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# DISSOCIATION OF HUMAN ADULT EPIDERMAL CELLS BY DISULFIDE-REDUCING AGENTS AND SUBSEQUENT TRYPSINIZATION[1]

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*Abstract.* Primary cultures of epidermal cells from human adult skin were dissociated by dithiothreitol (DTT) with subsequent trypsinization. After treatment with disulfide reducing agents, the desmosomes were either destroyed or showed a homogeneous ultrastructure. Trypsin separated the homogeneous desmosomes, whereas cell membranes, organelles, and nuclei remained undamaged. Trypsin did not influence normal desmosomes. A suspension of human epidermal cells was recultivated in new flasks. Two types of cells grew in the cultures—polygonal and spindle-shaped. The polygonal cells were maintained for 30 to 60 days and grew slowly. The spindle-shaped cells were leucine-amino-peptidase negative, grew actively, and were subcultured.

*Key words:* Epidermal cell culture; Dithiothreitol; Desmosome; Culture technique; Epidermal dedifferentiation

After skin has been cultivated for 2 or 3 days, epidermal cells always grow in membranous sheets around the explants, while fibroblasts grow out after 1 to 3 weeks. Trypsin dissociates the fibroblasts but not the epidermal cells (11). Previous authors have separated whole sheets of epidermis from trypsinized grafts prior to cultivation. Dissociated cells were obtained from the separated epidermal layer by mechanical action such as shaking, flushing, or scratching (2, 15, 22, 28).

The present study introduces the disulfide-reducing agent, dithiothreitol (DTT), for splitting the disulfide groups of desmosomes. The agent was used successfully, together with trypsin, for dissociating cultivated epidermal cells.

[1] Parts of this study were presented at the Fourth Annual Meeting of the European Society of Dermatological Research, Amsterdam, April 23–24, 1974.

## MATERIALS AND METHODS

*Human epidermal cells.* Skin of normal human adults was obtained from the Department of Plastic Surgery, Rigshospital, and cut into small explants sized 1–2 $mm^2$. The skin was cultivated in Falcon flasks (25 $cm^2$), each containing 6 to 9 small explants. Epidermal cells from a 4-day-old outgrowth were used for the experiments (Fig 1). The culture medium was minimum essential medium (Eagle) with Earle's salts, 100 ml; fetal calf serum, 20 ml; l-glutamine (200 mM), 2 ml; penicillin–streptomycin (5 000 i.u. per ml each), 2 ml; and was buffered at pH 7.4 with 7.5% w/v sodium bicarbonate. Five percent $CO_2$ in air was supplied to the flasks. The medium was renewed every 4 days. The cultures were incubated at 37°C.

*Trypsin solution.* Trypure® (NOVO, Copenhagen) crystalline trypsin, 4 500 000 NF units per g, was dissolved in Hanks' balanced salt solution (BSS) at a concentration of 1 mg per ml. The pH was adjusted to 7.8 with 6 N NaOH.

*DTT* was dissolved in Hanks' balanced salt solution (BSS) at concentrations of 1.5, 3.0, 6.0, 12.0 and 24.0 mg/ml. The pH was adjusted to 7.8 with 6 N NaOH. The optimal concentration of DTT was 3 mg/ml.

*Trypan blue* was used for vital staining at a concentration of 0.2% dissolved in normal saline. After removal of the original explants, the epidermal outgrowths (Fig. 1) were washed twice with BSS. Thereafter, 5 ml of DTT solution was poured into the flasks. The disulfide-reducing agent was left in the cultures for 10 to 15 min at 37°C. The cells were observed at regular intervals with an inverted phase contrast microscope (Leitz Diavert). When the intercellular spaces of epidermal cells had widened, the DTT solution was sucked out. Finally, the trypsin solution was added and the flasks gently shaken for 1–3 min. Solution and cells were transferred to a centrifuge tube with fresh culture medium and centrifuged at 300 *g* for 10 min. The supernatant fluid was discarded and the cells in the sediment were resuspended in fresh medium and cultivated. Treatment with trypsin solution alone was tried at the same concentration and time. Cultures of human adult skin fibroblasts were also treated with DTT for 15 min at pH 7.8.

A 4-day-old epidermal outgrowth was studied with an inverted phase contrast microscope and an electron

microscope under the following conditions. (*a*) Before treatment, as a control. (*b*) After treatment with 0.1% trypsin at pH 7.8 for 5–15 min. (*c*) After treatment with DTT at pH 7.8 for 15 min. (*d*) After treatment with both DTT for 15 min and 0.1% trypsin for 1 to 3 min at pH 7.8. The cells were also studied after 2 and 4 months of cultivation.

For electron microscopic studies the materials were fixed in a 4% glutaraldehyde solution in Veronal acetate buffer, pH 7.2, with 7.5% sucrose. After osmification, the materials were washed, dehydrated in a series of alcohols of increasing concentration, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and studied with a Siemens electron microscope (Elmiskop 1A) at 80 kV with a double condensor system.

The enzymatic pattern of both keratinocytes and spindle-shaped cells was studied. Dermal fibroblasts contain leucine aminopeptidase, in contrast to keratinocytes (6, 23). The leucine aminopeptidase was stained on coverslip cultures according to the technique of Nachlas et al. (20).

## OBSERVATIONS

### *Phase microscopic observations*

At 10–15 min after pouring DTT-solution into the culture flask, the epidermal cells became rounded and the intercellular spaces between the cells widened, though a few intercellular bridges still held (Figs. 2, 3). When the disulfide-reducing agent was replaced with the trypsin solution, all intercellular bridges ruptured immediately and the cells became detached from the flask. The dissociation was complete within 1–3 min. When the trypsin solution alone was supplied for 5 min, the cells did not become rounded, and no widening of the intercellular spaces took place. In a comparative study, skin fibroblast cultures were treated with DTT at pH 7.8, but none of the cells appeared rounded or freed from the bottom of the flask. DTT solutions were tested at various concentrations for comparable periods. The epidermal cells became rounded at concentrations of 3 mg/ml or more. However, no evidence of cell damage was seen after 15 min of exposure at a concentration of 24 mg/ml. The cells continued to grow after returning to a fresh culture medium. The vital staining with Trypan blue gave a viability within the range 83–92%. Cells treated with DTT plus trypsin were recultivated. They started to settle on the bottom after 1 hr (Fig. 4). After 15 hr of cultivation, most of the cells were attached and were polygonal in shape. Most of the cells appeared solitary, some in small groups. Occasionally, the cells showed long cytoplasmic protrusions (Fig. 5). The polygonal cells grew slowly and disappeared after 30–60 days. The spindle-shaped cells appeared after 24 hr and grew more rapidly than epithelial cells (Fig. 6). Both cell types showed distinct nucleoli and nuclear membranes. The keratinocytes and spindle-shaped cells were both found to be leucine aminopeptidase negative.

### *Electron microscopical observations*

Before and after trypsin treatment, the desmosomes appeared quite normal (Figs. 7, 8). The intercellular matrix of desmosomes contained a distinct intermediate layer (M-line, 14). After DTT treatment, most of the desmosomes were separated, while some showed a homogeneous intercellular matrix without M-line (Fig. 9). After treatment with DTT plus trypsin, all desmosomes ruptured. No intercellular matrix was seen, while cell membranes and attachment plaques were left intact (Fig. 10). Numerous finger-like protrusions of the cytoplasm were seen on the surface of the cells (Fig. 11). No definite differences in cell organelles were found after DTT plus trypsin treatment. The cytoplasm of the polygonal cells was like that of mature keratinocytes, with both desmosomes and tonofilament bundles (Figs. 12, 13). The spindle-shaped cells contained nuclei with nucleoli, while the cytoplasm showed only a few cell organelles, i.e. mitochondria, granular endoplasmic reticulum, ribosomes, lysosomes, Golgi complex and pinocytotic vesicles. In all contact areas the cells showed attachment—plaque-like thickenings of the cell membrane without obvious connection to the intra-

*Fig. 1.* Epidermal outgrowth from the explant, on the 4th day of cultivation. ×180.

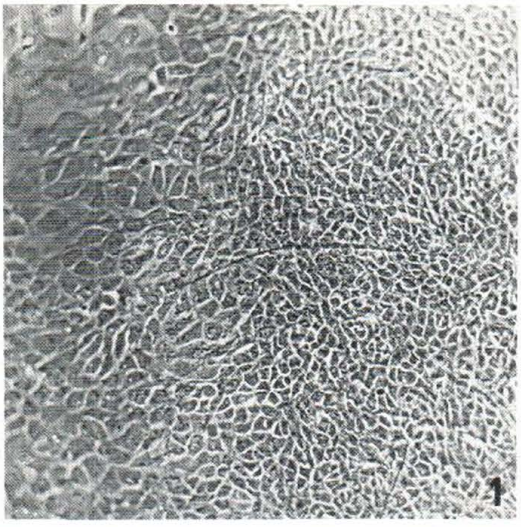
*Fig. 2.* Epidermal cells after 5 min of DTT treatment. The intercellular spaces appear widened. ×180.

*Fig. 3.* Epidermal cells at 15 min after DTT treatment. Numerous epidermal cells appear rounded, but none are freed from the bottom. ×180.

*Fig. 4.* Recultivated cell suspension, 1 hr after treatment. ×180.

*Fig. 5.* Recultivated cells after 24 hr. The cells show long, cytoplasmic protrusions. ×180.

*Fig. 6.* Recultivated cells after 4 months of cultivation. The cells are now more spindle-shaped. ×180.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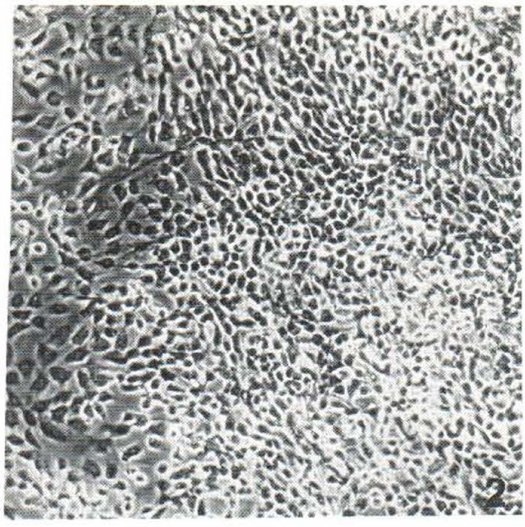
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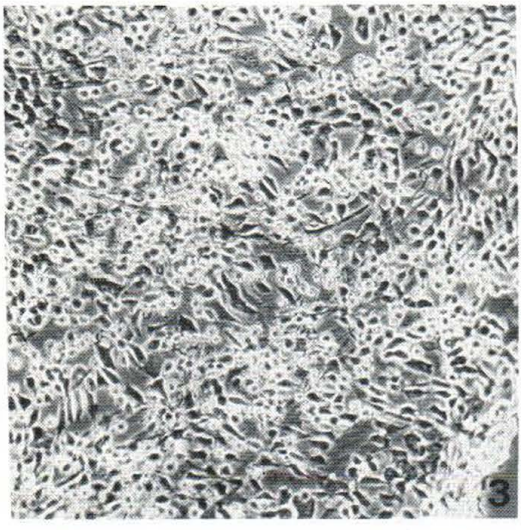
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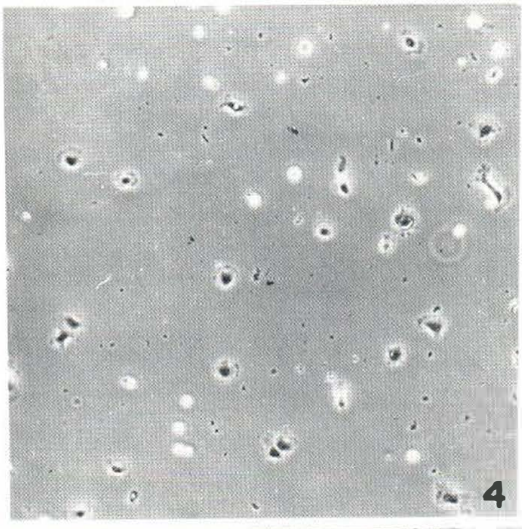
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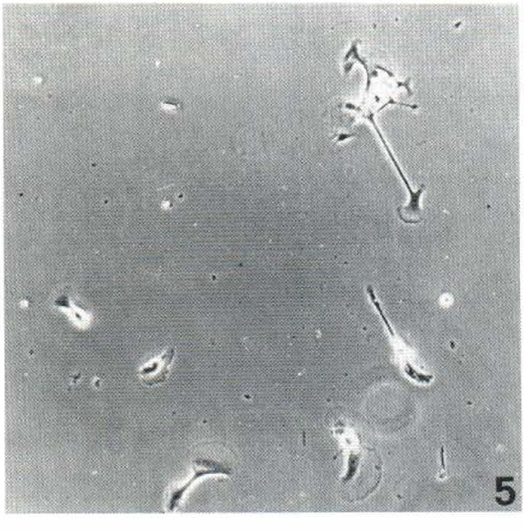
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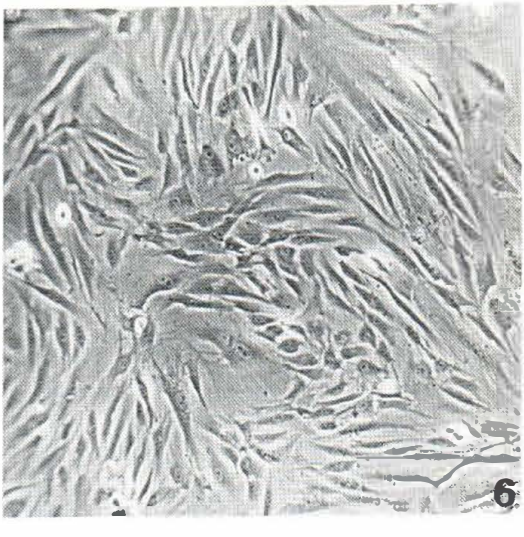
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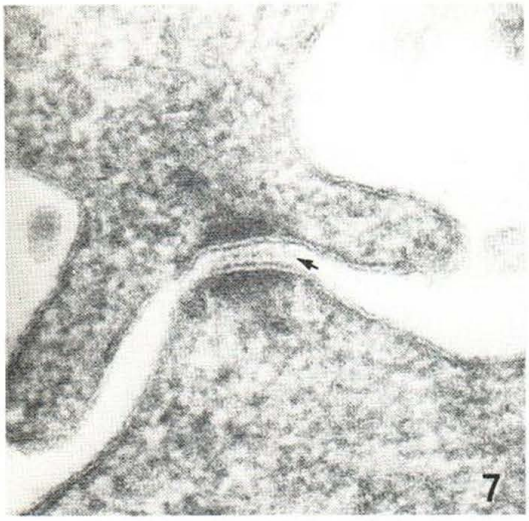
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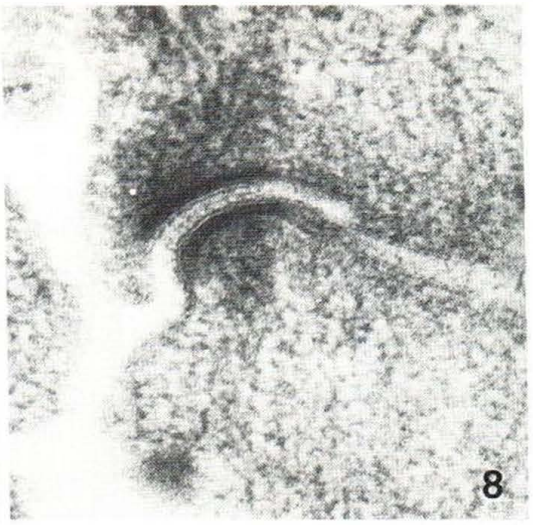
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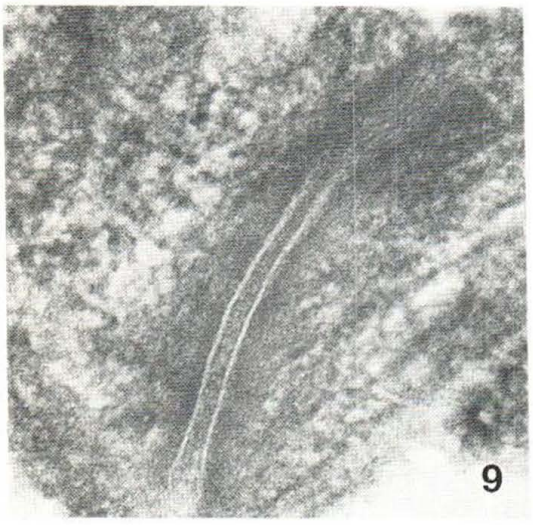
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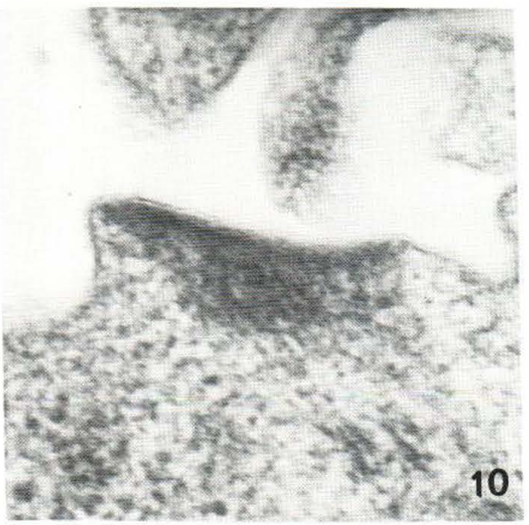
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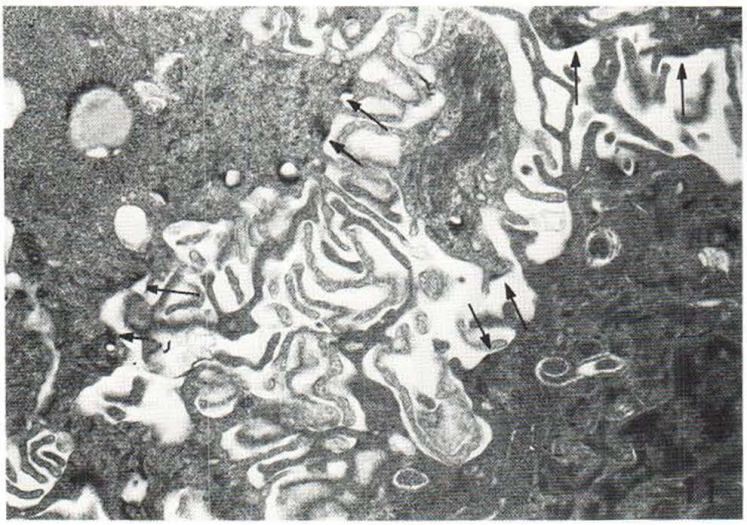


cytoplasmic microfilaments. Rough bundles of dense microfilaments were seen in the peripheral area of the cytoplasm, though no typical desmosomes were found (Figs. 12, 14). No collagen-like fibrils were found in the extracellular spaces of the spindle-shaped cells.

## DISCUSSION

Epidermal cells are firmly connected by desmosomes (1). Experimental acantholysis was hitherto produced in fresh skin specimens by disulfide-splitting agents, i.e. cysteine, reduced glutathione, sodium thioglycolate (pH 8.5) and borate buffer (pH 8.8) (1, 13, 14, 17, 19, 24, 26). However, several hours of incubation was required, and the cells did not seem to survive the treatment. The proteolytic enzymes trypsin and papain also produced acantholysis in fresh skin (1). The acantholysis was completed when the cells disintegrated due to long-term incubation. Other authors have previously found that the intercellular matrix of desmosomes is derived from the epidermal cell coat (17, 24) and contains disulfide groups and mucoprotein (13, 27). Sulfhydryl groups located on the cell surface seem to influence the ability of cells to adhere to other cells or to growth surfaces. The sulfhydryl binding reagent, *N*-ethylmaleimide, inhibited cell adhesion to a plastic flask, while DTT reversed this (8, 9). Dithiothreitol reduced disulfide groups to sulfhydryl groups and preserved them within a range of pH 7.0 to 8.0 (4).

Dithiothreitol is toxic for animals, the lethal dose for mice being 180 mg per kg body weight (10). For cultivated cells, DTT was applied in cell contact studies (8), for inhibiting the *N*-ethylmaleimide effect, at a concentration of 1.0 mM (0.122 mg/ml). Dithiothreitol inhibited fusion of karyomers in the telophase of sea-urchin egg mitosis and produced micronuclei. However, the change was reversible and the micronuclei fused in a few minutes after removal of DTT (29). To split disulfide groups in elastic fibres, 0.025 M (3 mg/ml) was used for 7 hr (3) Within a few minutes, trypsin suspended most cell types from monolayer cultures, though not epidermal cells (11). The present study shows that acantholytic epidermal cells produced by a disulfide-reducing agent are dissociated, after mild trypsinization, into a single-cell suspension.

Spindle-shaped cells also grow in epidermal cell cultures of guinea pig (2, 6, 23). These cells have an intracytoplasmic enzyme pattern similar to epithelial cells, and also have incomplete desmosomes. The mitotic cell cycle is shorter than that of fibroblasts. Bauer et al. (2) cultivated the cells on a Millipore filter with a nucleoprotein-enriched medium. They found keratinized cells in a lamellar arrangement. However, no electron microscopical identification of the keratinized cells was obtained. The spindle-shaped cells in the present study were isolated from outgrowths of epidermal cells within 1 week after cultivation, in which period no fibroblasts grew from the explants. In contrast to typical skin fibroblast cultures, no extracellular fibrils were found. Attachment-plaque like thickenings of the cell membrane and rough bundles of intracytoplasmic microfilaments are seen both in undifferentiated epidermal cancer cells (18) and cultured embryonic fibroblasts (21). Human skin grown in organ culture for 3–6 weeks showed an encapsulation of the dermis by epithelial cells of various degrees of differentiation (12). Many of these cells showed neither desmosomes nor tonofilaments and were quite similar to the spindle-shaped cells of the present study.

Besides keratinocytes, a primary epidermal cell culture also contained melanocytes and Langerhans cells (7). The latter survive for about 10 days in cultivation. Cultured melanocytes have been described in previous studies (5, 16, 25).

The present findings suggest that the spindle-shaped cells are dedifferentiated epidermal cells. A more detailed ultrastructural study of these cells in comparison with fibroblasts from the dermis is the subject of a separate paper.

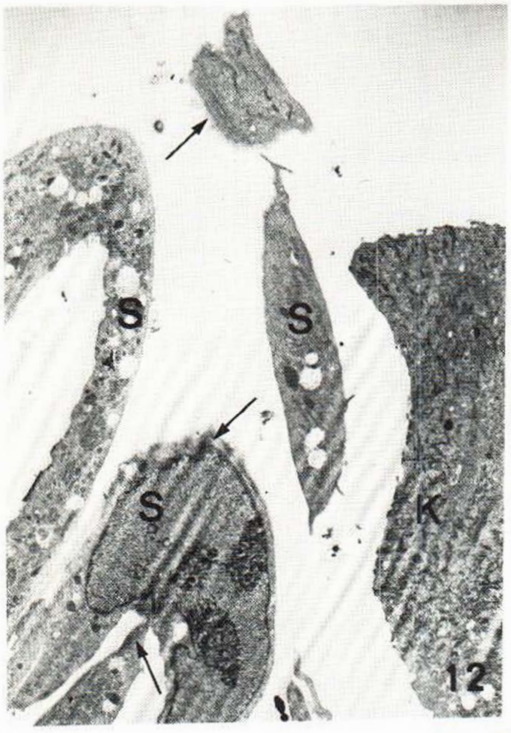
*Fig. 7.* Desmosome of 4-day-old epidermal outgrowth before any treatment. Arrow indicates the M-line. ×60 000.

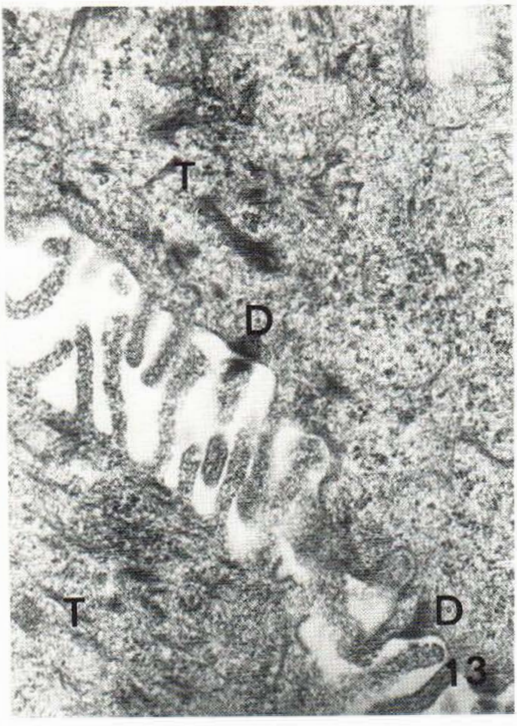
*Fig. 8.* Desmosome treated with trypsin. The intercellular matrix shows a distinct M-line. ×60 000.

*Fig. 9.* After DTT treatment some desmosomes showed a homogeneous, dense, intercellular matrix without M-line. ×60 000.

*Fig. 10.* Desmosome completely separated by DTT–trypsin. Cell membrane and attachment plaque are intact. ×60 000.

*Fig. 11.* Dissociated epidermal cells. Arrows indicate ruptured desmosomes. ×4 500.



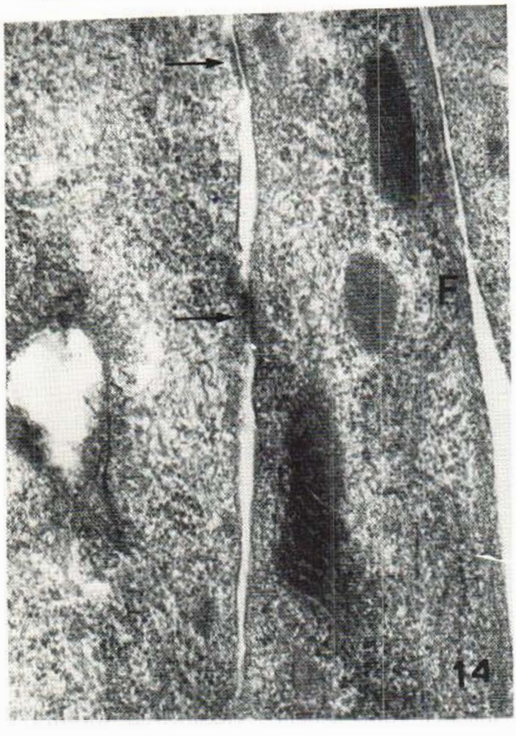




*Fig. 12.* Spindle-shaped cells (*S*) and a keratinocyte (*K*) after 4 months of cultivation. Bundles of intracytoplasmic filaments are indicated by arrows. ×4 500.

*Fig. 13.* Two keratinocytes after 4 months of cultivation. Desmosomes (*D*) and tonofilaments (*T*) are seen. ×30 000.

*Fig. 14.* Three spindle-shaped cells. Arrows indicate desmosome-suspect structures. Intracytoplasmic microfilament bundles (*F*) are seen immediately under the cell membrane. ×30 000.

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