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Histological study of sheep skin transformation during the recreation of historical parchment manufacture

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Abstract

We report a simple histological study on skin biopsies from young domestic sheep following each step in transformation from skin to parchment production. During the recreation of historical parchment manufacture, histological analyses were conducted; before and after lime treatment, hair removal, and stretching. Sections were fixed and stained using a variety of histological stains to identify the presence of different molecular classes and the fibrous proteins, collagen and elastin. The results reveal surprisingly few histological changes in most steps in the production process. However, very visible changes in the supramolecular ordering of skin macromolecules (elastin, collagen) occur during the final stage of parchment production when stretched on the frame. Collagen fibres and hair follicles were all strongly re-oriented in the direction of strain. Surprisingly despite the thinness of the lambskin and the exhaustive treatment in lime, not all fats were saponified and even in the final product Oil Red O stained fat bodies were detectable on the hair side of the skin. We believe this study will help compensate for the lack of sources on microscopic changes in parchment during the recreation of its historical manufacture.

Keywords: Sheep skin, Histology, Parchment, Collagen, Elastin, Lipid

Introduction

Studies on the histology of animal skins have mainly focused on living animals, exploring pathology [1, 2], differences between species [3, 4] or breeds [5, 6], structural features [5, 7] or specific constituents [1, 8]. Some are focused on hair follicles and parchment grain surfaces in leather [2, 3, 6, 9, 10] or parchments [10–12], both in terms of species differences and quality. However, the transformation of the skin, from rawhide to parchment, has rarely been reported, a notable exception being the work of Saxl [13, 14].

Recent research [15] has revealed that sheep skins have been widely employed for parchment production throughout history, typically for legal documents. In this paper we explore the histological changes that occur to fresh sheep skins during the different stages employed in parchment production. Using an old manufacturing recipe [16], and by studying the impact of changes in the ingredients and processes we hope to better interpret the results of previous studies of old manuscripts.

There have been few previous histological studies of parchment [e.g. 13, 17–19] and none have followed the process of parchment manufacture. By documenting how each stage is processed and by taking successive samples it will be possible to correlate with the non-invasive procedures currently used to decipher the production of parchment (SEM, XRF, Raman, etc.) from unknown historic production methods. The resulting data from experimental manufacture can then be compared with non-invasive methods applied to historical material [15, 20, 21], as it was in a recent article [22] in which the

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authors conclude that "the skin studied was first tawed with alum and then dressed with gypsum".

Skin structure

Sheepskin, like the skin of other mammals, is divided into two layers: the (outer) epidermis and the (inner) dermis (Fig. 1). The epidermis is an epithelial tissue made mainly by cohesive cells called keratinocytes. The differentiation of these cells generates four classical layers in the epidermis: stratum basale, stratum spinosum, stratum granulosum and stratum corneum.

The sublining dermis is a connective tissue rich in collagen fibers. Invaginations of the epidermis form hair follicles in the dermis, with their attached sebaceous glands, containing sebocytes rich in lipids. Smooth muscle cells from the hair erector muscle attach the hair follicle to the epidermis.

The greasiness that is noted in the processing of sheep skins stems from the distinct distribution of fat specific to sheep because of the abundance of secondary follicles in fine wooled sheep, to which are attached sebaceous glands [23]. The lipids produced and secreted by these glands (notably lanolin) generate a lipid layer below the epidermis separate from the usual subcutaneous fat. The greater the number of follicles, the more lipids that will be produced. It is this additional lipid layer that contributes to the 'delaminating' effect observed in sheep parchment [24–26], where the skin can be effectively split into two thinner layers.

Materials and methods

Parchment preparation

The skins from four lambs (*Ovis aries*) were taken from the Ovine Center of the University of Namur (natural deaths); the animals were cared for according to procedures conforming to the European requirements on farm animals (EC directive 86/609). The skins—two stillborn animals and two from animals less than 2 weeks old (approximately a rectangular shape and size of 16 cm by 20 cm or lower) were manufactured into parchment. Recipes for parchment production exist from the 8thC [14], the first well documented example being John Beale in "*The Art of making Parchment, Vellum, Glue* etc." read to the Royal Society in 1664 [27]. Here we used the later description of Diderot & D'Alembert [16].

Preparation

Fresh skins were washed in water then left in a light lime solution (3 g/l) for 5 days.

Dehairing

Two dehairing processes were used (each treatment was applied on one born dead animal and one animal less than 2 weeks old), both at ambient temperature, either:

1. The flesh side of a skin was uniformly covered with a milky lime solution and left for 1 week folded in two, flesh side together (pancake method).
2. The full skin was put into a lime bath and left for 1 week (bath method).

Skins were dehaired using successively stronger lime $Ca(OH)_2$ solutions (5 g $Ca(OH)_2$/l, 15 g/l and 30 g/l), equivalent to 'plein mort', 'plein gris' and 'plein vif' for 1 week each, with a wash in clean water for 1 day in between each different solution.

Scraping

The hair was removed by rubbing the skin with hands and finished with a semi lunar knife. The skins were then put on a framed, scraped, chalked and pounced, to produce the final parchment.

Box 1

Aussi-tôt que les peaux ont été pelées, on les lave à la rivière afin de les nettoyer, on les laisse ensuite égoutter quelque temps; après quoi on les met dans un

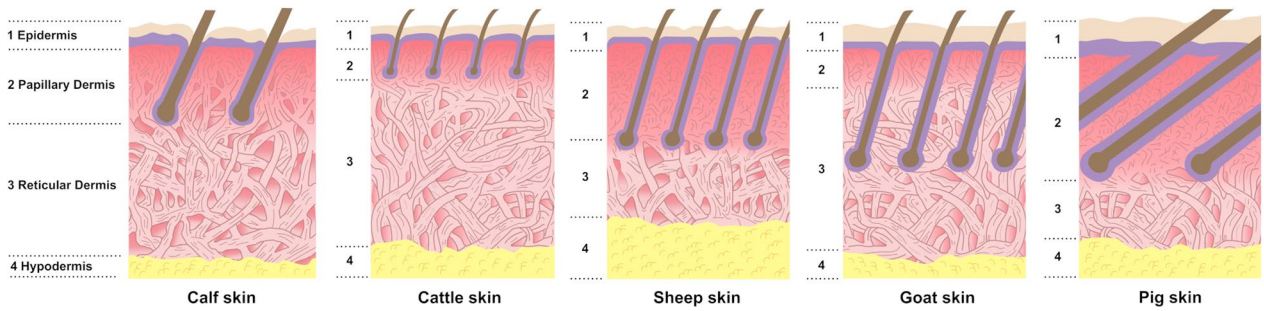

Fig. 1 Comparative skin histologies: Sean Doherty Wikimedia Commons

mort-plein, c'est-à-dire dans un plein qui a servi et dont la chaux *a presque perdu toute sa force*;

on les laisse dans ce *mort-plein environ* vingt-quatre heures;

d'où on les retire ensuite pour les mettre égoutter sur le plein….

Deux jours après que les peaux sont sorties du mort-plein, on les plonge dans un autre plein dont la chaux *est moins usée*, on les y laisse environ deux ou trois jours, après lesquels on les retire pour les mettre en retraite égoutter comme auparavant….

s'il en est besoin, on les replonge ensuite dans le plein,

on réitère cette opération pendant six semaines ou deux mois seulement, pendant les chaleurs de l'été; mais en hiver il faut les faire passer successivement de plein en plein au-moins pendant trois mois.

Diderot & D'Alembert (1765).

Once the skins are peeled, they are washed in the river, then allowed to drain for some time; next they are put into 'dead-lime' that is to say, lime which has lost almost all its strength.

Skins are left in baths of dead-lime for about 24 h;

They are removed and allowed to fully drain again.

Two days after the skins are removed from the dead-lime, they are immersed in another bath with less aged lime and left there for about 2 or 3 days with agitation, after which they are removed put to drain….

If necessary, they are then plunged back in the lime bath again, this operation is repeated for between 6 weeks to 2 months during the heat of summer, but in winter in the open it is necessary for the skins to soak for at-least 3 months.

loose translation, Matthew Collins.

Two subsamples of skins were taken at each step of the manufacturing processes described by Diderot and D'Alembert [16] and placed immediately in formaldehyde for subsequent histological analyses: one for the paraffin embedding and the other one for the frozen section to enable alternative histochemical staining to be used. Samples of approximately one square centimeter were taken in periphery of the skin, after specific steps of the manufacturing process: fresh skin (sample #1), skin after hair removal (sample #2), skin after the 3rd lime bath (sample #3), parchment (sample #4).

Method: Histological procedures:

The sheepskin and parchment samples were fixed in acetified formalin (formol 4%, acetic acid 1%). Six micron thick sections of the paraffin and of the frozen blocks were placed on SuperFrost + glass slides.

Sections from the paraffin blocks were dewaxed, rehydrated in graded alcohols and stained using the five following methods, (the two first being generalized topographic stains, while the three others are specific for specific macromolecules):

(1) *Hemalun, Erythrosin and Saffron* (HES) stains nucleic acid blue/purple, cytoplasmic proteins red and collagen yellow-orange.
(2) *Green trichrome* stains nucleic acid in blue/purple, cytoplasmic proteins red and collagen green;
(3) *Orcein and hematoxylin* stains elastin fibers red brown and nuclei in blue/purple.
(4) *Picro-Sirius Red and hematoxylin* stains collagen I fibers and nuclei in blue/purple.
(5) *Wilder and green light* stains reticulin fibers, mainly collagen III fiber black with a green background.

Sections from the frozen blocks were stained with *Oil Red O (ORO) and hematoxylin* which stains lipids red and nuclei blue.

Results and discussion

From a histological point of view, samples taken from the four skins shared similar changes during parchment production, there were no significant differences between stillborn and two-weeks old animals. Nor did the two different dehairing processes (i.e. pancake method versus bath method) result in any histological differences in the appearance of tissues (Fig. 2).

Pre-treated skins

Histological sections of the pre-treated skin (samples #1; Fig. 3) exhibited typical histological features, expected from fresh skin. The density of the hairs is highest in the grain layer, where they occupy at least half the total thickness of the skin. We did not observe any histological structure related to the subcutaneous tissue. This may be due to us sampling at the periphery of the skin (given the unevenly distributed layer thickness over the body), the (young) age of the animal and/or the way the skin was removed.

Orcein staining shows that the elastin fibers are preferentially located in the deep dermis, close to the (missing) subcutaneous layer and are more abundant around hair follicles. Picrosirius stain highlights the ubiquitous presence of collagen everywhere in the dermis in abundant bundles. The Wilder stain shows presence of reticulin fibers underlining the basement membranes, and perhaps some sparse fibers in the dermis. The specific staining of ORO reveals the presence of fats in the sebocytes of the sebaceous glands confirming previous observations by Haines [12]. Fats are present discreetly in the stratum granulosum and rarely in the stratum corneum.

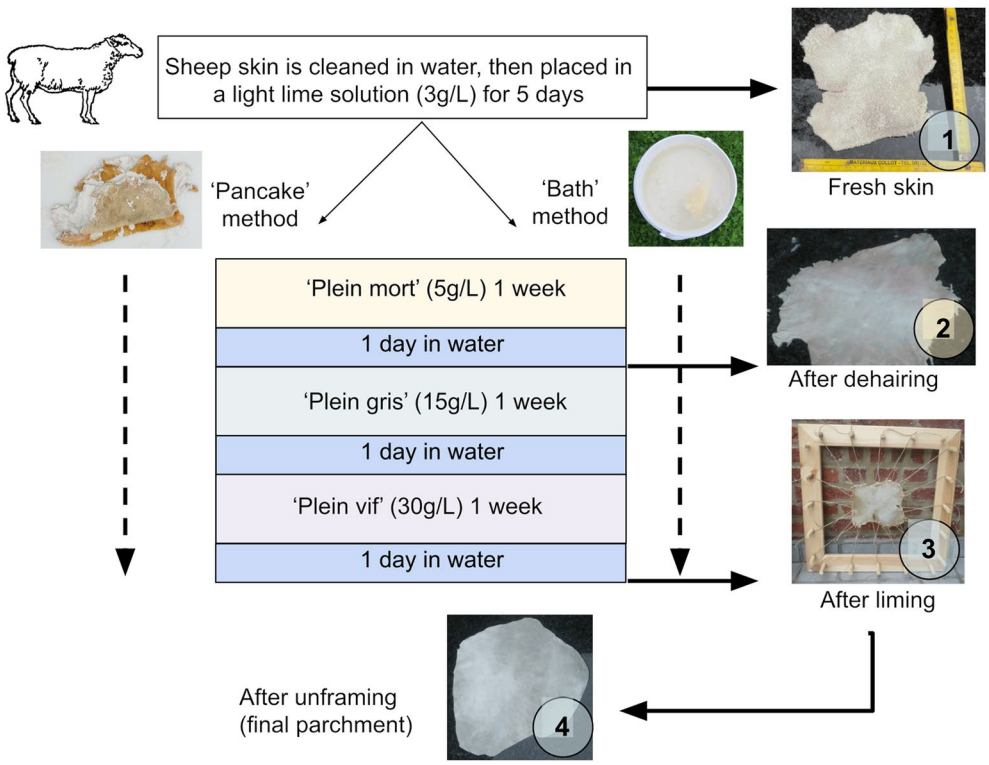

Fig. 2 Schematic of parchment production

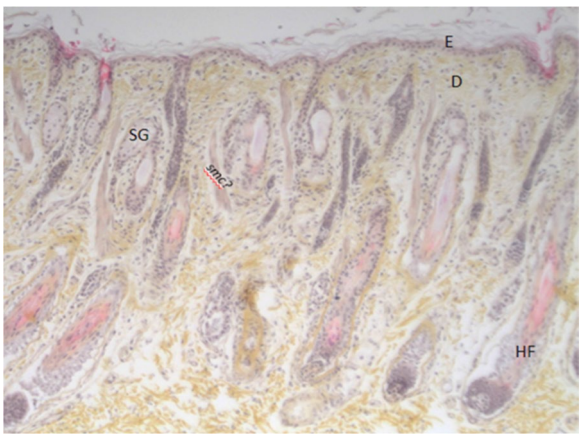

Fig. 3 Section of sample from fresh skin (Fig. 1) stained with HES (×100) (E, epidermis; D, dermis; SG, sebaceous Gland; HF = Hair Follicle; smc? = smooth muscle cells suspected)

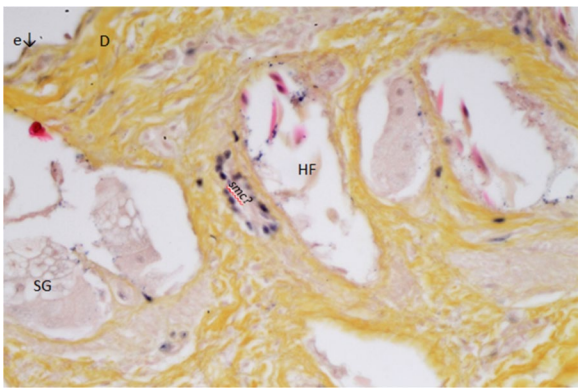

Fig. 4 Skin after hair removal, HES (×400) (e↓, rest of the epidermis; D, Dermis; SG, Sebaceous Gland; HF, Hair Follicle; smc, smooth muscle cells suspected)

Dehaired skins

The second samples were taken from the skin after light liming and hair removal (Fig. 4). It is known that after soaking in lime ($Ca(OH)_2$), the dermal fiber network in the skin expands [28]. We observed the loss of hairs, whose roots only persist here and there in the stratum basale of the epidermis (Fig. 4). All the stained components observed in the pre-treated skins were all still present after dehairing.

Prolonged liming

After the liming processes (three baths of increasing lime concentration; Fig. 5), collagen fibers seem now to have

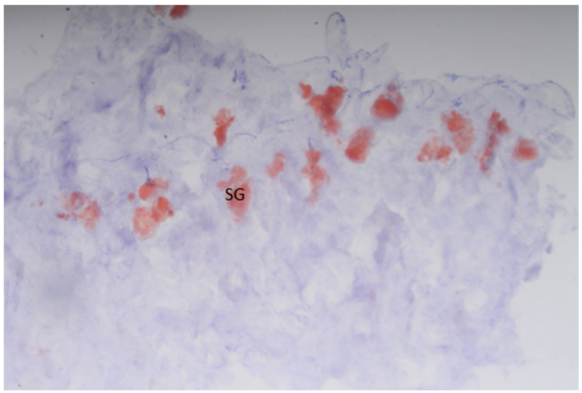

Fig. 5 ORO staining of skin after liming (×100) (SG, Sebaceous Gland)

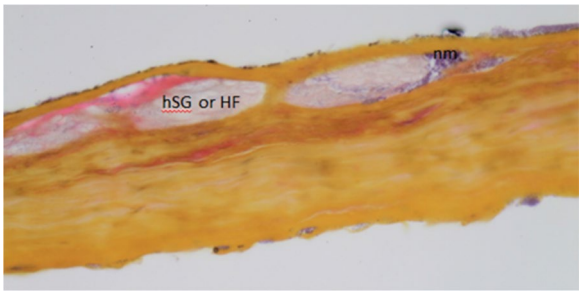

Fig. 6 HES staining of parchment taken out the frame (×100) (hSG or HF, horizontal Sebaceous Gland or Hair Follicle; nm, nuclear material

lost the characteristic fibrillar structure and appear to form a homogenous mass. Maxwell et al. [29] observed that "*after salting there is an increase in the distance between the collagen molecules in the hydrated state, and even more expansion after liming, which is carried through the hierarchical levels up to fibril packing; deliming did not appear to reverse these increases, indicating that the effects of salting and liming are permanent*". Elastin and reticulin fibers seem to be present only in traces. We observed that, despite remaining for three weeks in the lime solution, fat cells were still present, albeit most likely in a lower concentration (Fig. 5). The ability to remove fats from the skin is known to be related to duration in the lime bath (alkali causing saponification) and to the freshness (alkalinity) of the solution [30]; this aspect is worthy of further investigation.

Final parchment

The fourth samples were taken from the fully processed skin, i.e. the final parchment. During this final manufacturing step, the skin was put on a frame, scraped, bleached, cleaned and polished under tension (Fig. 6).

In the parchment samples, the bulk of the material took the form of a two dimensionally structured layer of uniformly oriented fibrous material, as observed here in cross-section (Fig. 6). This is consistent with our previous observations of optical birefringence patterns induced near the edges of parchment when stretched on a wooden frame [19]. The rest of the glands and hair follicles tend to reorient horizontally, i.e. parallel to the surface, in the direction of strain. Somewhat surprisingly—despite the fact that skins were taken from young or born dead animals and the final parchment was very thin < 0.1 mm—the production process did not remove all the fat cells (i.e. saponify all of the lipid). Oil red O staining revealed residual lipid deposits on the hair side of the skin.

The major histological changes observed at the different stages of skin transformation are revealed by the result of the six methods applied on samples and summarized in Table 1.

Persistence of lipids

The problem of residual grease bedevilled parchment makers in the past. Saxl [14] writes that "*Sheep parchments have often a high grease content in the grain layer*". "Magister Bernardus" (Bodleian Library Oxford. Canonici Misc. 128) recipe for parchment production deals with the subject of the removal of grease with warm dusting powders of chalk or other calcium salts or of wood ashes which melted 'the grease and acted like a "dubbin"; penetration was brought about by compressing the parchment.

Saxl's analysis of parchment recipes revealed that *"degreasing was extensively practised in Italy in the fourteenth and fifteenth centuries and in France"*, and the practice was still being used by Messrs. Russell of Hitchin using a mixture of wood ash and soda added to the stretched (sheep) parchment on the frame which was then heated in a warm stove.

From an optimal tissue preservation standpoint, it is assumed that high levels of lipid dripping from heated parchment initiates combustion of the membranes. Ghioni et al. [31] report variable, but in some cases, substantial levels of lipid in historical and modern parchment. We conducted species identification [15] on seven of the samples analysed by Ghioni et al. [31], and in support of Saxl confirmed that the two membranes with high levels of lipid were from sheep (Fig. 7).

The production of fine wool has resulted in the selection of breeds with larger numbers of secondary follicles, resulting in a greater density of sebaceous glands, and thus sebocytes in sheep when contrasted with goat- and calf-skin. Two side effects of the greater number of secondary follicles in sheep are the higher levels of residual lipid and the disruption in the dermal collagen network,

Table 1 The major histological changes observed at the different stages of skin transformation

Histological staining methods	HES	Green Trichrome	Orcein	Picro-Sirius red	Wilder	Oil Red O
Histological specificity			Elastin	Type I Collagen	Type III Collagen	Lipids
Sample #1: fresh skin	Classical topography	Classical topography	More Present at the limit dermis-hypodermis and just around hair follicles	Present everywhere Highest density just around hair follicles	Anchored in the basement membranes	At the apex of epidermis and in the sebaceous glands
Sample #2: skin after hair removal (1.Watery lime bath; 2. Milky lime bath)	Loss of epidermis, hair follicles without hairs Evidence for expanded network of collagen, Persistent nuclei	Like HES	Persistent in the described zones	Evidence of expanded network of collagen	Basement membranes less emphasized	Persistent in sebaceous glands
Sample 3: skin after 3 lime baths	Collagen gelled in a kind of pulp	Like HES	Present in traces	Collagen gelified in pulp	Present in traces	Persistent in sebaceous glands
Sample #4: parchment	Layers in gelled collagen Horizontalisation of the glands	Like HES	Present in parallel traces	Quite uniform	Present in parallel traces or artefact?	Persistent in sebaceous glands

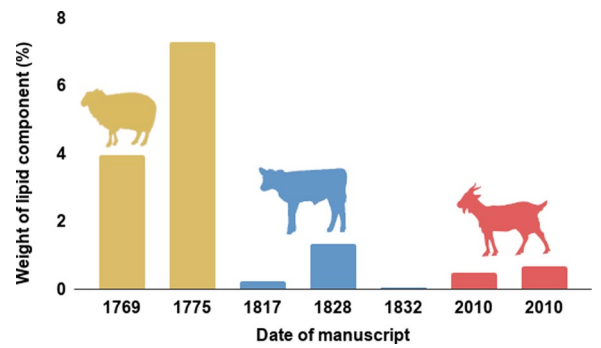

Fig. 7 Species identification of samples analysed for lipid content, data taken from historical manuscripts of known date as described in (Table 1 of [31]). Sheepskin parchment has the highest levels of lipid, consistent with the histological investigation reported here (see Fig. 5)

which means that there is a tendency for sheepskin to delaminate (that is for the parchment to split). Anecdotally, parchment maker Jessie Mayer (Pergamena, pers comm. 2015) concurs, and notes the difficulty of preparing parchment from wool sheep skins, due the excessive levels of lipid, which are not removed by the liming process.

Conclusions

Our histological study shows the modification of the biological material and more specifically the collagen fibrous material in the course of a parchment manufacturing experiment starting from skins of immature sheep. In particular, the modification and, ultimately, the disappearance of collagen type III fibers was observed (using the Wilder staining method) in addition to a progressive loss of elastin fibers. Another interesting observation is the persistence of fats, despite the decrease in quantity of the material stained with oil red O (so most likely lipids) as well as the genetic material observed with hemalaun staining.

Whilst we were unable to demonstrate any histological differences caused by the alternative deharing methods, the manufacture of parchment itself was shown to induce dramatic histological changes from the fresh skin. The transformation involved a progressive evolution from a classical full and complex three dimensional framework of biological material to a lamellar structure essentially composed of co-aligned collagen fibers. The most dramatic changes happen during the processes of drying and stretching on a frame. During this phase the thickness of the skin reduces and the collagen fibrils realign in the direction of the applied force.

With the reported recreation of historical parchment manufacture and tracking of skin transformation through histological analysis, we believe this study will help compensate for the lack of sources on microscopic changes in parchment during manufacture.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40494-020-00421-z.

Additional file 1. Table summarizing the details of histological analysis of sample "Still born lamb 1".

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Authors' contributions
MF: experimental design, manufacture of parchment and writing. DVV: histological processing and interpretation of histological results. MC and SF Analysis of ancillary data. Figure preparations. CC, MC, SF, YP and OD: writing and interpretation of histological results. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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