Chapter 12
Blood to Molecules: The Fossil Record of Blood and Its Constituents

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Abstract Contrary to prevalent assumptions, blood—the ultimate "soft tissue"—has a substantial fossil record. Although initial reports of blood remnants from the Holocene were deservedly controversial—and reports of blood cells and proteins in Cretaceous therapods remain controversial today—there is currently good evidence for original blood components in fossils more than 500 million years old. In this review, our knowledge of the fossil record of blood and its cellular and molecular constituents is documented and appraised. Cellular components have been described from both amber (e.g., erythrocytes and protozoan parasites such as *Plasmodium* and *Leishmania*) and mineralized bone tissue (erythrocytes and capillary vessels). Although small molecules such as hemoglobin-derived heme and hemocyanin-derived copper are documented in the fossil record, sequenceable polymeric molecules proteins and DNA have the greatest potential for informing us of ancient behavior and physiology—examples include the functionality of mammoth hemoglobin and the disease states of pharaohs.

Keywords Blood · Ancient biomolecules · Erythrocytes · Blood parasites · DNA · Protein · Fossil record

12.1 Introduction

Vertebrate blood, as an environmental milieu, a source of nutrition, and/or a means of transmission, has played a critical role in the evolution of parasitism (Lukashevich and Mostovski 2003; Mans and Neitz 2004; Perkins et al. 2010; Mans 2011; Peñalver and Pérez-de la Fuente 2014; O'Donoghue 2017). A parasite's interaction with blood may be transitory and limited to a single portion of an organism's life

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cycle or, as in the case of human-to-human transmission of the protozoan *Babesia microti*, the sole locus of a parasite's life cycle (Saito-Ito et al. 2000). Blood and its many constituents, including organismal (i.e., parasites), cellular, and molecular components, provide obvious candidates in the search for a fossil record of parasitism (De Baets and Littlewood 2015). Unfortunately, as a "soft tissue," blood is exceedingly rare in the fossil record. Nevertheless, it is of interest to note that copper found in fossils of *Marrella splendens*, an arthropod from the 508 Ma Burgess Shale, was reported to be derived from the blood pigment hemocyanin (Pushie et al. 2014)—but see Gaines et al. (2019) for an alternative interpretation. In this chapter, the fossil record of blood and its constituents is reviewed. Although it is not the intent of this review to catalogue all published data relevant to the molecular paleobiology of blood, it is hoped that the examples described will provide an adequate assessment of the history and current status of the science. Given the newly developing techniques of molecular paleobiology, the constituent molecules of fossilized blood have a large and exciting potential, with their informational content potentially transformational (Briggs and Summons 2014; De Baets and Littlewood 2015).

This review is not limited to the fossil record of blood as it relates to parasitism. Rather, it seeks to cover all aspects of ancient blood and its constituents. The field of ancient biomolecules is new and exciting and covers, as will this review, a very broad array of research topics, including, for example, the chemistries involved in the preservation of such molecules. Most sections of this review begin with a general discussion of the current status of ancient biomolecule research as it relates to the specific type of biomolecule (i.e., DNA, proteins, and small molecules) or structures (cells and blood vessels) addressed, so as to place the blood-related data in context.

Although there are rare exceptions due to taphonomic variables, data gleaned from the fossil and archeological records usually decrease in both quantity and quality with time. For the purposes of this review, a provisional timescale (Fig. 12.1) depicts the maximum ages currently known for preservation of various types of biomolecules. Small molecules such as heme can survive for billions of years (Briggs and Summons 2014). However, the polymeric sequences of DNA and protein are much more fragile. In the case of proteins, that age is controversial; the consensus is that proteins cannot survive for more than a few million years although some research groups have published polypeptide sequences from specimens over a hundred million years old. Records of blood and its constituents, including blood parasites, from relatively young time periods are rich in both ancient DNA and protein. Medieval and Roman-era skeletons provide obvious targets, and have provided
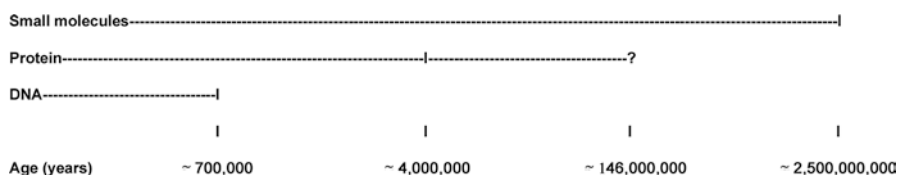

Fig. 12.1 A provisional timescale for the fossil record of various types of biomolecules

fascinating data, including diagnoses of diseases such as multiple myeloma, sickle cell anemia, tuberculosis, leprosy, and metastatic carcinoma (Maat 1991; Cattaneo et al. 1994; Schultz 2001). It is the wealth of mummified humans, both embalmed and desiccated, from Egypt and South America, however, that has provided the majority of information on ancient blood and blood-borne parasites. Although molecular archeology is still in its infancy, these data are both copious and varied. For example, Guhl et al. (1999) used PCR to amplify the multiple repeat minicircle DNA sequences of the kinetoplast of *Trypanosoma cruzi*, the causative agent of Chagas disease, from Chinchorro mummies. These particular mummies, preserved via burial in the dry sands of the Atacama Desert in Peru and Chile, are the world's oldest known mummies at approximately 9000 years in age (Marquet et al. 2012). Although the deep time fossil record of blood and its constituents lacks DNA data and contains limited examples of ancient protein, it is well documented by morphological data, such as that preserved in hematophagous arthropods and their parasites preserved in amber (Lukashevich and Mostovski 2003; Poinar and Poinar 2010; Peñalver and Pérez-de la Fuente 2014; Poinar 2018).

12.2   Blood Residues and Vessels

Residues of fossil blood per se are common within the archaeological timeframe and have been identified from a wide variety of specimens (reviewed in Smith and Wilson 2001 and Moore et al. 2016). In a particularly gruesome example, the lips of exceptionally preserved mummies of a 7-year-old boy and a 15-year-old girl, sacrificed as part of a religious ritual 500 years ago and subsequently buried under a half meter of volcanic ash at the summit of a 6739 m volcano in Salta, Argentina, were covered with blood (Corthals et al. 2012). The field of tool residue analysis was established by Thomas Loy in 1983 with his publication of data from an examination of 1–6 Ka obsidian, chert, and basalt tools from coastal middens and boreal forests in British Columbia and documented the presence of "residual blood films" on many tools (Loy 1983). Subsequently, portions of fluted projectile points from eastern Beringia were found to be coated with blood residues, and some of the blood specimens were purportedly identified as being from large mammal species; the data was used to support the hypothesis that indigenous North Americans engaged in big-game hunting at the close of the Pleistocene (Loy and Dixon 1998). Other examples include Loy and Wood (1989), who reported human blood from both a stone alter and an associated flint knife of Neolithic age from the Cayönü Tepesi site in Turkey, and Kononenko et al. (2016), who identified blood residues on the surfaces of Holocene obsidian tools used in tattooing in the Solomon Islands.

Even older blood residues were reported on stone tools from a cave in Israel, dated to 90 Ka (Loy and Hardy 1992), and Oldowan stone tools from the Sterkfontein Caves located in the Blaaubank river valley of South Africa and dated to approximately 2 Ma (Loy 1998; Williamson 2000). However, many of these early reports were widely criticized (reviewed by Odell 2001; Craig and Collins 2002). The

presence of blood was often based, in part, on a colorimetric assay for the presence of heme, an integral component of hemoglobin. This technique, and a modern version in which luminol (3-aminophthalhydrazine) is oxidized in the presence of sodium perborate and a peroxidase-like catalyst [e.g., the iron in heme] to produce luminescence (Blum et al. 2006), is subject to false-positive reactions due to interactions with nonspecific oxidizing agents such as other metals and the peroxidase enzymes of bacterial and fungal contaminants. Newman and Julig (1989), who identified human blood on a 9 Ka stone scraper from a paleo-Indian site in Ontario, reported that only a fraction of paleotools initially identified as having blood residues tested positively with polyvalent polyclonal antisera produced against an array of animal sera. Residues on the surfaces of the 2 Ma Oldowan stone tools from the Sterkfontein Caves initially reported by Loy (1998) and Williamson (2000) were in fact shown to be due to contamination (Langejans 2012). Matheson and Veall (2014) and Lombard (2014) have suggested and/or developed improvements relative to the specificity of peroxidase-dependent reagents. Modern protocols require multiple confirmatory biochemical and molecular assays.

Remnants of coagulated blood are often present within blood vessels in vertebrate specimens preserved in permafrost for hundreds of thousands of years. The very first histological examination of a mammoth was reported nearly 60 years ago by Ezra and Cook (1959); both blood vessels and bone marrow were observed. Histological examination of a 1-month-old baby woolly mammoth (*Mammuthus primigenius*) found on the Yamal Peninsula in northwest Siberia (Fisher et al. 2012) and dated to 41.8 Ka revealed blood vessels and remnants of blood within lung, liver, and cecal tissues (Papageorgopoulou et al. 2015). More recently, a large amount of coagulated blood was found in a hematoma in muscle tissue of the lateral wall of the abdomen of a 30 Ka mammoth from an island off the northern coast of Siberia (Grigoriev et al. 2017). The specimen also contained dark brown liquid adjacent to dark brown-colored ice; a blood vessel was reported to be filled with hemolyzed blood. Schweitzer et al. (2007a) demonstrated the presence of blood vessels in the much older Pleistocene mammals *Mammut americanum* and *Mammuthus columbi*, both 300 Ka in age; in the latter, "vascular contents"/"intravascular material" were observed.

There are a number of reports of fossil blood 10 Ma or older. McNamara et al. (2006) reported preservation of vascular structures and hematopoietic bone marrow in a 10 Ma frog from the Libros basin in Spain. This same laboratory, in a histological examination of skeletal muscle of an 18 Ma old salamander from lacustrine sediments of Ribesalbes, again in northeastern Spain, revealed "solidified blood residue" within the lumen of a blood vessel (McNamara et al. 2010). Of similar age are sand flies (Diptera: Psychodidae: Phlebotominae) in Dominican amber that were described as "replete, probably with a blood meal" (Grimaldi 1996). Another inclusion, in 99 Ma amber from Myanmar, is *Palaeomyia burmitis*, a female sand fly with a "dark area" suggested to be "remains of a blood meal" in its abdomen (Poinar 2004). Given the time course of blood meal digestion in extant phlebotomines, it was suggested that the fly had acquired its meal only 2–3 h before its death. Although blood per se was not preserved, Peñalver et al. (2017) argued that *Deinocroton draculi*, a 99 Ma female tick from Myanmar amber, was engorged based on its large volume, extruded genital area, and the smooth surface of its dilated integument and

Yao et al. (2014) have argued that elevated iron content indicated that a fossil hemipteran in the extinct family Torirostratidae may have taken a blood meal immediately prior to its death. And yes, dinosaurs suffered from parasitic infections; the tick *Cornupalpatum burmanicum* was found entombed in Myanmar amber and in intimate contact with a feather from its host, an unidentified Cretaceous feathered dinosaur (Peñalver et al. 2017).

Diptera of the superfamily Hippoboscoidea are all obligate blood feeders as adults but their eggs hatch and develop in utero; the adult fly gives birth to a prepuparium and pupation occurs soon after birth. The clade includes the tsetse and bat flies among others, and consists of four families: the Hippoboscidae, Glossinidae, Nycteribiidae, and Streblidae which are represented by 1, 2, 0, and 1 fossil species, respectively. Only the streblid is preserved in amber; the remaining specimens are in shale from the Rott and Florissant Formations (Statz 1940; Maa 1966; Grimaldi 1992; Poinar and Brown 2012). Unfortunately, none of the original descriptions, redescriptions, or photographs of the specimens available at the Florissant Fossil Database (2017) indicate preservation of remnants of blood meals.

Without question, the most interesting and the most controversial specimens reported to contain fossil blood and blood vessels are Late Cretaceous dinosaurs (Saitta et al. 2018a, 2019). Schweitzer et al. (2007b) identified blood vessels in specimens of *Tyrannosaurus rex* (65 and 68 Ma), *Triceratops horridus* (65 Ma), and *Brachylophosaurus canadensis* (78 ma). In one case, *T. rex* vessels with endothelial cell nuclei were reported (Schweitzer et al. 2005a). Schweitzer and colleagues have even developed protocols for the isolation of blood vessels from the fossil bones of *Brachylophosaurus canadensis* and other species (Cleland et al. 2015). Other groups have reported similar data. For example, "blood vessels and possible preserved blood products" were identified from the supraorbital horn of a Late Cretaceous *Triceratops horridus* (Armitage and Anderson 2013; Armitage 2016). Preserved blood vessels were also reported in an 80 Ma dinosaur (Pawlicki and Nowogrodzka-Zagórska 1998). In addition, Lindgren et al. (2010) reported a "large, reddish pigmentation" within the lower rib cage of an approximately 80 Ma mosasaur, *Platecarpus tympaniticus*, from Kansas, USA; no attempt at a chemical characterization of this area was attempted.

The oldest fossil with purported blood vessels is the 520-million-year-old arthropod *Fuxianhuia protensa* from the Yunnan Province of China. This spectacular fossil preserved the ancient organism's entire cardiovascular system—recognized based on pattern recognition—as a thin carbon film (Ma et al. 2014). Other examples include the 300 Ma hagfish *Myxinikela siroka* from the Carbondale Formation in Illinois, in which branchial blood vessels were identified (Bardack 1991) and, nearly as old, blood vessels of a 270 Ma *Nothosaurus* from Poland which were reported to contain both hydroxylysine and hydroxyproline, amino acids characteristic of collagen (Surmik et al. 2016). Less ancient are blood vessels in a Miocene whale from the 10–12 Ma Pisco formation in Peru (Vidal 2010) and the arteriosclerotic plaques imaged in Ötzi, a 5300-year-old mummy preserved in ice in the European Alps (Murphy Jr et al. 2003). The blood residues and vessels reported in the fossil record represent a gradation of compositions from largely molecularly intact, as in desiccated Andean mummies, to pliable Cretaceous era vessels as

reported by Schweitzer and colleagues, to permineralized replicas of vessels as in the hagfish. Although vessels in situ have a greater potential for preservation than blood per se, limits to the identification and characterization of original biomolecules in these and many other fossils have yet to be determined.

12.3 Blood Cells

A.L. Seitz (1907), in one of the first attempts at histological examination of fossil dinosaur bone, described, with a degree of reservation, "fossil blood" in a Cretaceous *Iguanodon*. Subsequent histological examination of a wide array of different kinds of fossils has revealed an astonishing array of preserved tissues and cells (reviewed by Schultz 2001; Ricqlès 2011; Houssaye 2014; Kolb et al. 2015). Perhaps most spectacular are the preserved cellular architecture of flight muscle in a fly (Diptera: Empididae) and a bee (Hymenoptera: Apidae: *Proplebeia*), both from Dominican amber, and an elopomorph fish from the Cretaceous Santana Formation in Brazil, in which individual sarcomeres and mitochondrial cristae are beautifully preserved (Martill 1990; Henwood 1992; Grimaldi et al. 1994). Unfortunately, preservation of larger polymeric biomolecules (e.g., chitin, DNA, and protein) has so far not been shown to occur in amber inclusions (Stankiewicz et al. 1998; Martínez-Delclòs et al. 2004; Kowalewska and Szwedo 2009). In fact, Penney et al. (2013) failed to recover ancient DNA from insects in copal (young and poorly or unpolymerized resin) as little as 60 years old (Peris et al. 2020).

A controversial report by Loy (1983) identified enucleated mammalian erythrocytes on the surface of a chert flake from northern British Columbia dated as 3.5 ± 2.5 Ka. Equally contentious was the work reported by Loy and Wood (1989) in an investigation of what has been called the skull building in Cayönü Tepesi Turkey. The building contains a large stone slab which they suggested served as a site of ritualistic or mortuarial dismemberment. Enucleated erythrocytes found on the slab were identified as belonging to *Homo sapiens*, *Ovis* sp., and *Bos primigenius*, an extinct species of cattle (aurochs). Species determinations were based on the shape of hemoglobin crystals produced from blood residues isolated from the surface of the slab. Since *B. primigenius* was extinct, Loy and Wood obtained museum samples of *B. primigenius* bones and reportedly extracted enough hemoglobin to produce crystals of that protein, the structures of which he thought to be species specific. Subsequent criticisms, which documented the inability to differentiate crystal structure at the microscopic level (X-ray diffraction is a far superior technique), the improbability of obtaining sufficient amounts of protein for crystallization, and the fact that even slightly degraded protein will impede crystallization and/or produce abnormal crystal structures, severely undermined Loy's work (Gurfinkel and Franklin 1988; Smith and Wilson 1992; Cattaneo et al. 1993; Remington 1994). Modern characterization of Holocene tool residues often utilizes immunological assays and nondestructive mass spectrometry (e.g., Heaton et al. 2009; Moore et al. 2016). Although examples of the recovery and use of DNA from ancient stone tools

have been reported (Hardy et al. 1997; Kimura et al. 2001; Shanks et al. 2005), such efforts have been limited due to the dual problems of contamination and rapid degradation of this biomolecule in unprotected environments (Damgaard et al. 2015; Hagelberg et al. 2015; Sarkissian et al. 2015).

Preservation of the physical architecture and morphology of fossil erythrocytes is a different matter. Early reports of intact ancient erythrocytes, from a 2 Ka mummy from Kentucky and the 3.2 Ka Egyptian mummy Nahkt, were made by Zimmerman (1973) and Hart et al. (1977), respectively. In an examination of the marrow cavity of a rib of a 2.1 Ka soldier from Failaka in the Persian Gulf, Maat (1991, 1993) visualized white blood cells complete with microvilli. In a very lengthy actualistic experiment, Hortolà (2002) covered the surface of a small piece of fractured chert with fresh human blood, dried it in a dark, air current-free room, and then stored the specimen for 122 months in closed non-sterile conditions with fluctuating temperatures (11–34 °C) and humidity (38–84%). Upon examination, many of the erythrocytes displayed a shape and size essentially identical to fresh cells (Fig. 12.2a). Erythrocytes from a Paleo-American several thousand years old (Loy 1983); a biopsy of a wound in Ötzi, the Tyrolean Iceman (Janko et al. 2012); a tick in Dominican amber (Poinar Jr 2017); and the tibia of a Late Cretaceous *Tyrannosaurus rex* (Schweitzer et al. 2007b) are also well preserved (Fig. 12.2b-e). A more recent report describes the preservation of erythrocytes on six 3 Ka obsidian tools from Polynesia (Kononenko et al. (2016). Other types of blood cells have also been reported in the fossil record. For example, Grigoriev et al. (2017) reported the presence of erythrocyte ghosts, as well as nucleated neutrophils, lymphocytes, and monocytes, with preserved chromatin, in a 30 Ka mammoth from Siberia.

Maat (1991) reported the presence of crescent-shaped sickle cells in the cortical bone of a 2 Ka human skull characterized by porotic hyperostosis; normal erythrocytes were absent, and it was suggested that the individual had suffered from sickle cell anemia (Maat and Baig 1990; Maat 1991). Diagnosis of sickle cell anemia has been a favorite activity of paleopathologists, especially when porotic hyperostosis, a condition in which the bone of the cranial fault is very porous, is diagnosed (Schultz 2001). However, Walker et al. (2009) argue that anemias per se are not responsible for the bone lesions and diagnosis of sickle cell disease in fossil skeletons is, in the absence of additional confirmatory information, impossible. A far more reliable detection of sickle cell anemia is based on ancient DNA. Marin et al. (1999) examined the DNA of six mummies from Egypt dated to approximately 5.2 Ka and identified the sickle cell hemoglobin mutation in three of the specimens.

The blood-feeding lifestyle is quite successful in insects and ticks. Such a lifestyle is thought to have evolved about 30 different times in insects alone and is practiced by over 16,000 species and members of four different orders, two of which, fleas (Siphonoptera) and sucking lice (Anoplura), are obligatory blood feeders (Lukashevich and Mostovski 2003; Grimaldi and Engel 2005). Despite the common occurrence of hematophagy in insects and ticks, the number of fossil arthropods with preserved identifiable blood is exceedingly small. Although amber does not appear to preserve the original protein and DNA constituents of insect inclusions, its preservation of morphological detail can be spectacular. Examples of erythrocytes

Fig. 12.2 Preserved and/ or fossilized erythrocytes. (**a**) Fresh human cells dried on a chert flake and stored, dry and unburied, for 10+ years (Hortolà 2002); (**b**) mammalian cells removed from the surface of a 3.5 ± 2.5 Ka flake from northern British Columbia (Loy 1983); (**c**) erythrocytes from a biopsy of a wound in Ötzi, the Tyrolean Iceman (Janko et al. 2012); (**d**) mammalian cells surrounding a tick entombed in Dominican amber (Poinar Jr 2017); (**e**) cells entrained within a blood vessel from a Late Cretaceous *Tyrannosaurus rex* (Schweitzer et al. 2007b). Scale bars in A–D and E = 10µm and 50µm, respectively
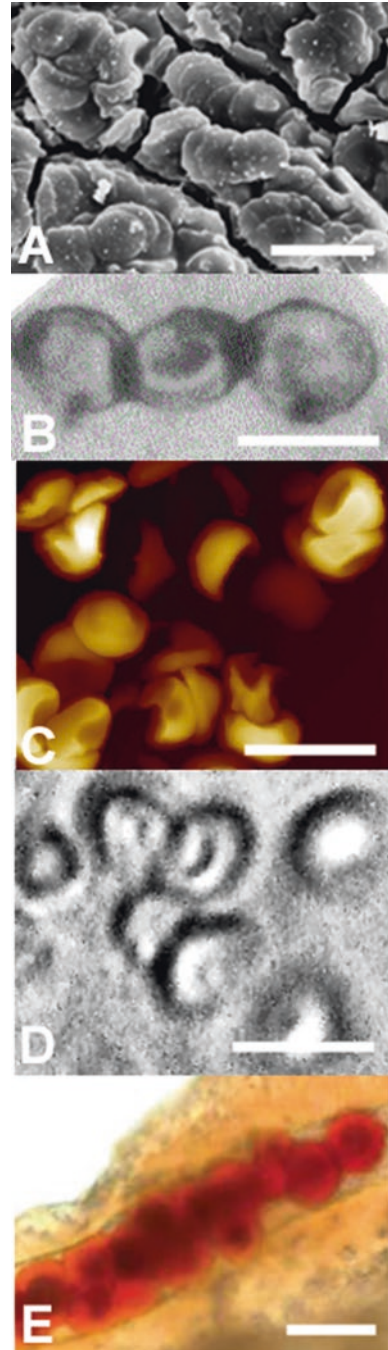
preserved in amber include enucleated red blood cells from a tick (Ixodidae: *Amblyomma*) in 20 Ma Dominican amber, nucleated (reptilian?) erythrocytes from the midgut of a sand fly (Psychodidae: Phlebotominae) in 100 Ma Myanmar amber, and blood cells near the mouthparts of a basal mandibulate chironomid fly in 130 Ma Lebanese amber (Poinar Jr and Poinar 2004; Azar and Nel 2012; Poinar Jr 2017).

Reports of blood cells in vertebrates from deep time are also few in number. Erythrocytes from a lizard in the Middle Eocene lignite of the Geiseltal near Halle in central Germany were reported by Voigt (1939, 1988) and Chin et al. (2003) described undigested tissue in a coprolite of an approximately 75 Ma tyrannosaurid (the volume of the coprolite was 6 L) from Alberta, Canada, that contained "tiny blocks" that resembled "red blood cells in capillaries"; the composition of this material was mostly carbon. Without question, the most well-known and influential work in the field is that of Schweitzer and colleagues. Initial reports of small red intravascular microstructures in bones of *Tyrannosaurus rex*, some of which were described as having "opaque central regions," were published by Schweitzer et al. (1997a, 1997b). Similar results were subsequently obtained with the same specimen and two different specimens of *T. rex* (Schweitzer and Horner 1999; Schweitzer et al. 2005a, 2007a). Schweitzer et al. (2005a) also reported the presence of structures "morphologically consistent with endothelial cell nuclei" in *T. rex* blood vessels. Small red microstructures/erythrocytes were also identified in bone tissues of a 1 Ka moa, a 300 Ka mammoth (*Mammuthus columbi*), and a 78 Ma specimen of *Brachylophosaurus canadensis*; however, such structures were not observed in the bone tissues of a 65 Ka mastodon *Mammut americanum* (Schweitzer et al. 2007a). More recently, Bertazzo et al. (2015) reported concave structures that resembled erythrocytes from a theropod dinosaur bone although, at approximately 2 nm in diameter, they were very small. Three-dimensional reconstructions of serial sections of these erythrocytes, when examined by scanning electron microscopy for backscattered electrons, revealed central areas of high density that were postulated to be nuclei. Interestingly, the bone specimens, from the Campanian Dinosaur Park Formation in Alberta, Canada, consisted of fragments that were "not exceptionally preserved." Nevertheless, mass spectrometric analysis of the "erythrocytes" identified peaks in common with whole blood from an extant emu (Chin et al. 2003).

Pawlicki and Nowogrodzka-Zagórska (1998) performed a scanning electron microscopic examination of femoral and toe bone fragments of an 80 Ma *Tarbosaurus bataar* from the Gobi Desert of Mongolia and reported the presence of endothelial cells lining the vessel walls. In addition, cells 15–18μm in diameter which "strongly resembled the erythrocytes in contemporary reptiles" were observed in vessel lumina. The identification of these structures as "erythrocytes" was supported by energy-dispersive spectroscopy which revealed that iron was concentrated 20-fold in the erythrocytes relative to the walls of the vessels. A more recent study described Haversian canals in the horn of a *Triceratops horridus* from the late Cretaceous Hell Creek Formation in Montana with permineralized (bi?)concave "red blood cell-like microstructures" 6–12μm in diameter (Armitage and Anderson 2013; Armitage 2016). Adjacent vessels contained large (≤50μm long) cuboidal crystals referred to as "possible blood products." It has been pointed out that all

structures that look like fossil erythrocytes are not necessarily cells; iron-rich pyritic framboids and bacterial films can mimic cell and vessel-like structures (Kaye et al. 2008). Martill and Unwin (1997), David (1997), and Cadena (2016) have reviewed the literature and registered warnings that still apply today.

12.4   Blood Parasites In Situ

About 14,000 species of extant arthropods have developed the capacity to feed on vertebrate blood (Graça-Souza et al. 2006). There are 28 species of mosquitos (Diptera: Culicidae) recorded in the Paleobiology Database (accessed/2017), 30 species of sand flies (Psychodidae: Phlebotominae), 17 species of fleas (Siphonaptera), 1 species of lice (Phthiraptera), 2 species of soft ticks, and 2 species of hard ticks (Ixodidae) and a single species of a "kissing bug" (Reduviidae: Triatominae). The majority of these organisms were undoubtedly hematophagic and many were vectors of other parasites but only a very few of them have been preserved as fossils with observable blood meals (Labandeira and Li 2021). Among those that have are sand flies from 15–20 Ma Dominican amber and *Palaeomyia burmitis* in Cretaceous amber from Myanmar which were both described as having preserved remnants of a blood meal (Grimaldi 1996; Poinar 2004). Similarly, a blood-engorged mosquito from 46 Ma lacustrine shale in Montana (Greenwalt et al. 2013) and a nymphal tick, *Amblyomma* sp., in Dominican amber with mammalian erythrocytes within its gut (Poinar Jr 2017), have been described.

Fossils of hosts with the blood stages of the organisms with which they are parasitized are, not surprisingly, rare as well. Even well-preserved mummies, although commonly preserved with the anatomical pathologies of parasite infestations, have not revealed the parasites themselves within blood vessels and/or blood cells. For example, although Ruffer (1910) reported calcified eggs of the blood fluke (*Bilharzia hemotobium*) in kidney tissue of a 20th dynasty Egyptian mummy, neither schistosomulae nor adults have been observed in blood vessels of mummies. Similarly, while "nests" of the amastigote stage of *Trypanosoma cruzi* have been observed in cardiac muscle of an Inca mummy from Peru, the blood stage of the parasite, the trypomastigotes, has not (Fornaciari et al. 1992).

The main mechanism for the preservation of deep time blood stages of parasites seems to be entombment of their hosts in amber (Poinar 2014, 2018, 2021). Such specimens have provided numerous examples of parasite blood stages in situ. In an examination of inclusions in 99 Ma amber from Myanmar, Poinar and Poinar (2010) found that approximately 50% of all fossil sand flies (Psychodidae: Phlebotominae) were infected with trypanosomatid parasites. The collective taxon *Paleoleishmania* was created by Poinar and Poinar (2004) for fossil "digenetic trypanosomes associated with sand flies." The genus contains two species, *P. proterus* from Myanmar amber and *P. neotropicum* from Dominican amber. The former's midgut contained promastigotes within the remains of a blood meal (Poinar Jr and Poinar 2004; Poinar and Poinar 2004). *P. neotropicum* contained amastigotes on its proboscis, preserved,

as the authors speculated, as a result of being entombed immediately after feeding (Poinar 2008). Amastigotes of another leishmanial trypanosome were localized within nucleated (reptilian) erythrocytes in the midgut of the 99 Ma psychodid *Palaeomyia burmitis* (Fig. 12.3a, b; Poinar 2004; Poinar Jr and Poinar 2004). Trypanosomatid parasites were also figured in the blood meal of a midge (Diptera: Ceratopogonidae: *Protoculicoides*?) from 99 Ma Myanmar amber (Fig. 12.3c; Poinar and Poinar 2005).

Blood stages of malarial parasites have also been reported as fossils. The mosquito *Culex malariager* described from Dominican amber by Poinar (2005a) was shown to harbor ookinetes and, possibly, microgametes of *Plasmodium dominicana* (Fig. 12.3d, e; Poinar 2005b). Although Poinar and Telford (2005) described oocysts and sporozoites in the abdominal cavity of *Paleohaemoproteus burmacis* from a 99 Ma ceratopogonid, these structures/cells may have been in the epithelial cells that line the gut of the insect and not a component of the blood meal per se. Finally, the prolific Poinar Jr (2017) also described an intraerythrocytic piroplasm in red blood cells of a tick (Ixoidea: *Ambylomma* sp.) (Fig. 12.3f).

12.5 Molecular Components of Blood

12.5.1 DNA

Integral to this review is an assessment of the fossil record of blood-related biomolecules, including DNA and protein. These biomolecules, and small molecules such as pigments, complex carbohydrates, and biochelates, vary dramatically in both the time span over which they survive and their informational content; unfortunately, these characteristics are inversely proportional. In a study of DNA preservation in fossil moas of ages ranging from 602 to 7839 years, Allentoft et al. (2012) calculated half-life values for a 30-base-pair fragment at 25 °C, 5 °C, and -5 °C of 500, 20,000, and 158,000 years, respectively, suggesting that survival of DNA fragments of informative lengths past 10^6 years of age is exceedingly unlikely. To date, that estimate appears accurate. However, preservation is dependent upon depositional environment and taphonomic conditions and recent advances in extraction technology, prevention and recognition of contamination, third-generation sequencing, etc. have vastly enhanced our ability to obtain valuable sequence information from ancient DNA (Schadt et al. 2010; Wood et al. 2013; Damgaard et al. 2015; Hagelberg et al. 2015; Sarkissian et al. 2015; Llamas et al. 2017; Wood 2018). Only 17 years ago the technical limit on DNA retrieval was thought to be 40 Ka (Smith et al. 2001). In striking contrast, the complete genome of a 560–780 Ka *Equus* fossil from the Yukon region of Canada currently stands as the oldest ancient DNA (Orlando et al. 2013). The report of DNA sequence from an 80 Ma unidentified dinosaur however is now regarded as inaccurate (Woodward et al. 1994).
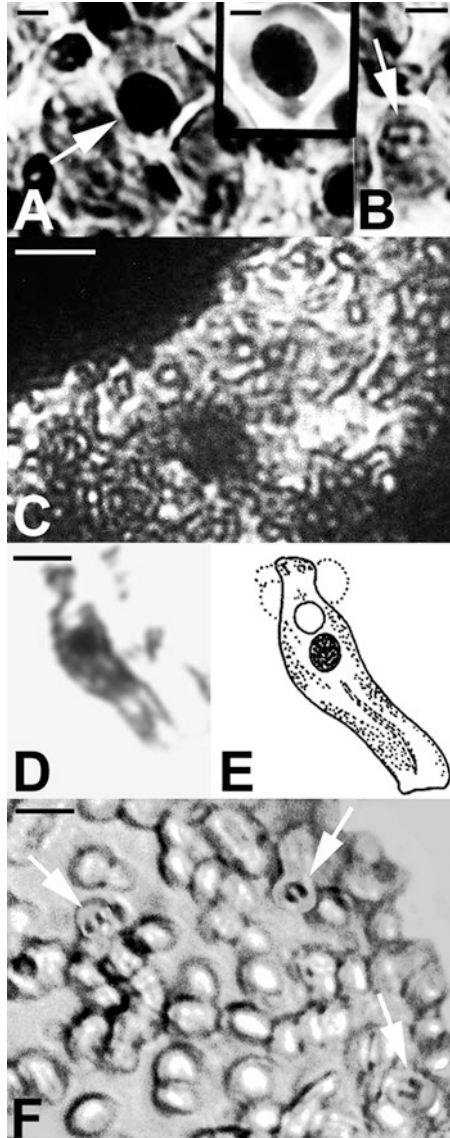
Fig. 12.3 Blood-stage parasitic organisms preserved as fossils. (**a**) A nucleated erythrocyte (arrow) in the thoracic midgut of a 99 Ma sand fly *Palaeomyia burmitis* (Diptera: Psychodidae: Phlebotominae). Insert depicts an erythrocyte of an extant lizard (Poinar and Poinar 2004); (**b**) putative parasitophorous vacuole (arrow) with developing amastigotes of a leishmanial trypanosomatid in an erythroid cell in the thoracic midgut lumen of the fly in A. Note chromatin clumps in the nucleus below vacuole; (**c**) trypanosomatid promastigotes in the midgut lumen of a biting midge (Diptera: Ceratopogonidae) preserved in 99 Ma Myanmar amber (Poinar and Poinar 2005); (**d**, **e**) microphotograph and drawing, respectively, of a putative oökinete of *Plasmodium dominicana* in a mosquito (Diptera: Culicidae: *Culex*) in Dominican amber (Poinar 2005b); (**f**) dividing stages of the piroplasm *Paleohaimatus calabresi* (arrows), some still connected at base, in erythrocytes of a tick (Ixodidae: *Ambylomma*) in Dominican amber (Poinar 2017). Scale bars in A–F = 3, 3, 5, 5, 5, and 10μm, respectively

Numerous studies have obtained blood parasite DNA sequence data from mummies and skeletons. For reviews, see David (2000), Araújo et al. (2009), Dittmar (2009), and Guhl (2017). These include detection of *Trypanosoma cruzi* kinetoplastid DNA in Brazilian, Chilean, and Peruvian mummies, some as old as 9 Ka (Guhl et al. 1997, 1999, 2014; Ferreira et al. 2000; Madden et al. 2001; Dittmar et al. 2003; Aufderheide et al. 2004; Fernandes et al. 2008; Lima et al. 2008), and *T. brucei* DNA in 4 Ka Egyptian mummies (Zink et al. 2006). The oldest parasite DNA sequence, of the gastrointestinal microsporidian *Enterocytozoon bieneusi*, was recovered from a 48 Ka specimen of *Homo neanderthalensis* (Weyrich et al. 2017). However, given the multiple stages of many blood parasites which themselves are often tissue specific, DNA-based confirmation of the actual presence of the blood form of a parasite may not be straightforward. For example, Matheson et al. (2014) reported amplification of *Schistosoma mansoni*- and *S. haematobium*-specific DNA sequences from liver and intestinal tissues from two approximately 3.9 Ka Egyptian mummies. One might assume that the PCR products confirmed detection of the schistosomulae and/or adults of these parasites, which are restricted to portal and mesenteric blood vessels of the host. However, the primers used were based on an intergenic spacer region in *S. haematobium* near the 28S ribosomal RNA gene (Kane and Rollinson 1998) and a 121 bp tandem repeat sequence from *Schistosoma mansoni* known as Sm1-7 (Hamburger et al. 1991); the latter could also detect schistosomal DNA sequences in infected snails. Neither probe appears therefore to be blood stage specific. The distinction however may be overfine and an unproductive exercise in splitting hairs.

A more definitive case is the detection of malaria in King Tutankhamun and his ancestors (Hawass et al. 2010). To test for *Plasmodium falciparum* DNA, PCR primers were designed to specifically amplify several *P. falciparum* gene fragments, one of which encoded a portion of the erythrocyte infective merozoite-specific merozoite surface protein 1. PCR products and cloned DNA fragments were sequenced to reveal that not only had Tutankhamun been infected, but two of his 18th-dynasty ancestors, Thuya and Yuya, also had malaria. In fact, detection of two distinct *P. falciparum* alleles of merozoite surface protein 1 demonstrated that both Tutankhamun and Yuya had been infected on two separate occasions. Tutankhamun is also thought to have had sickle cell disease (Timmann and Meyer 2010). The disease is caused by one of several different mutations in the hemoglobin gene thought to have evolved in response to malaria. The most common mutation results in the substitution of valine for glutamic acid at position 6 in the beta-globin chain to yield what is termed hemoglobin S, a variant of hemoglobin that, via multiple mechanisms, both confers host tolerance to the parasite and decreases the pathology of the disease (Ferreira et al. 2011). Sickle cell nucleotide mutations have been detected in several >5 Ka Egyptian mummies (Marin et al. 1999).

Much of the currently available data on ancient blood and blood cell DNA has been derived from recent whole nuclear genome sequences. The relative ease with which ancient genomes can be sequenced is demonstrated by the recent spate of research on human ancestry (reviewed in Haber et al. 2016). A short list of such genomes includes that of a 50–30 Ka Denisovan, a Paleo-American from a Clovis

site in Montana, a 50 Ka Neanderthal, and a 45 Ka modern human from Siberia and Ötzi (Keller et al. 2012; Meyer et al. 2012; Fu et al. 2014; Prüfer et al. 2014; Rasmussen et al. 2014). The oldest human genome sequenced to date is the mitochondrial genome of a >300 Ka hominin morphologically similar to *Homo heidelbergensis* from northern Spain (Meyer et al. 2014). Examinations of whole-genome sequence data have revealed that Otzi was blood group O+ while a 4 Ka Paleo-Eskimo from Greenland was blood group A (Rasmussen et al. 2010; Keller et al. 2012). Olalde and colleagues (Olalde et al. 2014) identified 24 genes for immune modulators, many of which are blood cell specific, such as cytokines, chemokines, and their receptors, from the genome of a 7000-year-old Mesolithic European.

Whole genomes of the blood-borne pathogen *Yersinia pestis* that, with the rat as its vector, causes the plague, have been obtained from teeth of numerous human skeletons dating from the sixth to the eighteenth centuries (Bos et al. 2011; Wagner et al. 2014). Upon infection, *Y. pestis* invades macrophage cells from which it then emerges resistant to subsequent phagocytosis by immune cells; the bacterium can reach blood concentrations as high as 100 million/mL. In 2015, Rasmussen and colleagues obtained whole genomes from 101 individuals that dated from approximately 3000 to 800 BC and demonstrated that, prior to 1686 BC, *Y. pestis* lacked a critical virulence factor (*Yersinia* murine toxin) without which it was incapable of causing the bubonic plague (Rasmussen et al. 2015).

In an instructive study of the last surviving population of woolly mammoths (*Mammuthus primigenius*) on Wrangel Island, Pečnerová et al. (2016) demonstrated a pronounced decrease in genetic variation relative to several loci of the MHC DQA gene. The major histocompatibility complex (MHC) class II protein heteromer consists, in part, of alpha (DQA) subunits anchored in the membrane of immune cells such as B lymphocytes, dendritic cells, and macrophages and is essential to immune reactions to foreign antigens. The small and isolated population of mammoths, having survived 6000 years longer than mainland populations, exhibited a 20% loss in autosomal heterozygosity and a 37% loss in allelic richness; inbreeding and genetic drift may have been largely responsible for the eventual extinction of the species.

In a study that exemplifies the power and potential of future molecular paleobiological research, Campbell et al. (2010), using DNA extracted from a 43 Ka Siberian woolly mammoth, isolated the genes for the α- and β/δ-globin chains of hemoglobin. The sequences differed in several positions relative to the Asian elephant. Specifically, the woolly mammoth β/δ-globin chain had acquired three amino acid substitutions: threonine to alanine at position 12, alanine to serine at position 86, and glutamic acid to glutamine at position 101. When the recombinant mammoth protein was synthesized and assayed for its ability to bind and release oxygen, the changes in the structure of the hemoglobin molecule that resulted from the three amino acid substitutions were shown to enhance the binding of chlorine ions and 2,3-bisphosphoglycerate, allosteric effectors that, while not directly involved in oxygen binding, functioned to promote the release of oxygen at cold temperatures. This phenotypic adaptation to colder arctic temperatures was probably critical to the success of the mammoths and allowed them to dominate the arctic Pleistocene for hundreds of thousands of years.

Can DNA sequence be obtained from fossils in rocks older than 1 Ma? As early as the mid-1990s, ancient DNA was purportedly detected in deep time fossils through the use of histochemical stains. For example, Pawlicki (1995) reported staining of DNA in osteocytes from bones of an 80 Ma specimen of *Tarbosaurus bataar* through the use of both ethidium bromide and Feulgen's method (Schiff reagent). Schweitzer and colleagues later reported localization of DNA in the lacunae (osteocytes) of a 66 Ma bone fragment of *Tyrannosaurus rex* with a bisbenzimide Hoechst DNA stain and subsequently repeated the observations in isolated "osteoclasts" with antibodies to DNA and the DNA stains propidium iodide and DAPI in both the *Tyrannosaurus rex* and an 80 Ma specimen of *Brachylophosaurus canadensis* (Schweitzer et al. 1997a; Schweitzer et al. 2013). Woodward et al. (1994) reported the isolation of DNA sequence from small (20 cm) bone fragments from an unidentified vertebrate preserved in the 80 Ma Blackhawk Formation in Utah. The interpretations of Woodward and colleagues were however questioned by numerous investigators (Hedges and Schweitzer 1995; Henikoff 1995; Allard et al. 1995; Zischler et al. 1995; Allentoft et al. 2012). There are other claims for the isolation of DNA from deep time fossils (e.g., Golenberg et al. 1990; Sutlovic et al. 2008) but here too, the consensus is that these data are artifactual (Allentoft et al. 2012 and references therein). While these data are intriguing, verifiable DNA sequence data from deep time have yet to be reported. The oldest fossil genome dates back approximately 700,000 years (Orlando et al. 2013); older DNA may well be buried, undiscovered, and waiting to be extracted and sequenced—particularly in permafrost.

Given the fidelity of the preservation of cellular and even subcellular structures in amber inclusions, amber would seem to be a plausible source of ancient DNA. Despite numerous attempts however, the consensus is that DNA is not preserved in such resins (Lindahl 1993; Howland and Hewitt 1994; Austin et al. 1997; Walden and Robertson 1997; Gutiérrez and Marin 1998; Hebsgaard et al. 2005; Peris et al. 2020).

12.5.2 Protein

The development of high-throughput shotgun protein sequencing technologies has dramatically increased the feasibility and potential of paleoproteomics (Altelaar et al. 2013; Mann et al. 2013; Zhang et al. 2013; Schweitzer et al. 2019). Current technology provides the ability to sequence large numbers of proteins simultaneously. For example, Cappellini et al. (2012) reported the determination of more than 100 partial protein sequences from a 43 Ka mammoth from Siberian permafrost, nearly 20% of which were blood cell or plasma proteins. The ability to more effectively characterize the fossil proteome is particularly important because the fossil record of protein is older than that of DNA (Allentoft et al. 2012; Tomiak et al. 2013; Briggs and Summons 2014). And while whole genomes provide a staggering amount of data of potential phylogenetic value, unlike the proteome, they do not allow analysis of that fraction of the genome that is actually translated into protein (i.e., levels of expression).

Abelson (1954, 1957) was the first to report the presence of amino acids in fossils—amino acid profiles similar to those of collagen and amino acids from fossils as old as 360 Ma. In 1974, De Jong et al. (1974) demonstrated cross-reactivity between antisera to decalcified extracts of the shell of the 70 Ma cephalopod *Belemnitella junior* and related extant species and cross-reactivity between antisera to extracts of the extant species and those of the fossil. Any attempt to identify the oldest fossil from which a protein has been identified must deal with numerous older controversial reports. For example, Gurley et al. (1991) reported unidentified non-collagenous proteins from a 150 Ma *Seismosaurus* (*Diplodocus*) from the Morrison Formation of New Mexico. Different fractions of proteinaceous material were defined by amino acid profiles but sequencing per se was not attempted. It must be kept in mind that the existence of "deep time" sequenceable protein is highly controversial with viable arguments that such material is not preserved for more than a few million years (Pevzner et al. 2008; Buckley et al. 2008, 2017). The consensus is that the oldest verifiable ancient protein sequences are those of the protein struthiocalcin in 3.8 Ma egg shells from Laetoli (Demarchi et al. 2016).

The first attempt to identify blood antigens in archeological and/or fossil specimens was that of Boyd and Boyd (1934) who identified blood groups in Egyptian mummies using standard serological techniques. Early (i.e., ≥20 years old) reports (albeit not an exhaustive list) of the detection of ancient blood proteins are listed in Table 12.1. Most of the proteins identified are among the most abundant blood proteins, i.e., albumin and hemoglobin. Nearly all of the reports utilized immunoassays, at that point the only viable technology available. In more recent studies, Lindgren et al. (2017) identified hemoglobin in the shell of a specimen of *Tasbacka danica*, a 55 Ma turtle from the Fur Formation in Denmark, and Schweitzer et al. (2009) reported identification of hemoglobin, again through the use of immunolocalization assays, in an 80 Ma *Brachylophosaurus canadensis*. Similarly, Schweitzer et al. (1997b, 2002) reported cross-reactivity of antiserum to extracts of bone tissues from both *T. rex* and a 200 Ka ± 100 Ka mammoth with extant hemoglobin. However, the design, application, and interpretation of antibody-based assays have many potential pitfalls that are compounded when applied to fossil material (Schweitzer et al. 2008; Potter et al. 2010; Saitta et al. 2018b). Histochemical techniques have also been used. For example, Grigoriev et al. (2017) used the hemoglobin cyanide method to detect hemoglobin at a concentration of 22 g/dL in dark brown fluid obtained from a 30 Ka frozen mammoth from Siberia.

Modern mass spectrometry-based protein sequencing technologies now provide the paleobiologist and paleoanthropologist with the ability to generate ancient, albeit incomplete, proteomes. Such potential is exemplified by the work of Cappellini et al. (2012) that produced partial sequences for 126 different proteins from a metapodial bone of a 43 Ka mammoth preserved in Siberian permafrost. Twenty-six of these were either plasma proteins or protein constituents of blood cells (Table 12.2). Cappellini's group obtained 47% of the sequence of albumin and went on to demonstrate two mutations, at positions 68 and 218. A comparison with the sequences of the African and Indian elephant albumins supported the

Table 12.1 Early reports of detection of ancient proteins

Protein	Specimen	Age	Site/country	Technique[a]	Reference
Blood group B antigen	Mummy	3.2 Ka	Egypt	IA	Boyd and Boyd (1934); Hart et al. (1977)
Albumin	Mammoth	40 Ka	Siberia	ID	Prager et al. (1980)
Albumin	Mammoth; Tasmanian wolf	Holocene	Siberia; Australia	RIA	Lowenstein et al. (1981
Albumin	Mammoth; mastodon	10 Ka	Siberia; Michigan	ID, RIA	Shoshani et al. (1985)
Hemoglobin	Human skeletal bone	4 Ka	Italy	IB	Ascenzi et al. (1985)
Albumin	Mastodon	13 Ka	Venezuela	IB	Tuross (1989)
Hemoglobin	Human skeletal bone	2 Ka	Italy	ELISA	Smith and Wilson (1990)
Albumin, IgG	Native American	1 Ka	United States	IB	Tuross (1991)
Albumin, IgG	Human skeletal bone	Iron and Bronze ages	Europe	ELISA	Cattaneo et al. (1992)
Hemoglobin	Stone tool	90 Ka	Israel	Crystallization	Loy and Hardy (1992)
IgA	Human cranium	Medieval	Europe	ELISA	Cattaneo et al. (1994)
Hemoglobin	Stone tool	1.5 Ka	Chile	ELISA	Tuross and Dillehay (1995)
Albumin	Hominid fossil bone	1.6 Ma	Spain	ELISA, RIA	Borja et al. (1997)
Hemoglobin	*Tyrannosaurus rex*	65 Ma	Montana	ELISA	Schweitzer et al. (1997b)
IgG	Hominid and equid	1.6 Ma	Spain	IB	Torres et al. (2002)
Albumin, a1-trypsin, a2-HSGP	Human skeletal bone	Pre-Columbian	Peru	ELISA	Brandt et al. (2002)

[a]*ID* Immunodiffusion, *RIA* radioimmunoassay, *IB* immunoblot, *ELISA* enzyme-linked immunosorbent assay, *IA* immunoassay

well-known monophyly of that group. Orlando et al. (2013) obtained sequence data for 73 different proteins from the femur of a 560–780 Ka *Equus* buried in permafrost in the Yukon of Canada, 15 of which were plasma proteins. Lists of the plasma proteins from the studies of Orlando et al. (2013) and Cappellini et al. (2012) show that nearly half (18/41; 44%) of the plasma proteins identified in the two studies were either coagulation factors or coagulation-related proteins (Table 12.2). Coagulation factors VII, IX, and X were common to both specimens. Hill et al. (2015) obtained sequences of 33 proteins from the skull of a 120 Ka specimen of

Table 12.2 Lists of mass spectrometry-derived ancient blood protein sequences from fossil Mammuthus, Equus, and Ötzi

Mammuthus (Cappellini et al. 2012)	*Equus* (Orlando et al. 2013)	Ötzi (Maixner et al. 2013)	Blood protein rank[a] (Farrah et al. 2011)
		Hemoglobin	1
Albumin		Albumin	2
		Fibrinogen alpha chain	3
	Apolipoprotein A-I precursor		5
Antitrypsin	**Alpha-1-antitrypsin**	**Alpha-1-antitrypsin**	8
C3 complement component		**C3 complement-like**	9
Alpha-2-HS-glycoprotein	**Alpha-2-HS-glycoprotein**		16
		Antithrombin-III	20
		C1 inhibitor	23
Apolipoprotein A-IV	**Apolipoprotein A-IV**		25
	Alpha-trypsin inhibitor heavy chain H1-like		31
Plasminogen			34
	Heparin cofactor 2		38
C9 complement			51
C8 complement, beta polypeptide			56
	Apolipoprotein C-II precursor		59
Apolipoprotein E			63
	Vitamin k-dependent protein S		68
C8 complement, gamma polypeptide			76
Coagulation factor IX	**Coagulation factor IX**		99
Coagulation factor X-like	**Coagulation factor X-like**		105
Thrombospondin 1			120
Coagulation factor II (prothrombin)	**Prothrombin**	**Prothrombin**	172
Vitamin K-dependent protein Z	Vitamin K-dependent protein Z		543
Coagulation factor VII	**Coagulation factor VII**		604
	Annexin A5	Annexin A5	829
		Leukocyte CD47	NA

(continued)

Table 12.2 (continued)

Mammuthus (Cappellini et al. 2012)	*Equus* (Orlando et al. 2013)	Ötzi (Maixner et al. 2013)	Blood protein rank[a] (Farrah et al. 2011)
	Annexin A5	Annexin A5	
	Immunoglobulin gamma 7 heavy chain		NA
C-type lectin domain family 11, member A			NA
Immunoglobulin gamma 1 heavy chain			NA
Immunoglobulin superfamily, member 3			NA
Kininogen A5			NA
Protein C			NA
Protein S alpha			NA
Serpin peptidase inhibitor			NA
Serpin peptidase inhibitor, D			NA
Unidentified immunoglobulin			NA

Clot proteome proteins are highlighted in bold

[a]In some cases the presence of a fragment or subunit of a protein is equated to the presence of the intact protein. The ranking of the most common proteins in blood is from those referenced in Farrah et al. as "Published"

Bison latifrons, from near Snowmass, Colorado. Of these, albumin, alpha-2-serum glycoprotein, and prothrombin are plasma proteins.

In contrast, an ancient proteome obtained from brain tissue of Ötzi, the 5.3 Ka mummy from the Tyrolean Alps, consisted of sequences from 502 proteins, only 11 of which were blood related (Maixner et al. 2013). Albumin sequences provided 87% coverage of that protein; other proteins included three different subunits of hemoglobin, fibrinogen, and leukocyte CD47 (Table 12.2). Partial sequences of prothrombin and anti-thrombin III, both coagulation-related proteins, were also obtained. Maixner et al. (2013) stated that their presence "could support the theory of an injury of the head near the site where the samples have been extracted." The most notable aspect of these data is the number of coagulation-related proteins, such as the coagulation factors, in the *Equus* and *Mammuthus* proteomic data. Stachowicz et al. (2017) produced a human clot proteome that consisted of 476 different proteins. In addition to the expected proteins directly related to coagulation, a major component of the clot proteome consisted of proteins characteristic of extracellular vesicles generated by platelet activation. Clot proteome proteins are highlighted in Table 12.2. While a number of the clot-related proteins are also some of the more prevalent proteins in plasma, several (e.g., coagulation factors II, VII, IX, and X, thrombospondin) are present in normal plasma at two or three orders of magnitude

less (e.g., Farrah et al. (2011) list coagulation factor VII as the 604th most prevalent protein in plasma). The presence of coagulation-related proteins in fossil bone proteomes is undoubtedly the result of postmortem thrombogenesis (blood clot formation). Whether or not clotting-based fractionation of proteins into aggregated and potentially more proteolytically resistant forms is responsible for survival of one or more of these ancient proteins is unknown.

Fibrinogen was absent from the *Equus* and *Mammuthus* proteomes but was recovered from the brain tissue of Ötzi. Fibrinogen makes up about 7% of plasma protein and polymerized fibrin is the major component of a clot. However, postmortem fibrinolytic activity can remove that fibrin. Janko et al. (2012) and Ferrón et al. (2014) both reported the presence of large blood clots near one or more wounds incurred immediately before Ötzi's death. Janko et al. (2012) concluded that fibrinolytic activity had occurred due to their inability to detect fibrin fibrils and/or meshwork.

Proteome analysis of a swab of the blood-covered lips of the 500-year-old Andean mummy of a young girl identified 67 different proteins. Partial sequences for albumin, immunoglobulins, hemoglobin, serotransferrin, apolipoproteins A-I and A-II, fibrinogen, and hemopexin as well as several neutrophil proteins (neutrophil defensins 1 and 3, neutrophil elastase) were obtained; coagulation factors were not among the proteins identified. The girl had apparently suffered severe inflammation due to a pulmonary bacterial infection; DNA amplification identified the presence of *Mycobacterium* sp. (Corthals et al. 2012). Barbieri et al. (2017) obtained 30 human protein sequences from dental pulp—a favored source of ancient protein and DNA—of several 300-year-old skeletons from France. Of these, prothrombin and coagulation factors IX and X were present along with the plasma proteins albumin, alpha-2-serum glycoprotein, IgA, and IgG.

Reports of blood proteins from dinosaurs and similarly old fossils are rare. Schweitzer et al. (1997b) reported reactivity of an antiserum made to *Tyrannosaurus rex* bone extracts to purified recent hemoglobin. Bern et al. (2009) reported sequences of very short peptides that matched bird hemoglobin in an analysis of bone from a 68 Ma specimen of *T. rex*. Isolated blood vessels from an 80 Ma specimen of the dinosaur *Brachylophosaurus canadensis* were prepared by Cleland et al. (2015) and analyzed for proteins. Partial amino acid sequences from actin, myosin, tropomyosin, and both α- and β-tubulin were reported. A series of actin sequences that contained 252 residues aligned with a consensus sequence 402 residues in length.

As previously indicated, the first attempts to identify blood-borne parasite antigens in archeological and/or fossil specimens utilized immunological assays. Records of detection of proteins and/or antigens on the surfaces of blood-borne parasites and pathogens in medieval skeletons, Egyptian and American mummies, etc. are dominated by work on three parasites, *Plasmodium*, *Schistosoma*, and *Trypanosoma*. Schistosomiasis is most often diagnosed with an immunoassay directed at circulating anodic antigen (CAA), a branched disaccharide that contains at least 30 repeating units composed of 2-acetamido-2-deoxy-β-D-galactopyranose and β-D-glucopyranuronic acid that is released from the parasite into the host's blood (Bergwerff et al. 1994). Although attached to a protein via O-glycosylation, it

is the carbohydrate portion of the protein that is both immunogenic and highly stable. Immunocytochemical and ELISA assays have been used to diagnose schistosomiasis in Egyptian mummies as old as 5.2 Ka by numerous laboratories (Deelder et al. 1990; Miller et al. 1992, 1994; Rutherford 1999, 2000, 2005, 2008; Lambert-Zazulak 2003; Hibbs et al. 2011).

The presence of *Plasmodium* is usually determined via the detection of either *P. falciparum* histidine-rich protein 2 antigen or Merozoite surface protein 1. The former is a large protein that is found both at the erythrocyte cell surface and soluble in the blood. It is unique in that it contains numerous repeats of alanine and histidine-rich sequences (e.g., AHHAAAHHEAATH, AHHAHHVAD, and AHHTHHAAD) (Baker et al. 2010). Merozoite surface protein 1 is assayed by detection of the highly immunogenic 19 kDa C-terminal domain of the protein which remains on the surface of the parasite after processing of the much larger 200 kDa precursor (Mazumdar et al. 2010). Both *Plasmodium falciparum* and *P. haematobium* have been detected by immunochemical assays in Egyptian mummies as old as 5.2 Ka (Miller et al. 1994; Bianucci et al. 2008; Rutherford 2008).

An immunoassay was used by Fornaciari et al. (1992) to detect *Trypanosoma cruzi* in a 0.6 Ka Peruvian mummy. Other blood-borne pathogens that have been detected in archeological and paleontological specimens by immunological assays include *Yersinia* in 0.3 Ka dental pulp from France (Barbieri et al. 2017) and *Treponema* in ancient human skeletal remains from North America (Ortner et al. 1992). The oldest specimen in which *Treponema* has been identified, by an immunochemical assay, is an 11.5 Ka Pleistocene bear (Rothschild and Turnbull 1987).

12.5.3   Small Molecules

The vast majority of all known ancient biomolecules are small molecules, defined here as everything other than polymeric DNA and protein. For example, both oil and coal consist largely of fragmented and derivatized biomolecules derived from plants. Similar molecules serve as biomarkers for specific types of organisms (reviewed in Eglinton and Eglinton 2008; Briggs and Summons 2014). For example, 24-isopropylcholestanes are derived from large sterol compounds synthesized by desmosponges and have been reported in rocks as old as 635 Ma (Love et al. 2009). Small molecules such as chitin, a homopolymer of N-acetylglucosamine, and a major component of arthropod exoskeletons, and the pigment melanin have been found in fossils ca. 505 Ma and >160 Ma old, respectively (Glass et al. 2012; Ehrlich et al. 2013).

Grigoriev et al. (2017) detected creatinine at a concentration as high as 1000-fold higher than normal in dark brown fluid obtained from a 30 Ka frozen mammoth from Siberia. The assumption was that the creatinine originated with massive degradation of phosphocreatine from the animal's muscle. This report of "blood" creatinine however is an exception. The preservation of blood-specific or even blood-associated small biomolecules in deep time fossils is typically limited to a

single compound, heme, and its derivatives. Heme ($C_{34}H_{32}O_4N_4Fe$) is a planar-conjugated heterocycle comprised of four 5-membered substituted pyrrole rings with a mass of 616. At its center is an atom of iron, bound by the four nitrogen atoms of the pyrrole rings. In the absence of iron, the molecule is referred to as a porphyrin. It is not known to be synthesized abiotically and is extremely stable—so much so that it has been proposed as a target in NASA's search for life on Mars (Suo et al. 2007). Metalloporphyrins are ubiquitous; they serve as redox centers that are essential for respiration and as charge-transfer centers in proteins such as cytochromes, catalases, and other enzymes; chlorophyll is a porphyrin. As one would expect, the fossil record of porphyrins is well documented; as early as 1933, Fikentscher (1933) reported the characterization of porphyrin from a crocodilian coprolite from the Middle Eocene lignite of the Geiseltal near Halle in central Germany. The oldest metalloporphyrins have been isolated from Carboniferous deposits (Izmailova et al. 1996).

Heme is the prosthetic group of hemoglobin, a protein that accounts for approximately two-thirds of the total protein in whole blood. After degradation of the proteinaceous portion of hemoglobin in deep time fossils, heme, or its components iron and porphyrin, often remains and serves as a proxy for the protein. Pawlicki and Nowogrodzka-Zagórska (1998) reported "dinosaur erythrocyte iron" in an 80 Ma specimen of *Tarbosaurus bataar* from the Gobi Desert. Elemental analysis documented a 20-fold higher level of iron in the contents of blood vessels vs. the bone of the dinosaur itself. The first report of heme in fossil dinosaur bones was that of Schweitzer et al. (1997b). Several spectroscopic techniques (nuclear magnetic resonance, electron spin resonance, and Raman scattering) were used to detect iron and heme or heme fragments in 66 Ma *Tyrannosaurus rex* bone tissue from the Hell Creek Formation in Montana. In addition, polyclonal antisera raised against tissue extracts of fossil trabecular bone from this specimen demonstrated binding to purified turkey and rabbit hemoglobin by immunosorbent assays, and to pigeon and rabbit (but not snake) hemoglobin in immunoblot assays. Geist et al. (2002) used mass spectrometry and X-ray diffraction to detect both iron and porphyrin derivatives within the body cavity of the mosasaur *Platecarpus tympaniticus* collected from approximately 83 Ma exposures of the Niobrara Chalk Formation in Kansas. The presence of iron in this same specimen was confirmed by Lindgren et al. (2010). Lindgren et al. (2017) subsequently reported the presence of iron, Fe(CN)2, Fe(CN)3, and higher molecular weight fragments of the heme prosthetic group in the sea turtle *Tasbacka danica* from the 55 Ma Fur Formation of Denmark. They also used immunohistochemical staining of fossil turtle tissues with polyclonal antisera to the hemoglobins of *Alligator mississippiensis* and the ostrich (*Struthio camelus*) to demonstrate the presence of the protein or fragments thereof; staining with the anti-alligator sera was more intense than that of the antisera against bird hemoglobin.

Mosquitos (Diptera: Culicidae) have existed for over 100 million years and 28 different fossil species have been described (Borkent and Grimaldi 2016; PBDB 2017). It was only recently however that a fossil of a blood-engorged mosquito was discovered (Fig. 12.4a; Greenwalt et al. 2013). The preservation of a mosquito, its abdomen distended with a blood meal, would seem improbable (Briggs 2013) but
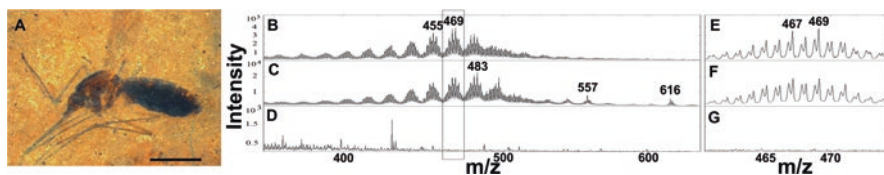
Fig. 12.4 Detection of the porphyrin heme in a 46-million-year-old blood-engorged mosquito (Greenwalt et al. 2013). (**a**) The fossil blood-engorged mosquito. Scale bar = 2 mm; (**b**) ToF-SIMS spectrum of the abdomen of the blood-engorged mosquito; (**c**) spectrum of heme (m/z = 616) and the heme-derived porphyrins in purified pig hemoglobin. Peaks m/z 455, m/z 469, and m/z 483 are three of numerous peaks present in both the abdomen of the female mosquito and pure hemoglobin; (**d**) spectrum obtained from the matrix adjacent to the abdomen of the female mosquito; (**e–g**) enlarged insets of mass region 460–474 of spectra B–D further illustrate the Gaussian distribution of peaks and near identity between the abdominal and hemoglobin control spectra

several such specimens have been discovered in the 46 Ma Kishenehn Formation in northwestern Montana (author, pers. observ.). In one of the specimens, the iron content was nearly tenfold greater in the insect's abdomen than in the abdomen of a male collected from the same site. Mass spectrometry provided a spectrum essentially identical to that of heme from reference hemoglobin (Fig. 12.4b-g); heme was not detected in the male mosquito, the thorax of the blood-engorged female, or shale matrix adjacent to the female. Factors that may have been involved in the preservation of the blood-engorged mosquito are discussed in the section on taphonomy below.

Recently, Yao et al. (2014) published interesting data suggesting that extant hematophagous true bugs (Hemiptera) could be distinguished from phytophagous and predatory species by their overall iron content. The authors then extrapolated these data to suggest that fossil Hemiptera could be identified as hematophagous based on this same criterion. *Flexicorpus acutirostratus* and *Torirostratus pilosus* (Hemiptera: Cimicomorpha: Torirostratidae), both from the 125 Ma Yixian Formation of China, were found to have iron concentrations twice that of four other fossil bugs identified as being either phytophagous or predatory, as determined by electron-dispersive spectroscopy. The authors also speculated that differences in iron content of the abdomen and rostrum (average = 7.31 wt %) vs. that of the legs, thorax, antennae, and head (average = 5.02%) of *Torirostratus pilosus* suggested that the insect had eaten immediately prior to its death. The presence of active transport systems for iron and the excretion of excess iron by insects are well documented (Nichol et al. 2002; Winzerling and Pham 2006). In tsetse fly (*Glossina*) feeding experiments with a radioactive isotope of iron, Kabayo et al. (1988) demonstrated that 98% of blood meal iron was lost after 5 days; the remainder was lost upon completion of the reproductive cycle. Similarly, Warren-Hicks et al. (1979) showed that fleas (*Xenopsylla Cheopsis*) allowed to feed on rats injected with ^{59}Fe incorporated "little of the iron … into the tissues." While the iron contents of the extant hemipterans measured by Yao et al. differed between the phytophagous and predatory insects (2.18 ng/g and 2.51 ng/g, respectively) and the hematophagous

insects (9.74 ng/g), there was no indication of the age, sex, or feeding status of the ten specimens examined; two specimens were reared in the lab; the others were collected from the wild. While Yao et al.'s work is intriguing, much larger, detailed, and more controlled experiments are required before their conclusions can be embraced. Wang et al. (2009) demonstrated iron concentrations in both a fossil fly and beetle from the Middle Jurassic (160 Ma) Jiulongshan Formation in China that were significantly higher than those in the matrix. However, the iron in the beetle was shown to be the result of pyrite deposition. Obviously, depositional environment and taphonomic conditions greatly influence iron retention.

12.6 Taphonomy

Given the relative lability of essentially all biomolecules, and, despite this, the preservation of some ancient proteins, pigments, DNA, etc. for hundreds of thousands if not millions of years (Briggs et al. 2000), are there identifiable factors and/or environments ("preservational niches," "privileged niches," "recalcitrant structures," "molecular refugia") responsible for their survival? Almost all ancient biomolecules have been recovered from bone, teeth, or shells. Although there are exceptions, the existence of DNA and blood proteins on 50,000-year-old tools buried in soil simply cannot be explained given our current incomplete understanding of tool-residue taphonomy. Amber on the other hand, which, at first glance, would appear to be a very effective preservational niche, is not. In any attempt to understand ancient biomolecule preservation, both physical and chemical environments as well as the innate chemical nature of the biomolecule itself must be considered.

Ancient vertebrate DNA is invariably isolated from bone and/or teeth although the isolation of DNA from eggshells has also been reported (Oskam et al. 2010). DNA binds to hydroxyapatite, a process which is thought to promote preservation of the nucleic acid (Grunenwald et al. 2014a, 2014b). Brundin et al. (2013, 2014) have shown that the enzyme DNase is less effective at hydrolyzing DNA when the nucleic acid is bound to either hydroxyapatite or dentin. Evidence for the preservation of DNA in the bones of both dinosaurs from Montana and human skeletons from Pompeii, as determined by fluorescent DNA-specific probes, has been presented by several laboratories although it is difficult to determine if staining is in ancient osteocytes or on the surfaces of osteocyte lacunae (Pawlicki 1995; Schweitzer et al. 1997a; Guarino et al. 2000). Interestingly, Brundin et al. (2014) also demonstrated that DNA bound to collagen, the major protein of bone tissue, was much less susceptible to cleavage by DNase.

Many of the more commonly preserved ancient biomolecules are those that exist at exceedingly high concentrations in the original organism (e.g., chitin in arthropod cuticles and hemoglobin in blood). Approximately 90% of all protein in bone is collagen and albumin is present in bone at concentrations 50- to 100-fold higher than in blood plasma (Owen and Triffitt 1976; Schmidt-Schultz and Schultz 2015). In a study of 31 Bronze and Iron Age skeletons, 23 contained albumin while only a single

specimen contained immunoglobulin (Cattaneo et al. 1992). Other factors that may contribute to the preservation of ancient proteins include their quaternary structure and the presence of posttranslational derivatization. The quaternary structure of several different types of collagen (i.e., the fibril) is highly resistant to protease activity (Perumal et al. 2008). In addition, Hill et al. (2015) recently demonstrated relatively high recoveries of collagen peptides that contained galactosyl- and glucosyl-galactosyl-modified hydroxylysine residues from 120 Ka bone tissue of *Bison latifrons*, collected from the Ziegler Reservoir fossil site in Colorado. Thought to be natural posttranslational modifications made while the animal was alive, their presence in the fossil was proposed to be the result of preferential survival during the fossilization process.

The preservation of albumin, which is not per se particularly resistant to proteolysis, and other proteins, may be due to their ability to bind to hydroxyapatite (Owen and Triffitt 1976; Masters 1987; Deniro and Weiner 1988; Tuross and Stathoplos 1993; Wadsworth and Buckley 2014). Wiechmann et al. (1999) demonstrated that nearly all preserved non-collagenous proteins from pre-Columbian Peruvian human skeletons had isoelectric points below 4.5. This may indicate the presence of relatively large numbers of negatively charged amino acid residues that could mediate binding to hydroxyapatite. Fossilization-mediated derivatization (e.g., deamidation of asparagine resides) may also be a factor. Poser and Price (1979) and Collins et al. (2000) have demonstrated that binding of osteocalcin to hydroxyapatite, via the γ-carboxylated glutamic acid residues at the middle of the protein, protects this portion of the protein from degradation. Similarly, Demarchi et al. (2016) demonstrated that the aspartic acid-rich domain of the ostrich egg protein SCA-1 is preferentially recovered from fossils as old as 3.8 Ma. Proteins that bind to hydroxyapatite (or to aragonite in turtle and mollusk shells and to calcite in bird egg shells) have been recovered from fossil egg shells (Schweitzer et al. 2005b; Demarchi et al. 2016), turtle shells (Muyzer et al. 1992), tooth enamel (Porto et al. 2011), and mollusk shells (Weiner et al. 1976).

Another mechanism through which proteins survive into deep time involves their cross-linking and subsequent polymerization. As early as 1999, Wiechmann et al. (1999) found that non-collagenous proteins were present in pre-Columbian Peruvian skeletons as very-high-molecular-weight components as determined by SDS-polyacrylamide gel electrophoresis. Briggs and colleagues (Briggs 1999; Gupta et al. 2007; Cody et al. 2011; Glass et al. 2013) have subsequently demonstrated that covalent "biopolymerization"/cross-linking is a nearly universal phenomenon and affects all types of biomolecules through deep time. Cross-linking of proteins contributes to resistance to proteolysis/degradation. Wadsworth and Buckley (2014) observed that while collagenase effectively removed collagen from modern bone tissue, it was much less effective at hydrolyzing resident collagen from bones dating from 10 Ka to 900 Ka. Most recently, Wiemann et al. (2018) have demonstrated that soft tissues, including blood vessels, isolated from an array of Mesozoic fossils, consist primarily of N-heterocyclic polymers derived from the oxidative cross-linking of proteins. They propose that the transformation of ancient proteins into nonproteinaceous polymers explains the very unexpected presence of soft tissues in

deep time fossils. Aggregation and cross-linking of proteins also lead to a loss of immunoreactivity which can confound immunoassay-based analysis of ancient proteins (Koch et al. 1998; Anderson and Waite 2000; Terwilliger et al. 2005). Despite these observations, the preservation of collagen and other proteins is exceedingly poor compared to other compounds (Saitta et al. 2019).

The ability of a variety of metals, including copper, zinc, and manganese, to induce the aggregation of proteins is well known (Bush 2003; Brown 2010). These metals also have bactericidal properties which have been suggested as a mechanism of the preservation of ancient biomolecules (Schultz 1997, 2001). Schweitzer et al. (2014) have proposed that iron, specifically that derived from hemoglobin, can induce oxygen radical-mediated cross-linking and subsequent protease resistance. Copper and zinc have been shown to mediate the auto-oxidation of beta-amyloid protein with a concomitant increase in protease resistance (Brown 2010). Metal binding alone, in the absence of oxygen radical formation, can also increase the resistance of proteins to proteolysis (Nielson et al. 1985; reviewed in Brown 2010). However, in some cases, the oxidation of proteins can actually make them more susceptible to proteolysis (Davies et al. 1987).

Racemization of the amino acid constituents of proteins (e.g., crystallins and amyloid proteins) can lead to conformational changes that lead to aggregation (reviewed in Gallart-Palau et al. 2015). The D enantiomers of most amino acids accumulate readily in preserved specimens; the D/L ratio of Asx in the bone tissue of a 45 Ka bison was recently found to be 0.39 (Buckley and Collins 2011). The degree to which amino acid racemization might lead to the potentially protective effect of protein aggregation is unknown.

There is no single best preservational niche. The best studied such niche, biofilms (reviewed in Krumbein et al. 2003), may not be particularly applicable to blood and its constituents although Peterson et al. (2010) have suggested that crystallization of biofilms may isolate internal areas of bones, a process they termed "microbial masonry," and be responsible for exceptional preservation of dinosaur soft tissue. Similarly, Rybczynski et al. (2013) described what could be termed "chemical masonry" in a >3.4 Ma tibia of *Paracamelus*, an ancestor of modern camels from Ellesmere Island, Canada—the bone was coated with fine-grain precipitates of iron oxyhydroxide and barium sulfate which infilled the pores of the bone and sealed in areas of carbon-rich organic material from which collagen was isolated and sequenced. Bone and teeth provide a physical mechanism for the isolation of tissue as well as chemical mechanisms of preservation such as iron-mediated cross-linking, bactericidal activity, and hydroxyapatite-mediated protein and DNA stabilization. Salamon et al. (2005) have suggested that sodium hypochlorite-resistant, nanometer-scale crystals from bone harbor high-quality DNA. Permafrost-mediated freezing and desert-mediated dehydration will continue to provide the most frequently utilized, albeit relatively young, sources of tissue for studies of blood-derived ancient biomolecules. Increases in our understanding of taphonomy, as provided by, for example, actualistic studies, will enhance our ability to discover new ancient biomolecules (e.g., Saitta et al. 2019).

12.7 Conclusions

The isolation of ancient biomolecules from fossil blood holds the promise of understanding the origins of blood types, blood diseases, and blood-borne parasites. Given advances in DNA extraction and sequencing technologies, the vast majority of future ancient blood and blood cell DNA sequence data will be derived from whole-genome studies. This revolution is exemplified by the recent work of Haber and colleagues (Haber et al. 2017) in which the complete genomes of five 3700-year-old individuals recovered from the city of Sidon, an ancient Canaanite city-state on the eastern coast of the Mediterranean Sea, and the genomes of 99 individuals from present-day Lebanon were sequenced in a study of genetic diversity. Whole genomes of organisms greater than a half million years in age are now routinely obtained from permafrost specimens. Given that sequenceable DNA in older specimens preserved in rock is currently only hinted at, the absence of verifiable DNA data older than the Ionian Age is due, in part, to the absence of specimens obtained from older permafrost. Much older permafrost does exist however, for example that found in the interior of the Transantarctic Mountains, dated to 20 Ma (Dobinski 2011). Prospects for advances in the identification of ancient molecules other than DNA and protein in much older sediments are even brighter. The identification of chitin in Burgess Shale, porphyrins from 340 Ma oil, and the routine isolation of numerous organic biomarkers from rocks more than two billion years old suggests that ancient biomolecules are far more commonly preserved than we might expect.

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