Ultrastructural Aspects of Skin after Cryoultramicrotomy

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Skin was studied by transmission electron microscopy after cryoultramicrotomy. Global and ultrastructural preservation of the tissue was obtained with satisfactory cohesion between dermis and epidermis. The main constitutive elements could be observed with good definition allowing easy recognition of all the organelles and therefore absolute identification of each cellular type encountered. The methodology provided a comparable quality to the conventional technique. Ice crystal artefacts were minimized but slight enlargement of intercellular spaces persisted. One of the main advantages of this procedure is that neither lipidic solvents nor embedding resins, are used, so that one can envisage cytochemical studies. *Key words: Skin; Epidermal cells; Keratinocyte differentiation; Dermal-epidermal junction; Dermal elements.*
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The theoretically superior retention of endogenous chemical components characterizes cryoultramicrotomy as a very powerful preparative method for Electron Microscopy (EM) of biological specimens (1–4), that can also make possible some interesting applications in cytochemistry (5–16) and X-ray analytic studies (1, 17, 18). However, the technique is not simple and it must be emphasized that skin is a very complex and heterogeneous tissue. This probably explains why the ultrastructural aspect of skin after cryoultramicrotomy has yet to be reported in the literature. Hence we used the method described by Morel (9) as adapted and developed from Tokuyasu (4) to verify if the general outline of skin and the main ultrastructural features were present and well preserved, when compared to what we observed from standard EM of skin prepared by conventional procedures. The present work will be used as a basis for further dermatological research and skin pathological investigations.

MATERIAL AND METHODS

Six mm skin biopsies from rat, mini pig, and human (mastectomy), were treated as previously described (9). Fixation as performed for 1 h in 2.5% glutaraldehyde in 0.09 M phosphate buffer pH 7.4 at +4°C. At the beginning of the fixation, biopsies were cut into small pieces of 1 mm^3. After washing for 1 h in phosphate buffer, tissues were post fixed for 1 h in 1% buffered osmium tetroxide at +4°C and washed again for 1 h in phosphate buffer, then rinsed with distilled water and stained (or not) for 20 min with 2% aqueous uranyl acetate at +4°C. After further distilled water rinsing, samples were incubated for 1 h at +4°C in a 0.4 M saccharose solution as cryoprotectant. Specimens were put in a saturated saccharose droplet and frozen in a cold gradient of fuming nitrogen to −4°C before being totally and rapidly immersed into the liquid nitrogen.

The ultrathin sections were obtained according to the method described by Tokuyasu (4) with an Ultracut (Reichert Jung) fitted with the cryokit FC4. Temperatures of sectioning were −90°C for the knife and −130°C for the specimen; the sections were collected with a droplet of saturated saccharose and mounted on formvar-coated nickel grids. The grids were then washed by floating, the section side on the surface of distilled water before staining either with 2% aqueous phosphotungstic acid (PTA) for negative staining, or with uranyl acetate and lead citrate for positive staining. Unstained sections were also observed after having been dried. Finally ultrathin sections were observed with a Jeol 1200 EX Electron Microscope operating at 80 kV.

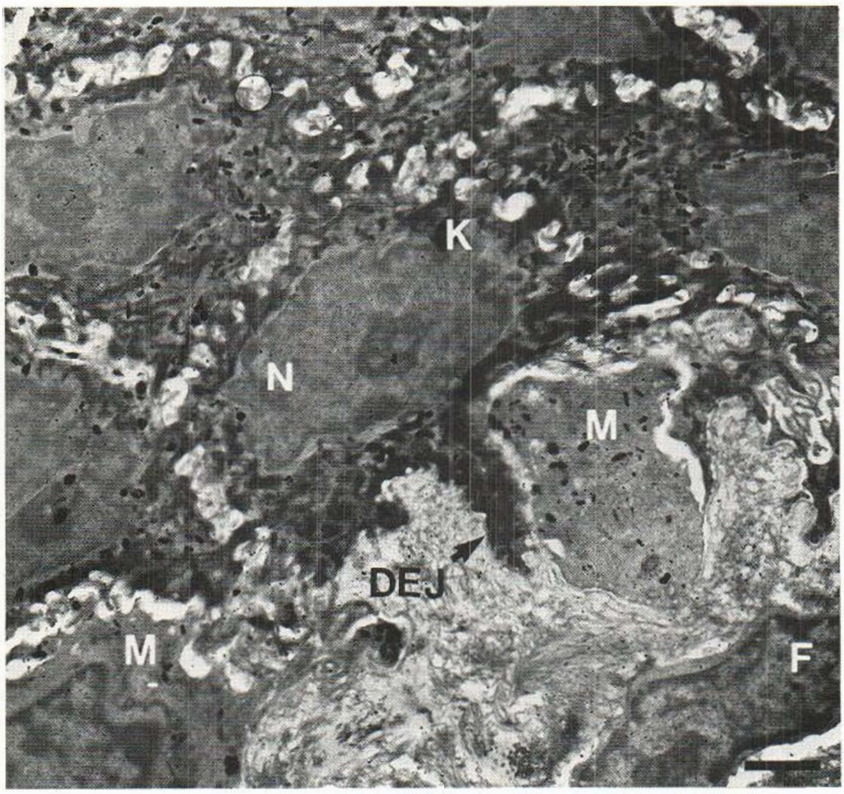


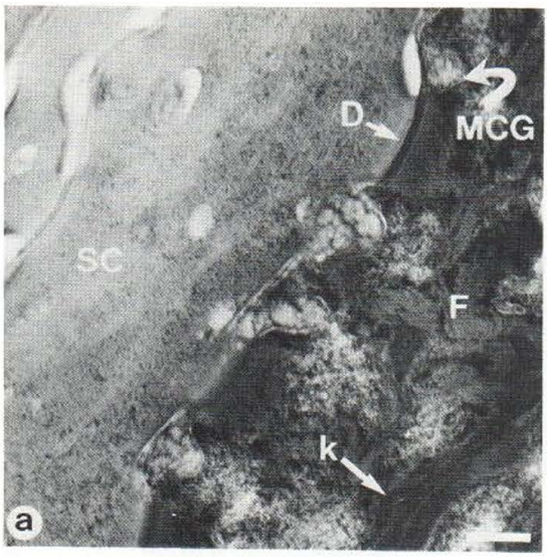
Fig. 1. General aspect of skin after cryoultramicrotomy around the dermal-epidermal junction. The left upper part is occupied by the sub and basal epidermis with keratinocytes and melanocytes. The right lower part shows the papillary dermis with collagen fibrils and one fibroblast. Bar: 2 μm. *K*, keratinocyte; *M*, melanocyte; *F*, fibroblast; *N*, nucleus; *DEJ*, dermal epidermal junction; *c*, collagen.

RESULTS

Since better results were obtained with negative staining using neutral PTA without the uranyl acetate prefreezing step, we present here only the micrographs obtained with this staining procedure.

The first observation was the satisfactory cohesion of the tissue between dermis and epidermis at low magnification (Fig. 1, Fig. 3*a, b*). However, in the epidermis we noticed for the basal and the first spinous layers, a slight enlargement of the intercellular spaces (Fig. 1) but without any disruption of the desmosomes. The distance between juxtaposed plasma membranes was normal in upper layers (Fig. 2*a, d*). The stratum corneum was well preserved and up to 10 keratin layers could be observed.

Fig. 2. Several important ultrastructural aspects of skin after cryoultramicrotomy. (*a, d*). Two aspects of junction between stratum granulosum (right) and stratum corneum (left). Bars: 100 nm (*a*), 200 nm (*d*). In *d* black arrow shows ice crystal. (*b*) Cytoplasmic area of a basal keratinocyte. Bar: 500 m. (*c, e*) Nuclear environment of keratinocytes. Bars: 100 nm. *SC*, stratum corneum; *PM*, plasma membrane; *MCG*, membrane coating granule; *D*, desmosome; *F*, keratin filaments; *k*, keratohyalin granule; *M*, melanosome; *m*, mitochondria; *E*, nuclea envelope; *N*, nucleus; *ch*, chromatin; *NP*, nuclear pore; *ER*, endoplasmic reticulum; *G*, golgi apparatus; *v*, vesicule; *bv*, budding vesicle; *tv*, transition vesicle.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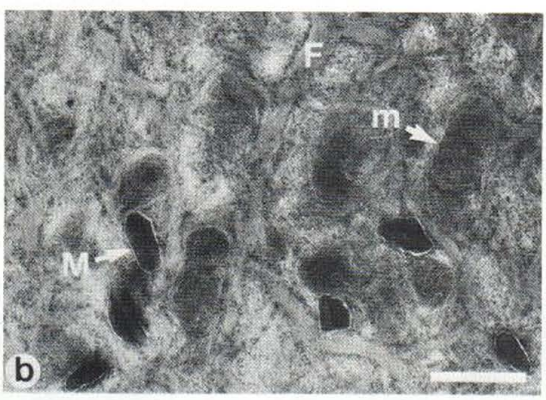
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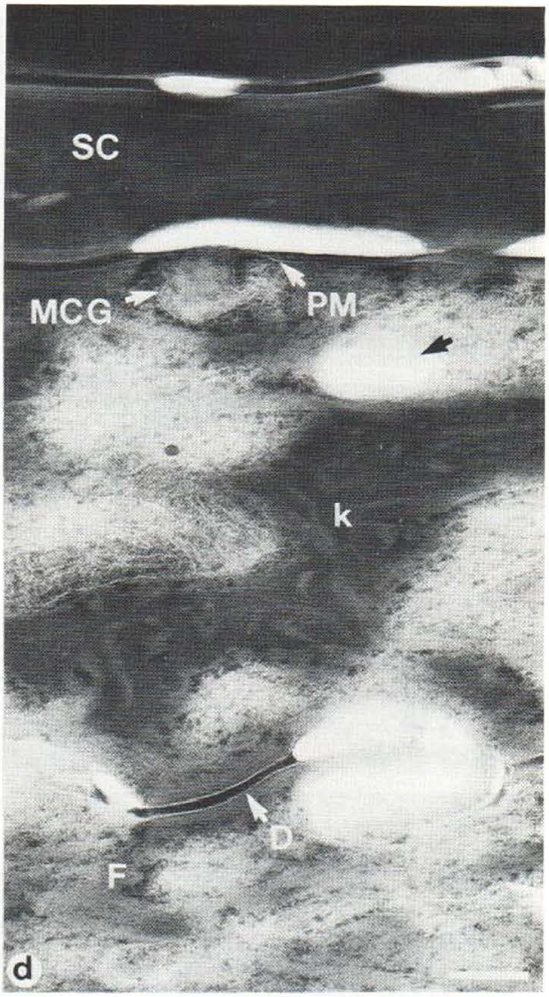
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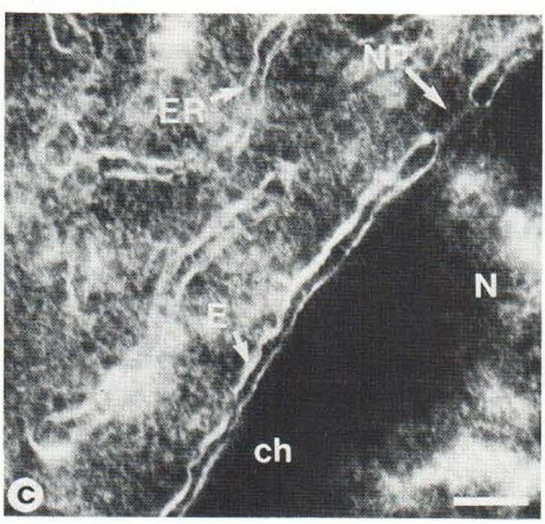
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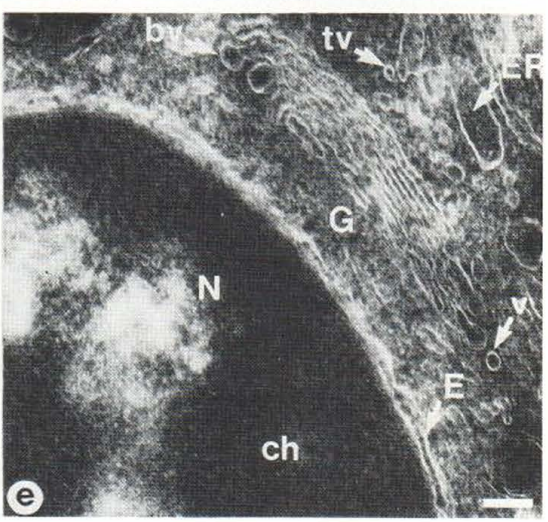
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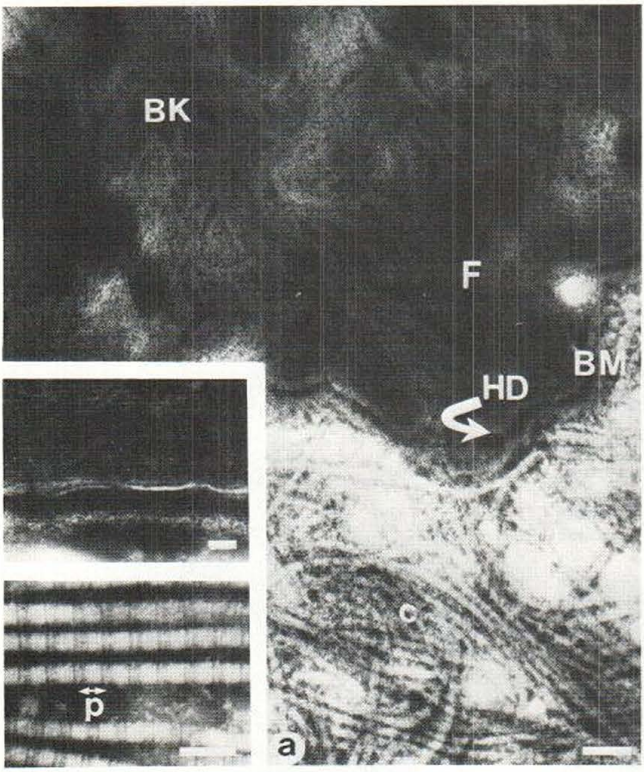
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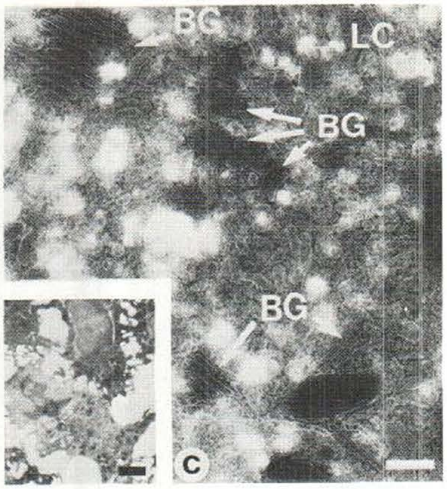
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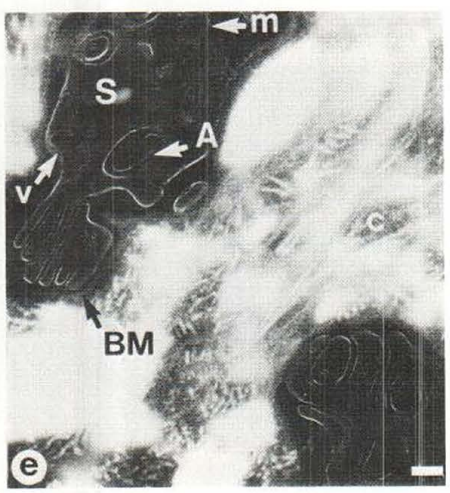
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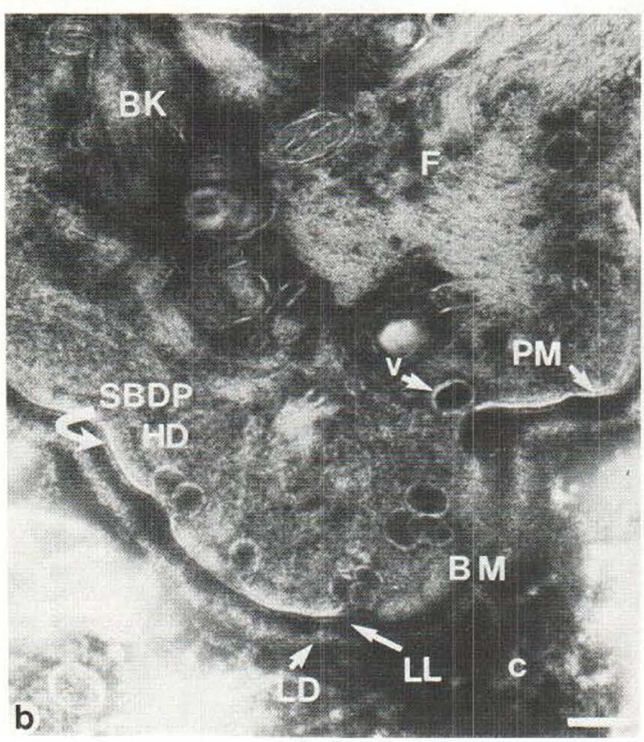
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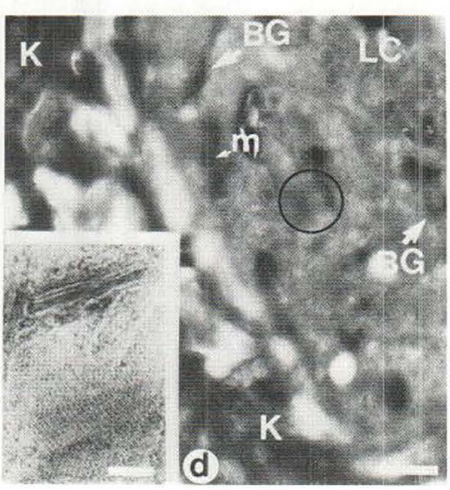
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In the dermis, the major observation was the transversal and longitudinal sectioning of collagen fibers in strong association with fibroblasts. Among the cellular population of the dermis we could observe endothelial cells in blood vessels, mastocytes, and nerve endings (Fig. 3*e*). But, due to the low and irregular cellularity of the dermis, empty spaces were frequent, altering the general architecture of the dermis (Fig. 1, Fig. 3*b*, *e*).

The common cellular organelles and ultrastructural details could be easily identified. The nucleus with the nucleolus, the chromatin, the nuclear envelope with its two leaflets were observed as well as some details of the nuclear pore structure, the endomembrane system with endoplasmic reticulum, golgi apparatus, vesicles, plasma membrane, mitochondria with well defined crest and matrix, and cytoskeleton with filaments isolated or in bundles (Fig. 2*b*, *c*, *e*, Fig. 3*a*, *b*).

In the basal and suprabasal epidermal compartments visibly unaltered melanosomes were encountered (Fig. 2*b*). All criteria of keratinocyte differentiation were detected. Desmosomes and hemidesmosomes were seen with all their ultrastructural elements (Fig. 2*a*, *d*, Fig. 3*a*). The presence of intra and extra cellular membrane coating granules (MCG) was noticed with their characteristic lamellar structure surrounded with a membrane (Fig. 2*a*, *d*). Keratohyalin granules often displayed a heterogenous aspect with clearer striation looking like keratin filament bundles and appearing in some places in continuity with external keratin filament bundles (Fig. 2*a*, *d*). The stratum corneum showed the same characteristics as with conventional technique with loss of dense plaque for desmosomes at the junction between granular and cornified layers and disappearance of all organelles except keratin filaments that could be distinguished (Fig. 2*a*, *d*).

Other epidermal cells than keratinocytes were easy to recognize due to their clearer aspect (Fig. 1, Fig. 3*c*). Intercellular spaces were enlarged. In the melanocyte, the external membrane of the pigment granule was very well preserved and the various stages of the melanosome maturation (melanization) could be observed. In partially melanized melanosome, the internal lamellar structure was well delineated. In fully melanized melanosome, the electron density was however not homogenous. The typical cytoplasmic organelles of Langerhans' cells, the Birbeck granules, were also very well preserved (Fig. 3*c*, *d*). Their internal striated structure could readily be distinguished. We oserved also a lattice arrangement (Fig. 3*c*, *d*) similar to the structure previously considered (19) as tangential sections of Birbeck granules. No Merkel cells were observed.

At the dermal-epidermal junction, the typical basement membrane structure was observed. This included laminae densa and lucida, in which basal keratinocytes were seen attached by hemidesmosomes, that exhibited the classical ultrastructural pattern. However, possibly due to the sectioning angle, the pyramids have yet to be clearly identified (Fig. 3*a*, *b*). In the dermis, collagen fibers displayed their classical striation (Fig. 3*a*) with a mean value of 57 nm for the major periodicity. The classical architecture of the vessels was preserved with unaltered lumen, endothelial cells, basal laminae and pericyte. This also applies

Fig. 3. (*a*, *b*) Dermal-epidermal junction zones. Bars: 200 nm (*a*), 100 nm (*b*). (*a*, *upper inset*) Details of the basal lamina with a hemidesmosome. Bar: 50 nm. (*a*, *lower inset*) Collagen periodicity. Bar: 50 nm. (*c*, *d*) Characteristic organelles of the Langerhans cell: the Birbeck granules. Bars: 200 nm (*c*), 500 nm (*d*). (*c*, *inset*) Low magnification of the Langerhan's cell. Bar: 3 μm. (*d*, *inset*) Enlargement of the circled area, reticular aspect of the Birbeck granule. Bar: 100 nm. (*e*) Dermis with nerve endings. Bar: 200 nm. *BK*, basal keratinocyte; *F*, keratin filaments; *HD*, hemidesmosome; *SBDP*, subbasal dense plaque; *LL*, lamina lucida; *LD*, lamina densa; *v*, vesicle; *PM*, plasma membrane; *c*, collagen fibril; *p*, periodicity; *BG*, Birbeck granule; *LC*, Langerhan's cell; *m*, mitochondria; *SC*, Schwann cell; *BM*, basement membrane; *A*, axone.

for the nerve endings (Fig. 3*e*). Mastocytes were also easily recognized due to their specific granulation.

DISCUSSION

Ultrathin cryosections of complete skin with dermis and epidermis closely linked by the dermal-epidermal junction, can be obtained relatively simply using the technique described here. For all the specimens observed, negative staining with neutral PTA provided a delineation of the structures superior to uranyl acetate with or without lead citrate. In addition, it is known that PTA is conducive to good preservation of biological samples (4). This technique provides a satisfactory conservation of the global cutaneous architecture and particularly of the weak attachment of the dermal epidermal junction. Practically no empty spaces due to ice crystal formation were encountered, suggesting that the freezing process we used appears to be well adapted to the skin tissue. Two main artefacts were noticed. The intercellular space enlargement for the first layers of the epidermis mainly appears when intercellular connexions are very weak as between melanocytes or Langerhans' cells and keratinocytes, as well as the lower cohesion of the dermis. This latter artefact is obviously inherent in the very heterogenous dermal structure devoid of any strong association between the constitutive elements. Dermal cohesion is attained with conventional technique using resins with progressive impregnation and embedding. Here, better cohesion could possibly be obtained by using methyl cellulose (16).

The discriminative power of the EM observations with this preparative technique is very important. Such adequate tissue and ultrastructural preservation, permits a sure identification of each cellular type in this very heterogenous tissue, as well as the characteristics of keratinocyte differentiation in the epidermis, due to the facilitated recognition of the characteristic organelles.

No Merkel cells were observed but their characteristics (basal localization and Merkel granules) should allow their identification. Furthermore, this procedure displays a particular feature at the keratohyalin level that was not observed using the classical method (Fig. 2*a, d*).

Concerning comparative measurements done so far, no major structural differences have been noticed between conventional and ultracryo samples. That is in agreement with results previously reported (4) for other tissues.

In most dermatological diseases, ultrastructural abnormalities have been described (20, 21). These could be reinvestigated using this technique. The same is true for the lipid contribution (intra and extracellular MCG) in the keratinization process (22), dehydration caused by the lipid solvent being avoided with this technique. If needed, ultrastructural preservation could be improved under experimental conditions, mainly at the freezing level, by using a vitrification procedure (23) for example.

The resolution obtained is sufficient to allow the study of very small subcellular domains and so the technique could be used for the cytochemistry of skin tissue. The definition of cellular organelles is much better than that obtained by conventional processing of 5–15 μm, cryostat sections previously immunolabelled and then treated up to epon embedding (24, 25).

The choice of sucrose 0.4 M as cryoprotectant is determined by its iso-osmolarity and its neutral chemical effect on numerous antigens (6, 9). The use of glutaraldehyde and osmium tetroxide as fixative increases the ultrastructural preservation of tissue after freezing and thawing. It is known that fixation can reduce antigenicity, or even in some cases abolish it (8, 16, 26). This technique has nevertheless been already used in order to detect many cellular antigens (6, 8, 9, 10, 11). Another appreciable advantage of cryoultramicrotomy is that it

enables immunolabelling on ultrathin sections devoid of resin thus facilitating the antigen accessibility. So this method should be highly relevant for labellings, when once fixation can be adapted to preserve the activity of the labelled entity (16).

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