The organic preservation of fossil arthropods: an experimental study

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Modern arthropod cuticles consist of chitin fibres in a protein matrix, but those of fossil arthropods with an organic exoskeleton, particularly older than Tertiary, contain a dominant aliphatic component. This apparent contradiction was examined by subjecting modern cockroach, scorpion and shrimp cuticle to artificial maturation (350 °C/700 bars/24 h) following various chemical treatments, and analysing the products with pyrolysis–gas chromatography/mass spectrometry (Py–GC/MS). Analysis of artificially matured untreated cuticle yielded moieties related to phenols and alkylated substituents, pyridines, pyrroles and possibly indenenes (derived from chitin). *n*-Alkyl amides, C₁₆ and C₁₈ fatty acids and alkane/alk-1-ene homologues ranging from C₉ to C₁₉ were also generated, the last indicating the presence of an *n*-alkyl component, similar in composition to that encountered in fossil arthropods. Similar pyrolysates were obtained from matured pure C₁₆ and C₁₈ fatty acids. Py–GC/MS of cuticles matured after lipid extraction and hydrolysis did not yield any aliphatic polymer. This provides direct experimental evidence that lipids incorporated from the cuticle were the source of aliphatic polymer. This process of *in situ* polymerization appears to account for most of the fossil record of terrestrial arthropods as well as marine arthropods that lacked a biomineralized exoskeleton.

Keywords: arthropod; lipids; experimental maturation; confined pyrolysis; taphonomy; fossilization

1. INTRODUCTION

Arthropod cuticles consist of chitin fibres embedded in a protein matrix, cross-linked by catechol, aspartate and histidyl moieties (Schaefer *et al.* 1987). Calcification, in the form of calcium carbonate, further strengthens the cuticle of many crustaceans and extinct trilobites; such biomineralized skeletons dominate the marine fossil record of arthropods. The fossil record of many arthropods, however, particularly in non-marine settings, relies on organic matter preservation, because they lack a biomineralized exoskeleton. Fossil eurypterids, scorpions and insects, for example, are abundant as cuticular remains (see Briggs 1999; Martínez-Delclòs *et al.* 2004, for review).

Decay of crustacean cuticle in earlier experiments resulted in an extensive loss of the protein component within the first two weeks, while chitin remained largely intact for the first eight weeks, attesting to its greater survival potential (Stankiewicz *et al.* 1998a). Traces of chitin are present in Pleistocene beetles (Stankiewicz *et al.* 1997a) and in weevils as old as 25 Myr (Stankiewicz *et al.* 1997c; Gupta *et al.* in press). However, the cuticles of fossil arthropods older than the Tertiary show no trace of chitin

or protein (Briggs *et al.* 2000), but have a dominant aliphatic component similar to type I/II kerogen (Briggs 1999) at times partially interlinked by fatty acyl moieties (Gupta *et al.* in press). This contradiction has intrigued palaeontologists and chemists alike, as it is difficult to explain the transformation of chitin (a carbohydrate) to the long-chain hydrocarbon polymer in the fossil. Selective preservation of resistant aliphatics is not a plausible explanation, as they do not occur in the exoskeletons of modern arthropods. Initially, the aliphatic composition was interpreted as the result of diagenetic replacement by aliphatic organic matter from an external source (Baas *et al.* 1995). However, recent research makes this argument untenable (see discussion in Briggs 1999; Gupta *et al.* in press). Arthropod cuticles have surface waxes composed of hydrocarbons and fatty acids (Howard & Blomquist 2005) that are labile, i.e. extractable/hydrolysable. Thus, it has been suggested that the aliphatic composition of the fossils was generated by *in situ* polymerization of constituent cuticular waxes (Briggs *et al.* 1998; Stankiewicz *et al.* 2000).

Taphonomic experiments have been used to investigate various parameters that control the preservation of arthropod cuticles in the fossil record (modern shrimp: Briggs & Kear 1993, 1994; Baas *et al.* 1995; Hof & Briggs 1997; Sagemann *et al.* 1999; cockroach: Duncan *et al.* 2003). The emphasis of these studies was on controlled necrolysis and/or the effect of transportation on disarticulation. Few experiments have addressed changes in

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the chemistry of modern arthropod cuticles to explain their composition in the fossil record. Stankiewicz *et al.* (2000) used artificial maturation techniques in a preliminary study of transformation of the cuticle of the emperor scorpion, *Pandinus imperator*. An aliphatic composition was generated, but the investigation did not determine the source of aliphatic components. Similar experiments have been used successfully to investigate the origin of aliphatic constituents in fossil plants (Gupta *et al.* 2005, submitted). Here, we describe an experimental investigation, using similar artificial maturation techniques, to determine the source of aliphatic component that accounts for the long-term organic preservation of fossil arthropods.

2. MATERIAL AND METHODS

The living arthropods, *P. imperator* (emperor scorpion), *Crangon crangon* (shrimp) and *Gromphadorhina portentosa* (Madagascan hissing cockroach), were investigated as they all belong to groups with a well-documented fossil record that have been the subject of taphonomic experiments (Briggs & Kear 1993, 1994; Stankiewicz *et al.* 1998a; Sagemann *et al.* 1999; Duncan *et al.* 2003). The large size and abundant cuticle of these organisms provide additional advantages for handling and experimentation. The specimens were obtained alive from Bristol Zoo and killed by freezing at -20°C for 24 h. They were dissected with a scalpel to remove the internal tissues and the cuticle was recovered. The cuticle was frozen in liquid nitrogen and crushed with a mortar and pestle. It was then boiled in double distilled water at 100°C , three times for 1 h each, to remove any remaining internal tissue, and then washed thoroughly with double distilled water. The recovered cuticles were subjected to two different preparations prior to artificial maturation. Cuticles from scorpion and cockroach were: (i) untreated or (ii) solvent extracted in 2 : 1 dichloromethane/methanol (v/v, five times, sonication for 30 min each) to remove extractable lipids, and then saponified (i.e. subjected to base hydrolysis) in 95% 1 M methanolic NaOH for 24 h to remove hydrolysable lipids. The shrimp cuticle was not subjected to any extraction/saponification prior to maturation. Commercially prepared pure chitin (derived from crab, Sigma Aldrich) was solvent extracted in the same way as the scorpion and cockroach cuticles to remove soluble impurities.

The cuticles and commercial chitin (0.1–0.15 g) were artificially matured at 350°C , 700 bars for 24 h in gold cell reactors (Monthieux *et al.* 1985; Landais *et al.* 1989; Stankiewicz *et al.* 2000; see electronic supplementary material). A successful experiment was indicated by no weight loss, i.e. no change before and after the experiment. No other quantitation was attempted. The volatiles and condensates generated were not characterized. The shrimp cuticle was also matured in two separate experiments in the presence of commercial powder CaCO_3 (1 : 1, w/w) and kaolinite (1 : 1, w/w) to explore the effect of different inorganic matrix materials on cuticle transformation. Pure C_{16} and C_{18} fatty acids (Sigma Aldrich, palmitic acid–stearic acid mixture, 40–51% as stearic acid, 49–54% as palmitic acid by weight) were matured to investigate the transformation of pure lipids (as detected in the extractable and hydrolysable fractions). Fresh unmatured samples were analysed without extraction to reveal the entire range of chemical constituents. The matured samples were analysed

Figure 1. Partial pyrolysis–gas chromatography/mass spectrometry chromatograms of (a) pure chitin and (b) cockroach cuticle. 1, Pyridine; 2, methyl pyridine; 3, acetamide; 4, 3-hydroxy-2-pyridone; 5, 2-pyridine carboxaldehyde; 6, acetylpyrrole; 7, acetylpyrroline; 8, amine derivative; 9, levoglucosenone; 10, unknown; 11, acetyldihydropyridine; 12, acetylpyridone; 13, 3-acetamidofuran; 14, 3-acetamido-5-methylfuran; 15, 3-acetamido-4-pyrone; 16, oxazoline structures; Pr_1 , methyl pyrrole; Pr_2 , phenol; Pr_3 , unknown; Pr_4 , indole; Pr_5 , methylindole; Pr_6 , diketopiperazines; C_nFA , fatty acyl moieties, where n refers to the carbon chain length and $n:1$ denotes mono-unsaturation in the alkyl chain. No n -alkanes/alkenes were detected. The analysis time was 55 min.

using thermal desorption (TD–GC/MS) at 300°C followed by Py–GC/MS at 610°C to reveal macromolecular information (see Gupta & Pancost 2004 for instrument parameters). Thermal desorption was used to remove volatile compounds (i.e. thermal extraction; Hartgers *et al.* 1995) in order to purify material prior to pyrolysis (i.e. to thermally extract compounds volatile at 300°C). It also provided a basis for direct comparison with the results of Stankiewicz *et al.* (2000). Aliquots of the artificially matured samples were subjected additionally to base hydrolysis by heating in 1 M methanolic NaOH for 1 h at 70°C to assess the degree of recalcitrance.

3. RESULTS

(a) Composition of samples used in experiments

Pyrolysis–gas chromatography/mass spectrometry (Py–GC/MS) of solvent-extracted commercial chitin (figure 1a) yielded 3-acetamido-4-pyrone, 3-acetamido-5-methylfuran, acetylpyridone and 3-acetamidofuran as the major products. However, protein and fatty acyl moieties were not observed. The pyrolysates of the untreated cockroach cuticle (figure 1b) contained not only these chitin markers, but also protein pyrolysis products (see Stankiewicz *et al.* (1997a,d) for identification and mass spectral characteristics). The straight chain fatty acids $n\text{-C}_{16}$ and $n\text{-C}_{18}$ (both saturated and unsaturated components) were also observed in the cuticle pyrolysates (and were also detected in modern stingless

Figure 2. Partial pyrolysis–gas chromatography/mass spectrometry chromatograms of (a) matured chitin, (b) matured cockroach cuticle and (c) matured shrimp cuticle. B_n, benzene derivatives (*n* refers to the number of carbon atoms in the alkyl component); C₁In, methyl indole; A, amide derivative (primarily C₁₆ and C₁₈ derivatives; C_nPy, pyridine pyrolysis products, where *n* is the number of carbon atoms in the alkyl substituent; P, phenol derivative; Id, alkyl indenenes (inset mass chromatograms *m/z* 83+85 reveal the presence of *n*-alkane/alkenes). The analysis time was 55 min.

bee cuticle; Stankiewicz *et al.* 1998b). No alkane/alkene homologues were detected. The modern scorpion and shrimp cuticle pyrolysates were similar to the cockroach cuticle (traces not shown).

(b) Composition of the cuticle and the lipid compounds matured without chemical pre-treatment

The pyrolysate of matured commercial chitin, analysed after thermodesorption at 300 °C (figure 2a), contained pyridine and its alkyl derivatives, and phenol and its mono-, di-, tri- and tetra alkyl derivatives. The phenol

derivatives are among the most abundant in the pyrolysate. Other important compounds tentatively identified include indene and its alkyl derivatives. Furans, pyrones, pyridones, pyrroles and oxazoline structures, which are the most important pyrolysis products of unmaturred chitin, were not detected, thereby confirming that the chitin has undergone chemical change. No *n*-alkane/alk-1-ene homologues were detected. Benzene derivatives were also not detected, but along with alkylated phenols, they were the primary thermally desorbed components.

The chitin-derived compounds are clearly evident in the pyrolysates of artificially matured scorpion, cockroach

Figure 3. Partial pyrolysis–gas chromatography/mass spectrometry chromatograms of (a) matured mixture of C_{16} and C_{18} fatty acids with kaolinite and (b) shrimp cuticle matured in the presence of calcium carbonate. Annotations as in figure 2. The analysis time was 55 min.

and shrimp cuticle (the latter two shown in figure 2*b,c*). The fatty acids, n - C_{16} and n - C_{18} , are abundant, as they were in the fresh cuticles. The relative abundance of fatty acids in the artificially matured shrimp cuticle (figure 2*c*) is less than in the cockroach (figure 2*b*) and scorpion (not shown), and n - C_{18} fatty acid was released only in trace abundances. The thermally desorbed products of all the matured cuticles consisted primarily of alkylated phenols, benzene derivatives and n -alkanes (primarily C_9 to C_{20}).

The matured cuticles also yielded a range of compounds upon Py–GC/MS that were not detected in either the matured commercial chitin (figure 2*a*) or the non-matured cuticles (figure 1*b*). These include n -alkyl amides, with C_{16} and C_{18} homologues being the most abundant (figure 2*b,c*). Although not readily apparent in the partial ion current chromatogram, the m/z 83+85 mass chromatogram reveals the presence of n -alkane/alk-1-ene homologues ranging at least from C_9 to C_{19} with the C_{14} to C_{17} components being dominant. Such n -alkane/alk-1-ene homologues (n - C_8 to n - C_{19} ; figure 3*a*) were also generated during the pyrolysis of matured pure C_{16} and C_{18} fatty acid mixture. However, base hydrolysis of the matured cuticle yielded no recoverable residue.

Shrimp cuticle, matured in the presence of clay and calcium carbonate, (figure 3*b*) yielded pyrolysates similar to the shrimp cuticle matured in the absence of any minerals.

(c) Composition of cuticle matured after chemical treatment

Cockroach cuticle, matured following lipid extraction and base hydrolysis, (figure 4) yielded pyrolysis products related to chitin and protein similar to those observed in the cuticle matured without chemical treatment. However, n -alkyl components, including n -alkanoic acids, n -alkyl amides and n -alkanes/alkenes, were not detected in the pyrolysate (inset, figure 4). Thermodesorbed products were similar to those released from matured chitin; in particular, no n -alkanes were detected.

4. DISCUSSION

The results of these artificial maturation experiments reveal the source of aliphatic component in fossil arthropod cuticles (table 1). Previous experiments (Stankiewicz *et al.* 2000) involved maturation of the cuticle of the emperor scorpion, which had been solvent extracted (but not saponified) following degradation for 8.5 months in a bacterial inoculum. The sample matured at 260 °C generated abundant phenol during Py–GC/MS, but markers directly related to chitin and protein were absent showing that thermal maturation alone can degrade chitin. C_5 to C_{20} n -alk-1-enes and n -alkanes were present, indicating the presence of an n -alkyl component. Thermal maturation at 350 °C resulted in

Figure 4. Partial pyrolysis–gas chromatography/mass spectrometry chromatograms of cockroach cuticle matured after lipid extraction followed by saponification. Annotations as in figure 2. The analysis time was 55 min.

Table 1. Products of maturation of different arthropod cuticle biochemical components.

starting material	composition	chemical treatment	products of maturation
commercial chitin	pure chitin	extraction	matured chitin products and no aliphatics
fresh scorpion, shrimp and cockroach cuticle	chitin + protein + lipids	none	matured chitin products + acids + amides + aliphatics ^a
fresh scorpion and cockroach cuticle	chitin + protein	extraction + saponification	matured chitin products and no aliphatics

^a Shrimp cuticle matured with CaCO₃ or clay and without any chemical pre-treatment also yielded these moieties.

an extensive alteration of the cuticle. Alkenes and alkanes with aliphatic carbon chain numbers up to n -C₃₀, significantly higher than those observed here, were the dominant pyrolysis products, and phenols were barely detected. Indenes and amides that occur in our experiments were not reported. These differences probably reflect alteration of the chitin–protein complex through decay prior to the maturation process; the cuticles used in the present experiments were not decayed.

In the present experiments, artificial maturation of untreated arthropod cuticle at 350 °C resulted in significant changes in the macromolecular composition (table 1). Thermally induced changes to the chitin–protein complex (deacetylation of chitin and formation of aromatic products of both chitin and protein) resulted in the presence of phenol and alkyl-substituted phenols in the pyrolysate. The presence of n -alk-1-enes and n -alkanes indicates that an aliphatic component was generated during maturation. These homologues are often encountered in the pyrolysates of fossil arthropods, including scorpions, eurypterids (Stankiewicz *et al.* 1998c) and shrimp (Stankiewicz *et al.* 1997b). Matured cuticle also revealed an abundance of n -alkyl amides along with compounds detected as alkylated indenes. While long-chain amides (e.g. ceramides) do occur naturally, the observed components are most probably formed by nucleophilic substitution reactions between N-bearing components in the chitin/protein complex and the fatty acyl components. They appear to be a product of accelerated maturation, since they have not been detected in

fossil arthropod cuticles. Interestingly, such amides did not form in the experiment of Stankiewicz *et al.* (2000), where the loss of more reactive nitrogen-containing compounds during decay may have prevented their formation, thereby leading to relative enhancement of the n -alkyl component. Shrimp cuticle matured in the presence of clay and calcium carbonate also yielded n -alk-1-enes and n -alkanes, demonstrating that lithological components do not inhibit the reaction.

Similarly, maturation of a mixture of pure C₁₆ and C₁₈ fatty acids (similar to those obtained after extraction and saponification of the lipid component from the cuticle) produced a distribution of n -alkane/alkene homologues similar to the matured untreated cuticles (for a discussion of the behaviour of unmatured C₁₆ and C₁₈ fatty acids during analytical pyrolysis; see Hartgers *et al.* 1995). In striking contrast, maturation of cuticle that had first been extracted and saponified (i.e. was devoid of labile aliphatic compounds) yielded no aliphatic component, but only moieties related to chitin and protein. These results provide direct experimental evidence that lipids present in the extractable and hydrolysable fraction of the cuticle are necessary for the formation of the aliphatic component. It is possible that aliphatic compounds from the cuticle become chemically bound to a macromolecular structure formed from chitin and proteins (as suggested by the presence of alkylamides). Indeed, fatty acyl moieties have been detected in the cuticle of fossil weevils (Gupta *et al.* in press).

When the matured cuticle was subjected to base hydrolysis, no residue remained, indicating that the polymer formed in the experiments is hydrolysable even though fossil cuticles are resistant to such hydrolysis. The recalcitrant nature of fossil material must be the result of further cross-linking and steric protection of functional groups that occurs over geological time. Formation of such a recalcitrant aliphatic macromolecule from the labile components of plant leaves was achieved using identical conditions to those reported here (Gupta *et al.* 2005, submitted). Comparison of the products derived from maturation of different pre-treated plant tissues demonstrated that soluble lipids are constituents of the macromolecular material generated, indicating that labile organic compounds are a potential source of the aliphatic component of fossil organic matter and kerogen, where decay-resistant aliphatic material (e.g. cutan) is not present in the living organism.

In the absence of a recalcitrant aliphatic polymer in living arthropods, its presence in fossils has been interpreted as a product of the polymerization of cuticular lipids (Briggs *et al.* 1998; Stankiewicz *et al.* 2000). The experiment described here provides the first test of this hypothesis. It shows that artificial maturation of arthropod cuticle in a closed system (eliminating contamination from an external source) can yield an aliphatic component generated from constituents within the cuticle itself. Lipids from the internal tissue of the organism could also contribute to the aliphatic component (this occurs in plants; Gupta *et al.* 2005, submitted). This demonstrates that the aliphatic component encountered in fossil insect tissues is not necessarily the result of migration from an external source (Baas *et al.* 1995), but rather a product of incorporation of lipids present in the organism itself. The major proportion of the fossil record of terrestrial arthropods, and of marine arthropods without a biomineralized exoskeleton, may be the result of this process of *in situ* polymerization.

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