

A Chemical Framework for the Preservation of Fossil Vertebrate Cells and Soft Tissues

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Abstract

Reports of preserved cells and other soft tissues in pre-Pleistocene vertebrates, including dinosaurs, have been met with controversy within the field of vertebrate paleontology. To explain such reports, Schweitzer et al. (2014) hypothesized that iron-mediated radical crosslinking preserves ancient soft tissues in a manner somewhat analogous to histological tissue fixation. In 2018, Wiemann et al. proposed a second hypothesis that these soft tissues were preserved as advanced glycation/lipoxidation end products (AGEs/ALEs). The biogeochemistry underlying these hypotheses, however, remains poorly described for fossil vertebrates.

This review posits a novel chemical framework describing the persistence of biological “soft” tissues into deep time. The prior iron-mediated radical crosslinking and AGE/ALE mechanisms are re-described in context of established chemistry from a diversity of scientific fields. Significantly, this framework demonstrates the hypotheses presented by Schweitzer et al. (2014) and Wiemann et al. (2018) are, in many cases, subsequent steps of a single, unified reaction mechanism, and not separate hypotheses. Knowledge of the chemical mechanisms underlying vertebrate soft tissue preservation has direct implications for molecular paleontology and archeology, including efforts at molecular sequence recovery within the ancient DNA and paleoproteomic communities. Such implications that are immediately apparent from examining the chemical framework are discussed.

I. Introduction

A growing number of studies have reported the recovery of cells and other soft tissues within ancient vertebrate remains, including those of dinosaurs (Armitage & Anderson 2013; Boatman et al. 2019; Cadena 2016; Cadena 2020; Lee et al. 2017; Lindgren et al. 2018; Lindgren et al. 2011; Manning et al. 2009; Schweitzer 2011; Schweitzer et al. 2007; Schweitzer et al. 2005; Schweitzer et al. 2013; Schweitzer et al. 2009; Surmik et al. 2016) (Figure 1(A-B)). These reported soft tissues initially generated controversy within the field of vertebrate paleontology and were challenged as being exogenous biofilms (Kaye et al. 2008; Saitta et al. 2019). Later experiments refuted claims that these structures were the result of biofilms, thus supporting their endogeneity (Armitage & Anderson 2013; Schweitzer et al. 2016; Wiemann et al. 2018). However, the notion that soft tissues are unlikely to preserve within mineralized vertebrate remains is questionable on its own. The fossil fuels used daily by society consist of original

biomolecules of ancient plants and microorganisms that have been chemically transformed into carbonaceous macromolecules referred to as kerogens (Tegelaar et al. 1989; Tissot & Welte 1984; Vandenbroucke & Largeau 2007). In cases such as with coalified fossil wood, for example, this conversion of biomolecules towards kerogen macromolecules can preserve original tissue morphology (Gupta 2015; Gupta et al. 2007a; Mustoe 2018). The fields of soil and petroleum science even accept that recalcitrant biomarkers can preserve through time as portions of these highly crosslinked kerogen macromolecules (Ferrer et al. 2018; Gupta 2014; Philp & Gilbert 1987; Westbroek et al. 1979). Further, the preservation of biological tissues is a phenomenon well known to occur within invertebrate fossils. For example, pre-Pleistocene arthropod cuticles have been experimentally shown to consist of hydrocarbons, and in some cases nitrogenous residues (Cody et al. 2011; Gupta et al. 2007d; Stankiewicz et al. 1997). Such findings support that the original cuticle is still present and has not been fully replaced by exogenous minerals. Multiple publications have hypothesized such preservation is the result of crosslinking and chemical transformation of the original cuticular chitin and proteins (Cody et al. 2011; Goth et al. 1988; Gupta 2014; Gupta et al. 2007d; Stankiewicz et al. 1997). The possibility that similar mechanisms are responsible for the persistence of endogenous soft tissues, and potentially even biomarkers or other biomolecules, protected within the mineralized remains of dinosaurs and other ancient vertebrates could thus be a viable hypothesis.

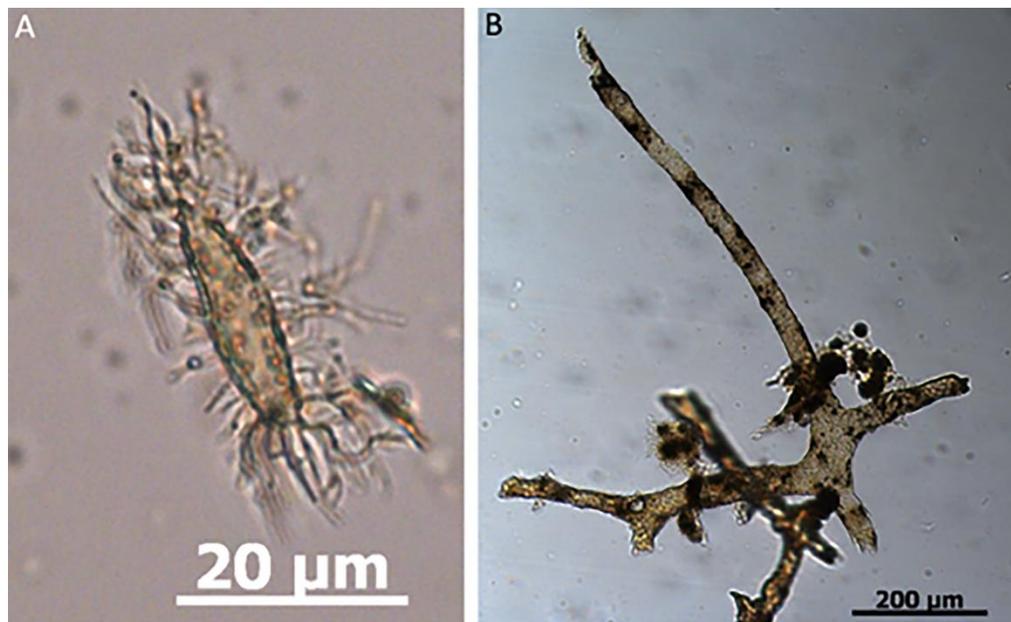


Figure 1. Microscopy images of soft tissues isolated from bones of the Cretaceous dinosaur *Brachylophosaurus canadensis*. (A) Light microscope image of a structure morphologically consistent with an osteocyte (bone cell) isolated from MOR 2598. Numerous filipodia are observed branching from the structure. Compare against light microscope images of extant *Struthio camelus* (ostrich) osteocytes shown in (Schweitzer et al. 2013). (B) Light microscope image of a structure consistent with blood vessels from GPD 328. A hollow, bifurcating, tubular structure is observable. Compare against light microscope images of *S. camelus* blood vessels from (Schweitzer et al. 2005) and (Schweitzer et al. 2007).

Indeed, two mechanisms (Boatman et al. 2019; Collins et al. 1998; Schweitzer et al. 2014; Wiemann et al. 2018) have been proposed to explain reports of soft tissues and biomolecules within ancient vertebrates. Both result in the intra- and intermolecular crosslinking and chemical transformation of reactant biomolecules, a process referred to as *in-situ* polymerization (Gupta et al. 2007a; Stankiewicz et al. 2000) (a comprehensive list of definitions is given in Table 1) within geological contexts. The first proposed mechanism, iron-mediated radical formation, hypothesizes that redox-active iron catalyzes the breakdown of hydroperoxides to free hydroxyl radicals (Boatman et al. 2019; Schweitzer et al. 2014). The free hydroxyl radical is unstable and highly reactive, and quickly abstracts a hydrogen atom (H·) from a biomolecule such as a protein, lipid, DNA, etc., and forms a radical on the biomolecule itself. The newly formed radical biomolecule can then react through one of several mechanisms with neighboring biomolecules to form intermolecular crosslinks (Catalá 2010; Roberfroid & Calderon 1995). The hypothesis of redox-active iron as a catalyst for this radical mechanism was supported by numerous reports of the presence of iron oxides, specifically goethite, in association with fossil vertebrate soft tissues (Boatman et al. 2019; Cadena 2016; Cadena 2020; Kaye et al. 2008; Schweitzer et al. 2014; Surmik et al. 2016).

Table 1. Glossary.

Authigenic mineralization	Diagenetic precipitation of mineral onto <i>in-situ</i> , organic templates
Biomarker; ancient biomolecule; taphonomically altered biomolecule	A biomolecule, whether bound or unbound, that has undergone taphonomic alteration but is still recognizable as to its biological precursor
Biomolecule	An organic compound of biological origin; refers to carbohydrates, lipids, proteins, and nucleic acids
Biostratinomy	The chemical and physical properties and/or processes that affect an organism's preservational state from death up until burial
Bound biomolecule	A biomolecule, either unaltered or taphonomically altered, that has undergone <i>in-situ</i> polymerization
Carbonization	The maturation of <i>in-situ</i> polymerization products towards aliphatic, and primarily aromatic, macromolecules lacking heteroatoms
Carbonized film; carbonized trace	Preserved soft tissue morphology with molecular compositions consistent with carbonization products

Diagenesis	The chemical and physical properties and/or processes that determine an organism's preservational state after burial and up until the potential onset of catagenesis
Heteroatoms	Atoms other than carbon or hydrogen, such as oxygen (O), nitrogen (N), or sulfur (S)
Humic-like macromolecule; immature tissue macromolecule (ITM)	Large, highly crosslinked macromolecules resulting from <i>in-situ</i> polymerization, but that have undergone only limited carbonization
<i>In-situ</i> polymerization; crosslinking	Taphonomic polymerization/crosslinking of unaltered or taphonomically altered biomolecules towards HLMs/ITMs
Kerogen-like macromolecule (KLM); mature tissue macromolecule (MTM)	Large, highly crosslinked macromolecules resulting from <i>in-situ</i> polymerization and extensive carbonization
Kerogenization	The overall process of converting biomolecules into kerogens, KLMs/MTMs, etc. Includes both <i>in-situ</i> polymerization and extensive carbonization
Molecular paleontology	A branch of paleontology concerned with the study of preserved biomolecules endogenous to extinct and/or prehistoric organisms
Non-polymerizing (NonP) taphonomic alteration; non-polymerizing (NonP) taphonomically altered	Taphonomic modification to functional group/s of an unaltered or previously taphonomically altered biomolecule that does not involve <i>in-situ</i> polymerization
Non-polymerized taphonomically altered biomolecule	A biomolecule that has undergone NonP taphonomic alteration but not <i>in-situ</i> polymerization: e.g. glutamine deamidation, fatty acid hydroxylation
Osseous soft tissues (OSTs)	Tissue structures that are derived from bone, not necessarily mineralized: e.g. collagen matrix, osteocytes, intra-osseous vascular tissue, intra-osseous nerve tissue
Soft tissues	The endogenous, organic portion of an organism/specimen's biological tissues

Soft tissue morphology	The structural form of soft tissue; does not always correlate with the presence of endogenous biomolecules
Taphonomy	The study of processes affecting the decay and preservation of an organism, including all processes of biostratinomy and diagenesis
Unaltered biomolecule	A biomolecule that has not undergone NonP taphonomic alteration or <i>in-situ</i> polymerization
Unbound biomolecule; free biomolecule; labile biomolecule	A biomolecule, either unaltered or taphonomically altered, that has not undergone <i>in-situ</i> polymerization

The second proposed mechanism (Boatman et al. 2019; Collins et al. 1998; Poinar et al. 1998; Poinar & Stankiewicz 1999; Schweitzer et al. 2014; Wiemann et al. 2018) is the formation of advanced glycation and lipoxidation end products (AGEs/ALEs) which involves the condensation of proteins, nucleotides, etc., with oxidatively damaged carbohydrates and/or lipids. Specifically, the conversion of carbohydrates to reactive dicarbonyl species, and the formation of aldehydes and ketones via lipid peroxidation generates substrates containing electrophilic carbonyl carbons with which nucleophilic groups of proteins and nucleic acids can condense. This results in the formation of large macromolecules generally termed melanoidins (Vistoli et al. 2013).

Both the iron-mediated radical and AGE/ALE pathways convert original tissue biomolecules into large, highly crosslinked macromolecules that are structurally analogous to melanoidins, humic substances (i.e. fulvic acids, humic acids, humins), immature kerogens, kerogen precursors, etc. (Harvey & Boran 1985; Harvey et al. 1983; Schweitzer et al. 2014; Vandenbroucke & Largeau 2007; Vistoli et al. 2013; Wiemann et al. 2018) (Figure 2). These highly crosslinked macromolecules are herein referred to as humic-like macromolecules (HLMs) or alternatively, more specific to soft tissues, immature tissue macromolecules (ITMs). Extensive heat/pressure and/or timespans can further transform ITMs into carbonaceous macromolecules with molecular structures analogous to kerogens. Herein, such carbonaceous macromolecules are referred to as kerogen-like macromolecules (KLMs) (Briggs & Summons 2014) or alternatively, more specific to soft tissues, mature tissue macromolecules (MTMs). Examples of potential molecular structures for kerogens are given in Figure 2.

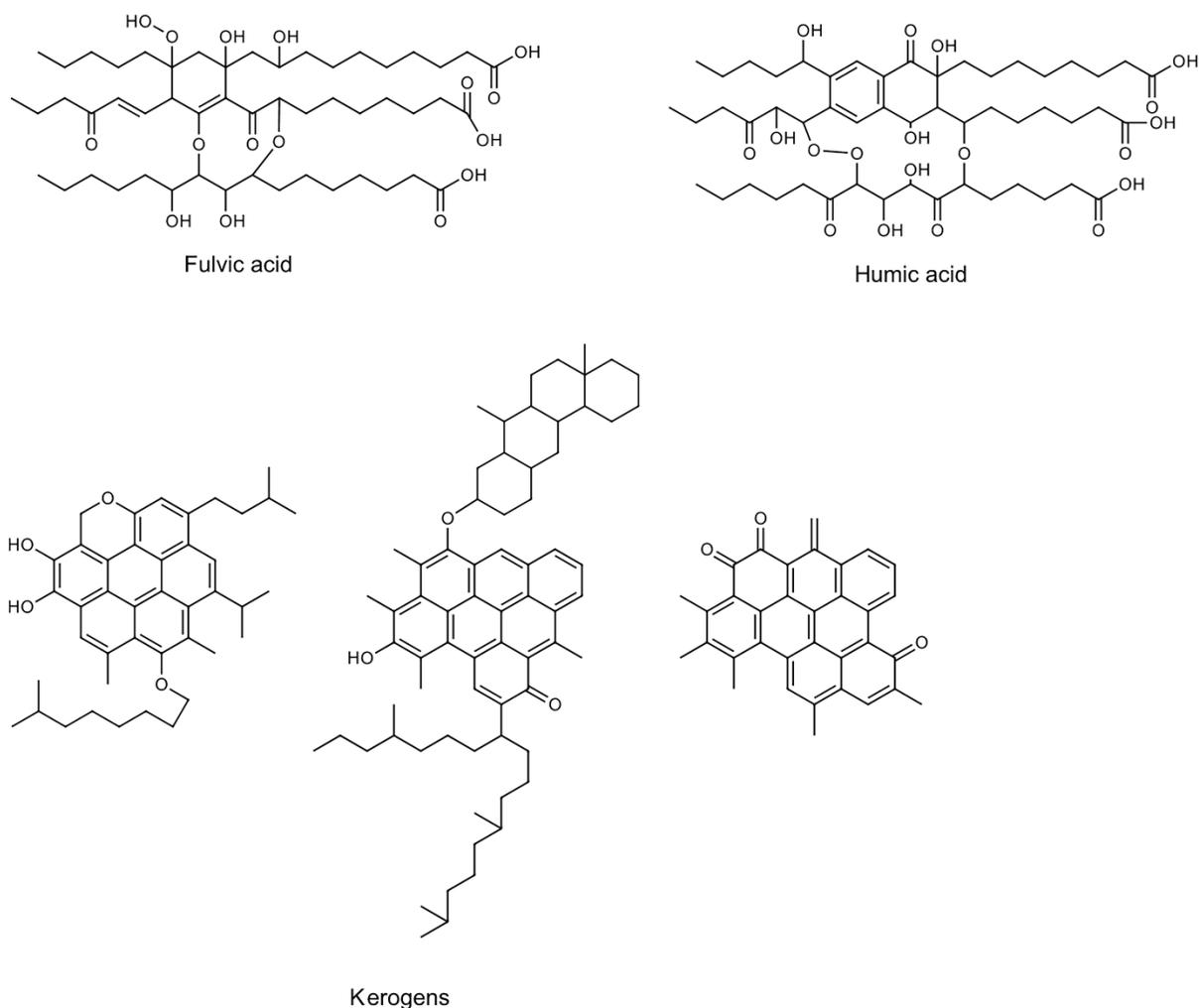


Figure 2. Potential molecular structures for humics and for various relatively mature kerogens. *Humics and kerogens typically consist of high-molecular weight aromatic ring systems functionalized to varying degrees with aliphatics and heteroatoms. The chemical composition of humics typically consists of a higher heteroatom:carbon ratio and possesses less aromaticity than kerogens. There is a broad range of potential molecular structures for humics and kerogens as the reactions that form them are complex and can incorporate a variety of biomolecules. Thus, the structures depicted here are only representative.*

A novel chemical framework (Figure 3) is herein hypothesized that explains reported cell and tissue preservation within vertebrate fossils. Currently, a detailed chemical framework does not exist within the primary literature for the fossilization of vertebrate cells and tissues. The iron-mediated radical (Boatman et al. 2019; Schweitzer et al. 2014) and AGE/ALE (Wiemann et al. 2020; Wiemann et al. 2018) mechanisms are currently understood to be competing hypotheses (Boatman et al. 2019; Wiemann et al. 2018) for vertebrate soft tissue preservation, and neither have been well-described in terms of vertebrate fossil biogeochemistry. Both hypotheses are herein re-described in terms of established chemistry across a diversity of scientific fields.

Importantly, this framework will demonstrate that in many cases, the previously hypothesized iron-mediated radical (Boatman et al. 2019; Schweitzer et al. 2014) and AGE/ALE (Wiemann et al. 2020; Wiemann et al. 2018) mechanisms are not separate hypotheses. Rather, the hypotheses presented by Schweitzer et al. (2014) and Wiemann et al. (2018) will, in several instances, be shown to be subsequent steps of a single, unified reaction mechanism. Further, the reaction mechanisms discussed in this paper apply not only to vertebrate cell and tissue preservation but rather to the biomolecules of organismal tissues in general. The chemical framework presented here has implications for any field investigating the biogeochemistry of ancient tissues, cells, and biomolecules, including molecular paleontology and archaeology, ancient DNA analyses, and soil and petroleum science. Particularly, direct implications of this framework for predicting degree of biomolecule preservation within fossil specimens, including for DNA and protein sequences, are discussed.

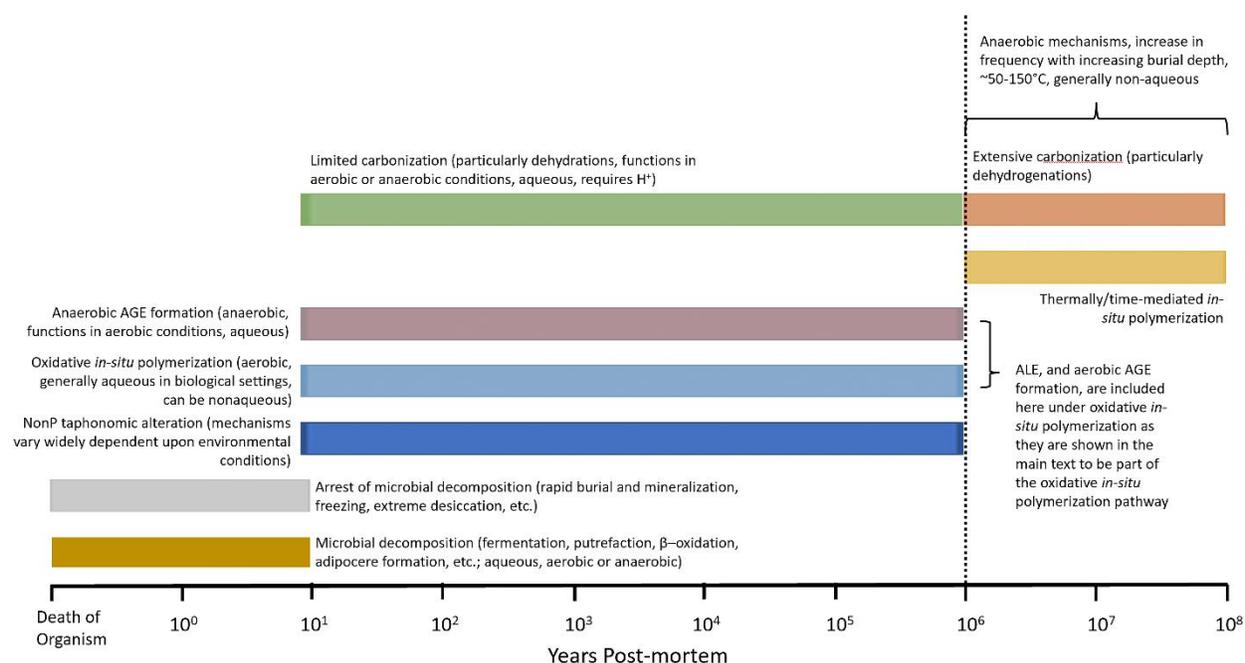


Figure 3. Proposed chemical framework for vertebrate cell and tissue preservation throughout time. Depicted above are approximate/relative timeframes over which the processes for vertebrate soft tissue preservation described within this review are hypothesized to have occurred under geologic settings. The sulfurization process is not shown as evidence supporting its applicability to vertebrate soft tissue preservation is currently limited (McNamara et al. 2016). A caveat, the time frames illustrated for each set of processes are approximations, relative, and can vary substantially depending upon environmental conditions. For example, total carbonization of biological tissues can occur in a matter of minutes or hours if enough heat/pressure is applied (Buseck & Beyssac 2014; Vandenbroucke & Largeau 2007). However, such conditions are generally not experienced during fossilization and longer time periods (10^6 - 10^8 years) at lower temperatures (~50-150°C) are generally hypothesized to have been typical, as shown in the diagram (Buseck & Beyssac 2014; Oberlin 1984; Stankiewicz et al. 2000;

Vandenbroucke & Largeau 2007). Adjustment of any such factors, such as local moisture and oxygen content, as well as heat and pressure, and even the species of dissolved ions present, can significantly accelerate or slow/inhibit the above processes. The dashed line sets an approximate boundary separating diagenetic processes from those that are catagenetic.

II. Chemical Framework

A. Post-Mortem Tissue Decay

Cellular tissues consist primarily of lipids, carbohydrates, proteins, and nucleic acids (Alberts et al. 2002). The exterior as well as interior compartments of a eukaryotic cell, including all vertebrate cells, are bounded by lipid membranes consisting largely of phospholipids and some sterols with various associated proteins that allow interaction with the environment. Bounded by these membranes, the interior of the cell is filled with an aqueous solution termed cytosol which contains dissolved ions, metabolites, biomolecules, etc. Additionally, an internal protein scaffolding, termed the cytoskeleton, is intimately associated with the lipid membranes and provides structural support for the cell. Finally, in general, the cellular genome, which consists of strands of millions to billions of polymerized nucleotide bases, resides within a double membrane bound organelle termed the nucleus (Alberts et al. 2002).

Upon the death of an organism, this cellular structure typically begins to rapidly decompose. Within minutes to hours (Donaldson & Lamont 2013; Prescott 1912), cellular autolysis occurs and fermentation increases. Endogenous bacteria migrate from the intestinal tract into surrounding tissues, expediting carbohydrate fermentation and initiating putrefaction (Butzbach 2010; Donaldson & Lamont 2013; Nolan et al. 2020; Prescott 1912). Phospholipids and glycerides are rapidly hydrolyzed, releasing their fatty acid moieties which are either aerobically metabolized via the β -oxidation pathway or, in the absence of free oxygen, are converted to free and crosslinked hydroxy fatty acids (adipocere) (Schoenen & Schoenen 2013). A buildup of gasses from these processes in the body cavity leads to bloat which stretches, deforms, and disarticulates tissues and skeletal elements, further exposing the body to external decomposers (Butzbach 2010; Donaldson & Lamont 2013; Nolan et al. 2020; Schoenen & Schoenen 2013). Unless organismal tissues are stabilized, these processes eventually result in complete breakdown and recycling of components, leaving no trace remains to be incorporated into the fossil record.

For original soft tissues and biomolecules to preserve in fossils, these degradative processes must have been slowed or arrested shortly post-mortem so that the original cells and tissues were not completely decomposed (Briggs et al. 2000). This can potentially occur via rapid burial and mineralization of organismal remains (Vandenbroucke & Largeau 2007) or via rapid freezing, as in the case of permafrost specimens (Jensen & Sheehan 2014), or via desiccation (Lennartz et al. 2020). Biologically mineralized skeletal elements such as bone offer an additional layer of protection to the soft tissues they harbor (Demarchi et al. 2016a). Regardless, the reaction mechanisms described within this framework assume that degradative processes were sufficiently arrested to allow for soft tissue preservation.

B. Non-Polymerizing Taphonomic Alteration

Post-mortem, biomolecules of soft tissues that are not completely decomposed undergo taphonomic alterations. Taphonomic alterations are herein defined as either being *in-situ* polymerizing (and thus termed *in-situ* polymerization) or as being non-polymerizing (NonP). NonP taphonomic alteration refers to biostratinomic and/or diagenetic chemical modifications to labile biomolecules that do not result in intra- or intermolecular crosslinking. One such example would be cytosine deamination occurring within a strand of DNA. Additionally, compounds within vertebrate tissues may undergo chemical reactions towards more stable NonP taphonomically altered forms that are sometimes recoverable and identifiable from fossil specimens of ancient strata (Shinmura & Sawada 2010; You-Xing et al. 2007); these are generally referred to as biomarkers (Farrimond et al. 1998; Ferrer et al. 2018; Grice & Eiserbeck 2014; Nguyen et al. 2019; Philp & Gilbert 1987; Toporski & Steele 2002).

NonP taphonomic alteration of proteins and nucleic acids in fossil vertebrates is not particularly well studied, especially in specimens of pre-Pleistocene strata, due in part to the assumption that these molecules will not preserve (Briggs et al. 2000; Evershed et al. 1997; Poinar et al. 1998; Schweitzer et al. 2019; Simoneit 2002). However, amino acid side chains of proteins are known to undergo NonP taphonomic alteration. Deamidation of glutamine and asparagine to glutamic and aspartic acid, respectively, is one such example in proteins (Schiffter 2011). Deamination of cytosine to uracil is an example in nucleic acids (Hofreiter et al. 2001; Lewis et al. 2016). Both proteins and DNA can also readily undergo degradative cleavages (Bada et al. 1999; Dabney et al. 2013; Jones et al. 1999; Lindahl 1993; Poinar & Stankiewicz 1999). Specifically, the peptide bonds of protein chains undergo hydrolytic cleavage (Poinar & Stankiewicz 1999). For DNA, depurination via hydrolysis of the purine-deoxyribose *N*-glycosyl bond leads to subsequent cleavage of the phosphate-sugar backbone via a β -elimination reaction (Bada et al. 1999; Dabney et al. 2013; Lindahl 1993).

Lipids also undergo NonP taphonomic alteration processes. For example, fatty acids are rapidly hydrolyzed from the glycerol unit in phospholipid and glyceride diagenesis (Briggs et al. 2000; Kostyukevich et al. 2018; Nolan et al. 2020); the resultant fatty acids can then be hydroxylated or methylated, among other NonP taphonomic alterations (Stefanova & Disnar 2000). While NonP taphonomically altered lipids and sterols often serve as biomarkers within sedimentary organic matter (Briggs & Summons 2014; Mackenzie et al. 1982; Mackenzie et al. 1981; O'Reilly et al. 2017; Philp & Gilbert 1987), such biomarkers have not often been reported within vertebrates prior to the Neogene (Briggs et al. 2000; O'Reilly et al. 2017). However, in one instance, lipid biomarkers were reported from the uropygial gland of an Eocene bird (O'Reilly et al. 2017).

C. *In-situ* Polymerization

In-situ polymerization occurs when the labile biomolecules of a soft tissue form intra- and especially intermolecular crosslink bonds that ultimately result in the formation of HLMs/ITMs (Harvey et al. 1983; Hatcher et al. 1983) that may or may not contain heteroatoms (Gupta et al. 2009; Osés et al. 2017; Schweitzer et al. 2014; Stankiewicz et al. 2000; Vandenbroucke &

Largeau 2007). Within laboratory settings, this process can be initiated by the addition of elevated heat and pressure (Gupta et al. 2007c; Stankiewicz et al. 2000; Vandenbroucke & Largeau 2007). In geological settings, *in-situ* polymerization has been proposed to proceed at lower temperatures and pressures over longer periods of time (Harvey et al. 1983; Kirschenbauer 1960; Stankiewicz et al. 2000; Tissot & Welte 1984; Vandenbroucke & Largeau 2007) or via other hypothesized pathways that are faster, such as catalysis by redox-active transition metals (Boatman et al. 2019; Harvey et al. 1983; Schweitzer et al. 2014; Smith et al. 2018; Wiemann et al. 2018) and AGE/ALE formation (Boatman et al. 2019; Schweitzer et al. 2014).

The following hypothesized pathways of *in-situ* polymerization are by no means mutually exclusive, nor comprehensive. Indeed, these may work in concert to facilitate preservation of fossil soft tissues. Furthermore, the proposed reaction mechanisms result from processes that are not fully replicable in the laboratory. However, they do provide a structured framework, in addition to NonP taphonomic alteration and carbonization, within which the conversion of unaltered biomolecules towards stable polymers within vertebrate soft tissues can be understood and tested.

1. Oxidative *in-situ* polymerization

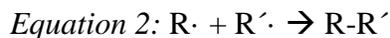
Lipids containing fatty acid moieties, including phospholipids and glycerides, are primary components of cellular membranes and the cytoplasm and thus must be accounted for in the preservation of ancient cells and tissues, including those of vertebrates (Alberts et al. 2002; Casares et al. 2019; Hulbert et al. 2014). An autoxidative mechanism was originally proposed and tested by Harvey et al. (1983) to explain the production of marine fulvic and humic acids from microbial phospholipids and glycerides and their eventual conversion to the kerogens of marine shales (Harvey & Boran 1985; Harvey et al. 1983). This same reaction mechanism should be extendable to vertebrates as phospholipids and glycerides are ubiquitous amongst eukaryotic cells. Indeed, outside of soil/petroleum science, this autoxidative mechanism, referred to as lipid peroxidation within biological fields (Catalá 2010), is recognized to initiate the oxidative crosslinking of cellular membranes. Further, lipid peroxidation is a focus of biomedical research as it has been linked to aging within the human body (Dmitriev & Titov 2010; Hulbert et al. 2014); it could function via similar mechanisms to preserve cellular tissues. Thus, lipid peroxidation is proposed to be a type of oxidative *in-situ* polymerization that may contribute to preservation of soft tissues throughout time; this was first suggested by Schweitzer et al in 2014 as a part of iron-mediated radical formation.

Lipid peroxidation begins with the formation of radical intermediates along the hydrocarbon tails of fatty acid moieties (Catalá 2010; Domínguez et al. 2019; Min & Ahn 2005; Roberfroid & Calderon 1995) (Figure 4). Under oxidative settings, transition metals, such as Fe²⁺ complexed with molecular oxygen (ferryl and perferryl ions), can directly catalyze the formation of radical intermediates



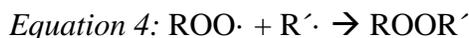
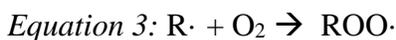
where R is the fatty acid moiety and R· is a fatty acid radical intermediate

on fatty acid carbons (Min & Ahn 2005; Repetto et al. 2010; Schafer et al. 2000). These intermediates can then combine directly with radical intermediates of neighboring fatty acid moieties, or even other biomolecules

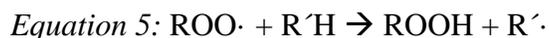


where R is a fatty acid moiety and R' is a radical biomolecule

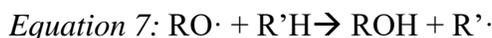
thus inducing crosslinking (2005; Gryn'ova et al. 2011; Harvey et al. 1983; Kirschenbauer 1960; Smith et al. 2018; Tegelaar et al. 1989). These radical intermediates form several orders of magnitude faster on allylic carbons, that is, carbons directly adjacent to an alkene pi-bond (for example, $C=C-C\cdot$ is preferred to $C-C-C\cdot$) (Shahidi & Zhong 2010; Smith et al. 2018). This is because the electron density of the unstable radical intermediate is delocalized via participation in resonance stabilization with the adjacent pi-bond (Catalá 2010; Domínguez et al. 2019; Min & Ahn 2005). Furthermore, the radical intermediates, instead of directly forming covalent bonds with intermediates of neighboring molecules, can react with O_2 to form peroxy radicals which then proceed to combine with adjacent radical intermediates (Gryn'ova et al. 2011; Harvey et al. 1983; Smith et al. 2018).



Alternatively, the peroxy radical can abstract a hydrogen atom from a neighboring molecule to form a lipid peroxide (Gardner 1986; Gryn'ova et al. 2011; Roberfroid & Calderon 1995; Smith et al. 2018). In the presence of heat and/or transition metals, this lipid peroxide can then decompose to form an alkoxy radical and a new hydroxyl radical (Min & Ahn 2005; Smith et al. 2018);



this results in rapid propagation of the overall autoxidation mechanism (Shahidi & Zhong 2010; Smith et al. 2018). The alkoxy radical in particular is significant as it can either abstract a neighboring hydrogen atom to form a hydroxyl group



or undergo β -scission (Gardner 1986; Gryn'ova et al. 2011; Smith et al. 2018). The β -scission pathway results in cleavage of the hydrocarbon chain, but importantly it can form aldehyde and ketone products (Gardner 1986; Nawar 1984; Vistoli et al. 2013).



where $R'=O$ and $R''\cdot$ are the cleaved halves of the original R hydrocarbon chain

Such aldehyde and ketone products serve as precursors for ALE genesis via crosslinking with nucleophilic (electron dense) functional groups of proteins, nucleic acids, and phospholipid head groups (Vistoli et al. 2013). Thus, the pathway of free radical formation proposed by (Schweitzer et al. 2014) generates the chemical precursors necessary for ALE formation proposed by (Wiemann et al. 2018). *ALE formation, and in this case oxidative in-situ polymerization, are subsequent steps of a single, unified reaction mechanism, and should not be viewed as competing hypotheses for soft tissue preservation.* Likewise, in some cases, dicarbonyl precursors necessary for AGE formation arise via autoxidation of reducing sugars, such as glucose and fructose (Wolff & Dean 1987) (Figure 5(A)). *This is another case in which oxidative radical formation is directly linked with AGE formation and in which the papers by (Schweitzer et al. 2014) and (Wiemann et al. 2018) form a single, unified hypothesis for soft tissue and biomolecule preservation through deep time.*

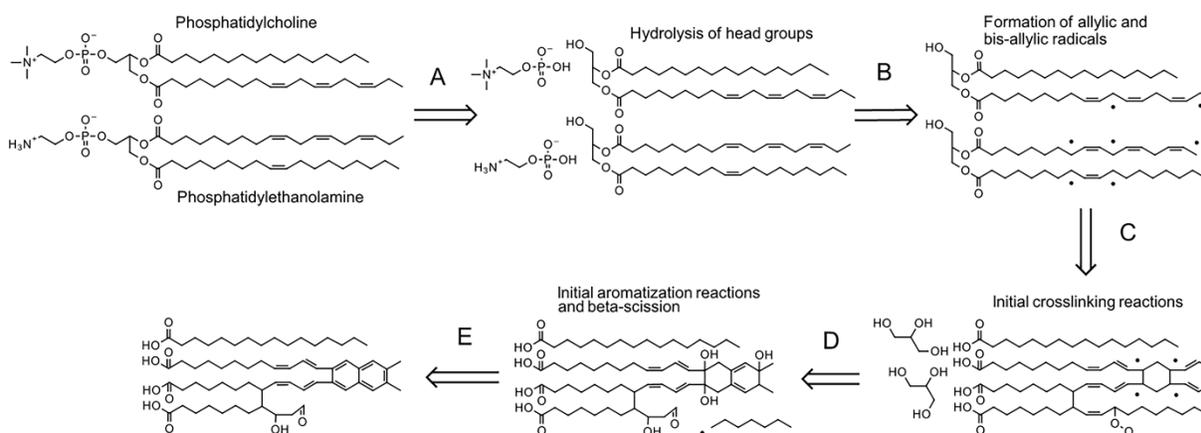
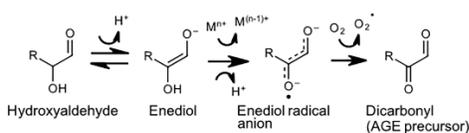
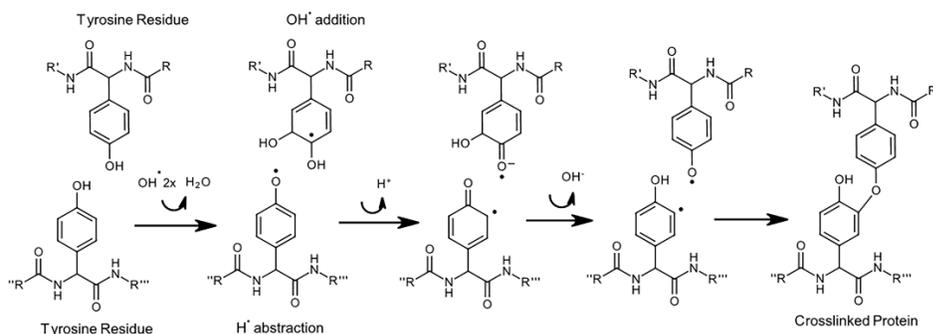


Figure 4. Representative mechanism proposed for the oxidative in-situ polymerization and initial aromatization of phospholipids. *The reactions depicted here are representative pathways. There is considerable variation as to how and when each reaction occurs within this sequence. For example, the six radicals of the intermediate between (B) and (C) would not likely form all at once, but rather one or two at a time; however, here they are depicted simultaneously for simplicity and clarity. Additionally, an oxidizing environment is assumed. (A) Hydrolysis of the phospholipid head groups occurs early during diagenesis. (B) Radicals form preferentially on allylic and bis-allylic carbons, either through hydrogen atom abstraction or C-H bond scission. Initiation of radical formation can occur via heat/pressure or catalysis via transition metals. (C) Radicals form covalent bonds (crosslinks) with neighboring radicals (crosslinks) or react with O_2 to form peroxy radicals. Glycerol is eventually cleaved from the individual fatty acids. (D-E) Some aromatization begins to occur, possibly through dehydration reactions. Additionally, peroxide functional groups can decompose to alkoxy radicals which undergo β -scission to form aldehydes/ketones and new hydrocarbon radicals.*

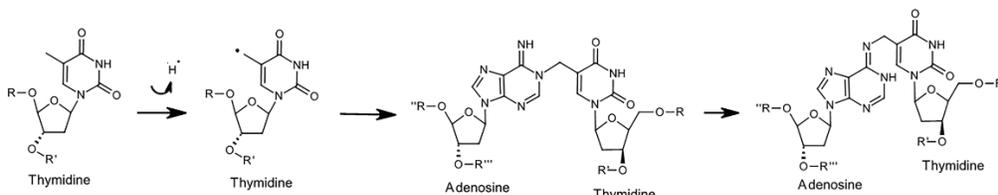
A.



B.



C.



D.

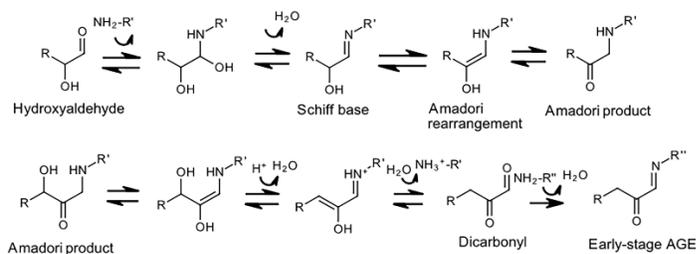
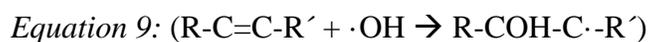


Figure 5. Representative *in-situ* polymerization mechanisms for proteins and nucleic acids. The crosslinking mechanisms depicted herein are only representative, many other mechanisms are possible depending on the reactants and environmental conditions. Additionally, the mechanisms only show the initial crosslinking and not further chemical transformation of the polymerized biomolecules. (A) The α -hydroxyaldehyde functional group of a linear reducing sugar can undergo a reversible transition to an enediol form. Then, transition metal (M^{n+}) catalyzed oxidation converts the enediol to a radical intermediate. Finally, transfer of the radical electron from the enediol to free oxygen forms an α -ketoaldehyde (dicarbonyl) functional group that can serve as a precursor for AGE formation. (B) Two tyrosine residues are depicted

undergoing crosslinking via formation of radical intermediates. One residue undergoes hydrogen atom abstraction at the side chain hydroxyl group while the other undergoes an addition reaction to the side chain aromatic ring. (C) Crosslinking reaction between inter-strand thymidine and adenosine nucleotides. (D) Formation of an early-stage AGE at the hydroxyaldehyde group of D-glucose. Only one potential pathway is shown after formation of the Amadori product, but multiple others have been well characterized and are dependent on environmental conditions, particularly pH. Additionally, the early-stage AGE, once formed, can then undergo a broad range of reactions dependent on local reactants and conditions.

In addition to lipids, proteins and DNA are found throughout biological tissues of all taxonomic groups (Alberts 1998; Alberts et al. 2002), and it is herein assumed this holds for prehistoric organisms (Cappellini et al. 2012; Orlando et al. 2013; Rybczynski et al. 2013). Attempts to recover protein-related data, and even that of DNA (Bailleul et al. 2020; Schweitzer et al. 2013), from soft, still-flexible tissues of pre-Pleistocene specimens have received interest within the field of molecular paleontology (Asara et al. 2007; Cleland et al. 2015b; Lee et al. 2017; Lindgren et al. 2018; Rybczynski et al. 2013; Schroeter et al. 2017; Schweitzer et al. 2009; Surmik et al. 2016). The potential preservation of such proteins has been hypothesized to occur via transition metal-mediated oxidative *in-situ* polymerization (radical formation). *In-situ* polymerization of proteins and DNA via the formation of radical intermediates is well known to occur within biological systems exposed to transition metal catalysts (Roberfroid & Calderon 1995). Additionally, substantial amounts of goethite have been reported associated with the OSTs (osseous soft tissues) of ancient vertebrates (Boatman et al. 2019; Cadena 2016; Cadena 2020; Kaye et al. 2008; Schweitzer et al. 2014; Surmik et al. 2016). This supports the hypothesis that at some point post-mortem the tissues encountered redox-active iron which could have functioned to catalyze *in-situ* polymerization. Taken together, such evidence supports the hypothesis that soft tissue preservation occurs at least in part through the *in-situ* polymerization of radical intermediates.

The transition metal catalyzed free radical formation hypothesis proposes that redox-active transition metals catalyze the breakdown of peroxide groups to free hydroxyl radicals ($\cdot\text{OH}$) (Boatman et al. 2019; Schweitzer et al. 2014); these peroxide groups can arise from free hydrogen peroxide (H_2O_2) (Catalá 2010; Gardner 1986; Repetto et al. 2010; Roberfroid & Calderon 1995; Winterbourn 1995), such as may be present in an organism immediately post-mortem, or a previous autoxidation pathway, such as with lipid peroxidation (Domínguez et al. 2019; Min & Ahn 2005; Smith et al. 2018). The free hydroxyl radical, being a highly reactive species, can quickly abstract a hydrogen atom from a biomolecule such as a protein, lipid (in the case of lipids this is equivalent to lipid peroxidation), etc., and form a radical on the biomolecule itself (Kehrer 2000; Roberfroid & Calderon 1995). Alternatively, the hydroxyl radical can bond covalently to an alkene bond via an addition reaction (Anbar et al. 1966; Roberfroid & Calderon 1995).



Hydroxyl radicals typically react via addition reactions with tyrosine, cysteine, phenylalanine, histidine, tryptophan, and methionine. In contrast, hydrogen atom abstraction is favorable at carbons directly adjacent to hydroxyl or amine functional groups such as those of serine, threonine, asparagine, glutamine, aspartic acid, and glutamic acid, as well as the guanidine group of arginine. For both addition and hydrogen atom abstraction, these differences are primarily due to the potential for resonance stabilization of the radical intermediate (Anbar et al. 1966; Hawkins & Davies 2001; Roberfroid & Calderon 1995). Newly formed radical amino acid moieties can covalently bond through one of several mechanisms with neighboring biomolecules to form intermolecular crosslinks (Equation 2), or preferentially react with O₂ to form peroxide functional groups (Equation 3, Equation 5) (Hawkins & Davies 2001). Examples of both hydrogen abstraction and an addition reaction facilitating protein crosslinking are depicted in Figure 5(B). Further, nucleic acids, specifically DNA, also readily undergo reactions with free hydroxyl ions to incur oxidative damage. Many such reactions result in inter-strand crosslinking (Figure 5(C)) as well the formation of crosslinks between DNA and proteins (Dizdaroglu et al. 1989; Hong et al. 2006; Nackerdien et al. 1991; Roberfroid & Calderon 1995).

2. Anaerobic AGE Formation

In addition to the mechanisms of radical crosslinking (oxidative *in-situ* polymerization) outlined above, a second proposed hypothesis explaining the preservation of fossil vertebrate soft tissues is the formation of AGEs and ALEs from individual biomolecules (Collins et al. 1998; Poinar et al. 1998; Poinar & Stankiewicz 1999; Schweitzer et al. 2014; Wiemann et al. 2018). Specifically, lipids and reducing sugars are oxidized to reactive carbonyl substrates with which nucleophilic groups of proteins and nucleic acids can irreversibly condense. The end products of such reactions are large macromolecules termed melanoidins (AGEs/ALEs) (Vistoli et al. 2013) which would be equivalent to HLMs/ITMs.

As discussed under the oxidative *in-situ* polymerization section, precursors for ALEs (Gardner 1986; Gryn'ova et al. 2011; Smith et al. 2018), and some AGEs (Wolff & Dean 1987), form directly from oxidative *in-situ* polymerization. Thus this review considers ALE and aerobic AGE formation to be equivalent pathways to oxidative *in-situ* polymerization. AGEs, however, can also form via a separate anaerobic mechanism independent of oxidative *in-situ* polymerization through the conversion of reducing sugars into dicarbonyls (Figure 4(D)). In this pathway, a primary amine reacts with the carbonyl group of the aldehyde or ketone of a reducing sugar to form a Schiff base (imine). The imine product then undergoes an Amadori rearrangement, and subsequent hydrolysis converts the imine back to a carbonyl group and the original primary amine. The original reducing sugar is now a reactive dicarbonyl species capable of condensing with nucleophilic groups of proteins, nucleic acids, etc. and thus crosslinking them (Vistoli et al. 2013).

Within biomedical research, AGE formation, as well as lipid peroxidation (including ALE formation), has been linked to the ageing of biological tissues. As tissues age, their proteins undergo crosslinking to form AGEs and ALEs. Such modifications inhibit protein functionality

and are involved in various disease pathologies (Gill et al. 2019; Moldogazieva et al. 2019; Nagaraj et al. 2012; Tessier 2010). Additionally, Maillard (Maillard 1912a; Maillard 1912b; Maillard 1916) hypothesized that AGEs were a source for soil humus (immature kerogens) and that these served as precursors for the formation of mature kerogens (Tissot & Welte 1984; Vandenbroucke & Largeau 2007).

Evidence of AGEs and ALEs have been noted within prehistoric biological tissues (Cleland et al. 2015a; Evershed et al. 1997; Poinar et al. 1998; Wiemann et al. 2020; Wiemann et al. 2018). Studies of Egyptian plant specimens dated to 1,500 B.C.E. have demonstrated AGE/ALE compounds and suggested these play a role in the preservation of biological tissues (Evershed et al. 1997). Data obtained via Raman spectroscopy has been used to support that OSTs of Mesozoic organisms are preserved in this manner (Wiemann et al. 2020; Wiemann et al. 2018). However, in a recent publication, the signal detected within some of these Raman spectra was suggested to be the result of instrumental artefacts; thus further experimentation with more diverse and appropriate methodologies is necessitated (Alleon et al. 2021). Additionally, this Maillard reaction pathway has, in at least one case, been observed as the initial mechanism for *in-situ* polymerization of DNA. Specifically, a coprolite recovered from a late Pleistocene cave deposit was tested for the preservation of endogenous DNA. Initial analyses yielded no DNA amplification; however, after incubation of extracts with N-phenacylthiazolium bromide, a chemical known to cleave glucose-derived Maillard product bonds (Dabney et al. 2013), DNA amplification was achieved (Poinar et al. 1998). Therefore, as biological tissues age post-mortem, assuming they have been stabilized against microbial degradation, their constituent biomolecules are hypothesized to undergo similar crosslinking towards AGEs and ALEs.

3. Thermally/Time Mediated *in-situ* Polymerization

Continued deposition of overlying sedimentary strata exposes an organism's remains to increasing levels of heat and pressure, and the burial environment becomes increasingly depleted of oxygen (Tissot & Welte 1984; Vandenbroucke & Largeau 2007). While this depletion of oxygen inhibits oxidative *in-situ* polymerization, the elevated heat and pressure eventually initiates hydrogen atom abstraction, or potentially even bond scission, and thus radical formation on a given biomolecule.



where *R* is the biomolecule

These intermediates can then combine directly with radical intermediates of neighboring biomolecules (Equation 2), thus inducing crosslinking (2005; Gryn'ova et al. 2011; Gupta et al. 2007b; Harvey et al. 1983; Kirschenbauer 1960; Smith et al. 2018; Tegelaar et al. 1989) (Figure 6). This pathway is slower relative to oxidative *in-situ* polymerization, partly because it lacks propagation via hydroxyl radical formation (Equation 6; the cleavage of the peroxide group

generates multiple new radicals), and radicals must generally combine directly with one another (*Equation 2*) (Gupta et al. 2007b; Hawkins & Davies 2001; Smith et al. 2018).

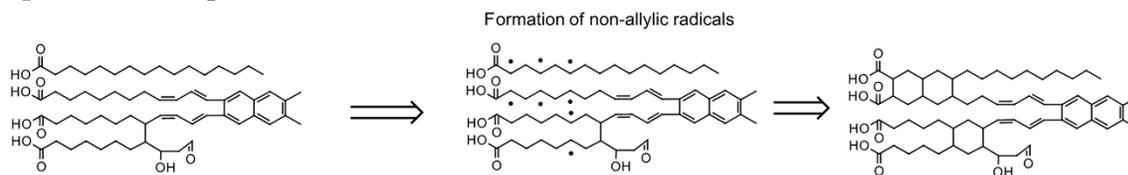


Figure 6. Thermally/time mediated crosslinking of HLM/ITM. As time goes on and/or pressure and temperature increases with burial depth, non-allylic carbons can also form radicals and undergo lipid peroxidation via thermally/time mediated crosslinking. This further crosslinks the original fatty acid chains. Environmental oxygen levels decrease as burial depth increases, limiting the potential for *in-situ* polymerization via oxidative reactions.

Experimental evidence shows that thermally-mediated radical formation results in the crosslinking of lipids (Gupta et al. 2007b; Harvey & Boran 1985; Harvey et al. 1983; Nawar 1978; Nawar 1984; Tian 2013); this likely can occur for other biomolecules as well (Tian 2013) although it is poorly studied. In laboratory settings, thermally mediated radical crosslinking occurs with increasing rate up to a temperature of $\sim 470\text{--}500^\circ\text{C}$ (Gupta et al. 2007b; Harvey & Boran 1985; Harvey et al. 1983; Tissot & Welte 1984; Vandenbroucke & Largeau 2007). However, in geological conditions, such temperatures generally are not encountered; rather, these radical forming reactions have been hypothesized to have occurred over longer timescales of a minimum of $\sim 10^6$ years and at temperatures up to $\sim 150^\circ\text{C}$ (Gupta 2015; Harvey & Boran 1985; Harvey et al. 1983; Vandenbroucke & Largeau 2007). Under such conditions, these reactions would have been slow, time-dependent processes requiring an initial stabilization of soft tissues against degradation to have taken place.

The oxidative *in-situ* polymerization and anaerobic AGE crosslinking pathways have been hypothesized to help stabilize vertebrate soft tissues against degradation within a relatively short time interval ($\sim 10^1\text{--}10^4$ years) post-mortem under oxidative conditions (Boatman et al. 2019; Schweitzer et al. 2014; Wiemann et al. 2018). Such stabilization would have given a potentially slower heat and/or time-dependent chemical mechanism a chance to proceed. Therefore, oxidizing conditions would be hypothesized to promote *in-situ* polymerization and thus soft tissue preservation, as has been suggested in prior studies (Muscente et al. 2019; Schweitzer et al. 2014; Wiemann et al. 2020; Wiemann et al. 2018). For organisms initially buried under conditions of anoxia, some mechanism not reliant on oxidative conditions would have been necessary to stabilize soft tissues and allow a slow thermally/time mediated mechanism of *in-situ* polymerization a chance to occur under the assumed geological conditions. AGE formation can function under anoxic conditions via the radical independent (anaerobic AGE) mechanism to crosslink proteins, nucleic acids, and phospholipid head groups with carbohydrate monomers (Vistoli et al. 2013). Combined with the generally recalcitrant nature of lipids (Briggs et al. 2000; Schweitzer 2011), this could stabilize soft tissues to some extent under anoxic conditions and allow thermally/time mediated *in-situ* polymerization to then take place. This would potentially account for reports of soft tissues from vertebrate specimens of anoxic, marine settings

(Boskovic et al. 2021; Lindgren et al. 2017; Lindgren et al. 2018; Lindgren et al. 2011). However, further exploration of the nature of such a mechanism is outside the scope of this paper. Alternatively, anoxic depositional environments enriched in reduced sulfur could also initiate rapid *in-situ* polymerization of soft tissues and biomolecules. Specifically, hydrogen sulfide ions can function via addition reactions at ketones and alkene groups to form intra- and intermolecular crosslinks (Adam et al. 2000; Damsté et al. 2007). Such a reaction mechanism, termed sulfurization, is well established to occur within sedimentary biomarkers (Adam et al. 2000; Damsté et al. 2007; Koopmans et al. 1997; McNamara et al. 2016), but evidence as to its significance within vertebrate soft tissues is limited (McNamara et al. 2016) and thus it is not further discussed herein.

Finally, complete desiccation of biological tissues shortly post-mortem would likely preserve them to the point that thermally/time-mediated *in-situ* polymerization could occur. Absent an aqueous environment, microbially-mediated enzymatic degradation, as well as degradative chemical reactions such as hydrolysis, cannot occur, and the result is the mummification of biological tissues (Lennartz et al. 2020). Tissues preserved via such a mechanism are known to persist indefinitely (Lennartz et al. 2020; Mustoe 2018) even if initial oxidative *in-situ* polymerization and anaerobic AGE crosslinking reactions are limited. The addition of elevated temperature and pressure with increasing burial depth over time would then cause such preserved tissues to undergo thermally/time-mediated *in-situ* polymerization.

D. Carbonization

A chemical process often referred to as carbonization can occur to a limited extent alongside *in-situ* polymerization and contributes to the formation of HLMs/ITMs from labile biomolecules, in addition to crosslinking reactions (Harvey & Boran 1985; Harvey et al. 1983). However, at higher temperatures and pressures and/or over longer timespans, carbonization-type reactions take place more extensively (Rouzaud et al. 2015). Extensive carbonization-type reactions within an HLM/ITM proceed beyond *in-situ* polymerization and result in the conversion of the HLM/ITM to a KLM/MTM (Figure 7). However, the exact point at which an HLM/ITM transitions to a KLM/MTM (or even at which humics are considered kerogens) on a structural/molecular basis is not well defined within the primary literature outside of operational definitions based on solubility (Vandenbroucke & Largeau 2007).

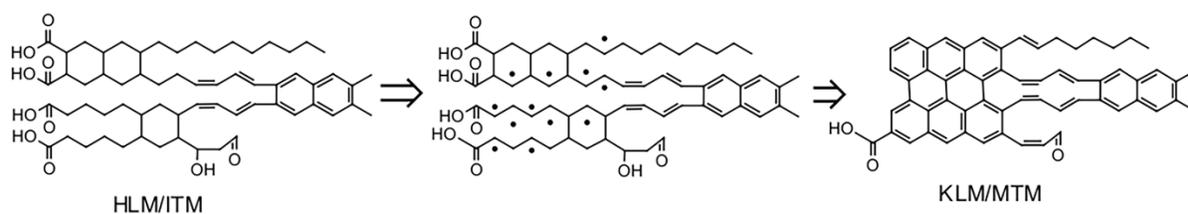


Figure 7. Maturation via carbonization towards a KLM/MTM. Shown is a potential carbonization pathway for the product HLM/ITM of Figure 6. Crosslinking via non-allylic

radical intermediates continues under heat/pressure and anoxic conditions. Dehydrogenation reactions drive aromatization, and heteroatoms are removed from the structure; these reactions result in a KLM/MTM.

During carbonization, the HLMs/ITMs of preserved cells and tissues undergo extensive conversion of aliphatic sp^3 orbitals to lower energy/more stable sp^2 orbitals of aromatic alkenes (Agrawal & Sharma 2018; Rouzaud et al. 2015; Wei et al. 2005). Additionally, heteroatoms are removed from the polymer structure, resulting in a disorganized system of interconnected aromatic rings (polyaromatic hydrocarbons, i.e., KLMs/MTMs) (Agrawal & Sharma 2018; Briggs & Summons 2014; Oberlin 1984; Rouzaud et al. 2015; Wei et al. 2005), thus reducing structural variation within the macromolecules. The sp^2 orbitals of the carbon-carbon bonds within the aromatics are further stabilized by resonance of the conjugated pi-bond system, resulting in electrochemical stability and low reactivity (Harvey 1997; Lawal 2017).

The chemical changes resulting in aromatization are likely due in part to elimination reactions, especially dehydrations (Bockisch et al. 2018) and dehydrogenations (Schimmel et al. 1999; Wang & Zhao 2020; Wei et al. 2005). Dehydration favorability begins at lower temperatures and pressures, approximately 150°C under experimental settings (Bockisch et al. 2018), and likely below ~50°C under predicted geological conditions (Gupta 2015; Harvey & Boran 1985; Harvey et al. 1983). Dehydrogenations, in contrast, would be associated more closely with the onset of catagenesis (Gupta 2015; Schimmel et al. 1999; Wang & Zhao 2020; Wei et al. 2005). Under geologic settings, temperature is hypothesized to be the primary factor influencing the onset and rate of catagenesis (Stanov 1981; Vandenbroucke & Largeau 2007). Pressure during geologic catagenesis is hypothesized to fall within the range of ~300-800 bar (Stankiewicz et al. 2000; Vandenbroucke & Largeau 2007), but currently this is not well studied.

This overall process of converting large, complex *in-situ* polymerization products, termed HLMs/ITMs, to aliphatic and aromatic macromolecules, termed KLMs/MTMs, occurs on a gradient, or maturity level, generally determined by applied heat and pressure (Hatcher et al. 1983; Oberlin 1984; Rouzaud et al. 2015; Stankiewicz et al. 2000). Under experimental conditions, one study reported that by 260°C, many protein-related functional groups had been removed from kerogens and KLMs. Up to at least a temperature of 350°C, the macromolecules still maintain a detectable aliphatic content (Stankiewicz et al. 2000). Other studies have likewise supported that the conversion of aliphatics to aromatics within kerogens and KLMs occurs gradually as temperatures increase from ~150°C to ~400°C (Ishiwatari & Machihara 1982; Wei et al. 2005). When a temperature of ~435°C is obtained, catagenesis begins in which the process of “cracking” releases some relatively small aliphatics and aromatics from the bound portions kerogens and KLMs (Buseck & Beyssac 2014; Tissot & Welte 1984; Vandenbroucke & Largeau 2007). These released molecules form the basis for oil and natural gas in geological sediments. In contrast, under geological conditions, catagenesis is hypothesized to have occurred between ~50-150°C over an approximate timeframe of 10^6 - 10^8 years (Gupta 2015; Tissot & Welte 1984; Vandenbroucke & Largeau 2007). At higher temperatures, up to 3000°C for experimental conditions, kerogens and KLMs convert to highly aromatic structures with some potential small,

interspersed aliphatic chains. Beyond 3000°C, the carbon of mature kerogens and KLMS crystallizes through the process of graphitization. Exposure to high pressure can reduce the temperature threshold for graphitization, to 1000°C (Buseck & Beyssac 2014; Rouzaud et al. 2015; Vandenbroucke & Largeau 2007). However, for fossil vertebrates of sedimentary strata, temperatures are generally hypothesized not to have exceeded 150°C (Tissot & Welte 1984; Vandenbroucke & Largeau 2007) and graphitization generally need not be considered.

The high carbon content observed in some fossil feathers, skin, and amber (Calede et al. 2018; Davis & Briggs 1995; Greenwalt et al. 2013; Ji et al. 2006; Ji et al. 2002; Mayr et al. 2002; Moyer et al. 2014; Xing et al. 2020) using energy dispersive X-ray spectroscopy (EDS) analyses (Davis & Briggs 1995; Vinther et al. 2010; Vinther et al. 2008) likely results from the carbonization-type reactions. However, EDS is only capable of detecting the presence of elements down to a concentration of 1,000ppm (0.1%) (Nasrazadani & Hassani 2016; Ngo 1999). Thus, EDS alone is not capable of distinguishing whether such films are truly carbonized, and thus considered KLMS/MTMs. As a result, the molecular composition of these carbonized remains is poorly explored for fossil vertebrate soft tissues. For example, examination of feathers purported to preserve as carbonized film revealed ultrastructure consistent with keratin protein and melanosomes (Moyer et al. 2014), suggesting total carbonization had not yet occurred. However, the actual molecular composition of such carbonized films, at least in fossil vertebrates, has not been fully explored.

The conversion of tissue biomolecules into ITMs and eventually MTMs has been examined widely within fossil invertebrates (Gupta et al. 2008; Gupta et al. 2009; Gupta et al. 2007d; Stankiewicz et al. 1997) and plants (Gupta et al. 2007a; Gupta et al. 2007b; Gupta et al. 2007c; Mustoe 2018; You-Xing et al. 2007) but less so in fossil vertebrates (Lindgren et al. 2018; Manning et al. 2009; O'Reilly et al. 2017; Thiel et al. 2014). One recent exception to this is an exceptionally preserved Jurassic ichthyosaur from the Holzmaden area of Germany (Lindgren et al. 2018). Authigenic mineralization via phosphatization was reported in epidermis recovered from the specimen. However, demineralization and subsequent analysis with mass spectrometry revealed epidermal soft tissues consisting of high molecular weight aliphatic, and particularly, aromatic hydrocarbons; this is consistent with a preserved soft tissue structure/composition intermediate to ITMs and MTMs (Lindgren et al. 2018). Such macromolecules composing the ichthyosaur epidermal structure would be expected based on the framework mechanisms described for *in-situ* polymerization and some extent of carbonization-type reactions. This is consistent with previous hypotheses of low preservation potential of labile lipids common within organismal tissues, specifically the unaltered fatty acid moieties of phospholipids, and supports that such molecules are chemically transformed via *in-situ* polymerization in ancient vertebrates (Briggs 1999; Harvey et al. 1983; Lindgren et al. 2018; O'Reilly et al. 2017).

Further, in a recent study on Oligocene whale bone of the El Cien Formation of Mexico (Thiel et al. 2014), extracts of ground bone were acquired and analyzed for their bound vs unbound fatty acid content. Substantially fewer fatty acids were recovered in the fossil whale bone than in the extant comparison. However, of those in a recoverable condition, anywhere from 21% to 70%

(dependent on the specific fatty acid) were found to have undergone *in-situ* polymerization (with the rest preserved in an unbound, or labile, state), thus further supporting the occurrence of *in-situ* polymerization within ancient vertebrate tissues. With more time and/or elevated burial temperature and pressure, these remnant fatty acids would be hypothesized to increasingly skew in proportion towards the bound state. The authors of the study did add a caveat, however, that a microbial source for the fatty acids could not be ruled out with the data collected therein (Thiel et al. 2014). Furthermore, because bound *in-situ* polymerization products generally transition from aliphatic to aromatic in character as they mature via the process of carbonization (Agrawal & Sharma 2018; Delarue et al. 2016; Oberlin 1984; Rouzaud et al. 2015; Wei et al. 2005), these bound fatty acid moieties (of the El Cien whale) would be hypothesized to, over time, undergo transformation towards polyaromatic hydrocarbons, leading to a molecular composition closer to that of the Holzmaden ichthyosaur.

While further testing on a broader range of samples is necessary, these data and reasoning suggest that ITMs/MTMs could potentially serve a role as proxies of diagenetic history within ancient vertebrates. As the conversion of biomolecules into ITMs, and subsequently MTMs, proceeds, the quantity and quality of biomolecule preservation (dependent on type of biomolecule) is hypothesized to decrease. Molecular methods can quantify the extent to which original biomolecules have undergone *in-situ* polymerization and carbonization. This is similar to the fields of soil and petroleum science using sediment maturity, which is essentially the degree of *in-situ* polymerization and carbonization that has occurred within sedimentary organics, as a marker for the quality of oil and/or natural gas present at a given locality within a geologic formation (Delarue et al. 2016; Ferralis et al. 2016; Lindgren et al. 2018; Schito et al. 2017; Sjövall et al. 2021; Thiel & Sjövall 2011). With fossil vertebrates however, instead of sediment maturity, soft tissue “maturity” can be measured and then correlated to quality or degree of biomolecule preservation. Establishing such correlations across a range of specimens spanning the fossil record would form the foundations of a chemical index with ITMs and MTMs as proxies of biomolecular preservation. Such an index could in turn be used to screen future specimens, from the Holocene to the Mesozoic, according to biomolecule/biomarker preservation potential, including for DNA and protein sequence information.

III. Biomolecule and Biomarker Preservation

The reactions described within this chemical framework, while potentially preservative for soft tissue morphology, are generally degradative of its constituent biomolecules. This is analogous to histological tissue fixation with chemicals such as glutaraldehyde (Singh et al. 2019; Srinivasan et al. 2002) or osmium tetroxide (Singh et al. 2019). Chemical fixation, depending on the fixative used, crosslinks tissue proteins, nucleic acids, and lipids, altering their chemical structure such that they are not amenable to degradation by most enzymes, including those of exogenous microbes. This preserves tissue morphology, but the alteration of chemical structure inherently degrades the crosslinked biomolecules. For example, histological fixation is known to inhibit DNA sequence recovery from fixed tissues (Srinivasan et al. 2002). Similarly, the

chemical reactions presented within this chemical framework, while preservative of soft tissue morphology, are predicted to gradually degrade soft tissue constituent biomolecules over time.

This begs the question of how reported biomolecule and biomarker preservation within early and pre-Pleistocene vertebrate remains is explained in context of this chemical framework.

Preservation of sequence-able DNA has generally not been reported beyond the range of ~130-240kA outside of continuously frozen permafrost sediments and a few instances of cave deposits (Mitchell & Rawlence 2021; Welker et al. 2019). This is consistent with the degradative nature of the chemistry described within this framework. Nucleic acids are hypothesized to be labile biomolecules (Briggs et al. 2000; Lindahl 1993) and thus far have not been observed to persist deep into the fossil record, at least not in a sequence-able form. For proteins, almost all sequences reported from early and pre-Pleistocene specimens originate from robust structural proteins, primarily type-1 collagen. Type-1 collagen, the biomineralized collagen of bone, consists of 3 tightly intertwined helical peptide chains. A repeating sequence of X-Y-Gly aids in forming tight helical turns (where the X and Y positions can be any amino acid but are often proline and hydroxyproline; Gly corresponds to glycine) (Jenkins et al. 2003; Lodish et al. 2000). Lysine residues in the collagen helix often undergo post-translational hydroxylation allowing the formation of inter- and intramolecular crosslinks between collagen fibrils, contributing to the stable nature of structures comprised of this protein (Yamauchi & Sricholpech 2012).

Biomineralization of this tightly interconnected matrix with (primarily) bioapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$) further enhances its structural stability and durability (Collins et al. 2000; Schweitzer et al. 2008). These chemical features lead to high preservation potential for type-1 collagen which, when combined with *in-situ* polymerization, is hypothesized to contribute to the protein's preservation through deep time. Small segments of the collagen peptides that avoided transformation via *in-situ* polymerization (possibly within protected regions of the collagen molecule (Schweitzer et al. 2019)) may explain reported collagen sequences from Pliocene (Rybczynski et al. 2013) and Mesozoic (Asara et al. 2007; Schroeter et al. 2017; Schweitzer et al. 2009) specimens. Sequences for proteins other than collagens have been reported from early (Welker et al. 2019) and pre-Pleistocene (Cleland et al. 2015b; Demarchi et al. 2016b) specimens, but in almost all cases the proteins are likewise of a robust, structural nature and/or intimately associated with a highly mineralized matrix.

Regarding lipids, their saturated hydrocarbon chains are generally unreactive under conditions of mild heat and/or pressure. Accordingly, saturated lipids have been shown to be relatively stable throughout time, albeit often in the form of *in-situ* polymerization products (Briggs et al. 2000; O'Reilly et al. 2017; Oberlin 1984; Rouzaud et al. 2015; Stankiewicz et al. 2000; Tegelaar et al. 1989). Unaltered phospholipids and glycerides are rapidly hydrolyzed post-mortem, releasing their constituent fatty acids from glycerol (Briggs et al. 2000; Kreps et al. 1981; Schoenen & Schoenen 2013). Intact phospholipids are generally not recovered even from permafrost frozen Pleistocene soft tissues (Kostyukevich et al. 2018; Kreps et al. 1981). Unbound, saturated fatty acids, on the other hand, can be recovered from permafrost specimens (Kostyukevich et al. 2018; Kreps et al. 1981). Volatile, unbound fatty acids have even been reported from whole bone extracts of Pliocene, Miocene, and Oligocene specimens (Shinmura & Sawada 2010; Thiel et al. 2014; You-Xing et al. 2007). However, some researchers have hypothesized unbound fatty acid

moieties to undergo *in-situ* polymerization relatively rapidly post-mortem (Briggs 1999; Briggs et al. 2000; Briggs & Summons 2014). The specific geological time frame for complete *in-situ* polymerization of fatty acids is unclear, but has likely not occurred at least for some specimens of Oligocene strata (Solli et al. 1984; Thiel et al. 2014).

Other lipids, particularly steroids, sometimes remain unbound under thermally mild conditions but undergo NonP taphonomic alteration and are recoverable as sterane biomarkers (Briggs & Summons 2014; Mackenzie et al. 1982; Mackenzie et al. 1981; O'Reilly et al. 2017). The saturated tetracyclic, hydrocarbon core of these sterane biomarkers has been shown to be relatively stable throughout geologic time (Mackenzie et al. 1982). Alternatively, under conditions of elevated heat and pressure, such biomarkers are often recovered as bound portions of sedimentary humics and kerogens (Briggs & Summons 2014; Mackenzie et al. 1982; Mackenzie et al. 1981) or potentially are even degraded beyond recognition (Mackenzie et al. 1982). Under such conditions, the saturated tetracyclic core of steranes can be converted to a system of aromatics as a part of the carbonization process (Mackenzie et al. 1982). Other lipid biomarkers, including squalanes (corresponding to squalene, a biological steroid precursor) and related compounds, are known to persist into deep time as well (Ferrer et al. 2018). Despite their predicted stability, such lipid biomarkers, while present within ancient sedimentary plant and microbial matter, have not been commonly reported within ancient vertebrates, at least prior to Neogene strata (O'Reilly et al. 2017; Thiel et al. 2014). One recent exception is the Jurassic ichthyosaur, in which C₂₇ cholestanes were reported to be preserved within the specimen's integument (Lindgren et al. 2018).

IV. Conclusion

Individual hypotheses have been proposed in prior studies that partially explain preservation of vertebrate cells and other soft tissues, either via iron-mediated free radical formation (Boatman et al. 2019; Schweitzer et al. 2014; Winterbourn 1995) or AGE/ALE formation (Collins et al. 1998; Schweitzer et al. 2014; Vistoli et al. 2013; Wiemann et al. 2018). However, this review proposes a novel chemical framework that re-describes these hypotheses in context of established chemistry across a diversity of scientific disciplines. These two pathways, while originally described separately (Boatman et al. 2019; Collins et al. 1998; Schweitzer et al. 2014; Vandenbroucke & Largeau 2007; Wiemann et al. 2018), are now shown in many cases to form subsequent steps of a single oxidative *in-situ* polymerization pathway. A key example of this is the generation of radical biomolecules via oxidative *in-situ* polymerization that ultimately form the reactive carbonyl precursors for ALE/aerobic AGE formation (Schweitzer et al. 2014; Vistoli et al. 2013; Wolff & Dean 1987).

To summarize this proposed framework, biomolecules of soft tissues (post-mortem), for which normal (microbial and other) decay pathways have been arrested, undergo a variety of NonP taphonomic alterations as well as *in-situ* polymerization/crosslinking reactions; these *in-situ* polymerization/crosslinking reactions are largely described by the oxidative *in-situ* polymerization (Boatman et al. 2019; Schweitzer et al. 2014) and anaerobic AGE formation

(Collins et al. 1998; Wiemann et al. 2018) pathways. Such reactions lead to the formation of ITMs from the original biomolecules of soft tissues, including those of vertebrates (Gupta et al. 2008; Gupta et al. 2009; Gupta et al. 2007c; Gupta et al. 2007d; Lindgren et al. 2018; Stankiewicz et al. 1997; Stankiewicz et al. 2000; Tegelaar et al. 1989). Preservation of segments of biomolecules, including protected, recalcitrant regions of proteins (Schweitzer et al. 2019; Wiemann et al. 2018), as untransformed portions of these ITMs may explain reported peptide sequences for Pliocene (Buckley et al. 2019; Demarchi et al. 2016b; Rybczynski et al. 2013) and Mesozoic (Asara et al. 2007; Cleland et al. 2015b; Schroeter et al. 2017; Schweitzer et al. 2009) fossils. Additionally, further chemical transformation of these ITMs, whether through heat, pressure, the passage of time, etc., facilitates the removal of heteroatoms and the conversion of their carbon backbones from aliphatic to aromatic in character, resulting in MTMs (Buseck & Beyssac 2014; Delarue et al. 2016; Rouzaud et al. 2015; Tegelaar et al. 1989; Vandenbroucke & Largeau 2007).

The underlying chemical reactions of this framework explain observations by some authors (Muscente et al. 2019; Schweitzer et al. 2014; Wiemann et al. 2018) that oxidizing conditions may be favorable for soft tissue preservation. Free oxygen can promote the formation of radical species that contribute to the initiation and rapid propagation of *in-situ* polymerization (Gryn'ova et al. 2011; Roberfroid & Calderon 1995; Smith et al. 2018; Vistoli et al. 2013; Wolff & Dean 1987). This does not rule out, however, that soft tissue preservation can occur within anoxic depositional environments. Rather, such soft tissues must be well-stabilized against microbial degradation as *in-situ* polymerization is generally a slower process under anoxic conditions. Anaerobic AGE formation could provide this stabilization relatively shortly post-mortem (Vistoli et al. 2013), as could sulfurization in the presence of substantial hydrogen sulfide (Damsté et al. 2007; Koopmans et al. 1997; McNamara et al. 2016). This would account for the observation that specimens exhibiting cell and other soft tissue preservation can be recovered from anoxic depositional environments (Boskovic et al. 2021; Lindgren et al. 2017; Lindgren et al. 2018; Lindgren et al. 2011).

Finally, soft tissues of vertebrates from strata experiencing lower thermal and/or time-dependent maturation are predicted to possess less aromaticity and a higher heteroatom percentage relative to more matured fossils, and to possess a greater potential to harbor endogenous biomolecules (Agrawal & Sharma 2018; Delarue et al. 2016; Oberlin 1984; Rouzaud et al. 2015; Wei et al. 2005), in agreement with reported data from Cenozoic, Mesozoic, and Paleozoic invertebrates (Briggs et al. 2000; Gupta 2014; Gupta et al. 2007d; Stankiewicz et al. 1997) and sedimentary kerogens (Agrawal & Sharma 2018; Rouzaud et al. 2015; Wei et al. 2005). To test this hypothesis for vertebrates, broader analyses of Cenozoic and Mesozoic vertebrate soft tissue chemical compositions would need to be performed and reported as this has only been done in limited studies. Conclusions drawn from such experiments (and compared against those from invertebrates and sedimentary kerogens) would provide insight as to why soft tissues of some strata yield biomolecules while others do not; this would serve as the foundation for a chemical index for screening fossils according to biomolecule preservation potential. This has potential to broadly affect the focus of biomolecule recovery efforts within the fields of molecular

archeology, molecular paleontology, ancient DNA research, and paleoproteomics, and to create a more unified understanding of soft tissue and biomolecule preservation throughout the fossil record.

Competing Interests

The author has no relevant financial or non-financial interests to disclose.

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