Evidence of a distinct lipid fraction in historical parchments: a potential role in degradation?

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Abstract Parchment, a biologically based material obtained from the processed hides of animals such as cattle and sheep, has been used for millennia as a writing medium. Although numerous studies have concentrated on the structure and degradation of collagen within parchment, little attention has been paid to noncollagenous components, such as lipids. In this study, we present the results of biochemical and structural analyses of historical and newly manufactured parchment to examine the potential role that lipid plays in parchment stability. The lipid fraction extracted from the parchments displayed different fatty acid compositions between historical and reference materials. Gas chromatography, small-angle X-ray scattering, and solid-state NMR were used to identify and investigate the lipid fraction from parchment samples and to study its contribution to collagen structure and degradation. We hypothesize that the origin of this lipid fraction is either intrinsic, attributable to incomplete fat removal in the manufacturing process, or extrinsic, attributable to microbiological attack on the proteinaceous component of parchments. Furthermore, we consider that the possible formation of protein-lipid complexes in parchment over the course of oxidative degradation may be mediated by reactive oxygen species formed by lipid peroxidation.—Ghioni, C., J. C. Hiller, C. J. Kennedy, A. E. Aliev, M. Odlyha, M. Boulton, and T. J. Wess. **Evidence of a distinct lipid fraction in historical parchments: a potential role in degradation? *J. Lipid Res.* 2005.** 46: **2726–2734.**

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Parchment is a material of biological origin that has been used for centuries as a foundation for historical documents and works of art, including most of the written history of Europe. Consequently, the conservation of parchment has frequently been a foremost concern. Many early methods of intervention did more harm than good to the original documents, and the work of modern conservators is often made more difficult because they have to not only preserve the historical material but also undo earlier treatments or cope with the damage caused by overzealous washing, flattening, or other methods applied in the past (1).

Some recent work has focused on the protein structure of parchment and changes to the material on the molecular level over time. Parchment, primarily originating from the hides of cattle, sheep, and goats, is predominantly composed of type I collagen, along with some associated macromolecules such as proteoglycans (2). The hierarchical structure of collagen in a feltwork, which provided mechanical strength to the skin of the animal when it was alive, is now responsible for maintaining the parchment as a viable storage medium. As the collagen degrades over time, the parchment loses strength, becomes brittle, and deteriorates to the point that it can no longer be used. The basis for the biodegradation of collagen within parchment is largely unresolved and is the focus of several current and past research programs, which intend to minimize the loss of cultural and historical artifacts resulting from the biodegradation of collagen in parchment (3–5).

The presence of a lipid fraction in parchment has not been examined extensively, nor has the role that lipids may play in the degradation of parchment been studied. A recent study found little spectroscopic difference between the lipids present in skin samples from three different species and those in degraded samples of parchment (6). These results indicated that the lipids present in parchment may have originated in the skin, survived the processing of converting skin to parchment, and were not subjected to alteration over time. However, excessive handling of documents, particularly at the corners and outer edges, over time could introduce a lipid fraction into parchment from users' skin, which may resemble the animal skin lipids in composition. The lipid fraction in parchment

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could also originate from microbial populations that can contribute to degradation in archaeological and historical textiles and leather samples (7, 8), but these lipids would be less likely to resemble the lipid component of skin.

A lipid fraction present in parchment samples may interact with the collagen and other macromolecules present, possibly contributing to the overall degradation of parchment. There is some evidence for the formation of conjugates between collagen and sphingomyelin liposomes (9). Other groups have identified some possible interactions between the polar head groups from phospholipid bilayers and collagen fibrils (10). The present multidisciplinary investigation, incorporating gas chromatography, small-angle X-ray scattering, microfocus X-ray diffraction, fluorimetry, and solid-state NMR, broadens the characterization of the interactions between collagen and lipids with specific reference to historical parchment. This appears to be a fairly untapped line of investigation, and the characterization of lipid features in parchment could be of interest to a diverse range of fields of inquiry.

MATERIALS AND METHODS

Parchment samples

Twelve historical parchments and two modern parchments were used in this study. The 12 historical samples, USH01–USH12, were obtained from the National Archives of Scotland, Edinburgh, and vary in age and level of preservation. The new parchments, NP3 and NP7, are reference samples, produced according to traditional methods within the last 3 years, obtained from the School of Conservation of the Royal Danish Academy of the Fine Arts, Copenhagen. **Table 1** provides details of the parchment ages and visual descriptions of all 14 samples as well as the weight and dimensions of each sample.

Lipid extraction

Samples of historical and reference parchments were cut into 1 mm strips, and the lipid fraction was extracted by immersion in 10 ml of a 2:1 chloroform-methanol mixture (C/M), as described elsewhere (11). The extraction was performed for 1 h at room temperature, then at −20°C for the following 48 h to ensure a thorough penetration of the solvent mixture into the samples. The C/M-extracted fraction was then dried and weighed to obtain a measurement of raw yield. The weight and color description of the desiccated lipid residues obtained from the complete extraction are also provided in Table 1.

The lipid extracts were diluted to 10 mg/ml in C/M (2:1, v/v), and 150 μl (1.5 mg) was loaded onto TLC plates (20 cm × 20 cm × 0.25 mm, precoated with silica gel 60; Merck, Darmstadt, Germany) on 1.5 cm origins for polar lipid separation. After elution in isohexane-diethyl ether-acetic acid (80:20:2), the origins were scraped and transmethylated at 50°C overnight in 1% sulfuric acid in methanol. All of the pigments remained in the polar lipid fraction at the origin, and very little or no neutral lipids were present. The yellow-brown color present in the polar lipid fraction disappeared after transmethylation, which suggests that lipid-protein conjugates may have been present in the original extract and were destroyed by the subsequent acid treatment.

Chromatography

Samples yielded low levels of fatty acid methyl esters (FAMEs; 0.1–0.3 mg), which is in accordance with the hypothesis that the majority of the original lipid extract is composed of protein-lipid conjugates. The FAMEs extracted from each sample were diluted in 0.1 ml (~1 mg/ml) isohexane and analyzed in a Fisons GC8000 gas chromatograph (Crawley, UK) equipped with a fused-silica capillary column (30 m × 0.32 mm × 0.25 mm; CP Wax 52 CB; Chrompack) using hydrogen as a carrier gas. The temperature regime progressed from 50 to 150°C at 40°C/min, then to 225°C at 2°C/min. Individual FAMEs were identified by comparison with known standards and published data, or were identified as other non-fatty acid compounds reported by GC-MS (Polars GC-MS-MS, EI+, full scan 60–650, 70 eV; GLC on a 30 m × 0.25 mm × 0.25 mm ZB-WAX column using helium as a carrier gas, splitless injection at 50°C hold for 0.5 min, 40°C/min to 150°C, 1.5°C/min to 195°C, 0.5°C/min to 205°C, and 40°C/min to 220°C).

High-performance thin-layer chromatography (HPTLC) was undertaken to quantify the lipids present in the parchments by class. Separation of the lipid classes was obtained on 10 × 10 cm silica gel 60 HPTLC plates (Merck, Darmstadt, Germany); all solvents used were of HPLC grade. The plates were washed with 20 ml of 1:1 isohexane-diethyl ether, and the ends were scraped of all impurities. Two microliters of each sample was then loaded onto 2 mm strips equally spaced out and run two-thirds of the way up with 20 ml of the first elution of methyl acetate-isopropanol-chloroform-methanol-0.25% KCl (25:25:25:10:9 by volume). The plates

TABLE 1. Visual and quantitative descriptions of the parchment samples analyzed and their C/M extracts

Code	Year	Parchment Description	Approximate Area	Weight	C/M Extract	Percentage of Weight	C/M Extract Description
			cm^2	*mg*			
USH01	1765	Corner; brownish; writing	16.0	485.9	4.4	0.91	Yellow-brown
USH02	1769	Corner; yellow; writing	12.3	515.2	20.4	3.96	Yellow-orange
USH03	1775	Handling corner; yellow-brown spots	14.0	486.4	35.4	7.28	Yellow-brown
USH04	1827	Side; yellow-red	12.3	541.6	60.3	11.13	Yellow-orange white
USH05	1824	Corner; brownish; writing	14.0	550.1	7.7	1.40	Brownish
USH06	1832	Internal edge; writing	7.5	547.0	0.2	0.04	Transparent
USH07	1828	Clean, not brown; writing	9.0	515.5	6.9	1.34	Transparent yellow
USH08	1817	Internal corner; clean; some writing	12.3	551.8	1.4	0.25	Transparent green
USH09	Year unknown	Internal corner; very thick; some writing	10.5	618.4	1.1	0.18	Transparent yellow
USH10	1765	Back page, bend; brown; some writing	9.0	428.8	29.9	6.97	Yellow-brown
USH11	1792	Back corner; brownish; some writing	10.5	434.7	2.3	0.53	Yellow-white
USH12	1740	Top; brownish; writing	12.3	486.2	15.0	3.09	Yellow-gray
NP3	New	Cream; clear	16.0	435.4	2.9	0.67	Transparent white
NP7	New	Cream; clear	20.0	428.5	2.1	0.49	Transparent white

C/M, chloroform-methanol mixture.

were then allowed to dry by desiccation and subjected to a second elution (20 ml) with hexane-diethyl ether-acetic acid (80:20:2 by volume). Compounds were identified by visual analysis as well as by ultraviolet and visible absorption spectra obtained with the use of a TLC Scanner 3 densitometer and the personal computer application winCATS (Camag, Muttenz, Switzerland).

X-ray diffraction

Small-angle X-ray scattering measurements on historical parchment samples and on C/M extracts from parchment were performed at the NanoSTAR (Bruker AXS) small-angle X-ray facility at Cardiff University. Parchment samples from before and after lipid extraction were mounted in the sample chamber with the primary plane of the parchment lying perpendicular to the direction of the X-ray beam. C/M extracts were placed between two sheets of mica and mounted into the sample chamber of the NanoSTAR. Measurements were taken at a sample-to-detector distance of 1.25 m, with an X-ray wavelength of 0.154 nm. The diffraction patterns were analyzed using the image-processing software FIT2D (A. Hammersley, European Synchrotron Radiation Facility, Grenoble, France) and in-house software, according to the procedure described previously (12).

Microfocus X-ray diffraction of thin sections of parchment, according to earlier procedures (10), was carried out at beamline ID18F at the European Synchrotron Radiation Facility at a camera-to-detector distance of 20 cm with an X-ray wavelength of 0.154 nm. Microfocus X-ray diffraction facilitated the calculation of the *d* spacing of the lipid at different areas within the parchment section.

The degradation of collagen in parchment was measured using previously published methods (10). The 0.85 nm^{-1} equatorial reflection of dry collagen was used to assess the degree of gelatinization of the collagen in parchment. The relative intensities of the peaks at 0.85 nm^{-1} and 2 nm^{-1} are capable of indicating the relative amounts of collagen and gelatin in a parchment sample. The 2 nm^{-1} peak is consistent in terms of intensity and width in collagen and gelatin samples, whereas the 0.85 nm^{-1} peak displays increased intensity and reduced width if the amount of fibrillar collagen in a sample is high and the degree of gelatinization is low. The 0.85 nm^{-1} and 2 nm^{-1} reflections in the samples were quantified using the one-dimensional peak-fitting program XFIT (Collaborative Computational Project 13). The integrated intensities of the 0.85 nm^{-1} reflections were then divided by the 2 nm^{-1} reflections to produce a ratio of collagen to gelatin in the samples (C/G).

Fluorimetry

Fluorescence emission spectra of the C/M parchment lipid extracts, diluted in methanol at 60 μg/ml (except USH06, USH07, USH08, USH09, and USH11, which were diluted at 1 μg/ml), were recorded from 300 to 600 nm on a Perkin-Elmer LS50B fluorimeter-luminescence spectrometer as described previously (13). An excitation at 345 nm with the slit window set at 10 nm, and an incremental wavelength increase of 75 nm/min, gave a maximum fluorescence emission at ~416 nm, as reported in the literature (13) (see Fig. 5 below).

Solid-state NMR

High-resolution solid-state ^{13}C spectra using cross-polarization (CP) from protons, high-power ^{1}H decoupling, and magic angle sample spinning (MAS) were recorded from a subset of parchment samples (USH01, USH02, USH04, USH08, USH10, USH12, and NP7) that displayed the greatest variation in color and flexibility at 75.5 MHz on a Bruker MSL300 spectrometer (7.05T) using a standard Bruker double-resonance MAS probe. The samples were fitted into cylindrical zirconia rotors (7 mm external diameter) and spun at a MAS frequency of 5.5 kHz with stability $>\pm 3$ Hz. Typical acquisition conditions for ^{13}C CP-MAS experiments were as follows: ^{1}H 90° pulse duration of 5 μs, a contact time of 2 ms, recycle delay of 1 s, 27 ms acquisition time, and 15,000 transients. In addition, ^{13}C CP-MAS experiments combined with dipolar dephasing (14) were used to identify quaternary and methyl carbons. The dipolar dephasing delay used in these experiments was 40 μs. All spectra were recorded at ambient probe temperature, and the ^{13}C chemical shifts are given relative to tetramethylsilane.

RESULTS

Chromatography

Table 2 shows the FAME composition of the parchment extracts. Only the saturated and monounsaturated fatty ac-

TABLE 2. Percentage fatty acid composition of the polar fractions from the C/M extract of new and historical parchments

Fatty Acid	NP7	NP3	USH01	USH02	USH03	USH04	USH05	USH06	USH07	USH08	USH09	USH10	USH11	USH12
14:0	1.0	1.7	1.4	1.8	1.5	0.9	1.7	1.0	1.7	1.2	0.8	1.3	0.9	2.0
15:0	0.7	1.1	0.9	0.3	0.4	0.4	1.0	0.9	1.0	0.7	0.6	1.1	0.6	0.3
16:0	18.1	21.0	21.0	35.7	28.5	9.5	15.9	27.3	27.5	22.4	16.1	13.3	17.9	31.0
16:1n-9	0.7	0.9	1.2	1.1	1.4	1.3	0.9	1.4	0.9	0.5	1.4	0.0	1.0	0.9
16:1n-7	0.4	0.1	0.6	0.1	0.0	0.1	0.2	0.5	0.5	0.2	0.2	1.4	0.1	0.2
17:0	0.6	1.0	0.9	0.9	1.4	0.5	0.1	0.4	0.8	0.5	0.2	1.0	0.4	0.7
18:0	7.0	7.4	7.5	16.3	10.4	3.9	6.4	7.6	6.2	7.1	5.6	5.1	5.7	8.0
18:1n-9	3.3	1.1	2.0	2.3	1.1	9.9	1.6	4.6	4.6	2.6	3.6	5.2	2.9	5.6
18:1n-7	1.2	0.9	1.4	0.5	0.6	0.9	2.6	1.0	0.8	1.3	3.4	2.1	2.1	0.7
20:0	2.4	4.8	1.8	0.7	0.7	1.8	1.7	2.0	2.3	4.9	3.2	1.8	1.7	1.0
20:1n-9	2.5	1.9	2.4	2.2	2.9	6.8	2.6	1.0	1.5	2.0	2.4	2.1	1.5	3.2
20:1n-7	2.6	2.0	1.9	8.4	2.8	8.0	2.5	1.0	1.4	2.2	3.1	2.6	1.6	3.5
22:0	5.8	5.2	5.7	0.3	1.4	0.8	4.8	6.5	4.5	5.1	4.6	2.1	4.9	0.4
22:1n-11	0.0	0.2	0.8	1.0	1.5	2.3	0.9	0.3	0.8	0.3	0.4	0.0	0.0	0.9
22:1n-9	1.5	0.4	0.8	0.3	0.7	0.9	1.2	1.2	0.9	0.5	1.8	0.7	0.8	0.4
22:1n-7	1.5	0.2	1.1	0.7	1.0	1.7	1.1	0.3	1.4	1.6	0.5	0.0	0.2	0.9
24:0	12.1	10.5	10.5	0.9	3.9	1.8	8.4	11.3	7.7	9.4	7.8	6.6	9.8	1.2
Total saturates	47.7	52.8	49.8	57.1	48.0	19.5	39.9	57.0	51.6	51.3	39.0	32.3	42.0	44.7
Total monoenes	13.8	7.7	12.2	16.4	12.0	31.9	13.6	11.4	12.7	11.1	16.8	14.2	10.3	16.3
Saturates/monoenes	3.5	6.9	4.1	3.5	4.0	0.6	2.9	5.0	4.1	4.6	2.3	2.3	4.1	2.7
Total	61.479	60.47	62.019	73.565	60.002	51.378	53.59	68.441	64.309	62.345	55.845	46.476	52.27	61.049

A recovery of 75% of initially methylated lipid extract is estimated, diluted to give 1 mg/ml solutions for GC samples.

ids have been reported. These account for the majority of the material present on the GC traces in most cases (between 46% and 73%), and a variety of branched, hydroxy, and epoxy fatty acids, together with other compounds with no fatty acid structure, were also evident. The fatty acid profiles of the two reference parchment samples (NP3 and NP7) are very similar. In contrast, the historical samples with the highest content of lipid extracts by weight (USH02, USH03, USH04, USH10, and USH12) present a very different distribution of fatty acids compared with the reference parchments. In the first instance, a variety of commercially available standards and lipid extracts of known composition obtained in the laboratory from various sources were cochromatographed with individual parchment extracts on separate plates to identify the lipid classes. Then, because of the limited number of samples loadable on a HPTLC plate, priority was given to comparison between different parchment extracts on the plates shown, and standards were omitted at this stage.

In addition to lipids, GC was able to elucidate additional materials present in the extracts. The GC peak partially coeluting with fatty acid 24:1 at 41 min retention time has been tentatively identified by mass spectrometry as podocarpa 6,8,11,13-tetraen-15-olic acid 13-propyl methylester. The relationship between the presence of this compound as a percentage of total FAME material and the percentage in weight of the gross lipid extract is presented in **Fig. 1**. A negative correlation is evident between the two in the historical samples, whereas the new parchments (NP3 and NP7) showed little lipid content and no resin. Despite the problems of coelution of the compound with the FAME 24:1, we can assume that when the peak represents >1% of total FAMEs, it is predominantly podocarpa 6,8,11,13-tetraen-15-olic acid 13-propyl methylester.

Abietic acid, a major component of the oleoresin synthesized by many conifers, was also found in trace amounts. It is an unsaturated alicyclic fatty acid obtained from heated rosin, the evaporated resin of pines and other conifers and a major component (13%) of Canada balsam, which is made from balsam fir (*Abies balsamea*).

The lipid composition of each sample was further quantified by HPTLC to determine the classes of lipids present and their relative amounts. A clear difference is evident between the newly manufactured parchments and the historical samples: in the new parchments, most of the lipid fraction remains uncharacterized by the techniques used here. This aspect of parchment lipid chemistry is worthy of further study but was outside the scope of the current investigation. This uncharacterized fraction is classified as unknown in the results shown in **Table 3**. The historical samples contained more lipid overall, and the unknown fraction of lipids in these samples was much smaller. These samples contained a significant amount of polar and nonpolar lipids compared with the new parchments. Table 3 shows the quantification of the lipid classes from the polar and nonpolar phases obtained from the HPTLC experiments. The main contributors to lipid content in historical parchments were free fatty acids, phospholipids, and cholesterol. Cholesterol and the other neutral lipids (free fatty acids, wax esters, and triacylglycerols) contribute a greater amount of the known lipids (up to 74.4%) compared with that of the polar lipids.

X-ray diffraction

Parchment samples examined by small-angle X-ray scattering show the distinctive collagen meridional diffraction pattern present in collagen-rich materials in which fibrillar collagen is retained. As the X-ray beam passes throughout the entire thickness of the parchment samples, where the collagen fibers lay at random azimuthal orientations, the meridional series appears as rings at small angles of diffraction. A broad ring at a *d* spacing of ~4.6 nm is also present in some lipid-rich samples (**Fig. 2**).

To determine whether the ~4.6 nm diffraction peak is attributable to lipid present in the samples, X-ray diffraction was carried out on C/M extracts from parchment sample USH01. The diffraction profile of the extracts indicates that no fibrillar collagen is present in the extract, as the collagen meridional series is no longer present in the samples. The ~4.6 nm peak observed in the parchment samples, which corresponds to the fundamental periodicity of partially hydrated phospholipid bilayers (15), was observed also in the diffraction profile of the extracts. Furthermore, analysis of two 1 mm strips of sample USH01, one obtained after delipidation with C/M extraction, showed that the

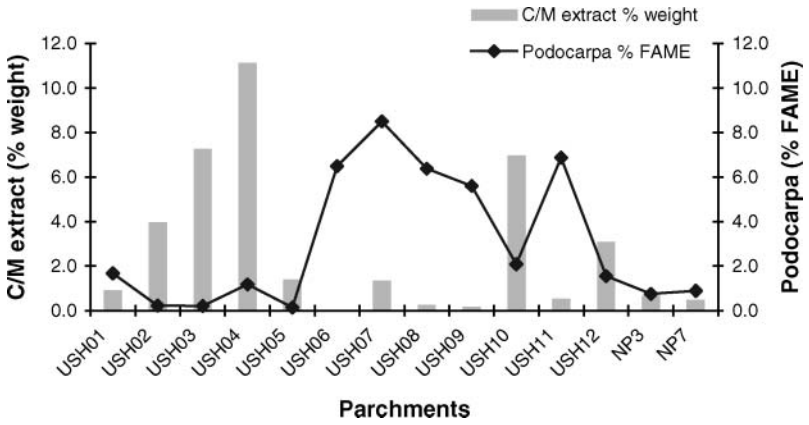

Fig. 1. Lipid extract and podocarpa resin content in historical parchments. A clear negative correlation exists where the level of chloroform-methanol mixture (C/M) extract is inversely proportional to the amount of podocarpa present in the sample. FAME, fatty acid methyl ester.

TABLE 3. Quantification of lipids in parchment samples by lipid class

Lipid	USH01	USH02	USH03	USH04	USH05	USH06	USH07	USH08	USH09	USH10	USH11	USH12	NP3	NP7
Sphingomyelin	—	—	0.2	—	—	—	0.4	0.3	—	—	—	—	—	—
Phosphatidylcholine	4.9	1.4	2.6	0.6	2.5	3.7	—	—	—	—	—	—	—	—
Phosphatidylserine	—	—	—	—	—	—	0.5	1.0	—	—	—	—	2.4	1.3
Phosphatidylinositol	—	—	—	—	—	—	1.0	1.3	—	—	0.4	—	3.1	3.7
Cardiolipin	0.9	—	—	—	0.8	1.4	0.6	—	—	—	1.0	0.5	—	—
Phosphatidylethanolamine	1.9	—	—	—	—	—	—	—	—	—	—	—	—	—
Cholesterol	34.6	28.1	27.4	24.3	28.2	24.9	—	—	—	—	—	—	—	—
Free fatty acids	29.4	32.4	28.2	32.0	41.4	35.4	50.1	51.1	33.1	32.3	46.4	40.8	6.6	—
Wax esters	6.6	6.2	11.1	14.2	—	—	2.5	—	14.4	3.6	—	—	4.9	6.8
Triacylglycerols	—	5.9	7.7	—	—	7.1	1.8	—	3.7	—	—	—	—	—
Total	78.2	73.8	77.2	71.1	72.9	72.6	56.8	53.6	51.2	35.9	47.8	41.2	17.0	12.0
Unknowns	21.8	26.2	22.8	28.9	27.1	27.4	43.2	46.4	48.8	64.1	52.2	58.8	83.3	87.7

~4.6 nm ring present in the original sample is absent after delipidation.

Microfocus X-ray diffraction analysis of parchment sections gives a spatially resolved analysis of the lipid presence in parchment. Within a single parchment section, the lipid *d* spacing varies between 4.4 and 4.8 nm (**Fig. 3**). This could be an indication of the hydration state of the lipid (16), or it could indicate that different biochemical compositions of lipid exist in different areas through a parchment section (17).

The correlation between the degradation of collagen and the level of lipids present is shown in **Fig. 4**. From this figure, it appears that the sample set clusters into two main regions: samples with lipid contents of <1.4% display higher C/G values (0.13–0.27), whereas samples with lipid contents of >1.4% display lower C/G values (0.07–0.13). Thus, it appears that the most damaged samples have a higher proportion of lipids present.

Fluorimetry

Transmethylation of the polar lipid fraction produced a far lower yield of FAMEs than was expected and also caused an acid-catalyzed discoloration of the organic material recovered from the origin of the TLC plate. This effect may be explained by the presence of proteolipid aggregates in the samples; to investigate this possibility, fluorescence spectra of the total lipid extracts were measured (**Fig. 5**). The peak recorded at 420–430 nm was in accordance with that reported in the literature for a variety of proteolipid complexes, particularly of the ceroid type (18).

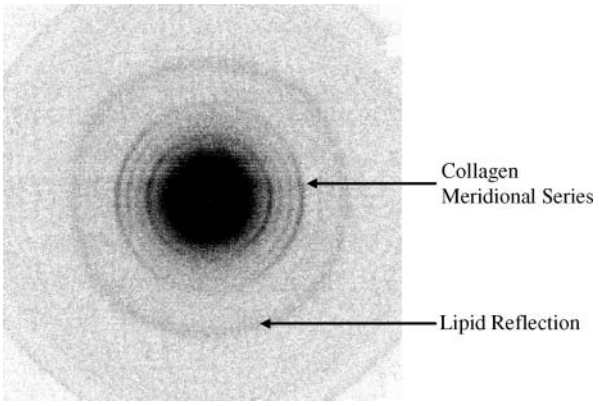

Fig. 2. Small-angle X-ray scattering image from parchment sample USH01. The main features shown are the meridional series of collagen and the broad ring at ~4.6 nm, which arises from the presence of crystalline lipid.

Solid-state NMR

The aliphatic portions of the NMR spectra, between 0 and 85 ppm, are shown in **Fig. 6**. The spectra for the new reference parchment NP7 was very similar to that of USH01; therefore, it is not included in Fig. 6. For some of the historical parchment samples studied, an additional sharp and intense peak at 33 ppm is observed. It is most evident in samples USH02, USH04, and USH12 (Fig. 6). **Figure 7** shows the difference spectrum between the ^{13}C CP-MAS spectra of USH02 and USH01, providing a clear illustration of this peak. Close inspection of this difference spectrum reveals that the observed peaks and their relative intensities are consistent with those expected for the mixture of saturated and unsaturated fatty acids, which can exist either in the free or in the esterified form (e.g., glycerides of palmitic, oleic, stearic, and other acids). The strong peak at 33 ppm and its nearby satellites in the region of 10–40 ppm can be assigned to the -$(CH_2)_n$- group of the -$(CH_2)_n$-CH_3 fragment in fatty acids (e.g., palmitic or oleic acids). There are other peaks in the difference spectrum, which also agrees well with the presence of the fatty acid residues in the USH02 sample, as well as other parchments that display a peak at 33 ppm, relative to samples that do not show a clear peak in that region. The origin of the lipids that produce the peak at 33 ppm is unclear, although it has been found that untreated pig skin displays a similar peak (A. E. Aliev and M. Odlyha, unpublished results). Assignments of the peaks based on the lipid component are shown in Fig. 7. Simple or mixed glycerols are assigned to lipids persisting from calf skin; this is confirmed by the observation of broad peaks near 70 ppm in the difference spectrum, which are assigned to the glycerol carbons [-OCH_2-$CH(O)$-CH_2O-].

Based on close examination of the ^{13}C CP-MAS spectra, the lipid content as estimated relative to the collagen content in the historical samples displays a clear trend: samples USH02, USH04, USH10, and USH12 display higher proportions of lipid than samples USH01 and USH08. This trend is similar to that of the extracted levels of lipid from

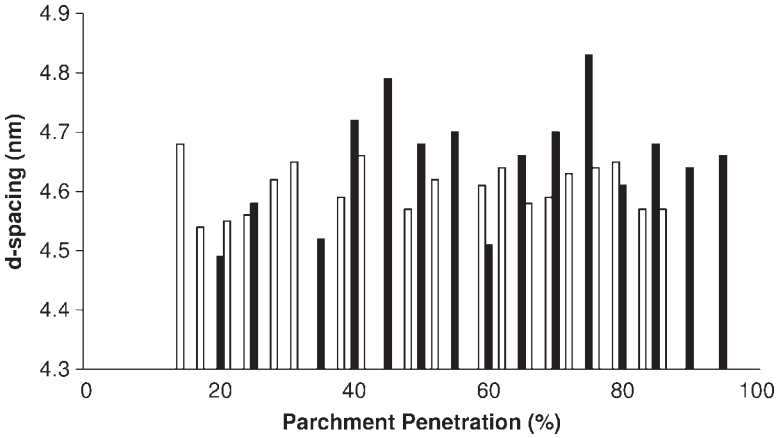

Fig. 3. Lipid *d* spacings (columns) from parchment samples USH01 (white columns) and USH04 (black columns) calculated from microfocus X-ray diffraction images of thin sections of parchment. The scan originated from the grain surface and progressed to the flesh surface. The *d* spacing in the samples appears variable between 4.4 and 4.8 nm.

these samples. These results were obtained from the whole samples, because no preliminary sample treatment was required for the solid-state NMR measurements, which may account for any discrepancy between the two techniques.

DISCUSSION

Previous investigations have highlighted the potential presence of a lipid component in parchment, including a Fourier Transform-Raman spectroscopy study of animal skin (6). In the current study, the presence of lipid in parchment was initially determined by the identification of a characteristic lipid diffraction pattern (corresponding to a *d* spacing of ~4.6 nm) present in the small-angle X-ray scattering patterns obtained from parchment samples. This spacing corresponds to the approximate distance between the polar head groups of lipid bilayers as determined in earlier work (19). Upon extraction of the lipid fraction, this lipid ring disappeared from the parchment scattering pattern, but it became apparent in the X-ray profile of the extract. Microfocus X-ray diffraction of parchment sections determined at an enhanced spatial resolution that the lipid within parchment is not uniform in terms of the distance between polar head groups. Within each parchment section, the *d* spacing of the lipid is variable between 4.4 and 4.8 nm (Fig. 3), which can be an indication of differing hydration levels or biochemical composition of the lipid within a sample. Furthermore, the lipid diffraction patterns obtained from the parchment samples indicated a high degree of spatial organization, which suggests that the collagen structure lends order to the lipid phase.

With the initial identification of a lipid diffraction pattern in parchment samples, biochemical analysis was un-

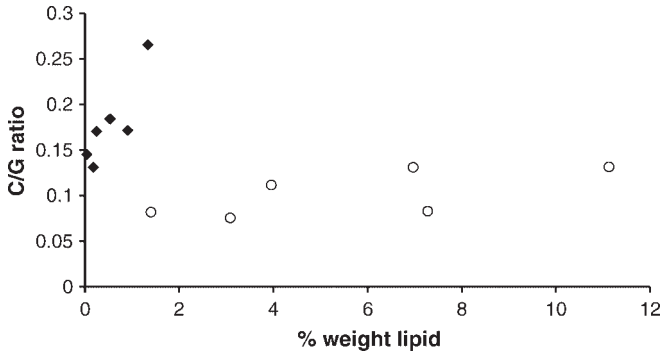

Fig. 4. Scatterplot of parchment samples determined by the proportion of lipid present (% weight) and the collagen-to-gelatin (C/G) ratio. The relationship between the level of lipid present and collagen damage is not linear; there appears to be a clustering of the results. Samples with low levels of lipid present (<1.4%) display the most intact collagen (USH01, USH06, USH07, USH08, USH09, and USH11; diamonds), whereas samples with higher proportions of lipid present (>1.4%) display damaged collagen (USH02, USH03, USH04, USH05, USH10, and USH12; circles).

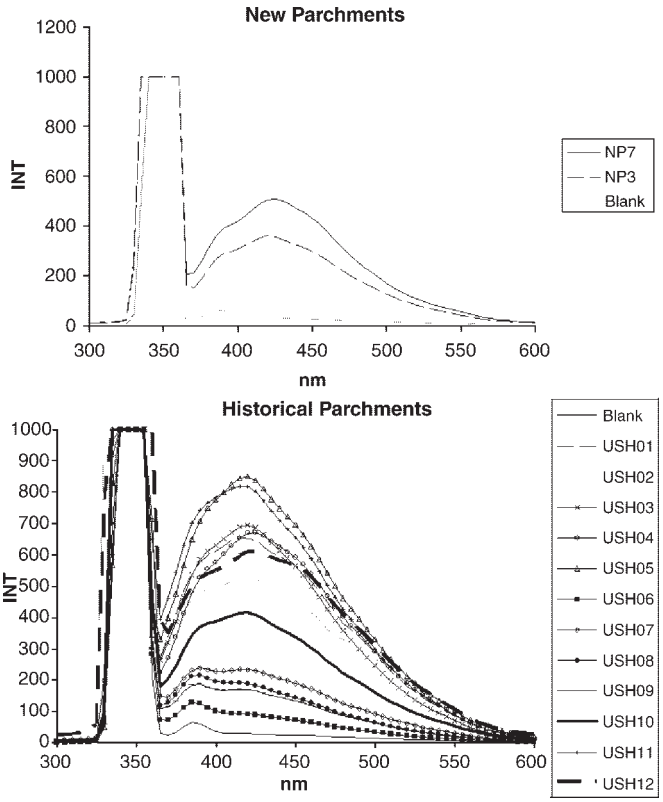

Fig. 5. Fluorescence emission from C/M extracts of parchment samples. The peak observed at 420–430 nm is in agreement with Nilsson and Yin (18), corresponding to a variety of proteolipid complexes of the ceroid/lipofuscin type. Int, intensity.

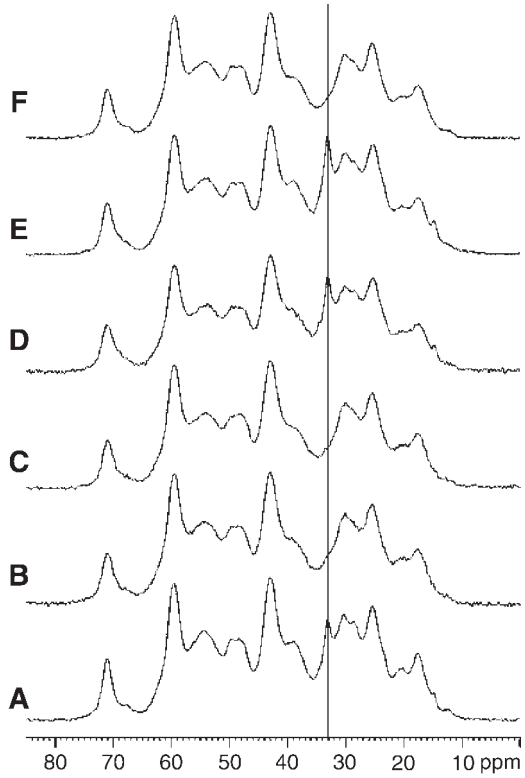

Fig. 6. ^{13}C cross-polarization-magic angle sample spinning (CP-MAS) NMR spectra of parchments USH12 (trace a), USH10 (trace b), USH08 (trace c), USH04 (trace d), USH02 (trace e), and USH01 (trace f). The vertical line highlights the position of the most intensive peak from the lipid component.

dertaken to verify these results and characterize the lipid fraction present. The lipid component was found to be a significant portion of many of the historical samples investigated. The primary lipid fraction obtained from C/M extraction was yellow-brown in color and constituted up to 11% by weight of the original sample. Degraded parchment samples yielded a higher lipid component (Fig. 4); reference samples of new parchments and well-preserved historical samples contained very little lipid extract in comparison.

There is substantial variance between the types of lipid extracted from new and historical parchments (Table 3).

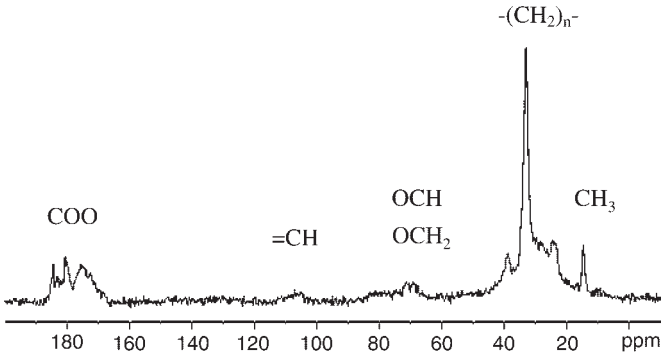

Fig. 7. Difference spectrum between ^{13}C CP-MAS NMR spectra of USH01 and USH02. Assignments of fatty acid carbons are shown.

The lipids extracted from new parchment were largely unclassified, with >80% classed as unknown, which is a significant difference between the historical and new parchment samples. This may be attributable to changes in the method of parchment production, which has been modified over time (2). Newly produced parchments are often treated with bleach, scalded with boiling water, and polished extensively in an effort to produce thinner, whiter parchments; such treatments would not have been used on the historical samples. This may lead to the lipids in recently manufactured parchments taking on different chemical characteristics, making analysis of the lipid types more difficult.

After extraction, samples were subjected to acid treatment to produce FAMEs that could be analyzed via GC-MS, to determine the lipid composition of the samples. It was observed at this stage that samples with high lipid yields produced comparatively little FAME. In addition, the yellow-brown color also disappeared with acid treatment. Together, these results may indicate the presence of protein-lipid conjugates that are destroyed by acid treatment. The protein-lipid complexes present in the raw extract may hinder further investigation by preventing esterification of the samples for the purposes of GC-MS analysis, resulting in low FAME yield even from samples with high amounts of raw lipid extract. They may also impede successful analysis of the proteinaceous component of the parchment. The extracted FAMEs were subsequently used to determine the compositional profile of the lipid extract, which was shown to consist primarily of polar moieties.

The presence of protein/lipid conjugates as measured by fluorescence spectra in certain samples, in particular USH01, USH02, USH03, USH04, USH05, and USH11, does not correlate with the amount of lipid extracted from the parchment samples: USH02, USH03, and USH04 have relatively high amounts of extractable lipid present, whereas USH01, USH05, and USH11 have very little extractable lipid available. Other researchers (20) used fluorescence to recognize various types of proteolipids; samples in this study display lipids of the ceroid type. NMR spectroscopy identified peaks that can be attributed to the presence of fatty acids in the parchments and indicated significant differences between historical and reference parchment samples, mainly in the formation of a new peak at 33 ppm. It remains to be determined whether this is a degradative change or is attributable to differences in the species of origin. It is not clear whether the historical parchments studied here could have come from different species. The origin of the peak at 33 ppm is the subject of further study in our laboratories, as this line of inquiry is intimately linked to the question of the origin of the lipid fraction in parchment.

A peak that coeluted with one of the FAMEs present has been tentatively identified as podocarpa 6, a diterpenoid resin derived from some types of pine trees, particularly from the subgenus *Pineaceae*. The origin of this resin in parchment is unclear, however. Similar resins have been used in the conservation of paintings for centuries (21), and oxidized pine resins similar to podocarpa 6, such as dammar resin, have been used for centuries for their fun-

gistatic and bacteriostatic properties, both inhibiting spore germination and mycelial growth (22), but there is no evidence that they were ever applied to parchments. Other possible sources for this resin include the historical storage of parchment documents in pine boxes, allowing the resin to transfer to the parchment, or the use of wax seals on historical documents.

Lipids in parchment could potentially originate in the dermal layer of the animal and persevere through the parchment preparation process. Lipids from the epidermis are unlikely to be represented in parchments, as the stratum corneum is effectively removed from the skin during the manufacturing process. However, the samples with high amounts of lipid show a compositional change in the fatty acids present, such as higher levels of monounsaturated fatty acids (Table 2). This result, coupled with the observed degradation of collagen in lipid-rich samples (Fig. 4), indicates a potential microbial degradative process resulting in the loss of protein structure and the formation of protein-lipid aggregates. Similar conjugates have been observed in disaggregated skin tissue (23). The negative correlation between the presence of traces of podocarpa 6 in the historical samples and the high levels of lipid extract is a matter of interest when considering this. The interactions between diterpenoids and lipids have been documented (24, 25). One explanation for this correlation is that the parchments contain very little lipid when new and acquire them over time through microbial attack; early addition of the resin, either deliberately or from other means, would then act as a protective barrier preventing the microbes, and hence the lipids, from entering the parchment. Another explanation could be that the lipids primarily originate from the skin from which the parchment was created, surviving the preparation process. In this case, the addition of resin at a later date may remove the lipid from parchment, thus preventing possible collagen damage from lipid peroxidation. Further studies are required to determine which of these scenarios is most likely.

The role of lipids in the degradation of collagen within parchment is a matter that has not been investigated extensively. Physiologically, the action of lipid peroxidation has been investigated, as this leads to atherosclerosis (26). Lipids are subject to peroxidation by sulfur dioxide and copper ions, which may act as a catalyst (27, 28). It is possible that a mechanism exists whereby atmospheric light (29) or sulfur dioxide interacts with the lipid fraction of parchment, causing its peroxidation and subsequent generation of reactive oxygen species with the capacity to damage the collagen structure of parchment. This hypothesis is in agreement with the assessment that the parchment samples here that display greater levels of lipid show a reduced level of structural integrity as measured by X-ray diffraction.

Conclusions

In this study, we investigated the significant lipid fraction of historical parchments by gas chromatography, X-ray diffraction, fluorimetry, and solid state NMR. The lipid extracted from the samples displayed a great deal of difference between reference and historical samples. Gas chromatography characterized the FAMEs present in extracts, and GC-MS results displayed a diterpenoid resin known to derive from trees of the subgenus *Pineaceae* in historical samples with low lipid content. The presence of this resin is an interesting result: it may indicate possible antibacterial action by conservators, or provide information on the historical storage conditions of the samples. X-ray diffraction was used to assess the removal of the lipid fraction by C/M extracts and to examine the *d* period of the lipids in thin sections of parchment. It was shown that C/M extraction effectively removed the lipid fraction from parchment, and microfocus X-ray diffraction showed that the *d* spacing within a sample is variable from 4.4 to 4.8 nm, indicating differences in the hydration state or biochemical composition of the lipid present. Solid-state NMR spectra of high-yield lipid samples contain peaks that appear to be caused by the presence of fatty acids.

The origin of this lipid fraction is either intrinsic or extrinsic, or possibly a combination of the two. The role of lipids in the degradation of collagen within parchment is considered here. The presence of lipids acting as a free radical generator upon interaction with atmospheric sulfur dioxide is suggested as a possible means of the degradation of the collagen. The formation of protein-lipid complexes may be an indication of collagen degradation, as similar conjugates are found in the aging process. Our results suggest a relationship between collagen degradation and the increased lipid content of parchment.

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REFERENCES

1. Peckstadt, A., L. Watteeuw, and J. Wouters. 1996. The conservation of parchment manuscripts: two case studies. *In* ICOM Committee for Conservation: Preprints from 11th Triennial Meeting, Edinburgh, Scotland. Vol. II. James and James, London.
2. Kennedy, C. J., and T. J. Wess. 2003. The structure of collagen within parchment: a review. *Restaurator.* **24:** 61–80.
3. Larsen, R., editor. 2002. Microanalysis of Parchment. Archetype Publications, London.
4. Faccini, A., M. F. Fancinelli, A. Bairati, C. E. Bottani, P. L. Cavallotti, D. Fessas, A. Schiraldi, and G. Zerbi. 2001. Restoring process and characterisation of ancient damaged parchments. *Quinio.* **3:** 51–70.
5. Fessas, D., A. Schiraldi, R. Tenni, L. Vitellaro Zuccarello, A. Bairati, and A. Faccini. 2000. Calorimetric, biochemical and morphological investigations to validate a restoration method of fire injured ancient parchment. *Thermochim. Acta.* **348:** 129–137.
6. Edwards, H. G. M., D. W. Farwell, E. M. Newton, F. M. Perez, and S. Jorge-Villar. 2001. Application of FT-Raman spectroscopy to the characterisation of parchment and vellum. I. Novel information for

paleographic and historiated manuscript studies. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **57:** 1223–1234.
7. Strzelczyk, A. B., L. Bannach, and A. Kurowska. 1997. Biodeterioration of archaeological leather. *Int. Biodeterior. Biodegradation*. **39:** 301–309.
8. Peacock, E. E. 1996. Characterization and simulation of water-degraded archaeological textiles: a review. *Int. Biodeterior. Biodegradation*. **38:** 35–47.
9. Choglay, A. A., I. F. Purdom, and D. J. S. Hulmes. 1993. Procollagen binding to sphingomyelin. *J. Biol. Chem.* **268:** 6107–6114.
10. Kennedy, C. J., J. C. Hiller, D. Lammie, M. Drakopoulos, M. Vest, M. Cooper, W. P. Adderley, and T. J. Wess. 2004. Microfocus X-ray diffraction of historical parchment reveals variations in structural features through parchment cross-sections. *Nano Lett.* **4:** 1373–1380.
11. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
12. Wess, T. J., M. Drakopoulos, A. Snigirev, J. Wouters, O. Paris, P. Fratzl, M. Collins, J. Hiller, and K. Nielsen. 2001. The use of small-angle X-ray diffraction studies for the analysis of structural features in archaeological samples. *Archaeometry.* **43:** 117–129.
13. Gildenast, T., and J. Lasch. 1997. Isolation of ceramide fractions from human stratum corneum lipid extracts by high-performance liquid chromatography. *Biochim. Biophys. Acta.* **1346:** 69–74.
14. Opella, S. J., and M. H. Frey. 1979. Selection of nonprotonated carbon resonances in solid-state nuclear magnetic resonance. *J. Am. Chem. Soc.* **101:** 5854–5856.
15. Chen, F. Y., W. C. Hung, and H. W. Huang. 1997. Critical swelling of phospholipid bilayers. *Phys. Rev. Lett.* **79:** 4026–4029.
16. Zhang, R., W. Sun, S. Tristram-Nagle, R. L. Headrick, R. M. Suter, and J. F. Nagle. 1995. Critical fluctuations in membranes. *Phys. Rev. Lett.* **74:** 2832–2835.
17. Nagle, J. F., and S. Tristram-Nagle. 2000. Structure of lipid bilayers. *Biochim. Biophys. Acta.* **1469:** 159–195.
18. Nilsson, E., and D. Yin. 1998. Preparation of artificial ceroid/lipofuscin by UV oxidation of subcellular organelles. *Mech. Ageing Dev.* **99:** 61–78.
19. Petrache, H. I., N. Gouliaev, S. Tristram-Nagle, R. Zhang, R. M. Suter, and J. F. Nagle. 1998. Interbilayer interactions from high-resolution X-ray scattering. *Phys. Rev. E.* **57:** 7014–7024.
20. Yin, D. 1996. Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. *Free Radic. Biol. Med.* **21:** 871–888.
21. Van den Berg, K. J., J. J. Boon, I. Pastorova, and L. F. M. Spetter. 2000. Mass spectrometry methodology for the analysis of highly oxidised diterpinoid acids in Old Master paintings. *J. Mass Spectrom.* **35:** 512–533.
22. Feio, S. S., B. Gigante, J. C. Roseiro, and M. J. Marcelo-Curto. 1999. Antimicrobial activity of diterpene resin acid derivatives. *J. Microbiol. Methods.* **35:** 201–206.
23. Lopez, O., M. Cocera, L. Campos, A. de la Maza, L. Coderch, and J. L. Parra. 2000. Use of wide and small-angle X-ray diffraction to study the modifications in the stratum corneum induced by octyl glucoside. *Colloids and Surfaces A: Physicochemical and Engineering Aspects.* **162:** 123–130.
24. Mateo, C. R., M. Prieto, V. Micol, S. Shapiro, and J. Villalain. 2000. A fluorescence study of the interaction and location of (+)-totarol, a diterpenoid bioactive molecule, in model membranes. *Biochim. Biophys. Acta.* **1509:** 167–175.
25. Evans, G. B., R. H. Furneaux, M. B. Gravestock, G. P. Lynch, and G. K. Scott. 1999. The synthesis and antibacterial activity of totarol derivatives. I. Modifications of ring-C and pro-drugs. *Bioorg. Med. Chem.* **7:** 1953–1964.
26. Steinbrecher, U. P., H. Zhang, and M. Lougheed. 1990. Role of oxidatively modified LDL in atherosclerosis. *Free Radic. Biol. Med.* **9:** 115–168.
27. Meng, Z., G. Qin, B. Zhang, H. Geng, Q. Bai, W. Bai, and C. Liu. 2003. Oxidative damage of sulfur dioxide inhalation on lungs and hearts of mice. *Environ. Res.* **93:** 285–292.
28. Patterson, R. A., D. J. Lamb, and D. S. Leake. 2003. Mechanisms by which cysteine can inhibit or promote the oxidation of low density lipoprotein by copper. *Atherosclerosis.* **169:** 87–94.
29. Boulton, M., A. Dontsov, J. Jarvis-Evans, M. Ostrovsky, and S. Svinstunenko. 1993. Lipofuscin is a photoinducible free radical generator. *J. Photochem. Photobiol. B.* **19:** 201–204.