*J. Anat.* (1973), **114**, 1, pp. 65–81
*With* 12 *figures*
*Printed in Great Britain*



# Freeze-fracture replication of cells of stratum corneum of human epidermis

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(*Accepted* 20 *October* 1972)

## INTRODUCTION

The technique of freeze-fracture replication has particular advantages as a method for studying the functional morphology of biological tissues at the ultrastructural level (Steere, 1957; Weinstein & Someda, 1967; Koehler, 1968; Bullivant, 1969; Moor, 1969, 1971; Branton, 1971). Chemical fixation, dehydration, infiltration with embedding media, and staining, all of which may produce artefacts, are not required, so tissues can be examined in a condition closer to the living state than is possible with conventional techniques employing thin sections. Glycerinated tissue, rapidly frozen to −180 °C, is fractured *in vacuo*, and the exposed surface is then shadowed from an angle and replicated with platinum and carbon. The replica is then separated from the tissue, placed upon a grid, and examined in the electron microscope.

With a frozen cell, the plane of fracture traverses the cytoplasmic matrix in an apparently random fashion, and the two resulting fracture faces appear identical. With membranes (plasma, nuclear, and those surrounding organelles), however, the fracture plane frequently deviates and runs parallel with the membrane for variable distances, thus exposing extensive areas *en face*. Membranes may also be fractured straight across, thus appearing as they would in thin sections. In epithelia such as the epidermis, which are composed of closely apposed cells, it has been established that two fracture faces associated with the plasma membrane appear in replicas (Bullivant, 1969; Chalcroft & Bullivant, 1970; Scott McNutt & Weinstein, 1970; Breathnach, Stolinski & Gross, 1972*a*). One of these faces can be identified as being directed towards the interior of the cell; the other as being directed towards the exterior (Fig. 1). The two faces are separated by a step which includes the intercellular space. These two faces, each belonging to one or other of the apposed cells, are complementary, two similar type faces having been fractured away. Each fracture face of epidermal, and other, plasma membranes carries small (5–7 nm) particles – 'membrane-associated particles' – and in the epidermis the face directed towards the interior of the cell exhibits, in addition, aggregations of small particles which coincide with desmosomes (Breathnach, Stolinski & Gross, 1972*a*). Gap junctions and tight junctions also exhibit particular features but this study is not concerned with them. It is still uncertain exactly what these fracture faces represent. Some authors (e.g.

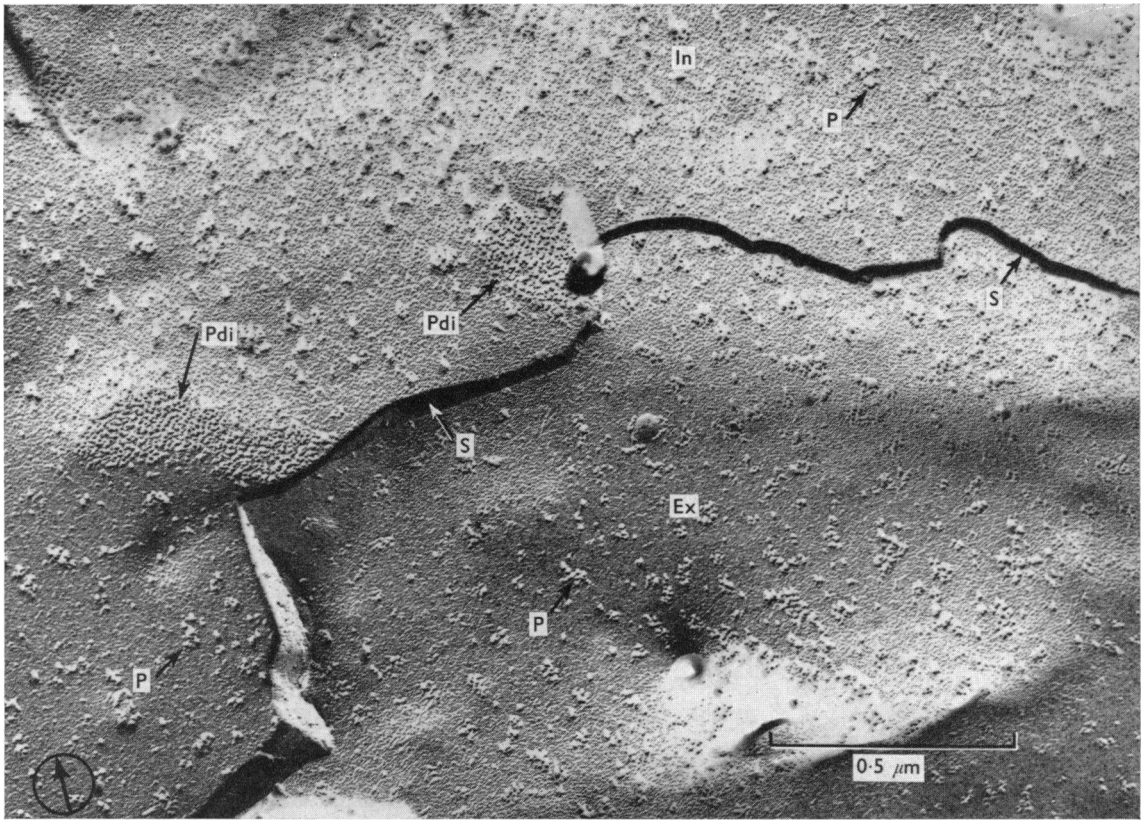


Fig. 1. Replica from basal layer of human fetal epidermis to illustrate the two types of fracture face associated with the plasma membranes of closely apposed cells. In, fracture face directed towards the interior of one of the cells; Ex, fracture face of plasma membrane of apposed cell, directed towards its exterior; S, step demarcating the two faces and including the intercellular interval or material; P, membrane-associated particles present on both faces; Pdi, aggregated desmosomal particles present only on fracture face In. × 64 500. NOTE. In this and succeeding micrographs the direction of platinum shadowing is indicated by encircled arrow at left lower corner.

Moor, 1969) have maintained that they represent the true inner and outer surfaces of the plasma membrane. But the majority (Branton, 1971; Chalcroft & Bullivant, 1970; Scott McNutt & Weinstein, 1970) believe that the fracture does not reveal true surfaces, and that its plane of passage invariably lies within the membrane, in effect 'splitting' it to reveal complementary internal faces. According to this view 'membrane-associated' and desmosomal particles would occupy the interior of the membrane, and would not be attached to its surface. The balance of present evidence seems to support the latter viewpoint, which implies that the freeze-fracture technique is capable of revealing some features of the internal structural organization of membranes. Recently (Breathnach, Stolinski & Gross, 1972*b*) it has been shown that the appearances in replicas of certain intracellular membranes (those of nuclei, mitochondria, and rough endoplasmic reticulum) differ from those of plasma membranes,

suggesting either a basic structural difference or a different plane of fracture. Whichever is the case, it is clear that the technique can reveal some differences between membranes which appear identical in chemically fixed and stained thin sections. It might therefore be expected to reveal further differences between membranes whose appearances in thin sections differ significantly.

The plasma membrane of the stratum corneum cell in the epidermis is thought to differ structurally from the plasma membranes of cells of the basal, spinous, and granular layers (Matoltsy & Parakkal, 1967; Martinez & Peters, 1971). In thin sections it appears 'thickened', and there is uncertainty as to the nature of this thickening, and as to how it has been brought about. Desmosomes in the stratum corneum are also thought to differ in structural arrangement from those present at lower epidermal levels. In previous investigations the appearance in freeze-fracture replicas of the plasma membrane and specialized contacts of cells of the basal and spinous layers has been established (Reed & Rothwell, 1970; Breathnach, Stolinski & Gross, 1972*a*), and it was considered desirable to make similar observations on the cells of the stratum corneum. Preliminary work (Breathnach, Goodman, Stolinski & Gross, 1972) indicated significant differences. The appearance of the cytoplasm of the cell and of lamellar granules (Odland bodies, membrane-coating granules) would also prove of interest, and the observations might be expected to throw some light upon the general process of keratinization.

## MATERIALS AND METHODS

Fresh (not chemically fixed) pieces of whole skin, or superficial shavings of epidermis, were immersed for $\frac{1}{2}$ hour in 20 % aqueous glycerol, and for a further $\frac{1}{2}$ hour in 40 % glycerol, at 4 °C. Glycerol serves as a cryoprotective agent. Specimens were then rapidly frozen in an isopentane bath, and transferred in a special chuck to the freeze-fracture apparatus. This comprised a special fracturing device, designed in this laboratory, and attached to a standard Genovac Vacuum Coating Unit; details of the apparatus will be published elsewhere. After evacuation of the apparatus specimens were fractured, and replicas of the fracture surfaces were made by evaporating platinum on to them from an angle of 40°. A backing layer of carbon was then evaporated normally to the surface. Specimens were then removed from the vacuum chamber and allowed to reach room temperature before the procedure for separating replica from tissue was instituted. Separation of the two was particularly difficult with skin, and involved, in effect, complete digestion of the tissue. During this process the tissue swells, leading to fragmentation of the replica, and several procedures were adopted in an attempt to keep this to a minimum. Best results were achieved with the following method: after the specimen had reached room temperature, it was placed in a small dish of distilled water, and the water was gradually replaced by adding, drop by drop, a mixture of equal parts of 20 % KOH and 40 % DMSO. After 1 hour this fluid was pipetted away, and replaced by neat KOH-DMSO mixture for 2 hours. The specimen was then heated in this mixture for 4 hours at 60 °C, after which it was transferred to water. Concentrated nitric acid was next added drop by drop until full strength was achieved, and the specimen was left in this overnight. Finally, the specimen was heated to 60 °C in concentrated nitric acid for 2 hours. By this time the

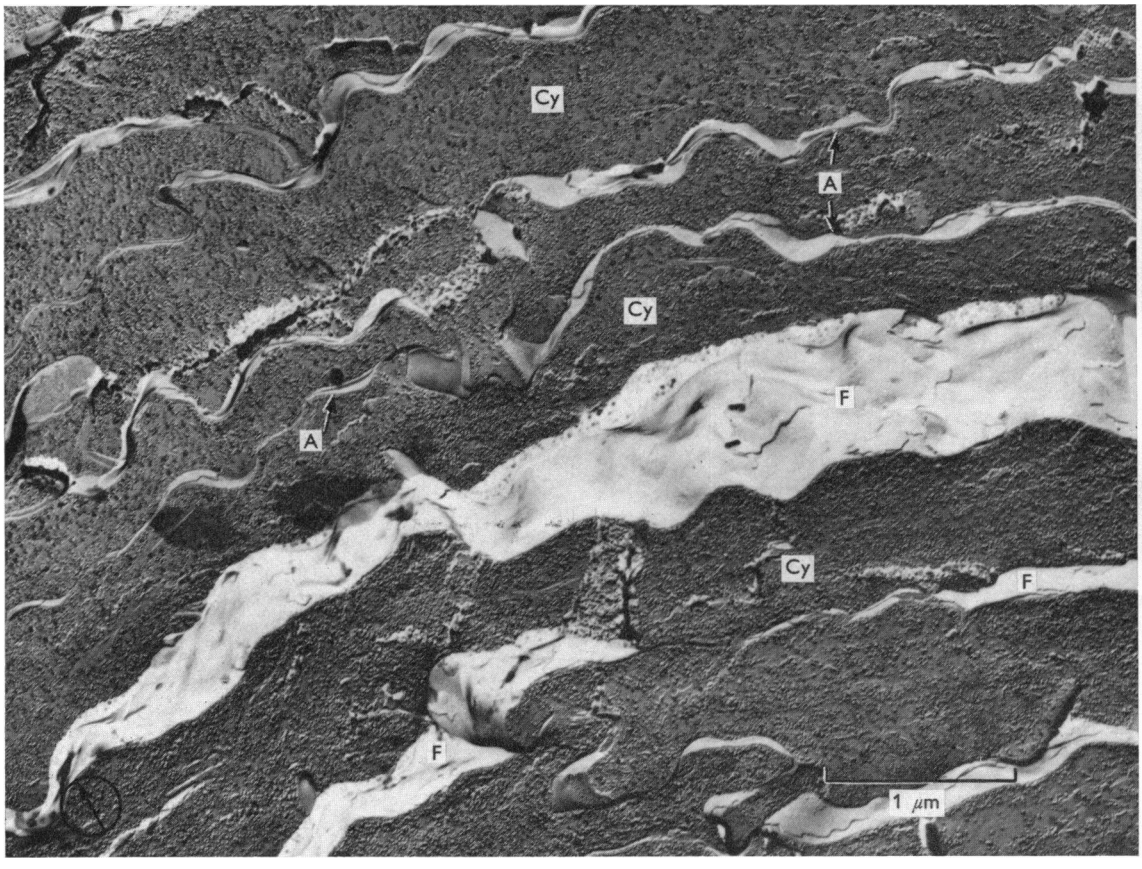


Fig. 2. Replica of freeze-fractured stratum corneum of adult human epidermis. Cells in the upper part of the field have been fractured almost at right angles revealing the cytoplasm (Cy) and cross-fractured cellular appositions (A). In the lower part of the field, extensive fracture faces (F) associated with plasma membranes are seen. ×22500.

tissue was completely digested away, and sizeable segments of replica were recoverable. These were finally washed in water, and mounted on small-hole copper or gold grids for examination. A Philips EM 300 electron microscope equipped with a goniometer stage was used in this investigation.

It should be emphasized that 'etching' (or more correctly, 'sublimation') of the specimens was not performed. This involves sublimation of ice by raising the temperature of the fractured specimen up to −100 °C for several minutes before shadowing and replication. It is only applicable to specimens suspended in water or to organized tissue pre-treated with chemical fixatives before freezing. It is not applicable to non-chemically fixed organized tissue treated with glycerol as cryoprotective agent, since in this situation sublimation does not occur. Chemical fixation leads to loss of fine detail and vitiates one of the major advantages of the technique, and sublimation can produce artefacts which complicate interpretation (Moor, 1971; Staehelin & Bertaud, 1971; Lickfeld, Achterrath & Hentrick, 1972).

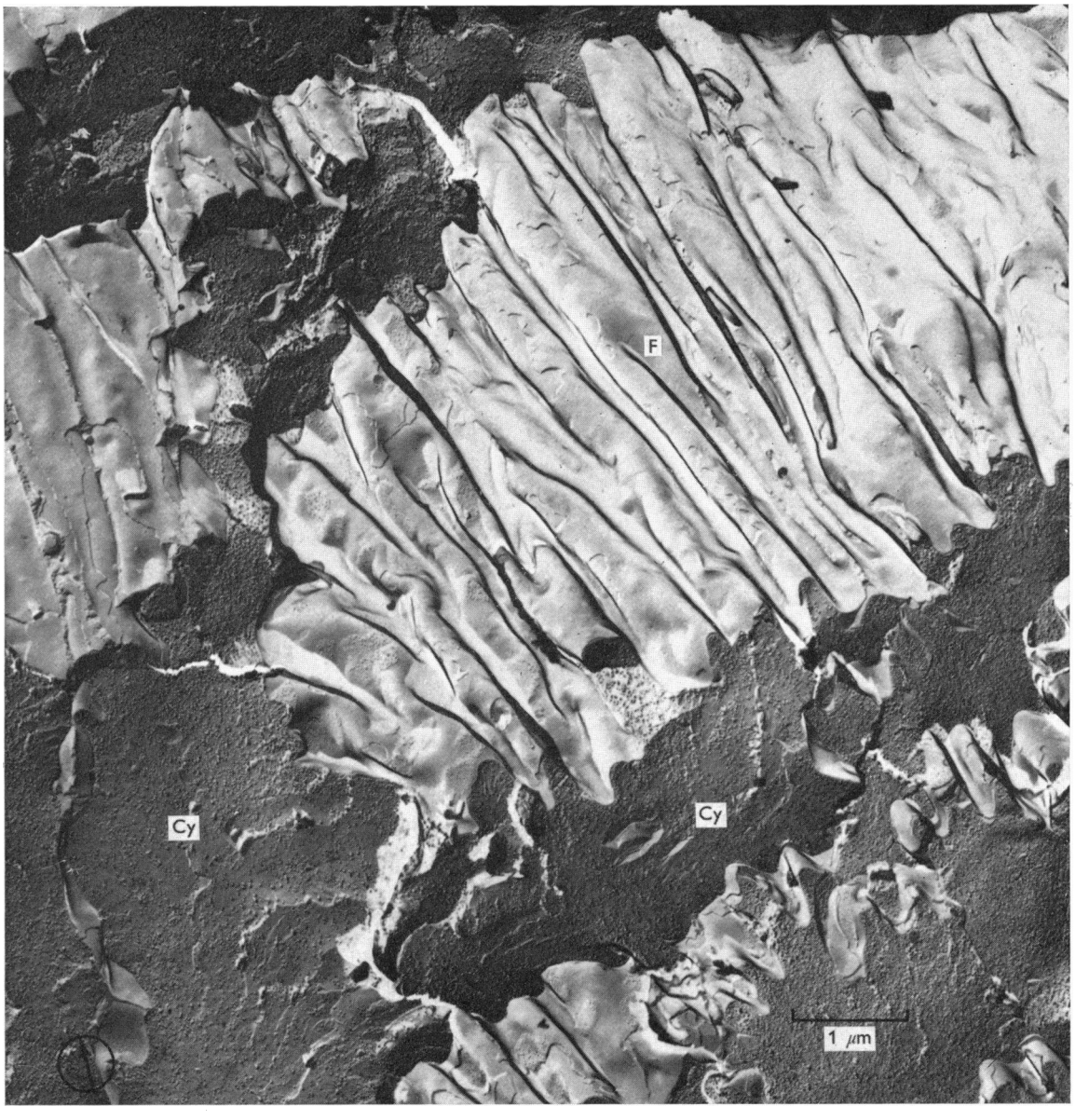


Fig. 3. Replica of freeze-fractured stratum corneum of adult human epidermis. Note corrugated appearance of extensive area of fracture faces (F). This area includes both types of fracture face associated with plasma membranes but they are not easy to distinguish at this magnification. Cy, cytoplasm. × 16000.

## RESULTS

### *General features of freeze-fractured stratum corneum cells*

Fig. 2 presents a replica which is readily comparable with micrographs of thin sections of stratum corneum. The cells have been fractured almost at right angles,

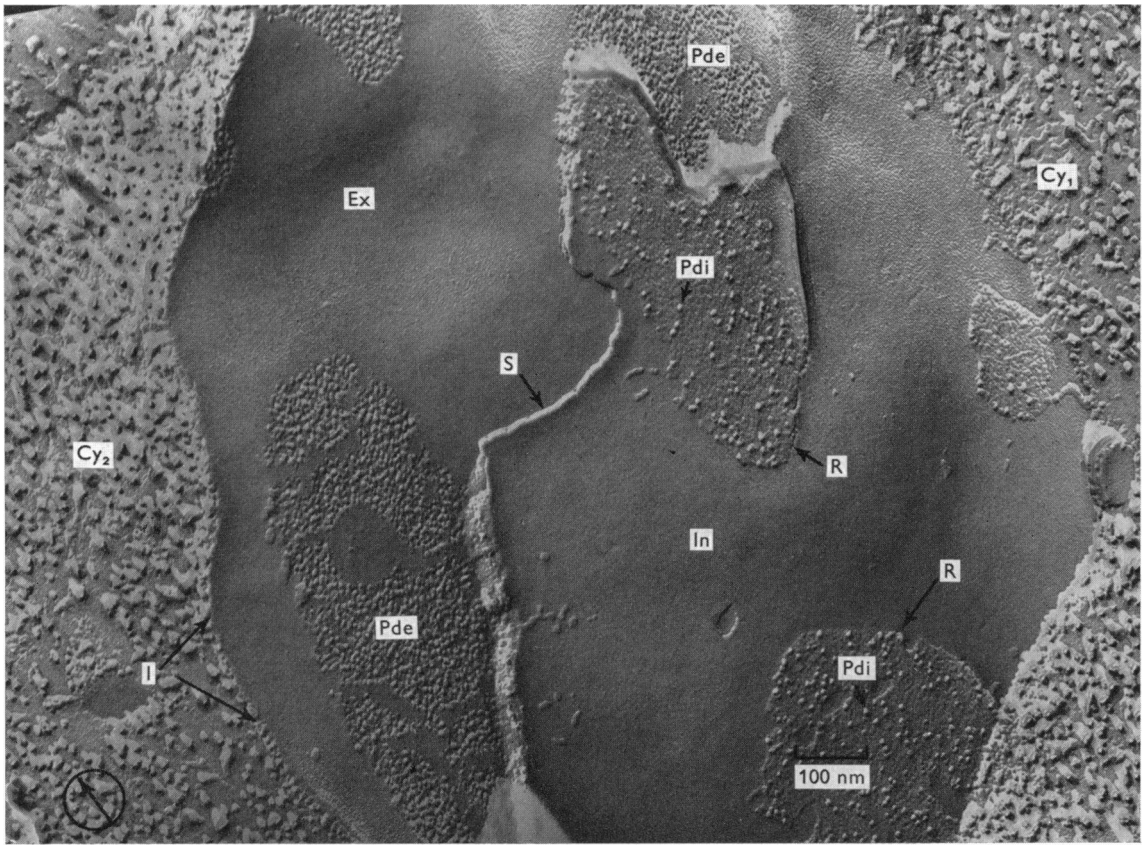


Fig. 4. Replica of freeze-fractured stratum corneum of adult human epidermis to show features of fracture faces associated with the plasma membrane. Two apposed cells are seen. $Cy_1$, cytoplasm of one cell with tonofilaments; In, fracture face of plasma membrane of this cell, directed towards its interior. Note absence of membrane-associated particles from the face (cf. Fig. 1), and the presence of desmosomal particles (Pdi) which rest upon a shallow raised plaque, the edges of which are indicated by R; S, step which includes intercellular material; Ex, fracture face associated with plasma membrane of apposed cell and directed towards its exterior. Note again the absence of membrane-associated particles from the general fracture face, but the presence of desmosomal particles (Pde). $Cy_2$, cytoplasm of the second cell. A linear array of tonofilaments (l) is seen bordering the fracture face (Ex). × 83 500.

and appear stacked in layers. The cytoplasm at this magnification has a fairly uniform granular appearance, which from previous observations (Breathnach, Stolinski & Gross, 1972*a*) can be attributed to fractured tonofilaments projecting above the general level, as seen in Figs. 4 and 12. At cellular appositions, variable extents of fracture faces associated with the apposed plasma membranes are revealed, and detailed features of these are described below. In the particular field illustrated, planes of apposition can be described as gently undulant, with little interlocking of apposed cells, but in other fields (Fig. 3) a considerable degree of interlocking may be evident, and this is manifest by the corrugated appearance of membrane faces.

In replicas obtained from superficial shavings of epidermis, the cells appear very

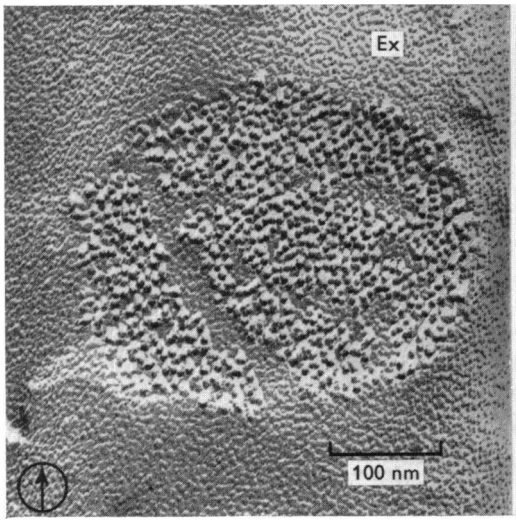


Fig. 5

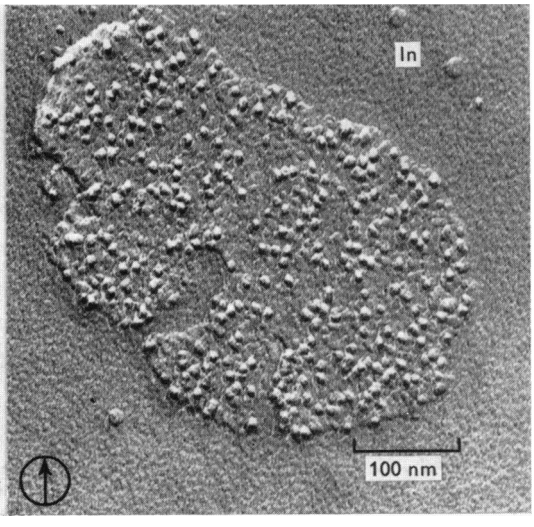


Fig. 6

Fig. 5. Desmosomal particles on fracture face Ex of stratum corneum cell membrane. The aggregated particles lie in a slight depression, so that their tops are approximately at the level of the general fracture plane (Ex). ×129000.

Fig. 6. Desmosomal particles on fracture face 'In' of stratum corneum cell membrane. The aggregated particles lie on, or embedded in, a shallow plaque with their tops above the level of the general fracture plane 'In'. ×129000.

closely apposed, with a narrow intercellular interval, estimated as approximately 11–14 nm. However, in some replicas obtained from specimens of whole skin the cells are sometimes separated by wider intervals. Where the intercellular interval is narrow, and the apposition appears in cross-fracture, desmosomes are not obvious in low power micrographs (Fig. 2).

### *Fracture faces associated with the plasma membrane*

As at lower epidermal levels, two fracture faces associated with apposed plasma membranes of stratum corneum cells are seen in replicas (Figs. 4, 7). Due attention being paid to the direction of shadowing, it can be established from micrographs that one of these faces (face 'In') is directed towards the interior of one of the cells, and that the second one (face 'Ex') is directed towards the exterior of the other cell involved in the apposition. These two main faces may be separated by a variable number of other faces and steps (Fig. 10) which represent intercellular material. Since fracture faces at deeper levels of the stratum corneum are not identical with those seen at more superficial levels, their appearance in the two situations is considered separately. The particular level being examined was identified by low magnification survey of the replicas.

At deeper levels of the stratum corneum the general appearance of both fracture faces associated with the plasma membrane is finely granular, and isolated membrane-associated particles are almost entirely absent (Figs. 4, 7–9). These particles are highly

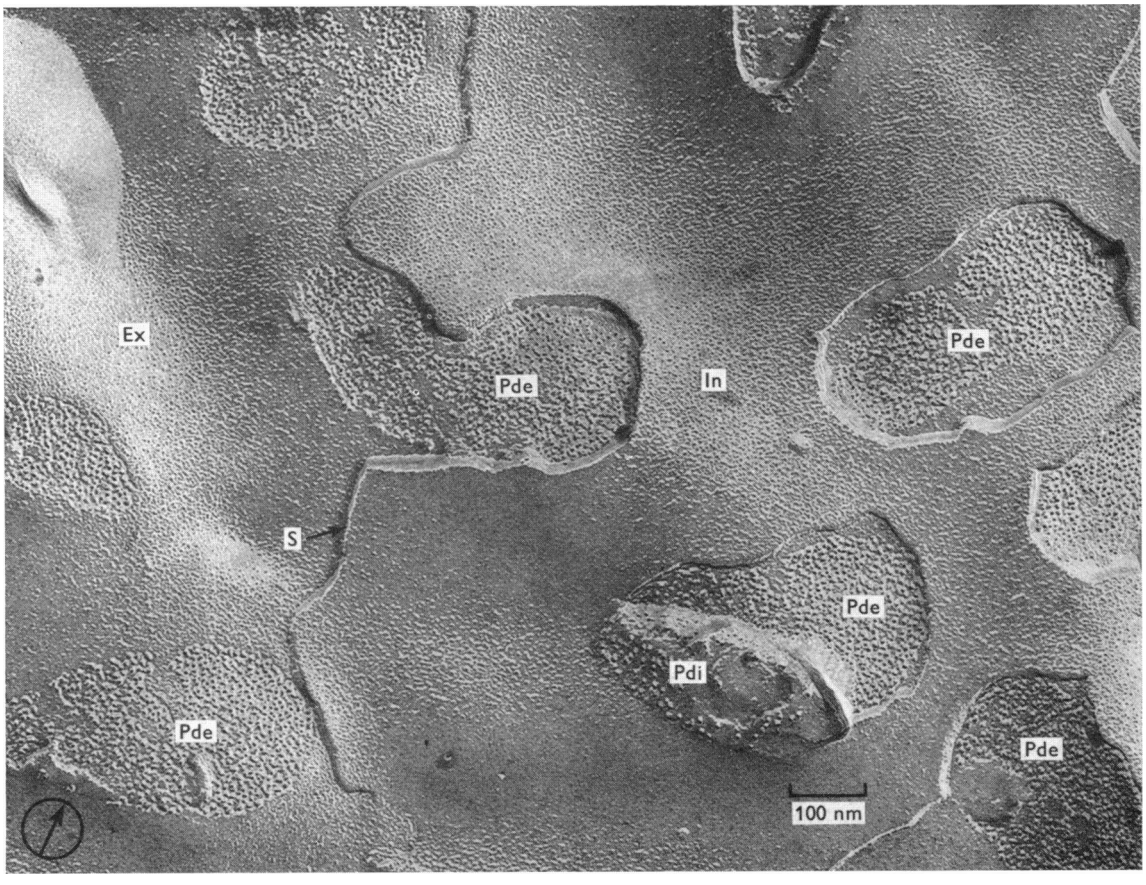


Fig. 7. Replica from a mid-level of stratum corneum. Fracture faces 'In' and 'Ex' are seen delimited by the intercellular step (S). Note in the general area of fracture face 'In' that, with one exception towards the lower part of the field, the aggregated desmosomal particles (Pdi) have become detached, exposing those present (Pde) on the underlying face (Ex). Note again the absence of membrane-associated particles from the fracture faces. × 86000.

characteristic of fracture faces of plasma membranes at lower epidermal levels and of cells generally, and their absence in the stratum corneum is therefore a striking feature. At lower epidermal levels aggregations of closely packed particles are seen on fracture face 'In' at desmosomes, but not on the complementary fracture face 'Ex'. In the stratum corneum, by contrast, desmosomal particles are seen on both fracture faces (Figs. 4–7). On fracture face 'In' the particles are associated with slightly raised plaques which they do not cover completely. On fracture face 'Ex' the aggregated particles lie on a lower level with respect to the general fracture plane, and their tops are approximately at the level of this plane. This is evident from consideration of their shadows, and the shallow step delineating the desmosome, as seen in Fig. 5. In general, desmosomal particles are more numerous on fracture face 'Ex' than on fracture face 'In'.

At more superficial levels both fracture faces present the same general finely

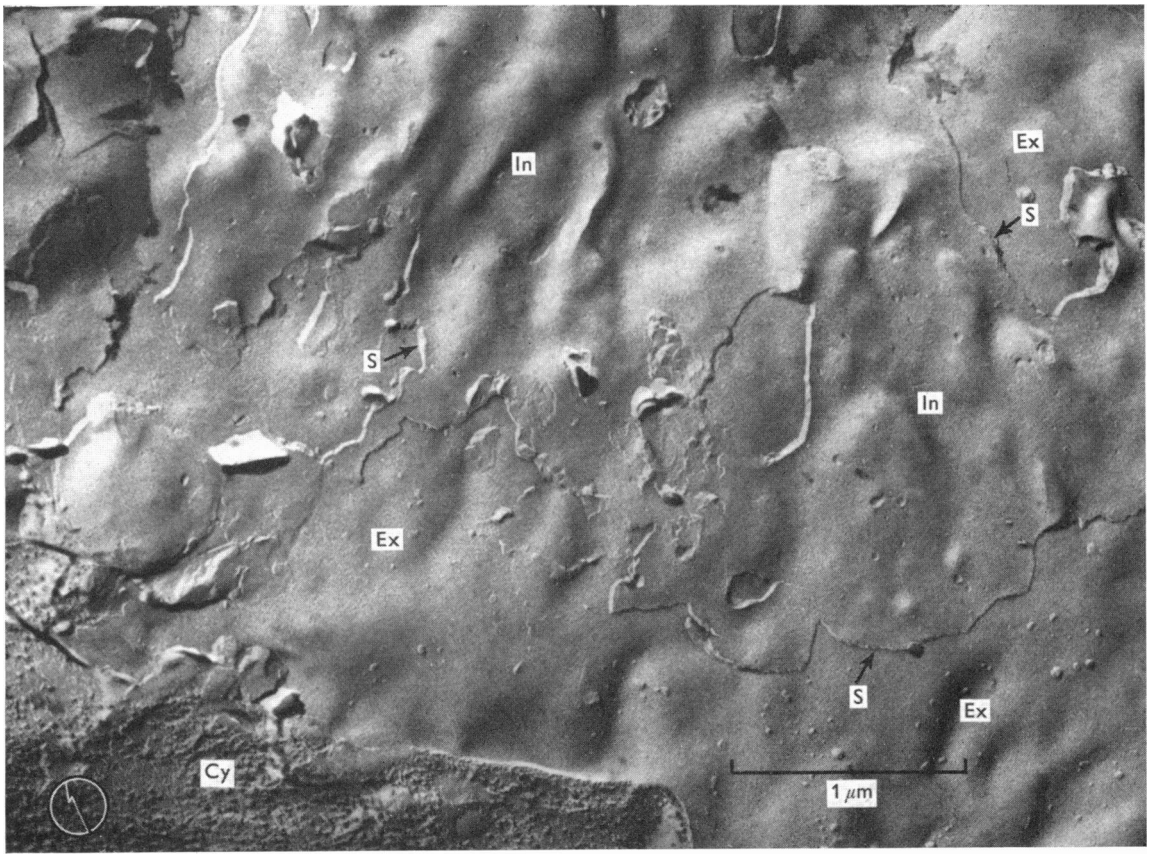


Fig. 8. Replica from a high level of stratum corneum showing extensive areas of fracture faces 'In' and 'Ex'. Aggregated desmosomal particles are not present on either face. Cy, cytoplasm; S, intercellular step. × 31000.

granular appearance, and likewise lack isolated membrane-associated particles. In some replicas many of the plaques carrying desmosomal particles, which are characteristic of face 'In' at lower levels, appear to have become entirely detached (together with underlying intercellular material), thus exposing the desmosomal particles on the underlying face 'Ex' (Fig. 7). In other replicas, which we believe to be derived from cells of the most superficial levels of the stratum corneum, desmosomal particles are not present on either fracture face (Fig. 8).

No evidence of gap junctions or tight junctions was seen at any level of the stratum corneum.

### *Intercellular material*

Laminated intercellular material is seen at intervals between the stratum granulosum and stratum corneum (Fig. 9), and between the deeper cells of the stratum corneum (Fig. 10). Depending upon the orientation of the laminae, this material can present as variable numbers of faces and steps between the two main fracture faces ('In' and

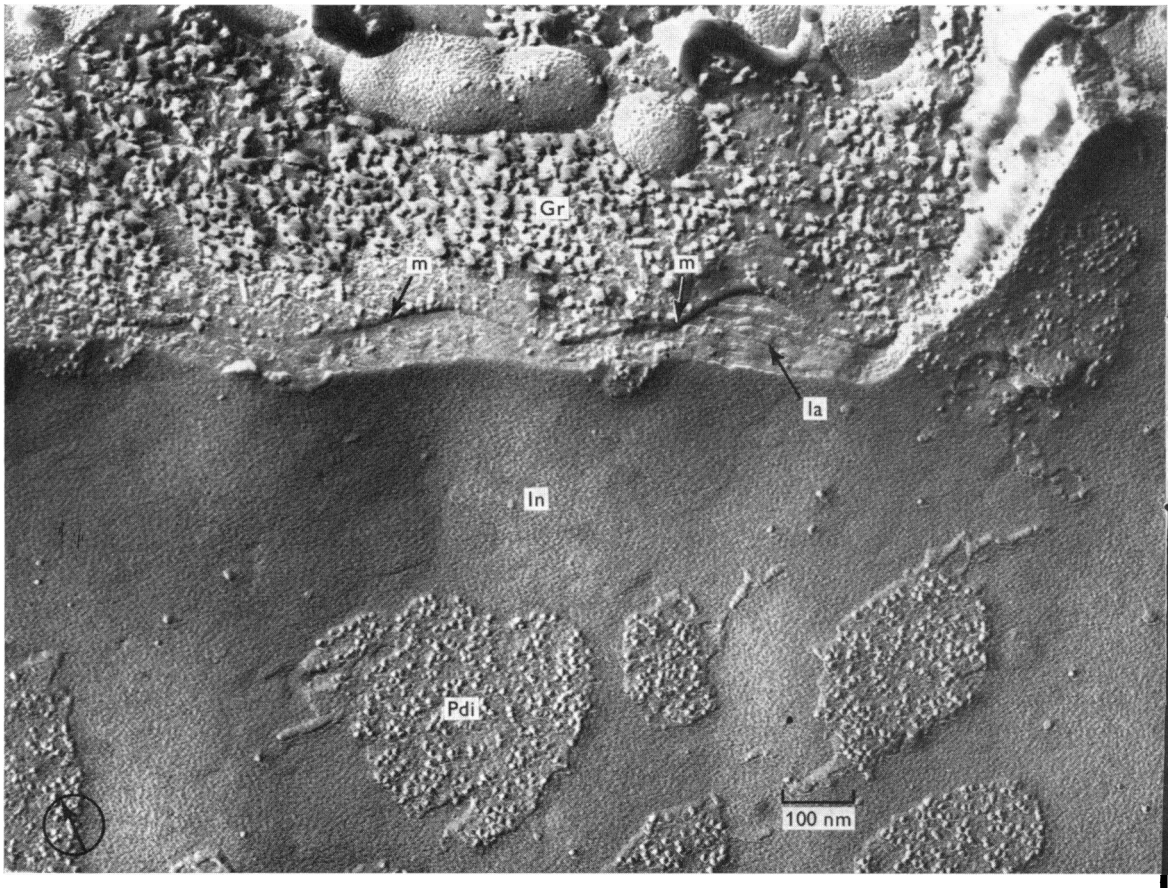


Fig. 9. Replica showing cytoplasm of uppermost stratum granulosum cell (Gr) and fracture face (In) of apposed stratum corneum cell membrane, with desmosomal particles (Pdi). The plasma membrane of the granulosum cell (m) appears on cross-fracture, and laminated material (la) is present in the intercellular interval. × 89000.

'Ex') associated with the plasma membrane (Fig. 10). Similar laminated material is seen within the cytoplasm of the cells of the stratum granulosum (Fig. 11), and also in an intercellular position at this level. There seems little doubt that this material represents the lamellar granules (Odland bodies, membrane-coating granules) seen in stained thin sections (Matoltsy & Parakkal, 1967; Martinez & Peters, 1971). Where multilaminated material is not present between the cells, the intercellular material on cross-fracture is represented by a step approximately 11–14 nm in height (Figs. 4, 7). The step representing the intercellular material at desmosomes is somewhat higher (Fig. 4).

*Cytoplasm of stratum corneum cells*

The interior of the fractured stratum corneum cell exhibits filaments (10 nm) which, to judge by the length of the shadows cast, project somewhat above the general level of the fracture plane (Figs. 4, 12). In general, considerably fewer of

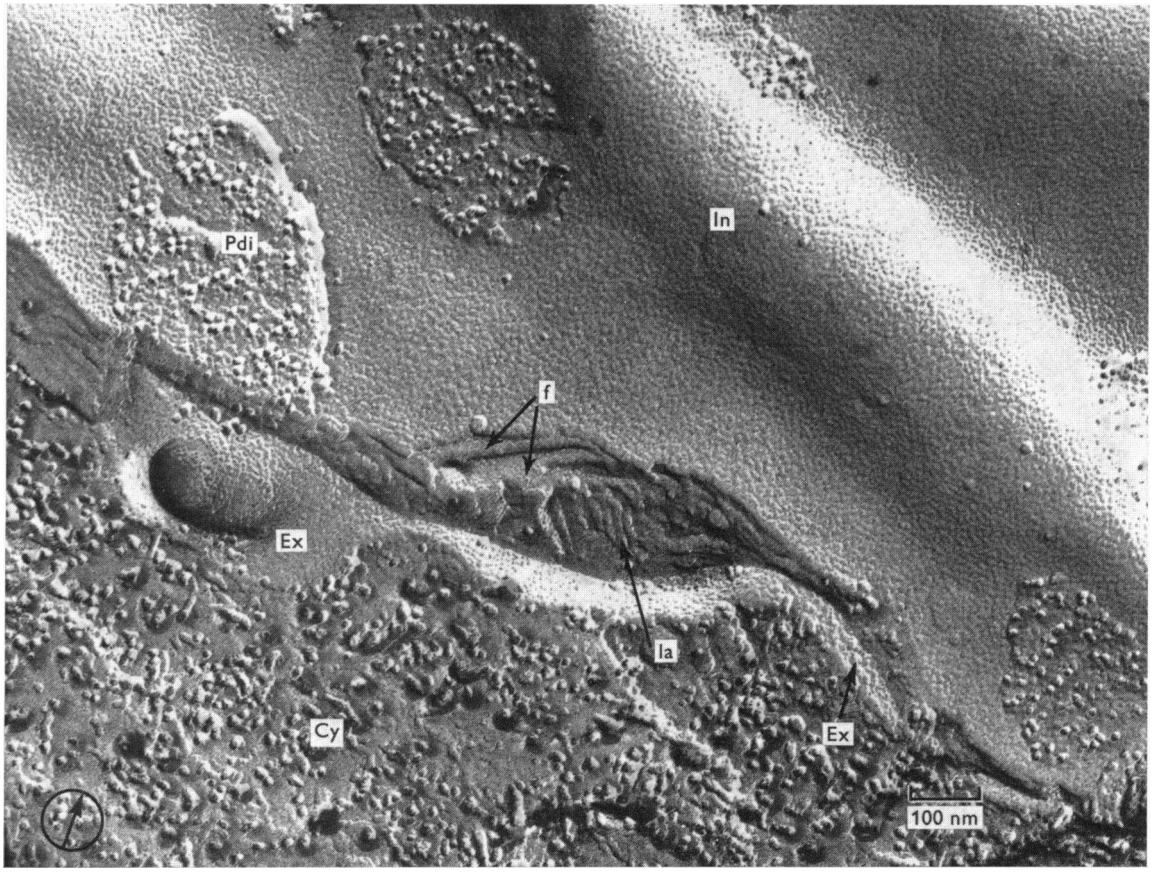


Fig. 10. This replica from a deep level of stratum corneum illustrates lamellated material (la) between the cells. Cy, cytoplasm of cell with tonofilaments; Ex, fracture face of plasma membrane of this cell directed towards the exterior; In, fracture face of apposed cell directed towards its interior; Pdi, aggregated desmosomal particles. Note fracture faces (f) of laminated material between the two main fracture faces (In and Ex). ×89000.

these filaments are seen in replicas than in thin sections exhibiting a typical 'keratin pattern', and their distribution is less uniform; in some areas they are particularly sparse. This apparent discrepancy also applies to tonofilaments at lower epidermal levels. Filament density is greatest in the immediate neighbourhood of the plasma membrane, and in this situation they are frequently arranged in close linear array (Figs. 4, 12). In some situations, and depending upon the angle of shadowing, which can vary somewhat from place to place due to undulation of the overall fracture surface, a step of height 8 nm is present between the row of filaments and the fracture face 'Ex' (Fig. 12, inset). In chemically fixed and stained thin sections, a dense layer 14–18 nm wide is present on the cytoplasmic aspect of the plasma membrane; this has been interpreted as a 'thickening' of the plasma membrane, and regarded as an essential element of the process of keratinization. The linear array of filaments, and the step referred to above, both fall within the territory of this dense zone.

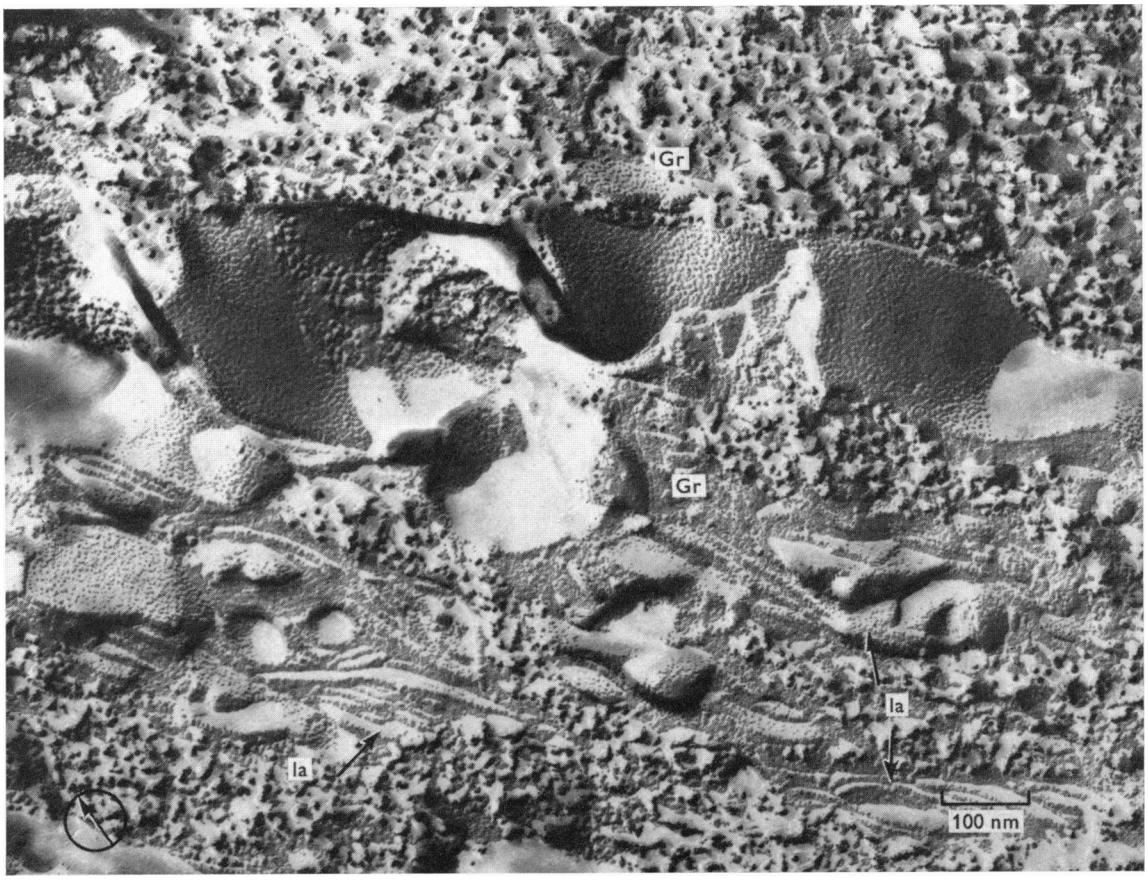


Fig. 11. Replica of freeze-fractured stratum granulosum cells, Gr. Note laminated material (la), probably representing lamellar granules, in cytoplasm of lower cell. × 101 000.

Apart from filaments, the only other cytoplasmic constituent of the stratum corneum cell is an occasional lipid droplet.

## DISCUSSION

Main interest in the observations reported here concerns the extent to which they provide additional information on the ultrastructural morphology of the stratum corneum cell. Keratinization is thought to involve alterations in both plasma membrane and cytoplasm of the cell (Brody, 1964; Matoltsy & Parakkal, 1967).

Fracture faces associated with the plasma membrane of the stratum corneum cell differ in two significant respects from those associated with plasma membranes of the non-keratinized cells of lower epidermal levels. The first difference concerns the virtual absence of membrane-associated particles from the faces, and the second relates to desmosomes. As already stated, membrane-associated particles are a constant feature of fracture faces of plasma membranes of cells of a variety of tissues, and

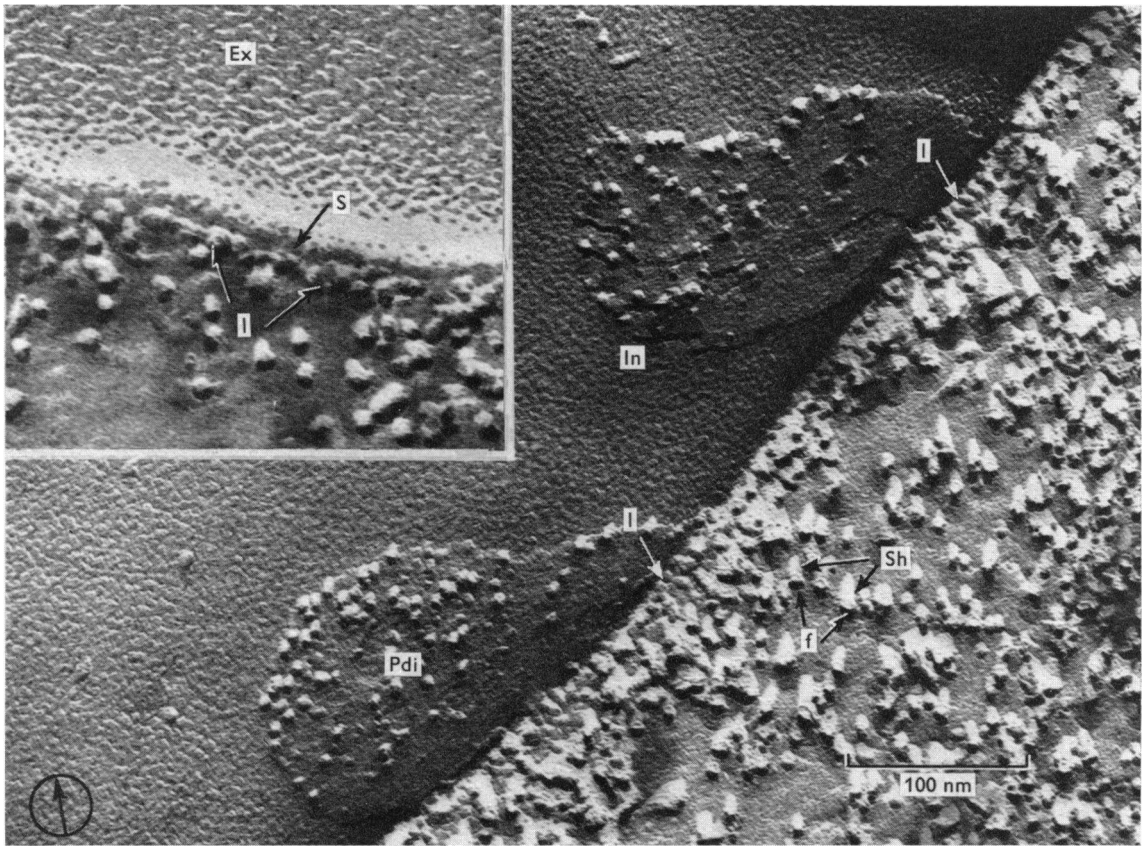


Fig. 12. Replica showing tonofilaments in cytoplasm of stratum corneum cell. Individual fractured filaments (f) cast long shadows (Sh), indicating that they project above the general plane of fracture of the cytoplasm. Note linear array of filaments (l) close to fracture face (In) of plasma membrane. Pdi, aggregated desmosomal particles. × 163000. Inset: replica showing shallow step (S) between linear array of tonofilaments (l) and fracture face (Ex) of plasma membrane (× 248000).

they are thought to constitute an internal component of the membrane, revealed by passage of the fracture within it. Their absence in the stratum corneum could be explained by suggesting that the plane of fracture is different in this situation, or alternatively, that the plane of fracture is similar, but the particles are either not present or not capable of being revealed. Either possibility would be indicative of some structural alteration of the membrane.

The path of the fracture in relation to the plasma membrane is probably the same in the stratum corneum as at lower levels, since it reveals desmosomal particles in both situations. Why then does it not also reveal membrane-associated particles on the general fracture faces of stratum corneum plasma membrane? The suggestion that the particles become detached during processing might appear plausible if one were to regard the stratum corneum cell, as is commonly done, as a 'dead' cell. On this view, internal structural constituents of the plasma membrane (membrane-

associated particles) could be regarded as being more easily detachable on being exposed by fracturing *in vacuo*. An alternative explanation would be that the general plasma membrane of the stratum corneum cell is so altered internally that it no longer appears particulate on fracturing. One might envisage some kind of disintegration and/or fusion of particles to present a more homogeneous appearance. Whichever be the case, it is clear that the internal structural arrangement of the plasma membrane differs from that of cells at lower levels. It is equally clear, from consideration of the desmosomal particles, that the internal structure of the membrane at desmosomal sites is also altered in some way. At lower levels these particles appear only on the fracture face 'In', whereas in the stratum corneum they appear on both fracture faces. In the strata basale, spinosum and granulosum the fracture does not enter among the particles, so that all of them adhere to one face, whereas in the stratum corneum it can evidently pass between particles, leaving some attached to each face. This indicates a difference in internal cohesion, the significance of which is not apparent. The significance of the shallow plaque of material associated with desmosomal particles seen on fracture face 'In' of the stratum corneum cell is likewise not evident. It may be emphasized that this plaque in no way corresponds with the 'desmosomal plaque' seen in stained thin sections. The latter lies within the cytoplasm, and not within the plasma membrane. The absence of desmosomal particles at more superficial levels of the stratum corneum is presumably indicative of termination of the function of the desmosome as a specialized zone of intercellular adhesion, in order to allow final desquamation of the cells. Desmosomes are not evident at this level in stained thin sections.

Examination of chemically fixed, stained thin sections has led to the conclusion that the plasma membrane of the stratum corneum cell is thickened. This is held to be due to deposition of material derived from lamellar granules on its outer aspect and of undefined material on its inner aspect (Matoltsy & Parakkal, 1967; Martinez & Peters, 1971). As regards the addition of material to the outer aspect of the membrane, the present observations show clearly that lamellar granules are not artefacts of fixation and staining, as is sometimes suggested. They do occupy an intercellular position, and can quite reasonably be regarded as contributing to or affecting the intercellular material of the stratum corneum. Whether they are sufficiently numerous to provide a complete coating to the plasma membranes seems doubtful; such a coating would be very thin in view of the narrowness of the general intercellular interval (11–14 nm). It may be that their main function is to modify the intercellular material, so that it constitutes a stronger cohesive bond between the cells; they may also affect its barrier properties.

Examination of stained thin sections has also failed to establish the nature of the dense zone evident in such material on the cytoplasmic aspect of the plasma membrane of the stratum corneum cell. This, as already stated, has been attributed to a thickening of the internal leaflet of the membrane (Martinez & Peters, 1971). In a comparable situation in replicas, a closely packed linear array of tonofilaments is evident, separated by a distinct step from the fracture face of the plasma membrane. Filaments and step appear to occupy the entire width of the dense zone seen in thin sections. This zone therefore cannot belong entirely to the membrane, since it includes tonofilaments. (This conclusion can be reached from a study of thin sections

alone, since the attachment plaque of the desmosome – which is cytoplasmic – lies within it.) The step seen in replicas could represent either the edge of the 'split' plasma membrane, or a narrow zone of amorphous cytoplasm between this edge and the row of filaments. If the former be the case, then this might be taken as evidence for some thickening of the membrane, though not to the extent suggested by stained thin sections. However, it must be said that there is no evidence in replicas to support the suggestion that the membrane is thickened on the cytoplasmic aspect. One can only assume that the linear array of filaments on its immediate cytoplasmic aspect is responsible for the particular staining properties of this zone. In conclusion, therefore, the present observations indicate that the plasma membrane of the stratum corneum cell undergoes some internal structural modification, but that it is not 'thickened' to any appreciable extent. Associated modification of the intercellular material and a particular arrangement of peripheral tonofilaments could be regarded as contributing to its strength, and thereby to the 'shell' which contains the cell contents. But this is an entirely different matter.

The diameter of tonofilaments in chemically fixed, dehydrated, and stained thin sections of human stratum corneum is usually given as about 7 nm. Measurements taken from replicas give a higher figure – 10 nm – and the same figure applies to filaments of deeper epidermal strata. The difference may possibly be due to effects of fixation. The fact that the concentration of tonofilaments is apparently less in replicas than in thin sections could be explained by suggesting that as the fracture process traverses the cell, filaments of particular orientation are pulled slightly out of the matrix or stretched before they actually fracture. These would appear on the fracture faces projecting above the general level – as actually seen. Their other ends, lying flush with or just below the general level, would not cast shadows, and therefore would not be visualized in replicas. In addition, a proportion of filaments, perhaps again depending upon their orientation in relation to the plane of fracture, might break off flush with this, and therefore, casting no shadows, would not be represented in replicas. Close examination of the linearly arranged filaments illustrated in Figs. 4 and 11 reveals that they are not quite as prominent, nor do they cast quite as long shadows, as the general cytoplasmic filaments. This would indicate that they are not pulled out of the matrix to the same extent before they fracture, and would suggest, in conformity with what has been written above, that these particular filaments may have a close association with, or even attachment to, the inner aspect of the plasma membrane.

It may be added that, to date, no organized structure which could positively be identified as a keratohyalin granule has been observed in replicas of cells of the stratum granulosum.

## SUMMARY

The stratum corneum of human epidermis has been examined by the freeze-fracture replication technique, without 'etching' or sublimation. Fracture faces associated with the plasma membrane of the stratum corneum cell differ in two respects from those associated with plasma membranes of cells of lower epidermal strata. First, 'membrane-associated particles' characteristic of fracture faces of membranes of basal, spinous, and granular cells (and of plasma membranes of cells

generally) are virtually absent from the fracture faces in stratum corneum. Secondly, aggregations of closely packed particles which at lower levels are present at desmosomal sites only on the fracture face directed towards the interior of the cell are present on both fracture faces in the stratum corneum. Since the fracture probably traverses the interior of the membrane, and the various particles revealed therefore represent internal structural components, the findings indicate that the internal structure of the stratum corneum cell membrane differs from that of non-keratinized cells.

No evidence emerged that the plasma membrane of the stratum corneum cell is 'thickened' on its internal aspect, as suggested by appearances seen in thin sections of chemically fixed, dehydrated, and stained material. Within the alleged zone of thickening, a more or less linear array of tonofilaments is seen in replicas; this might be regarded as strengthening the membrane from within. The diameter of tonofilaments as they appear in replicas is estimated as 10 nm.

Laminated material present within the cytoplasm of cells of the stratum granulosum, and at localized sites in an intercellular position both here and in the stratum corneum, very probably represents the lamellar granules (membrane-coating granules) seen in thin sections. The general intercellular interval in stratum corneum is narrow – approximately 11–14 nm.

Grateful acknowledgement is due to the following for grants to the Department of Anatomy for purchase of equipment used in this study: to the Wellcome Trust for a Philips EM 300 electron microscope; to the Joint Standing Research Committee of St Mary's Hospital for items used in constructing a freeze-fracture apparatus, and to the Peel Medical Trust and the Fitton Trust for items of ancillary equipment. Costs of consumable materials were covered by a grant from the Medical Research Council.

Dr Goodman was supported by the Medical College of Georgia Foundation, the Simon Greenberg Foundation, and by a Research and Education Associate Award from the Veterans' Administration.

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