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Calorimetric, biochemical and morphological investigations to validate a restoration method of fire injured ancient parchment

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Abstract

Differential scanning calorimetry, thermogravimetry, biochemical tests, morphological investigations, via electron, polarizing and phase contrast microscopy have been used to validate the effects of a restoration method for fire injured ancient parchment codices, requiring parchment softening, regeneration, stretching and drying. The results allowed understanding of modifications induced by the heat, namely collagen denaturation, and those by the restoring process on heat damaged ancient parchments. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Calorimetry; Ancient parchments; Restoration; Collagen; Electron microscopy

1. Introduction

Wild fire in libraries usually produces the irreversible loss of precious and unique documents among which ancient parchments can represent a relevant part of the cultural wealth and heritage therein stored. Although not wholly burnt, some seriously injured parchment manuscripts show shrunk and fragile sheets which can no longer be legible or even split off (see Fig. 1a). Fortunately this condition is reversible to some extent since suitable recovery procedures are nowadays available [1–4].

Ancient parchments are handcrafts of animal skin. The main component of the skin connective tissue matrix is collagen, a family of related proteins [5]. Different collagen types are present in skin, the most abundant ones belonging to the fibrillar subfamily. Type I collagen is present at a relative proportion of about 80–90%, type III collagen at about 8–12% (the percentage is age-dependent) and type V collagen at about 5% [6]. The structure unit of all collagen types is formed by three polypeptide chains, called alpha helices, with a repetitive sequence pattern, Gly-X-Y, where X is frequently proline and Y is hydroxyproline. Some collagen types are heterotrymeric, such as type I collagen (that is constituted by two alpha chains named α1(I), and by a different one named α2(I)), whereas other types are homotrimeric, such as type III collagen, $[\alpha 1(III)]_3$. The structure formed has a characteristic coiled coil triple helical conformation that is stabilized by weak non-covalent interchain bonds,

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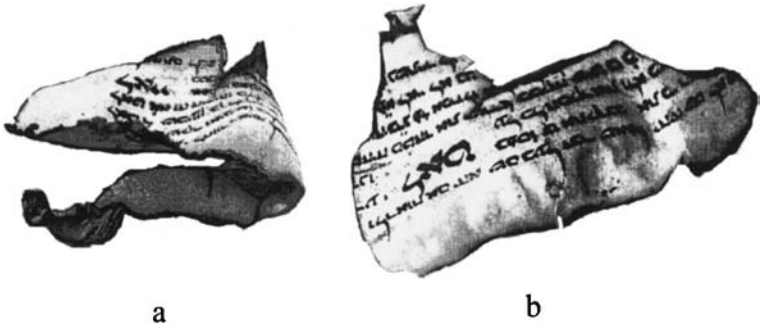

Fig. 1. Fire injured (a) and restored (b) parchments from ancient manuscript which experienced a wild fire in 1904 at the National Library of Turin, Italy.

mainly hydrogen bonds. In fibrillar collagens, there are no interruptions of the repetition of Gly-X-Y triplets and the result is a thin, long, rod-like molecule with a length of 300 nm and a diameter of about 1.5 nm.

On a higher organization level, molecules of the fibrillar collagens align side by side in a quater staggerd array with their main axis parallel, forming fibrils and fibers. These supramolecular structures are stabilized by cross-linking [7] and give characteristic banding patterns on staining [8]. The thermal stability of such a structure depends on the amino-acid composition of the proteins, in particular hydroxyproline [9], and on the environmental conditions (concentration, pH, ionic strength, denaturing agents, etc.).

When the temperature is raised above a given threshold (T_d) the protein undergoes denaturation with a mechanism which depends on a number of factors: basically, tropo-collagen chains dissociate with consequent degradation of the fibrous texture of the material. A number of works have been so far devoted to the study of collagen and its aging and heat induced modifications, but only few of them deal with parchments. Furthermore, all these imply extraction of collagen and do not offer results of investigations on the material as it is [10].

The present work reports the characterization of injured and restored parchments from ancient manuscripts which experienced a wild fire in 1904 at the National Library of Turin, Italy [11]. Well preserved ancient parchments were also investigated to support the reliability of the approach followed. The effects of the exposition to the fire and those of the restoration method were carefully checked with differential scanning calorimetry (DSC).

DSC is a powerful technique to investigate the conformational transitions of biological macromolecules (collagen in the case of parchment), as it allows the direct measurement of the enthalpy change, ΔH, associated with the process from samples of the material as it is. The measured property is the heat capacity at constant pressure, $C_p=(\partial H/\partial T)_p$, which reveals all the state modifications of the system (e.g., protein denaturation and aggregation, gel–sol transitions, fusion, crystallization, glass transition, etc.) when it undergoes some temperature change. None the less calorimetry is a 'blind' technique that requires side information from other experimental approaches which can support the interpretation of the results. For this reason, thermo-gravimetric analysis (TGA), biochemical tests, and morphological investigations, via electron, polarizing and phase contrast microscopy were included in the present research. The extra information obtained supported and confirmed the DSC results and allowed understanding of modifications induced either by the damaging events, or by the recovering treatment.

2. Materials and methods

The parchments considered were those sampled from

1. codices of the National Library of Turin
   1.1. fire injured (ITP) [11],
   1.2. restored sheets of the same codices (RTP).

2. XVI century goat parchments

   2.2. original (OGP),
   2.3. injured (IGP): the wild fire effects were reproduced by heating the fresh parchment in a spoon hanging above a flame,
   2.4. restored (RGP).

2.1. Restoration method

The details of the method are not inherent with the present paper, where we briefly summarize its main steps, namely, parchment softening, regeneration, stretching and drying. The codex sheets could be split off after a preliminary softening treatment, performed at room temperature in a glove box under water/ethanol/*n*-butanol atmosphere preventing any liquid condensation on the parchment. Each separated sheet was then 'regenerated' by immersion in a urea-sodium chloride hydro-alcoholic solution (water 48% (w/w), ethanol 48% (w/w), urea 2% (w/w), NaCl 2% (w/w)) and then washed with a 50% (w/w) hydro-alcoholic solution; it was finally stretched and dried to restore the original size and shape (see Fig. 1b).

2.2. Biochemical analysis

OGP, IGP, and RGP samples were analyzed. The following analyses were performed.

(1) Hyp (hydroxyproline) content was determined according to [12], after acid hydrolysis in 6 M HCl at 105°C for 24 h and solvent evaporation.

(2) SDS-PAGE (polyacrylamide gel electrophoresis in denaturing conditions for the presence of sodium dodecylsulfate) was performed according to [13]. Gels were stained with Coomassie Brilliant Blue R250 according to [14].

(3) Pepsin treatment. Parchment fragments were suspended in 0.5 M acetic acid and kept at 4°C for 5 days, in order to let the samples to swell. Pepsin was then added at a substrate: pepsin ratio of 35–55:1 w/w. The digestion was performed at 4°C for 17 h. After centrifugation, the residue was subjected to a second pepsin treatment. After a final centrifugation, Hyp was determined on the residue and on aliquots of both supernatants. Aliquots of both supernatants were also freeze-dried and subjected to SDS-PAGE, as above.

(4) Cyanogen bromide cleavage. A preliminary step was performed in order to reduce the oxidized methionines [15] by treatment of parchment fragments with 10% v/v 2-mercaptoethanol at 60°C for 30 min. The whole suspension was freeze-dried and then resuspended in 70% v/v formic acid and kept at 4°C for 3 days. CNBr was added (0.3–0.6 g/g of parchment) as a 10-fold concentrated solution in 70% HCOOH, the samples were flushed with nitrogen and the digestion was performed at 45°C for 12 h followed by 8 h at room temperature. The samples were freeze-dried and residues were subjected to SDS-PAGE, as above.

2.3. Differential scanning calorimetry (DSC)

Real and simulated damaging effects and modifications produced by the restoring process were investigated on ITP, RTP, OGP, IGP, and RGP samples (20 mg) with a Perkin–Elmer DSC-6 calorimeter. The samples were sealed in aluminum pans and an empty pan was used as the reference cell. DSC runs at a 5°C min^{-1} heating rate spanned the whole 20–120°C temperature range. Some samples were cooled down to 20°C (at a 5°C min^{-1} cooling rate), annealed at this temperature for 20 min, and reheated up to 120°C. The raw data were analyzed (subtraction of the instrumental base line, calibration, smoothing, etc.) with the software IFESTOS [16], to obtain the trend of the apparent heat capacity per gram of matter, $C_p^{ap}(T)$/J g^{-1} K^{-1}.

2.4. Thermogravimetric analysis (TGA)

The TGA instrument was a SETARAM TG-DSC111 which allows the simultaneous output of the TG trace (mass loss vs. time), its time derivative, DTG, and the thermal effect (heat flow). ITP and RTP, samples (30 mg) were analyzed. Measurements were carried out throughout the 20–200°C temperature range with a 2°C min^{-1} heating rate The raw data were analyzed with the software IFESTOS [16], to obtain TG(T), in %(sample weight/initial sample weight) units, and DTG(T), in %(w/w) K^{-1} units. The enthalpies observed indicate that the observable mass loss is related to water evaporation.

2.5. Polarizing and phase contrast microscopy

Transversal 20 μm thick cryostat sections were obtained from hydrated parchment samples. The sections, collected on glass slides and mounted on water

with a cover-slip, were examined with a Carl Zeiss polarizing microscope or with a phase contrast microscope.

2.6. Electron microscopy (EM)

Hydrated parchment samples were kept overnight in 1% aqueous osmium tetra-oxide at 4°C and stained in block with 2% uranyl acetate in 70% aqueous ethanol (2 h at room temperature). After dehydration with graded ethanols, samples were cleared in propylene oxide, embedded in Epon-Spurr resin and polymerized at 60°C for 48 h. Ultrathin sections (60–80 nm) were cut with a Reichert ultramicrotome, collected on copper mesh grids, counter-stained with lead citrate and uranyl acetate, and examined with a Zeiss 902 electron microscope.

3. Results and discussion

3.1. TGA and DSC investigations

The main heat damage suffered by the collagen network is a shrinkage (see Fig. 1a) coupled with some water loss. TGA was used to quantify the extent of the latter. Fig. 2a and b show the TG(T) and the relevant DTG(T) traces, respectively, for (a) a ITP, and (b) a RTP sample.

Although the general trend of water release was substantially analogous in either case, with a maximum water loss rate at about 70°C (see Fig. 2b), the sample (a) revealed a smaller water content than the sample (b), namely 12 vs.14% w/w (see Fig. 2a). For either sample the DTG trace showed a broad single peak which might suggest a single mechanism acting throughout the T range investigated: none the less, because of the large width of this signal, a multi-step process could not be ruled out. Fig. 3 shows the DSC traces obtained from (a) a ITP and (b) a RTP sample. It has to be stressed that the DSC runs were carried out with sealed pans which did not allow any water release from the samples: this means that the effects observed took place at the same moisture content throughout the T range scanned and no signal might be attributed to water loss.

The trace of the damaged parchment (a) was significantly different from that of the restored parchment

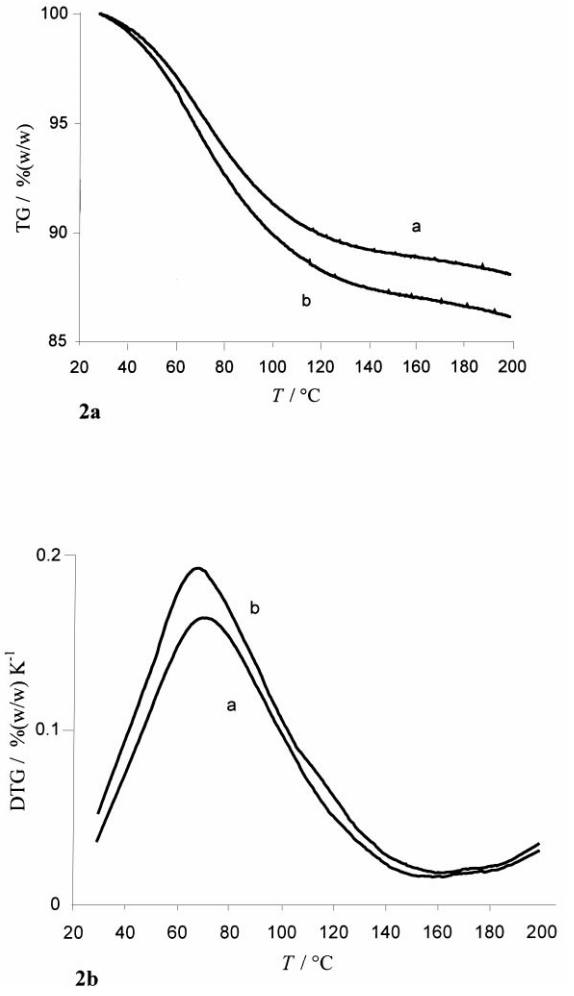

Fig. 2. TG (Fig. 2a) and DTG trace (Fig. 2b), respectively, for (curve a) a ITP, and (curve b) a RTP sample (see text).

(b). For a quantitative interpretation of this finding a reliable base line was necessary to define the excess heat capacity with respect to the not damaged material. Since the parchment samples were those which had experienced the effects of the wild fire, no 'native' parchment was available to define a reliable base line. To overcome this difficulty, experiments were carried out with samples of a different ancient parchment (see below) which had not undergone any wild fire. After the first heating run, the DSC pans were allowed to cool down and then reheated. The new DSC traces showed a neat endothermic shift, with the typical shape of a glass transition signal (Fig. 4): the corre-

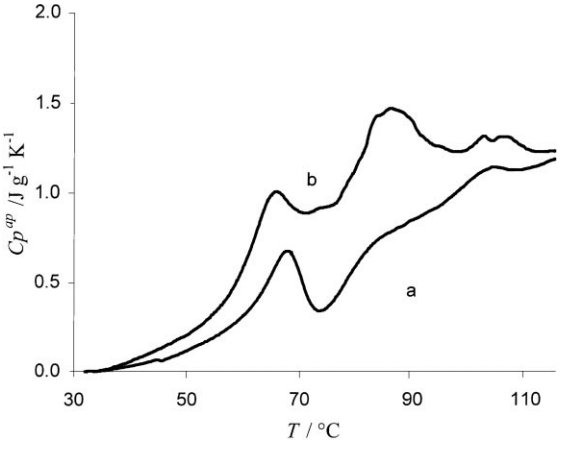

Fig. 3. DSC traces for (curve a) a ITP, and (curve b) a RTP sample.

sponding transition temperatures T_g (taken at the flexus point) were 50 and 70°C for the damaged and restored parchment, respectively.

These traces were representative of the thermoset state produced by the previous heating, and the difference between the T_g values could be matched with the corresponding difference in water content: for moisture levels below 15% w/w in a number of biopolymer materials, a 10°C T_g drop is indeed observed per 1% increase of water content [17].

In order to assess the reliability of the approach used and to rule out any doubt about the generality of the results in characterizing samples of ancient parchments, a different lot of samples from ancient parchment (goat skin XVI century) taken from another library (which did not undergo any wild fire) were considered. OGP samples to define a reliable base line of the relevant DSC traces (Fig. 5a); by subtracting this, the relevant profile of the excess heat capacity with respect to the native state was obtained (Fig. 5b). A main peak with maximum at about 65°C and a large high T (85°C) shoulder and a minor low T (52°C) skew were recognized.

The wild fire effects were reproduced by heating the goat parchment in a spoon kept above a flame. The non-burnt region was sampled (IGP samples) and part of it subjected to the restoration treatment (RGP): the relevant DSC traces are reported in Fig. 6.

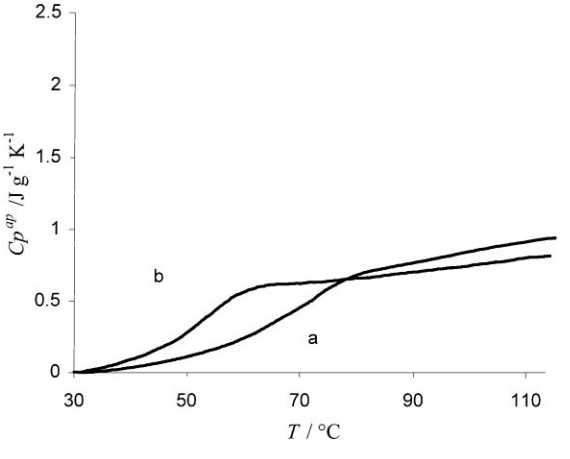

Fig. 4. $C_p^{ap}(T)$ from the second heat DSC run for (curve a) the ITP, and (curve b) the RTP samples showed in Fig. 3.

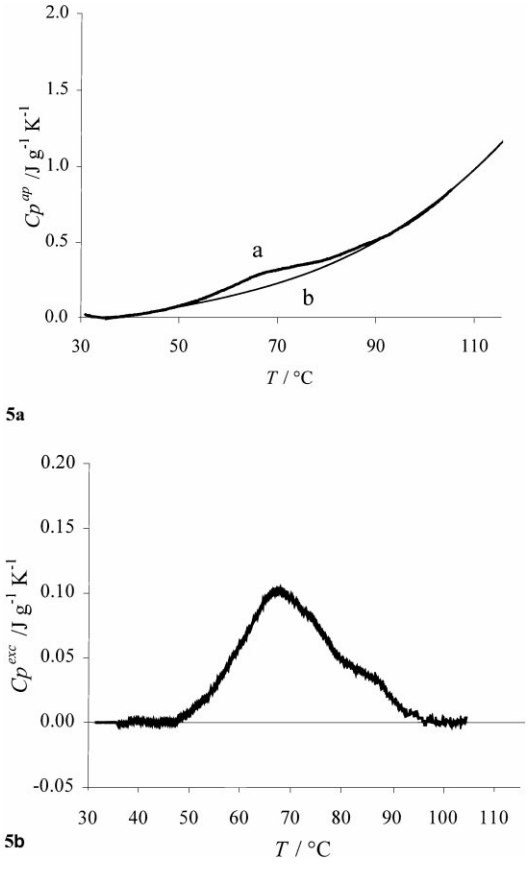

Fig. 5. (a) $C_p^{ap}(T)$ for (curve a) a OGP sample and (curve b) base line obtained by linear regression on the low and high T range of the trace. (b) Excess specific heat capacity, $C_p^{exc}(T)$, respect to the collagen native state of a OGP sample (see text).

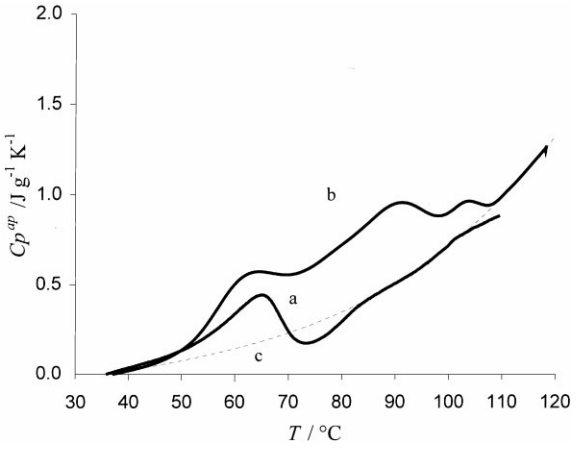

Fig. 6. DSC traces for (curve a) a IGP, and (curve b) a RGP sample. The curve c is the reference line showed in Fig. 5b.

The close similarity with the traces shown in Fig. 3 suggested that wild fire damages would be similar for any parchment, no matter the chemical peculiarities of the collagen in each of them. The excess heat capacity traces with respect to the native state, obtained after subtraction of the base line (Fig. 5a, curve b) are reported in Fig. 7, where original, fire damaged and restored parchments can be compared.

The trace of the original parchment was analogous to that observed for aged collagen samples by other authors [10,18]: this supports the statement that parchment would mainly contain collagen in its native conformation. This assumption was also confirmed by EM investigations (see below). The collagen network of an ancient parchment would none the less be more extensively cross linked than the native collagen, since it remained rather insoluble after SDS treatment (see below). The main endothermic peak could be thus attributed to the denaturation of less tightly cross-linked triple helices, while the fraction of the most cross-linked collagen triple helices would undergo denaturation and produce the shoulder of the DSC signal at higher T. This interpretation was in line with the suggestions by other authors [10].

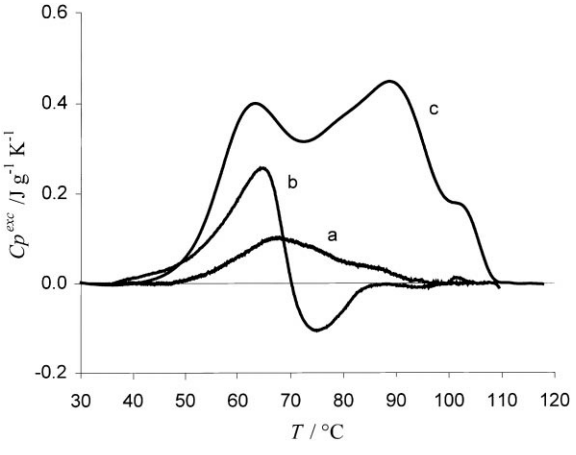

Fig. 7. Excess specific heat capacity, $C_p^{exc}(T)$, respect to the collagen native state of (curve a) a OGP, (curve b) a IGP and (curve c) RGP sample.

IGP samples gave a trace where an endothermic signal, which was larger and occurred at a lower temperature with respect to the main peak of OGP trace (maximum at 63 instead of 65°C); this was immediately followed by an exothermic aggregation signal (partially overlapped to the former). No high T shoulder was observed.

In the DSC traces of RGP samples any aggregation process seemed inhibited, while the endothermic signal reappeared split in two main components which were much better resolved than in the case of OGP samples: the high T shoulder was now a well defined peak. Both these endothermic components were larger than those observed in the DSC traces of OGP and IGP samples, and the first of them occurred at lower temperature (60°C).

The second heating DSC run again revealed glass transitions of the thermosets produced during the first heating. The results (see Fig. 8) showed that original and restored parchment were able to produce the same thermosetting and therefore had practically analogous macroscopic starting properties.

The DSC measurements were therefor adequate to put in evidence some differences between parchments. However, since the denaturation mechanism of the collagen molecules is irreversible (see Figs. 4 and 8), it was not possible to apply equilibrium thermodynamics to obtain the relevant denaturation enthalpy. In these cases the systems follow one of the Laumry–Euring models [19] where the 'activation energy' of the irreversible step is a main parameter which can change from sample to sample. It is indeed related to the peculiar kinetics of the irreversible steps and may affect either the temperature of the DSC peak or the

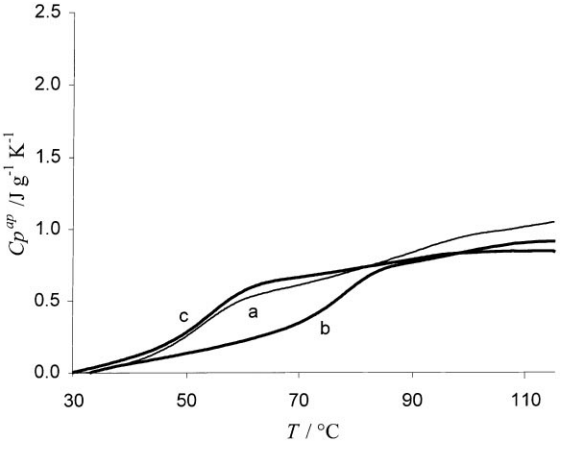

Fig. 8. $C_p^{ap}(T)$ from the second heat DSC run for (curve a) the OGP, (curve b) the IGP and (curve c) the RGP samples showed in Figs. 5 and 6.

underling area. For this reason, the only elements of comparison between DSC traces from different parchment samples were the presence, the shape and the relative proportions of the endothermic and exothermic components of the signal.

3.2. Biochemical findings

The results of Hyp determinations were the following:

- OGP: 95 μg Hyp/mg of parchment,
- IGP: 105 μg/mg,
- RGP: 104 μg/mg.

These values can be converted into collagen content by using a type I collagen/Hyp ratio of 6.41 g/g. The three parchments thus contained 61, 67 and 66% w/w of collagen, respectively. These data were in line with the amino acid analysis reported in [20], who did not determine the Hyp content, but were able to show that the parchments they analyzed had a composition similar to that of type I collagen, save for a gross overestimation of the aspartic acid content.

The parchment fragments were highly insoluble in Laemmli's [13] sample buffer (a denaturing solution containing 2% w/v sodium dodecyl sulfate) also at 85°C for 30 min. In fact, no bands for α1(I) and α2(I) chains of type I collagen or no whatsoever band was detectable by SDS-PAGE electrophoresis and gel staining, even in the presence of overloading (the detection limit is less than 1 μg of α1(I) or α2(I) chain).

On pepsin treatment, no collagen was solubilized from the OGP, the whole amount remaining with the residue, as determined by Hyp analysis. On the contrary, 18% of collagen was dissolved from the IGP and 10% from the RGP. However, the collagen dissolved was also degraded by pepsin and thus was not in the triple helical conformation, that is stable to pepsin proteolysis. In fact, SDS-PAGE analysis of the dissolved material from IGP and RGP did not show any α1(I) and α2(I) bands, even after overloading which produced only dye smears along the electrophoretic lanes.

On CNBr treatment, the parchment fragments did dissolve, but no bands attributable to CNBr peptides of type I collagen were detected with SDS-PAGE electrophoresis which showed only high molecular weight band(s) at the top of the gel and some dye smears along the gel. This finding suggests that the peptides formed by the CNBr treatment are cross-linked to each other.

The elevated insolubility of collagen in the parchments analyzed is very likely due to a large degree of cross-linking. Our data do not allow a quantitative valuation of the cross-linking in any sample but reveal a large presence in any case. Cross-links, other than those of physiological origin [7], could have been formed during the original treatment of the animal skin to produce the parchment texture and/or during the several century aging.

3.3. Morphological data

Polarizing microscope of cryostat sections obtained from (i) OGP, (ii) IGP, and (iii) RGP samples were performed. Both the OGP and RGP samples showed a typical architecture consisting of intensely birefringent fiber bundles embedded in abundant granular material (see Fig. 9B). Rotation of the sections in respect to the plane of polarization revealed crossing of birefringent structures at different angles. Fiber bundles were no longer recognizable in IGP parchment (Fig. 9A) which only displayed wide isotropic areas and strongly birefringent granules. At the phase contrast microscope (data not shown) isotropic areas appeared as homogeneous, densely packed structures,

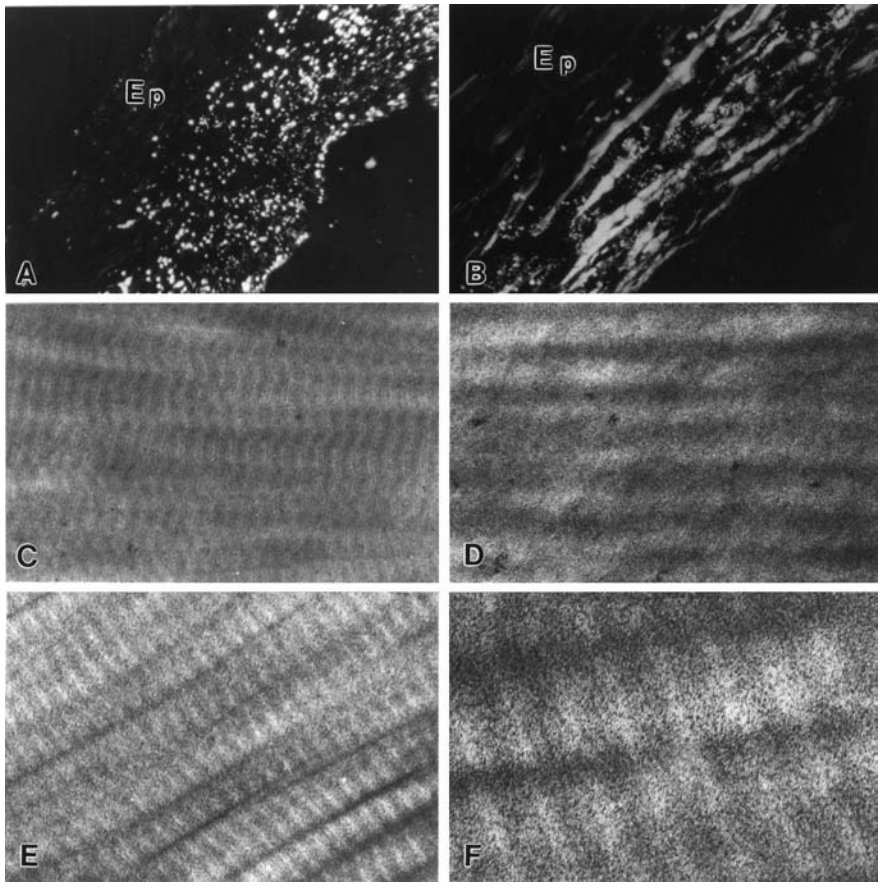

Fig. 9. Parts A, B=cryostat sections of parchments examined with polarized light microscope. Section of fire damaged parchment (Part A, × 33) and restored parchment (Part B, × 82.5) . Ep=epidermal surface of parchements. Parts C–F=ultrathin sections of parchments examined in the transmission electron microscope. Part C=bundles of banded collagen fibrils within native parchment, × 52,200. Part D=damaged parchment, × 57,200. Part E=banded collagen fibrils in restored parchment, × 71,500. Part F=detail of Part D, × 228,570.

either round-shaped or in form of short wavy segments.

To state that the birefringent structures observed in simply hydrated parchment samples correspond to the bundles of collagen periodical fibrils EM investigations were performed.

OGP sample sections examined by EM show a dense texture of fibers with periodically banded fibrils (Fig. 9C).

IGP samples appeared to mostly consist of irregular clumps of a dense cotton-like material, that only rarely displayed a more organized pattern suggesting the presence of fibrous structures (Fig. 9D). Periodic banded segments were never observed.

A similar structure was also present in RGP samples that, however, contained numerous bundles of fibrils (Fig. 9E) exhibiting a periodic structure. At higher magnification (Fig. 9F), the period showed a fine structure of regular alternating electron dense zones, formed by extremely thin packed filaments, and of homogeneous electron lucent zones. The period length varied from 45 to 60 nm. This is in line with the typical periodic ultra structure of collagen fibrils recognized in ancient parchments examined with several techniques, such as sample fragmentation and fibril isolation, and ultra thin sectioning [21]. The typical periodic structure of collagen fibrils observed in restored parchment indicated the presence of regular

arrangements of proto-filaments and collagen molecules, like those in native collagen.

4. Conclusions

According to the literature, collagen structure depends on specific interactions strongly affected by the environment and water content [9,22]. A detailed discussion of the specific interaction of the restoring solution components with the collagen molecules is out of the scope of this article. None the less a tentative description of the damaging and restoring mechanism matching with our data might be put forward.

The fire damage produced denaturation of a fraction of collagen triple helices which none the less could not achieve a complete chain separation, being under the constraint of cross-links, as biochemical analyses suggested. Denaturation and the moderate water depletion enhanced the packing of the polymer that shrank. The mutual orientation of the residual triple helices would largely differ in these samples from the original one: the EM investigations did not reveal any cross band image in fire injured parchment, which should accordingly be a disordered material. The further heat treatment experienced during the DSC scan sustained a progress of denaturation followed by aggregation of the molecular moieties.

The restoring treatment allowed a rearrangement of many denatured collagen segments with restoration of the triple helix conformation. This would be possible since these segments were under the constraint of neighboring cross links. EM investigations confirmed this statement, since banded fibrils were observed in these samples. On the other hand, the high T peak of the relevant DSC trace was proportionally larger than the high T shoulder in the signal of non-damaged parchment: this would therefore suggest that restored parchment was richer in cross linked collagen molecules arranged in the triple helix conformation.

In conclusion this study validates the restoration method in as much as it allows recovery of the 'native' properties of the collagen molecules within fire injured parchments, and demonstrates that DSC is a suitable technique to detect the effects produced.

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