Collagen decay

The decay rate of bone collagen under ideal conditions has been well characterised experimentally. Its energy of activation (*Ea*) of 173 kJ/mol equates to a half-life of 130 ka at 7.5°C [9,10]. Typical kinetic experiments use a two-step strategy to determine the decay rates of various proteins, including collagen. First, elevated temperatures are used to accelerate bone collagen decay, typically in a sealed glass vial. Three different experimental temperatures are required to construct an Arrhenius plot. Molecular decay is measured by various means including protein extraction and weighing, protein extraction and SDS-PAGE analysis, or protein concentration estimates using immunofluorescence. Each technique essentially delivers a fraction or percent protein remaining at certain time points during the typically four to eight week-long experiment. The resulting data are then plotted as the natural log of the percent remaining (or concentration of reactant) versus time in days for each of at least three temperatures. The slopes, obtained via linear regression analysis, are then used to calculate decay constants (*k*) for each temperature using: $k = -[\text{slope}]$.

The three resulting *k* values, one for each of three tested temperatures, are then plotted in a second logarithmic curve, the Arrhenius plot. It shows the natural logarithm of each decay

constant, ln(k) versus the inverse temperature, 1/T. The slope of the line of best fit through those three points is used to obtain the variables E_a and A for the Arrhenius equation. E_a is the activation energy, and A is a pre-exponential factor unique to each reaction and partly expresses frequency of collisions between reactants. The slope of the Arrhenius plot equals $-E_a/R$, with R being the gas constant, 8.31446 J/(mol·K). The y-intercept of the slope from that same Arrhenius plot equals ln(A). Finally, with all variables of the Arrhenius known, a form of the Arrhenius equation is then solved algebraically for the rate constant k at any given temperature.

The Arrhenius equation relates chemical reaction rates to energy (in this case thermal) of the system. In the Arrhenius equation,

$$k = Ae^{-Ea/(RT)} \qquad (1)$$

k is the rate constant and T is the absolute temperature. The other variables are described above. The expression $e^{-Ea/(RT)}$ estimates the fraction of collisions leading to chemical reactions, where e= 2.718 (the base of natural logarithms).

The decay constant k for any reaction, like the decay of collagen, that undergoes a first order logarithmic decay and is unique to a given temperature can be expressed in terms of a half-life using:

$$t½ = \ln(2)/k \qquad (2)$$

The activation energy Ea for the decay of collagen, and biomolecules in general, remains constant, whereas the rate constant (and hence half-life) varies with temperature. An average annual temperature for the history of a buried bone is substituted for T in the Arrhenius equation (Equation 1) in order to calculate a decay rate estimate for that temperature. Buckley *et al* (2008) used 7.5°C, the average annual temperature of regions in Montana where collagen has been recovered and sequenced from dinosaur bone [9]. However, those dinosaur-containing sediments captured swamp-like flora and fauna that represent higher past temperatures [11]. Colder temperatures afford fewer molecular collisions, which equate to fewer chemical reactions and higher fidelity protein preservation.

Collagen decay rate experimental results build a temporal expectation that restricts bone collagen to archaeological time frames, yet many reports of collagen and other proteins in older-than-archaeological samples have sprinkled the paleontological literature for decades.

Tension between the expectation of lability and observations of longevity has fueled steady debate over the veracity of original biochemistry remnants in fossils [12,13].

Even strictly archaeological samples exhibit disparity between thermal age and standard age assignments. The molecular integrity of bone collagen in a sample is compared to a collagen decay curve to determine its thermal age. In one study, Buckley and Collins determined thermal (collagen-based) ages for 65 archaeologically dated bones [7]. Every one showed a higher standard age than thermal age. The standard ages in their samples (range 2,500a to 1,500,000a) exceeded collagen content-based ages (range 1,584a to 144,862a) by two to tenfold. Original bone collagen in samples bearing even older standard ages highlights the mystery that underlies this disparity. By “original” is meant that the biochemical or even whole tissue under investigation came from the fossilized animal and not some contaminant like recent microbial growth. Hence, “The idea that endogenous molecules can be preserved over geological time periods is still controversial” [14]. Recent reports have sparked new interest in modes of collagen preservation and in technologies with very high collagen detection sensitivity. This question remains an active area of research and additional data should add new insights to questions related to this controversy, such as the expected longevity of particular proteins in particular settings.

Some mechanisms proposed to extend protein preservation beyond experimental expectations center around mineral interactions. Collins *et al* (2000) [15] found that close association of osteocalcin—a common bone protein—with bone mineral enhances its preservation potential far beyond that in aqueous solution. However, proteins in aqueous solution do not match any realistic burial and long-term protein survival scenarios. Thus, mineral association may explain how osteocalcin lasts longer in bone than in a watery ‘soup’, but falls short of predicting that original proteins in fossils should remain detectable until today.

Given the well characterized kinetics of bone collagen, researchers have investigated possible mechanisms of collagen preservation. Schweitzer and colleagues argued in 2013 for iron-mediated cross-linking via Fenton reactions [16]. In this reaction series, Fe II produces a free radical as it oxidizes to Fe III. The Fe III then makes another free radical via reduction back to Fe II. As non-selective oxidants, the free radicals become available to potentially cross-link nearby organic molecules and produce intermolecular bonds. Cross-linking would produce a more resistant biopolymer. The report of Bertazzo *et al* 2015 of collagenous material in a dinosaur claw invoked this mechanism [17].

No serious rebuttals of iron preservation have yet emerged, but the proposition does leave unanswered questions. For example, Bertazzo *et al* reported evidence of methionine. Since free radicals are non-selective, they would have oxidized methionine to methionine sulfoxide, for example. Even without free radicals it is highly implausible that native methionine would persist through geologic time. The same argument arises with each amino acid sequenced in degraded collagenous peptides described below. Along these lines, Fenton reactions are known as highly destructive to organic compounds. What mechanism would select preservation-friendly cross-linking over the more stoichiometrically preferred destructive reactions remains unknown.

More recently, Wiemann and colleagues proposed Maillard reactions that cross-link carbohydrates with proteins to generate Advanced Glycosylation Endproducts (AGEs) [18]. Fig 2 outlines a generalized Maillard reaction. In this scenario, AGEs form on a tissue surface. The recalcitrant polymer, which has characteristic Raman spectra, stiffness, and darkening, shields adjacent organic remains. This mechanism may help explain dark, stiff tissue, but not discoveries of white or clear, flexible apparently proteinaceous residues in fossils. More research into AGE kinetics would help evaluate the feasibility of this model. Last, synchrotron FTIR and small-angle X ray scattering (SAXS) helped characterize collagen and elastin in *T. rex* blood vessel structures [19]. These authors seemed to have stacked iron-mediated Maillard reactions onto Fenton chemistry to cross-link collagen molecules in epithelial vascular tissue. They report evidence of a thin distribution of the iron-rich mineral goethite on fossil blood vessel surfaces in support of the combined mechanisms.

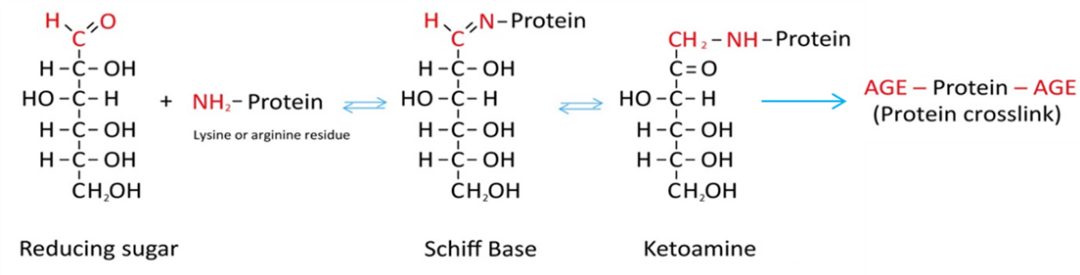

Figure 2 | Generalized Maillard reaction. Carbonyl groups on a sugar reduce neutrophilic amino groups on a protein. The resulting reversible Schiff base can stabilize, then yield glycation end products that include protein crosslinks.

Calculations that blood has sufficient iron, a mechanism to extract iron from erythrocytes and blood vessels, and a means of distributing iron across vessel outer surfaces would add

feasibility to the cross-linking mechanism. Also, kinetics that suggest extraordinary longevity for the resultant AGE's and intermolecular cross-link products (which after all are still essentially organic and therefore chemically labile) remain lacking. Even the most recalcitrant proteins have shelf-lives [20]. Although cross-linked polymers may last longer, abundant evidence including sequencing suggest the presence of highly (but not yet totally) degraded proteins. Thus, descriptions of degraded proteins in Mesozoic strata, summarized below, remain enigmatic [21]. New technologies and applications have arisen that have the potential to probe biomolecular deterioration from new angles.

4. Original biochemistry claims in archaeological and Cenozoic specimens

A survey of older techniques for bone protein characterization sets a backdrop against which the potential virtues of new techniques can be better appreciated. Historically, bone proteins from archaeological remains have been targeted much more often than those from paleontological remains. Subfossil bone proteins can vary from abundant and easily detectable to barely present, and even absent, from archaeological settings. These observations, coupled with short protein lifespans relative to geologic time, have dissuaded investigations of bone proteins from more deeply buried fossils, for example from Mesozoic or Paleozoic Erathems. Thus, archaeological and even Upper Cenozoic bone samples often harbor abundant and dense bone proteins, all time-altered to one degree or another. Such high abundances lend themselves to analysis by crude and inefficient techniques like protein extraction and weighing on an analytical balance. More sensitive technologies are required to detect faint and highly degraded protein traces that may persist in certain rare fossil samples.

Classic techniques like immunohistochemistry have been used to identify such proteins as hemoglobin [22] and albumin [23] in archaeological bone. These techniques take advantage of antibody-antigen specific molecular interactions. Antibodies of targeted biochemicals are added to usually demineralized bone tissue. If the target biochemical is present, antibody binding takes place. Unbound antibodies are washed off. Fluorescent markers are then attached to the exposed end of the antigen-bound antibody molecules, and excess markers are washed off. Micrographs record fluorescence of the biomolecular *in situ* patterns.

Decades of radiocarbon dating have supplied a robust and longstanding verification of the presence of endogenous protein in ancient bone. Collagen is routinely extracted for radiocarbon and other analyses, typically using some variation of the Arslenov method. Briefly, extraction involves dissolution and removal of bone mineral in acid, followed by

gelatinization of collagen via heating under a weak acid. Researchers require analytical evidence that the collagen extracted from archaeological bone has little or no contamination in order for radiocarbon age dates to be considered valid. Therefore, rigorous and repeated tests have demonstrated the reliability of extracting primary, as opposed to external secondary or exogenous, sourcing of bone collagen. The thousands of published radiocarbon ages obtained from extraction of proteins (collagen) from mineralised tissues including shell, tooth, and bone attest to the general abundance of proteinaceous material in archaeological sites.

In addition, the presence of bone collagen is regularly confirmed by protein sequencing of archaeological samples. This process begins with protein extraction protocols similar to those used in preparation for radiocarbon dating. Protein extracts are purified then digested with a selected enzyme (a protease) known to preferentially catalyze protein backbone hydrolysis at specific amino acid sites. The resultant mixture is separated and mass analysed typically by high performance liquid chromatography/ tandem mass spectrometry (HPLC-MS-MS) or MALDI-TOFMS. The measured molecular masses from the digested extract can then be compared with known collagen fragments so that original collagen sequences can be digitally reconstructed.

A single Siberian mammoth bone yielded 126 unique, partly intact protein types, detected by tandem mass spectroscopy. This exemplifies many Cenozoic proteins including the ‘gold standard’ technique of sequencing [24]. This one discovery contains far more unique protein remnants than the total collection of biochemicals found in the entire Mesozoic so far sampled, illustrating both the potential abundance of original biochemistry in Cenozoic fossils, and the disappearance of that biochemistry due to degradation over time. Another more recent report describes the oldest original animal lipid. The pygidial (preening) gland from an extinct bird captured in Germany’s Messel Shale preserved still-yellow oil, now waxy [25]. Overall, the abundance of published protein sequences from recent fossil and subfossil specimens attests again to the general abundance of protein remnants in archaeological and even some Cenozoic settings. Some of the same technologies used to reaffirm primary collagen in archaeological bone may be applied to older bone samples.

Few would question the reality of collagen in archaeological bones of high-quality preservation. However, reports of protein remnants including collagen in paleontological bone samples—especially Mesozoic and even older—have been met with skepticism on the basis of collagen decay model projections [9].

5. Original biochemicals and intact tissues from Mesozoic specimens

The vast majority of fossiliferous remnants of soft tissues such as skin, internal organs, body outlines, and nervous tissue occur in the fossil record as mineralisation that records only the shape, but no original biochemistry. However, there are reports of original biochemicals (rarely) in Mesozoic and even more rarely in Paleozoic fossil remains, as Table 1 documents below.

The phrases “soft tissue,” and “soft tissue preservation” appear throughout paleontological literature, but in most cases authors intend to convey “mineralised tissue.” Often, soft parts preserve via phosphatization, sulphurization, pyritization, or kerogenization [26]. Authors also note preservation by silicification, carbonization, phyllosilicate metamorphism, or apatite permineralisation [26]. Except for kerogenization and carbonization, these modes describe minerals that replace the original tissue. The resulting preservation records body organ outlines in whitish, reddish, and golden colors. These and other minerals can co-mingle in the same fossil, depending on mineral availability and complicated internal chemistries likely determined very early in diagenesis. More often than not, authors do not plainly distinguish “mineralised” versus “original” when they refer to soft tissue in fossils. In specimens with mineralised soft tissue, the minerals represent chemical transformations of the original, labile biochemistry into a more resistant material. Mineralisation can occur by hydrothermal action or groundwater precipitation, and even by bacterial degradation of organic components. Partial biodegradation blankets each organ with acid, which causes preferential precipitation of nearby minerals on the order of weeks to months in laboratory experiments [27]. Extreme care is therefore required in sifting literature for those rare instances of original biochemistry.

These common mineralisation modes of soft animal part preservation do preserve gross anatomy [28,29], and in some cases microanatomy [30], but none preserve as much biological or taphonomic information as do primary protein sequences, isotope analyses of primary biominerals, or original organically preserved residual or whole tissues. In addition to suggesting how future studies might apply new technologies to test diagenetic scenarios, an even more significant task is to test whether or not new applications of established technologies or altogether new technologies can increase protein detection sensitivity and efficiency enough to help resolve current controversy on bone collagen (and by extension proteins in general) longevity.

Despite the expected rarity of original biochemistry in fossils, a diligent literature search yields dozens of reports. Selected examples from the longer list are described next, followed by a more full compilation in Table 1.

Surprisingly, soft and pliable tissue was described in a *Tyrannosaurus rex* femur in 2005 [31], shown in Fig. 3, with a follow-up report that identified specific biochemicals, including collagen sequence [32]. It included results from immunofluorescence. Schweitzer and colleagues imaged proteins specific to vertebrates, including PHEX and histone H4. The specimen revealed whole osteocytes, whole epithelial and erythrocyte cell-like elements, and extracellular fibrillar connective tissue. Reactions to such fresh-looking biomaterial cited bacterial contamination, and in particular that mucilaginous bacterial biofilm was mimicking vertebrate tissues [33]. In response, the original team added more colleagues who sequenced collagen from the *T. rex* (Museum of the Rockies specimen MOR 1125), found in the Cretaceous Hell Creek Formation of Montana [34].

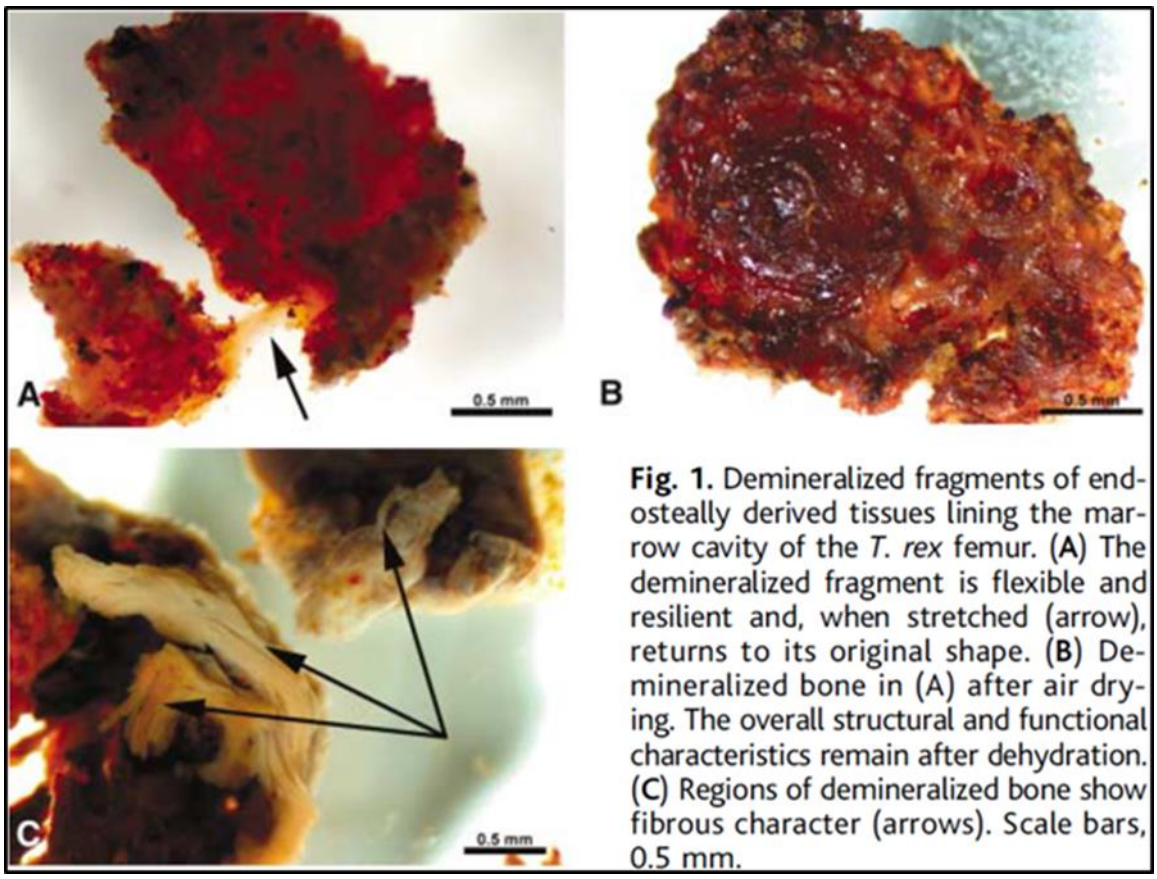

Fig. 1. Demineralized fragments of endosteally derived tissues lining the marrow cavity of the *T. rex* femur. (**A**) The demineralized fragment is flexible and resilient and, when stretched (arrow), returns to its original shape. (**B**) Demineralized bone in (A) after air drying. The overall structural and functional characteristics remain after dehydration. (**C**) Regions of demineralized bone show fibrous character (arrows). Scale bars, 0.5 mm.

Figure 3 | Fig. 1 from Schweitzer et al, Science, 2005.

They applied the same vertebrate-specific protein detection procedures to a *Brachylophosaurus canadensis* (hadrosaur) femur that was extracted using sterile handling techniques from the Cretaceous Judith River Formation. In collaboration with an external laboratory to perform collagen sequencing, the report confirmed elastin and laminin bone proteins as well as collagen [35]. An additional publication on this specimen described phenomena characteristic of modern blood vessels, including translucence, pliability, and reaction to immunological staining for collagen and other epithelial proteins.[36] In these cases and others below, tissue morphology is consistent with endogenous proteinaceous remnants, despite the counter intuitive protein longevity that such conclusions imply.

Another study published low magnification micrographs of a large, soft and pliable brownish sheet of fibrillar tissue exposed in a *Triceratops horridus* (HTC 06) horn core from the Hell Creek Formation of Northern Montana [37]. The study authors noted that no known bacterial biofilm rebounds after stretching. They also used scanning electron microscopy (SEM) to image osteocytes that retain their slender processes.

Polish researcher Pawlicki's investigations of dinosaur biomaterial included electron micrographs of bone tissue from a Gobi Desert *Tarbosaurus bataar* in 1966 [38], 1998 [39], and are ongoing [40]. The 1998 report noted, "the descriptions presented confirm that the morphology of the vascular canals in dinosaur bones and the bones of modern reptiles is the same" [39]. Tissue morphology is consistent with, but not proof of, original biochemistry. His papers reveal osteocytes, collagen fibers, and unusually, a positive immunoassay for DNA in dinosaur osteocyte nuclei [41]. Thus, although the early literature reported results from less technologically advanced detection methods than are available today, the reported results are consistent with results from more sensitive, sophisticated and accurate techniques of today.

Electron microscopy was again used in 2008 to compare naturally mummified *Psittacosaurus mongoliensis* skin to modern collagen fiber bundles [42]. The same researcher, South Africa's Theagarten Lingham-Soliar, published images of original skin coloration in a separate *Psittacosaurus*, also from China. Apparently unaltered pigments including carotenoids and melanins were described in the specimen [43]. Researchers working in southern China reported spectra consistent with endogenous protein from a Jurassic embryonic sauropod *Lufengosaurus* femur [44].

A well-preserved nodosaur *Borealopelta* found upside-down in shale sand preserves the outline of skin and shows every spike and nodule of the animal's dorsal surface.

Spectroscopy and mass spectrometry revealed melanin pigments from which the animal's coloration scheme was reconstructed [45]. Given their large molecular weights, insolubility, and stable molecular geometries, pigments are expected to last longer than many proteins, but skin pigment decay rate studies are lacking.

6. Cretaceous paleoproteomics

The gold-standard for protein detection is sequencing by mass spectrometry. A half dozen papers report short sequences from Cretaceous samples, though they remain contentious . Asara and coworkers published the tryptic peptide collagen sequence GVQPP(OH)GPQGPR and five others from *T. rex* femur bone extract [46]. Ancient protein experts Buckley and Collins led a study to refute that claim. Their rebuttal paper showed experimental decay data to substantiate the argument that collagen cannot last that long [9]. In response, Asara and Schweitzer relisted all six *T. rex* collagen peptides and noted the multiple separate contaminations required to produce their data as unlikely [47]. Mass spectrometrist Bern and colleagues reanalyzed the *T. rex* mass spectra and found no problems with the initial results [48]. Their re-analysis also revealed fragments of hemoglobin sequence. More recently, Saitta and coworkers did not find collagen, but did find microbes in a Cretaceous Centrosaurus bone [49]. They concluded that published reports mistook bacterial contamination for Cretaceous collagen despite the possibility of collagenous signals in bones other than their selected sample.

The 2009 *B. canadensis* referenced above included for example the collagenous peptide ion GLTGPIGPP(OH)GPAGAP(OH)GDKGEAGPSGPPGPTGAR [35]. Additional dinosaur peptides from Schweitzer's lab showed collagen α1 and α2 remnants plus actin, tubulin α-1A, and histone H4 sequence data [50]. Improved techniques then expanded the *B. canadensis* proteome [51].

More examples of apparently endogenous fossil bone proteins include non-collagen protein fragment sequences from an *Iguanodon* bone housed at the Natural History Museum of London [52]. Researchers long ago described amino acids from a New Mexico *Seismosaurus* [53] and even in fossil shells [54]. The now-dated 1980 book *Biogeochemistry of Amino Acids* noted, "work with dinosaur remains demonstrated that enough protein for analysis could often be recovered from bones and teeth as old as the Jurassic" [55]. The history of reported fossil proteins and possible protein constituents therefore contrasts with

the widespread concept that Mesozoic and earlier-deposited fossils all represent mineralised artifacts that contain no original biological material.

Many workers rightly maintain skepticism despite direct and indirect protein detection, using multiple techniques including mass spectrometry to describe proteinaceous or other biochemicals endogenous to Mesozoic strata. The skepticism is usually on the basis of kinetics e.g. “The identification of still-soft tissues and cellular structures in a suite of Mesozoic fossils, and claims of endogenous proteins preserved within these materials, is controversial because it challenges both conventional wisdom and theoretical kinetics, which preclude the persistence of proteins over geological time scales” [19]. Therefore efforts to explore mechanisms for protein preservation increase and are summarized below.

7. Original biochemistry from Paleozoic and older specimens

Most Paleozoic soft tissue data are recorded from mineralized or completely altered fossil material. A recent review of the 13 most prominent Burgess-Shale Type BST fossil sites concluded, “However, in the great majority of cases it is a carbonaceous film alone that defines the overall morphology of the fossils” [56]. Preservation modes like the BST also occur in more recent geologic settings, including the Cretaceous. Possibly subtle differences in sedimentation rates vary the time during which a carcass experiences sulphate-reduction microbial action that leads to pyritization versus methanogenic microbes that lead to kerogenization and the production of carbonaceous films [57].

A handful of Paleozoic fossil biomaterial claims appear in the literature. Their lower numbers in lower strata could result from workers not focused on original biochemicals in those settings, or more likely from their actual rarity.

Graptolites occur in Paleozoic strata and today. They are small, worm shaped marine creatures that secrete tube-shaped organic thecae, thought to be composed of collagen or chitin. The periderm of some Ordovician graptolites exhibited collagen-like structures imaged by wide-angle X-ray diffraction in 1972 by Towe and Urbanek [58]. The researchers found a few amino acids, but not the 4-hydroxyproline or 5-hydroxylysine characteristic of collagen. The Towe and Urbanek results were thus not definitive for original collagen, but were consistent with altered collagenous or chitinous residues, and suggest that graptolite fossils, which occur worldwide, warrant further investigation. Much later, X-ray absorption near edge structure (XANES) spectromicroscopy showed organic functional group distributions in

Paleozoic scorpion and false scorpion exoskeletons that were consistent with original chitin and chitin-associated protein [59].

The prevailing paradigm for British Columbia's Burgess Shale fossils holds that the flattened soft-bodied creatures consist merely of impressions, mineralised (for example pyritized) outlines of soft tissue, or kerogen. However, a German and Russian team used fluorescence microscopy, Fourier Transform Infrared (FTIR) microscopy, high-performance capillary electrophoresis, high pressure liquid chromatography, and mass spectroscopy to identify chitin in the Burgess sponge, *Vauxia gracilenta* [60]. Although not a protein, chitin is labile enough for its presence in fossils to be considered problematic, although chitin kinetics are not known.

Surprising preservation was also described in still-flexible, proteinaceous marine tube worm tubes. Extracted from Siberian drill core samples of Ediacaran strata, Moczydlowska described the worm casings as not mineralised, and original to the worms [61]. Comparison revealed direct correspondence with the chitin-structural protein composition of worm casings in extant siboglinid counterparts.

Plant fossils also may retain original organics. FTIR spectra match altered biomolecular signatures between extant and extinct Cretaceous Aroucarians, cycads, and Ginkgoales leaves [62]. Similarly, FTIR and Raman mapping of a completely permineralised Jurassic fern (Osmundaceae) revealed diagenetically altered organic cellular components 'frozen' in various stages of cell growth [63].

8. Persistent Controversy

Table 1 lists dozens of biochemical detection reports throughout the scientific literature. Despite the large number of reports, skepticism over original biochemical fossils persists. A 2017 report attempted to refute dinosaur-specific collagen sequences published in 2009 by showing a mismatch between expected and reported sequences, and by suggesting instrument contamination with modern sample [13]. However, it is difficult to define an expected sequence when no living samples exist for comparison, and the instrument contamination scenario calls into question the protein sequencing methodology *per se.* It is likely that contention will persist until some explanation is agreed that includes protein kinetics.

8.1 Five trends in 85 biomaterial fossil reports

Table 1 lists the biochemical identified in each fossil. Where morphology instead of biochemistry was identified, the table lists the apparently organic component. Table 1 also gives a Genus or more general taxon of the animal from which the listed biomaterial derived. The Table also notes geologic and geographic settings for each find. Following this, Figs 4, 5, and 7 summarize chronologic, geographic, and stratigraphic trends, respectively, that 85 apparently original biochemistry fossil publications reveal. The table emphasizes Mesozoic fossils for two reasons. A list of Cenozoic fossils would grow long and unwieldy. Also, Mesozoic and older fossils are the most challenging to explain and are thus the focus of the most intense scrutiny. This compilation reveals five trends that suggest research questions for future investigation.

Taxon and associated biochemical	**GSA* System, Age**	**Formation, Geography**	**Year Published**
Dinosaur bone collagen & vessels	Cretaceous, Campanian	Gobi Desert, Mongolia	1966 [38]
Megalosaurus egg shell protein	Jurassic, Bathonian	Rognacian Fm., S. France	1968 [64]
Sauropod limb hydroxyproline	Jurassic, Kimmeridgian	Morrison Fm., CO	1968 [65]
Dinosaur proteins and polysaccharides	Cretaceous, Maastrichtian	??	1974 [66]
Dinosaur gelatinised collagen	Cretaceous	Western US	1976 [67]
Mollusk shell glycoproteins	Cretaceous	Coon Creek Fm., Tennessee	1976 [68]
"Dinosaur bones" parallel collagen fibers	Cretaceous, Campanian	Mongolia	1985 [69]
Seven hadrosaurs' unfossilised bones	Cretaceous, Campanian	Upper Colville Grp., Alaska	1987 [70]
Tyrannosaurus tooth hydroxyproline	Cretaceous, Campanian	Judith River Fm., Alberta	1990 [71]
Diplodocus vertebra proteins	Jurassic, Upper	Morrison Fm., Brushy Basin member, NM	1991 [53]

Lambeosaurus osteocalcin	Cretaceous, Campanian	Alberta	1992 [72]
Various dinosaurs, organic material	Cretaceous, various	Judith River Fm., Alberta	1993 [73]
Hymenaia protera (extinct tree) chloroplast DNA	Eocene, Upper	La Toca mine, Hispaniola	1993 [74]
Amber insects with unaltered amino acids	Cenozoic, Eocene, Miocene	Dominican Amber	1994 [75]
Tarbosaurus osteocyte DNA	Cretaceous, Campanian	Gobi Desert, Mongolia	1995 [41]
Tyrannosaurus bone heme	Cretaceous, Maastrichtian	Hell Creek Fm., MT	1997 [39]
Tyrannosaurus DNA, amino acids	Cretaceous, Maastrichtian	Hell Creek Fm., MT	1997 [76]
Tarbosaurus blood vessels	Cretaceous, Campanian	Gobi Desert, Mongolia	1998 [39]
Shuvuuia feathers β-Keratin	Cretaceous, Upper	Ukhaa Tolgod, SW Mongolia	1999 [77]
Rahonavis (extinct bird) keratin	Cretaceous, Maastrichtian	Madagascar	1999 [78]
Scelidosaurus skin layers, cells, dermal scales	Jurassic, Pleisenbachian?	Lias Group, England	2000 [79]
Tyrannosaurus collagen SEM scans	Cretaceous, Maastrichtian	Lance Fm., Newcastle, WY	2001 [80]
Jeholopterus skin, fibers	Cretaceous, Barremian	Yixian Fm., Ningcheng, Mongoloa	2002 [81]
Iguanodon osteocalcin protein	Cretaceous, Aptian	UK	2003 [52]
Micrococcus (non-spore-forming bacteria) alive in amber	Cretaceous, Aptian	Lebanese amber, Mt. Hermon, Israel	2004 [82]
Tyrannosaurus soft, flexible connective tissue	Cretaceous, Maastrichtian	Hell Creek Fm., MT	2005 [31]

Titanosaur egg ovalbumin	Cretaceous, Upper	Rio Colorado Fm., Bajo de la Carpa Member, Argentina	2005 [83]
Enantiornithine embryo collagen	Cretaceous, Upper	Rio Colorado Fm., Argentina	2005 [84]
Frog bone marrow	Miocene, Tortonian	Libros Basin infill, NE Spain	2006 [85]
Tyrannosaurus collagen	Cretaceous, Maastrichtian	Hell Creek Fm., MT	2007 [46,86,87]
Triceratops blood vessels	Cretaceous, Maastrichtian	Hell Creek Fm., MT	2007 [88]
Feather melanocytes	Cretaceous, Albian	Crato Fm., Brazil	2008 [89]
Leaf fragments in mummified *Brachylophosaurus* gut	Cretaceous, Campanian	Judith River Fm., MT	2008 [90]
Psittacosaurus skin fibers	Crecaceous, Barremian	Jehol Biota, Yixian Fm., Liaoning	2008 [42,91]
Brachylophosaurus blood vessels, collagen sequence, elastin, laminin	Cretaceous, Campanian	Judith River Fm., MT	2009 [35]
Bird feather, purple pigment	Eocene, Bartonian	Germany, Messel shale	2009 [92]
Hadrosaur skin cell structures	Cretaceous, Maastrichtian	Hell Creek Fm., ND	2009 [93]
Salamander muscle, whole	Miocene, Burdigalian	Ribesalbes Lagerstätte, NE Spain	2009 [94]
Stegosaurus plate keratin	Jurassic, Upper	Howe Quarry, Morrison Fm., WY	2010 [95]
Sinosauropteryx melanosomes	Cretaceous, Aptian	Jehol Group, China	2010 [96]
Psittacosaurus skin scales and pigment	Cretaceous, Aptian	Jehol Biota, Yixian Fm., Liaoning	2010 [43]

Mammal hair in amber	Cretaceous, Albian	Archingeay-Les Nouillers, Charente-Maritime, France	2010 [97]
Archaeopteryx original elements	Jurassic, Tithonian	Solnhofen, Bavaria	2010 [98]
Penguin melanosomes	Eocene, Priabonian	Yumaque Point, Paracas Reserve, Peru	2010 [92]
Mosasaur humerus Type I collagen	Cretaceous, Maastrichtian	Ciply Chalk, Belgium	2011 [99]
Scorpion chitin and chitin-associated protein	Pennsylvanian, Moscovian	Cave fill, N. Illinois	2011 [59]
Eurypterid chitin and chitin-associated protein	Silurian, Upper	Williamsville Fm., Ontario	2011 [59]
Pterodactylus actinofibrils	Jurassic, Upper	Solnhofen, Bavaria	2011 [100]
Lizard tail skin breakdown products	Eocene, Bartonian	Green River Fm., Wyoming	2011 [26]
Tyrannosaurus and *Hadrosaur* Type I collagen	Cretaceous, Maastrichtian	Hell Creek Fm., MT	2011 [6]
Cuttlefish ink sac	Jurassic, Oxfordian	Blue Lias Fm., Lyme Bay, England	2012 [101]
Turtle osteocytes	Jurassic, Tithonian	Mongolia	2012 [102]
Tyrannosaurus and *Brachylophosaurus* actin, tubulin, histone, PHEX, DNA	Cretaceous, Maastrichtian; Campanian	Hell Creek Fm.; Judith River Fm., MT	2013 [32]
Lufengosaurus embryo bone protein	Jurassic, Sinemurian	Upper Lufeng Fm., Yunnan	2013 [44]
Triceratops osteocytes; soft sheets of fibrillar bone	Cretaceous, Maastrichtian	Hell Creek Fm., MT	2013 [37]
Mosquito gut hemoglobin	Eocene, Lutetian	Kishenehn Fm., MT	2013 [103]
Crinoid original organics	Mississippian, Tournaisian	Edwardsville Fm., IN	2013 [104]

Vauxia (sponge) chitin	Cambrian, Age 3	Burgess Shale, British Columbia	2013 [60]
Gastropod egg chitin	Jurassic, Hettangian	Skłoby Fm., Poland	2014 [105]
Sabellidites (tube worm) chitin, protein	Proterozoic, Ediacaran	Nekrasovo & Rovno Fm.'s, Russia	2014 [61]
Mosasaur melanin, melanosomes	Cretaceous, Coniacian	Boquillas Fm., TX	2014 [106]
Icthyosaur eumelanin in melanosomes	Jurassic, Sinemurian	Blue Lias Fm., Lyme Bay, England	2014 [106]
Brachylophosaurus blood vessels, collagen sequence	Cretaceous, Campanian	Judith River Fm., MT	2015 [36]
Dinosaur collagen and red blood cells	Cretaceous, Campanian	Dinosaur Park Fm., Alberta	2015 [17]
Dinosaur melanosomes and pigment	Jurassic, Tithonian	Tiaojishan Fm., Liaoning	2015 [107]
Rhamphyorhynchus orange claw material	Jurassic, Tithonian	Solnhofen, Bavaria	2015 [108]
Proteinaceous amide groups in chert	Paleoproterozoic, Orosirian	Gunflint Fm., Ontario	2016 [109]
Nothosaurus, *Protanystropheus* blood vessels, etc.	Triassic, Olenekian	Gogolin Fm., S. Poland	2016 [40,110]
Psittacosaurus keratin in skin	Crecaceous, Barremian	Jehol Biota, Yixian Fm., Liaoning	2016 [111]
Citipati (oviraptor) claw Beta-keratin	Cretaceous, Campanian	Djadokhta Fm., Mongolia	2016 [76]
Dinosaur eggshell protoporphyrin, biliverdin	Cretaceous, Maastrichtian	Hougang, Tangbian, & Nanxiong Fm.'s, China	2017 [112]
Lufengosaurus adult rib collagen	Jurassic, Sinemurian	Upper Lufeng Fm., Yunnan	2017 [113]

Borealopelta (Nodosaur) melanin plus other biochemicals	Cretaceous, Aptian	Clearwater Fm., Alberta	2017 [45]
Mammalian erythrocytes in tick gut	Upper Paleogene	El Mamey Fm., Dominican Amber	2017 [114]
Brachylophosaurus collagen re-confirmed	Cretaceous, Campanian	Judith River Fm., MT	2017 [51]
Araucaria cuticle	Cretaceous, Late	Shag Point Fm., New Zealand	2019 [62]
Messelirrisorid bird uropygial gland, yellow oil	Cenozoic, Eocene	Messel Shale, Hesse, Germany	2017 [25]
Hadrosaur bone collagen images	Cretaceous, Maastrichtian	Hell Creek Fm., MT	2018 [115]
Stenopterygius skin α-keratin and collagen, blubber, liver	Jurassic, Toarcian	Holzmaden, Germany	2018 [116]
Diplodocid bone matrix	Jurassic, Tithonian	Howe-St. Quarry, WY	2018 [18]
Hadrosaur vessels, osteocytes, fibrous bone matrix	Cretaceous, Maastrichtian	Hell Creek Fm., SD	2019 [117]
Osmundaceae (Royal fern) organelle remnants	Jurassic, Sinemurian	Korsarod, Sweden	2019 [63]
Tyrannosaurus elastin	Cretaceous, Maastrichtian	Hell Creek Fm., MT	2019 [19]

Table 1 | Publications of Original Biochemistry claims in Fossils. 85 publications which show the results of more than a dozen different techniques used to detect biochemical signatures. Reports that named “soft tissues” but specified mineralisation, and reports that did not address mineralisation versus original organics, were rejected. Cenozoic original biochemistry fossils are under represented here, since publications that dealt with the oldest, most difficult to explain, samples from Mesozoic, Paleozoic, and lower layers were instead targeted. * GSA = Geological Society of America. In publications that omitted the GSA Age, it was inferred by matching the published age date to the GSA Geologic Time Scale, version 4.0 [118].

The first trend from Table 1 is that taxa from which researchers extract biochemistry vary widely. It seems that no particular taxon is exempt from having organic remnants preserved as fossils. The list includes biochemistry from plants, microbes, amber-encased insects, clay-encased arthropods, sandstone- and mudstone-embedded dinosaurs, shale-bound semiaquatic reptiles and birds, fully aquatic reptiles in limestone, and perhaps most surprisingly, seafloor worm and sponge tissues found below the Cambrian. The list includes representatives from the phyla Monera, Porifera, Annellida, Plantae, Arthropoda, Mollusca (classes Gastropoda and Cephalopoda), Echinodermata, and Vertebrata (classes Mammalia, Aves, Amphibia and Reptilia.) This trend suggests that original biochemistry from additional phyla and lower level taxa await discovery.

Nor do those ancient animals' environmental niches seem to play much of a role in selection of primary organics for fossil preservation. Fossil assemblages and adaptive features of fossil forms suggest their origins from benthic, neritic, lacustrine, tropical, swampland, and perhaps semiarid terrestrial habitats. Taxa representing terrestrial biomes such as arid desert, savanna, temperate forests, taiga and tundra are rare among Paleozoic and Mesozoic fossil biochemicals. This is to be expected from the wetland, marine, and lacustrine environments that Mesozoic strata captured in general, and not to any specific taphonomy or diagenesis that favored or disfavored biochemical preservation. More dry land-living taxa occur in Cenozoic than Mesozoic deposits worldwide. Also, few temperate dwelling taxa—with or without biochemical preservation—occur in Paleozoic strata. If another table was constructed that included Cenozoic biochemistry, it would undoubtedly reveal abundant and widespread biochemical preservation of upland-living taxa [25,67]. In summary, fossilized creatures from land, sea, and sky retain signatures consistent with original biochemical remnants.

A third trend that Table 1 reveals is that of an increased interest in, and investigation of, original biochemistry fossils in recent years. What accounts for the increase in published papers on this topic? A 2005 *Science* paper showed full-color, clear photographs of blood red tissue and still-red erythrocyte-like elements inside blood vessels extracted from the sectioned femur of "B rex" (MOR 1125), a *T. rex* named after discoverer and dig volunteer Bob Harmon [31]. That paper, plus three follow-up reports published in a 2007 issue of *Science* [46,86,87], invigorated fascination in those investigating dinosaur phylogenies as well as controversy in those familiar with the lability of biochemicals. The recent increase in publications shown in Fig. 4 appears to have stimulated some negative reactions [9,119] while also sparking paleoproteomics research. Researchers have investigated proteins and

other biochemistry such as lipids [25], nucleic acids [32], and biological pigments such as melanin [120] and protoporphyrin [112] in fossils.

New techniques with the potential to nondestructively analyze labile organic fossil components have also come online of late. Workers have availed themselves of these techniques in order to gain unprecedented insights into fossil organics and what those data imply for physiology, diet, diagenesis, systematics, and other realms. These reports, including results presented in this paper, aim to solve the mystery of the apparent longevity of labile organic structures, and to explore how far afield and how deeply buried those organics extend.

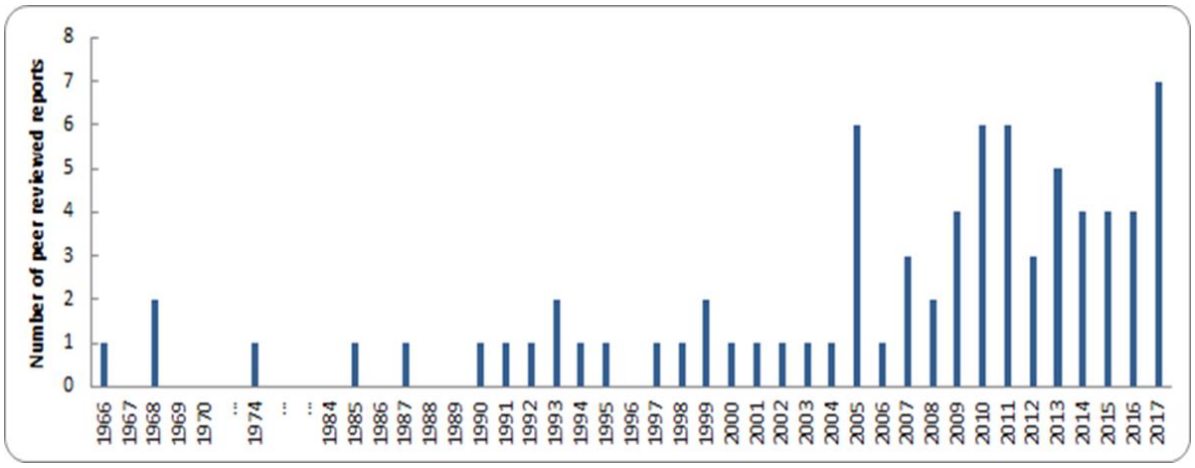

Figure 4 | Original Biochemistry Fossil Publications by Year. The years 1970-1973 and 1975-1984 are represented by ellipses in order to display the data legibly. This list is not comprehensive, but represents the literature accurately enough to reveal temporal trends. Investigators merely dabbled in fossil biochemistry from the 1960's until 2004. Beginning with dinosaur whole tissue discoveries in 2005, and in conjunction with the arrival of new techniques (see text), research has surged.

Fourth, Fig. 5 reveals a preliminary geographic distribution of original biochemistry in fossils worldwide. In Fig. 5, the bone proteins collagen and osteocalcin were marked separately from other biochemicals in fossils because this review focuses on bone fossils. The marked locations of original collagen versus other organics suggest future research into the extent of their overlap. Present data appear insufficient to answer this. Overlap does occur in the American West, the Gobi, and Northern Europe. However, a lack of overlap may simply mean researchers have not yet looked for, or do not have the tools to detect, collagen in locations such as China's Jehol Biota and Southern Europe. For comparison, Fig. 6 shows the distribution of fossils in general using the Paleobiology Database.

The occurrence of original biochemical fossils on most continents suggests that others await discovery on remaining continents Australia, Antarctica, and mainland Africa. Regions with few biochemistry fossils, such as Amazonia, the Sahara and Congo, and the deserts of Western Australia, coincide with regions where few fossils occur in general. This suggests that fossil biomaterial finds may continue to populate many more fossil sites.

The fifth and final trend that emerges from Table 1 pertains to the distribution of apparently original biochemistry fossils throughout the geologic column. The diagram shown in Fig. 7, here taken from the GSA Geologic Time Scale v. 4.0 [118], shows (mostly sedimentary) rock layers not found in any single earth location, but compiled graphically from many separate locations. All three Cenozoic rock Systems were reduced to a single bar at the top merely to show that it was not entirely forgotten in this present analysis, which focuses on earlier material. If the Cenozoic bar displayed a more accurate number of reports, it would stretch across many pages at the scale shown here. Therefore, Fig. 7 more accurately represents the number of reports from strata deposited prior to Cenozoic deposits. Similarly, the entire Precambrian assemblage was reduced to the two Systems from which original biochemistry was reported.

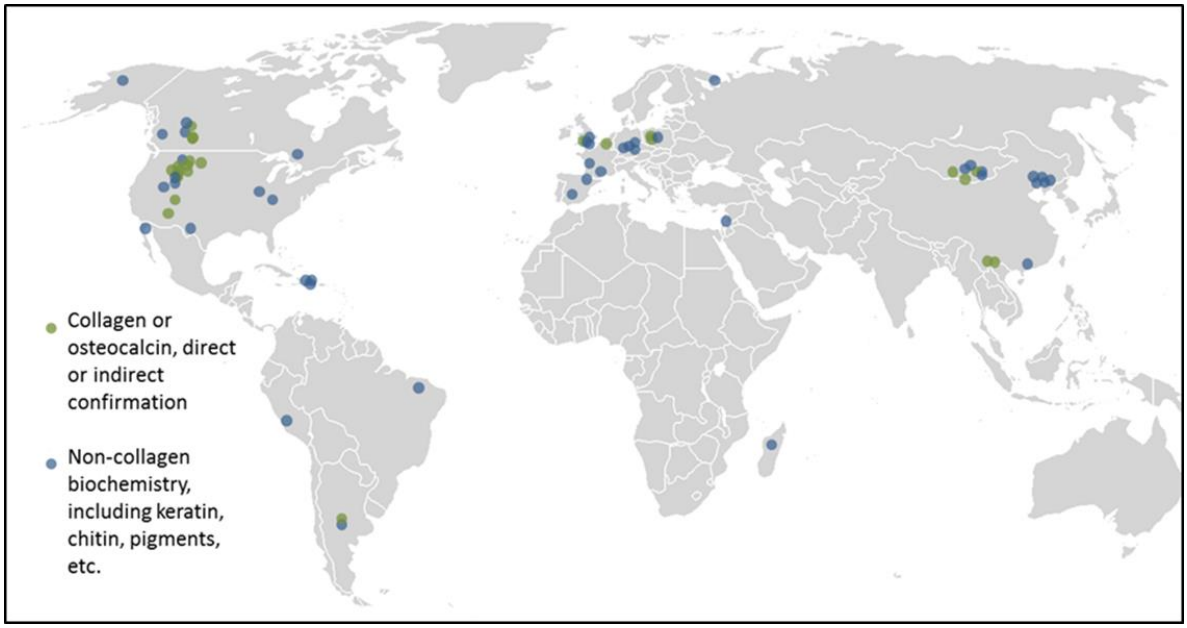

Figure 5 | Global Distribution of Original Biochemistry Fossils. Approximately seventy original biochemistry fossil locations show a non-random worldwide distribution. High concentrations likely reflect a combination of sample accessibility and general fossil distributions (see Fig. 1.5).

Ignoring the Cenozoic, the Cretaceous System has more than double the number of original biochemistry fossil reports than from all other geologic Systems combined. This could be partly due to the lack of heating that these rocks experienced since deposition. Higher temperatures and intense hydrothermal action can transform and redistribute ancient buried vertebrate proteins. The occasional biochemicals reported from pre-Cretaceous fossils suggest that detrimental factors avoided certain pockets of earth's crust over time.

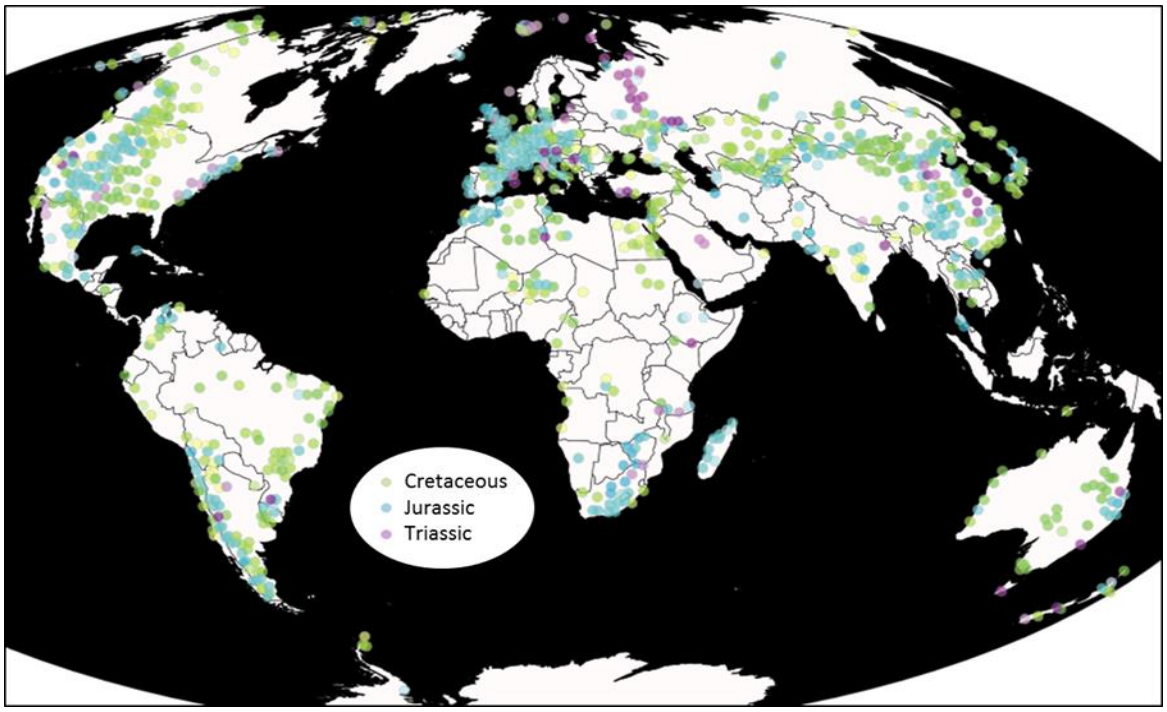

Figure 6 | Global Distribution of 28,834 Mesozoic Reptilia. The Paleobiology Database at paleodb.org was accessed to generate a distribution map of general fossils to compare with the distribution of biochemistry fossils from Fig. 1.4. The data were downloaded on 25 February, 2018, using the filters "Mesozoic" and "Reptilia." Comparison reveals that original biochemical fossils tend to occur wherever fossils are generally found. The 28,834 individual plotted specimens represent, in order of descending abundance, ornithischians, testudines, saurischians, theropods, avetheropods (includes birds), and other reptiles.

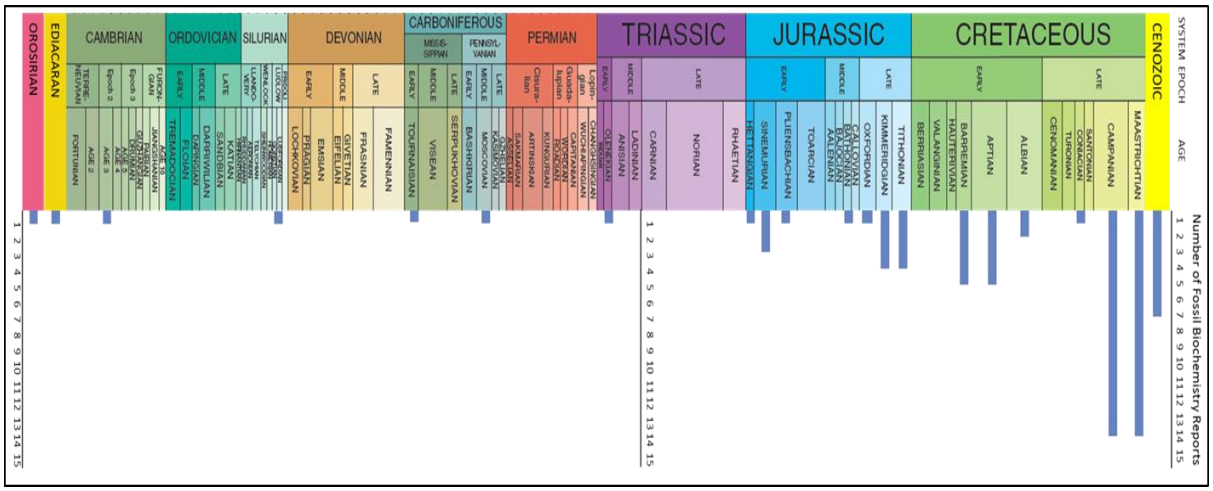

Figure 7 | Geological Distribution of Reported Original Biochemistry in Fossils. This chart emphasizes Mesozoic and Paleozoic rock Systems, as it condenses the entire Cenozoic at the top, and the entire Precambrian at the bottom down to merely the Ediacaran and Orosirian Systems. The data reveal a predominance of biochemistry in Cretaceous System rocks, and a persistent trickle of biochemistry elsewhere.

This geologic distribution shows that there are claims of original biochemistry also in some of the oldest strata that contain any fossils. No reports have yet described them from Permian or Devonian Systems. However, Systems that do preserve original organics or at least decayed remnants of those organics, flank both. The trend of increasing discoveries seen in these reports suggests that original biomaterial discoveries could, assuming adequate research focus and detection tools, begin to fill empty stratigraphic positions in the column diagram.

The five trends gleaned from Table 1 show that apparently original biochemistry is geologically extensive, geographically global, and taxonomically wide-ranging. These published results suggest two hypotheses. First, geographic and stratigraphic ranges for ancient collagen will continue to increase. Second, novel instrumentation and techniques will continue to characterize existing ancient proteins and help detect them in new places.

9. Techniques used to verify biochemistry in fossils

Table 2 notes many of the techniques used to detect original biochemistry in fossils as described in the literature listed in Table 1. Techniques in addition to those shown here, and especially techniques that directly and non-destructively target specific biochemicals, would help to either confirm or annul the hypothesis that published spectroscopic and spectrometric techniques have indeed detected original biochemistry in Mesozoic and lower strata. In addition, most of the techniques used so far involve costly instrumentation and/or

time-intensive preparation as well as considerable expertise to operate and interpret. The development of new technologies or new and accessible applications of established techniques will help add more data from the fossil record in an effort to determine the accuracy and distribution of these discoveries. Increased detection efficiency could also enable future studies to test diagenetic and mechanistic hypotheses proposed for various sites and tissues. Therefore more user-friendly, inexpensive, and rapid techniques would facilitate further increase in fossil biochemical analysis in this research area which is attracting increasing interest.

Technique	**Principle of operation**	**Typical organic targets**	**Advantages and disadvantages**
Collagen extraction	Dissolution of biomineral, separation and purification for weight	Collagen	Specific, low cost technique
Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)	Determination of protein masses by separation according to mass/charge	Any biomolecule with an analogue in database; widely used in protein sequencing	Highly sensitive and specific making MS both qualitative and quantitative over a wide mass range, expensive
Quantitative X-ray Fluorescence (XRF*)*	Displacement of inner shell electrons by X-ray, and detection of resultant fluorescence energy differences	Multi-element analysis, Oxide compound detection	Rapid, non-destructive technique with simple sample preparation, but non-linear and requires knowledge

			of element composition
Light microscopy	Magnification of intact tissue	Whole, intact tissues	Non-specific, general purpose low cost, visualization tool
Immunofluorescence	Antibody-antigen localization in situ, visualized by fluorescent dye	Any targeted proteins	Specific and low-cost technique but can cause sample photobleaching damage
Scanning Electron Microscopy (SEM*)*	Focused electron beam interacts with sample; backscattered electrons and characteristic X-rays detected	Position and morphology of structure revealed	Produces 3D and topographical images rapidly, but instruments are bulky and costly.
Synchrotron radiation Fourier transform infrared spectroscopy (SR-FTIR)	High brightness synchrotron light interacts with molecular structures at submicron resolution to form images by tomography	Quantity, composition, and distribution of proteins; lipid functional groups	Rapid, direct and non-destructive and high resolution, but requires a specialized synchrotron light source
X-ray absorption near edge structure (XANES*)*	Similar to SR-FTIR, but X rays target coordination structures	Multi-element mapping of chelating metals	Specific and sensitive but can be destructive to samples
Fourier Transform InfraRed (FTIR) spectroscopy	IR light absorption by certain molecular arrangements generates characteristic spectra	Vibrational modes of specific bonds	Rapid, direct, versatile, low cost and non-destructive, but limited in sensitivity
Raman spectroscopy	Similar to FTIR but detects Stokes	Vibrational modes of specific bonds	Rapid, specific and non-destructive, but weak Raman signal

	scattered light instead of Raleigh scatter		needs optimized instrumentation
Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)	Ion beam ionizes molecules from sample surface; mass of ions determined by retention time in detector	Organic molecules, e.g., amino acids	Highly sensitive, specific MS technique, analysis time can be long, expensive and bulky equipment
X-ray photoelectron spectroscopy (XPS)	X-ray excites atoms to emit electrons from characteristic shells; detects binding energy of electrons	Multi-element mapping and composition of surface chemistry	Non-destructive, highly sensitive and specific but slow; equipment is expensive and bulky
Energy dispersive X-ray spectroscopy (EDS)	High energy beam induces sample surface to emit X-rays; electron voltage spectrum produced	Multi-element composition information including surface chemistry	Raster imaging over spatial range ($1\mu m^2$ - $1mm^2$), poor energy resolution and low signal-to-background ratio
Matrix-assisted laser desorption ionization (MALDI) mass spectrometry	Laser impinges on sample surface at an angle, ionizes particles; ions enter mass spectrometer	Total surface ions of sample, including protein fragments	Highly sensitive, specific MS technique, including 2D and 3D imaging capability, analysis time long, equipment is expensive and bulky
Second Harmonic Generation (SHG) microscopy	High energy laser in a confocal microscope directly interacts with highly ordered peptide bonds, e.g. collagen	Images tissue in including endogenous proteins *in situ*	Rapid, non-destructive 2D and 3D imaging of specific proteins, equipment is expensive and bulky

Table 2 | Overview of Biochemical Detection Techniques from Table 1 Reports.
Techniques in italics represent more direct biochemical detection methods, and those not in

italics represent less direct detection methods including their advantages and disadvantages.

10. Expert opinion

Research into biochemical remains in fossils has become an important area of proteomics and is the focus of some high-profile research programs. Much of this research has been fuelled by differences in interpretation of protein decay rates and remnants within fossils. It was easier to doubt or dismiss the few descriptions of organic remnants endogenous to fossils when the field was in its infancy, but the profusion of similar discoveries coupled with an increase in sensitive and non-destructive molecular technologies in recent decades has rendered trivialization of original fossil biomaterials increasingly difficult.

As novel and even unforeseen technologies find application in this field, it is our opinion that an increasing weight of evidence will enable researchers to move beyond questions of the mere existence of biomolecular remnants in fossils to reveal unanticipated and fundamental connections across fields including taphonomy, geochemistry, diagenesis, and cladistics. So far, the protein collagen has received attention partly because of its widespread natural abundance in living things, its resistance to degradation over long periods and the relative ease by which it may be detected. As techniques progress, other less abundant and more ephemeral proteins such as globin may become the focus of greater attention.

Of the fifteen techniques noted in our paper (Table 2) none has yet emerged as a widely accessible, standardised test to confirm bone collagen in ancient and especially fossil samples, and several are partially or wholly destructive to the sample. Therefore, an ongoing goal of ancient proteomic research will be to explore inexpensive, user-friendly, broadly accepted, minimally- invasive, and minimally-destructive techniques that will facilitate future searches into the geologic and geographic extent of primary organics in the fossil record.

A weakness of some detection techniques (e.g. Second Harmonic Generation) is that they interact only with highly structural collagen fibres. Different approaches are required to analyse smaller collagenous remnants like collagen subunits. For example, a matrix-assisted laser desorption ionisation (MALDI) Orbi-Trap mass spectrometer generates an ion image of a specified area of a sample surface. It has not yet been used on ancient or fossil bone and offers at least three benefits. First, it could provide an independent verification of the specific distribution pattern of collagen as revealed in the various collagen-specific imaging techniques. Second, it would precisely identify small molecules on the bone surface such as

collagen subunits and collagenous breakdown products. Last, the high sensitivity of Orbi-Trap instruments suggests that a MALDI Orbi-Trap could map even smaller collagenous remnants than the collagen fibres that SHG visualizes and detect even smaller concentrations. It would thus non-destructively help verify SHG or other imaging results of ancient bone collagen while providing new insights on bone protein decay *in situ*.

Infrared spectroscopy, including FTIR and Raman, are attractive to ancient proteomic research because of their low cost, ease of use, low sample destruction requirements, and because they can target very specific chemical bonds in situ. A particularly useful operating mode for FTIR studies called Attenuated Total Reflectance (ATR) interfaces with finely powderised material. Its application to ancient bone collagen has found use through the recently established collagen-specific carbonyl-to-apatite peak ratio in the forensic analysis of bone.

Although IR techniques can confirm the presence of amide bonds in a sample via the amide carbonyl, they share a weakness in their inability to identify specific proteinaceous sources. In conjunction with published techniques such as protein sequencing by GC-MS/MS, MALDI Orbi-Trap would address this by identifying specific ionised proteinaceous biomaterials.

Neither infrared spectroscopy nor SHG imaging can as yet rapidly screen bone samples or regions within a bone most likely to preserve primary protein. Energy-dispersive X-ray spectroscopy (EDS) examines the distribution of elements at various points of an exposed bone surface. EDS can identify the elemental composition of original bone mineral, as opposed to secondary mineralisation, to explore general bone contexts that suggest likelihood of original bone protein preservation. It has been used as an independent verification of molecular techniques, and could be used to verify other spectroscopic, microscopic, and spectrometric techniques when applied to the same samples. In addition, the elemental mapping potential of EDS would more comprehensively establish the composition of the sample material.

How broadly do ancient bone proteins extend geographically over the earth? How deeply do they extend beneath earth (in the geological column)? The results so far reported show that from the seven continents, only two have not yet divulged fossils with original biochemistry. The widespread geography of proteinaceous materials reported within fossils suggests that it may only be a matter of time until fossil biochemistry is found on all continents. Similarly, the extensive stratigraphic coverage of ancient proteins suggests that strata long thought to have no chance of protein preservation deserve renewed attention.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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