CELL JUNCTIONS IN AMPHIBIAN SKIN

MARILYN G. FARQUHAR, Ph.D., and GEORGE E. PALADE, M.D.

From the Department of Pathology, University of California School of Medicine, San Francisco, and The Rockefeller Institute, New York

ABSTRACT

Cell junctions have been investigated in the amphibian epidermis, a stratified squamous epithelium, and compared to those described previously in simple columnar epithelia of mammalian cavitary organs.

In adult frogs and toads, and in larvae approaching metamorphosis, belts of membrane fusion or *zonulae occludentes* of considerable depth are regularly found between adjoining cells of the outermost layer of the *stratum corneum*, binding the cells together into a continuous, uninterrupted sheet. Another set of occluding zonules appears in the second cornified layer (when such a layer is present), and a third set usually occurs in the outermost layer of the *stratum granulosum*. Specialized elements described as "modified" and "composite" desmosomes are encountered along the lateral and basal aspects, respectively, of the cornified cells; ordinary desmosomes and *maculae occludentes* (*i.e.*, spots of membrane fusion) are found in all other strata. The usual 200 A intercellular gap is generally maintained between the cells of the *stratum germinativum* at the basal ends of the intercellular spaces. Hence, the intercellular spaces of the epidermis form a largely continuous network, closed to the external medium and open to the dermal interstitia. The situation is comparable to that found in columnar epithelia, except that the intercellular spaces are much more extensive, and an extracellular subcompartment (or two) apparently exists in the *stratum corneum* and between the latter and the *stratum granulosum*. The last subcompartment is usually filled with a dense substance, probably derived from discharged secretory granules. The tripartite junctional complex characteristic of lumen-lining epithelia (*i.e.*, a *zonula occludens* followed by a *zonula adhaerens*, and desmosome) is seen only in early larvae; in adults and in larvae approaching metamorphosis, the occluding zonule is followed directly by a series of modified desmosomes.

Interpreted in the light of current physiological data, these findings suggest that the diffusion of water, ions, and small, water-soluble molecules is impeded along the intercellular spaces of the epidermis by *zonulae occludentes* while it is facilitated from cell to cell within the epidermis by *zonulae* and *maculae occludentes*.

In a previous paper (1), we surveyed the epithelia lining a number of glands and cavitary organs of the rat and guinea pig and, in all cases investigated, found a characteristic tripartite junctional complex between adjacent epithelial cells. The complexes are located at the luminal end of the intercellular spaces and consist of three morphologically distinct junctional elements identified as *zonula occludens* (tight junction), *zonula adhaerens*, and *macula adhaerens* (desmosome). Along the occluding zonule, the intercellular space is obliterated by the fusion of the outer leaflets of the adjacent cell membranes; by contrast, a distinct intercellular gap is maintained along the other elements of the complex. Since the occluding zonules are apparently continuous around each cell and

throughout the epithelium, we suggested that they serve as "closing belts" which seal off the intercellular spaces from the lumen, and we postulated that they play a passive role in the maintenance of chemical and electrochemical potential gradients across epithelia by impeding back diffusion (leaks) along the intercellular spaces. This assumption was tested and confirmed for large molecules by using concentrated protein solutions (hemoglobin and zymogen) as mass tracers. The solutions filled the lumina but did not penetrate the intercellular spaces beyond the occluding zonules (1). The impermeability of these zonules to water, ions, and small water-soluble molecules remained, however, to be demonstrated. Since no direct means of investigating this problem is available, we approached it indirectly by examining the frog epidermis, an epithelium known to be practically impermeable to certain small molecules. The frog skin is an extensively studied biological membrane, frequently used in studies of transport functions of epithelia (2). It is known to exhibit a unilateral osmotic response (3) and to maintain marked electrochemical potential gradients, and both properties have been localized to the epidermis (2). If occluding zonules are impermeable to water, ions, and small water-soluble molecules, they should be present in the frog epidermis, binding together the cells close to its outer surface. The observations to be presented[1] fully confirm these expectations.

MATERIALS AND METHODS

Skin taken from the abdomen and back of adult frogs (*Rana pipiens* and *Rana catesbiana*) were the principal tissues studied. For comparison, more limited observations were carried out on abdominal skin from adult toads (*Bufo marinus*), tails of young *Xenopus laevis* larvae (stage 55), and tail fins of salamander larvae (*Amblystoma punctatum*) approaching metamorphosis.

Pieces of skin taken from pithed animals, or amputated tails, were fixed for 1½ to 2 hours at 0° in 1 per cent OsO_4 in either acetate-Veronal or phosphate buffer (pH 7.6). Other pieces were fixed in 2 per cent glutaraldehyde (6) in either 0.1 M phosphate or 0.1 M cacodylate buffer (pH 7.4) for 4 to 16 hours, followed by "postfixation" for 2 hours in OsO_4. Araldite or Epon was used for embedding. Some specimens were stained in block with phosphotungstic acid (PTA) or $KMnO_4$ (*cf.* 1), during dehydration, to enhance the contrast of fibrillar and membranous structures, respectively. More recently we have extensively used staining in block, before dehydration, in aqueous uranyl acetate solutions.[2]

Sections from all these blocks were stained for 30 minutes with lead alone (8) or doubly stained with the latter preceded by 1½ hours in 5 per cent uranyl acetate. They were examined in a Siemens Elmiskop I, operating at 80 kv with a double condenser. Further details of the embedding and staining procedures can be found elsewhere (1). For light microscopy, sections of 1 to 2 μ were cut from the same blocks and stained with azure II and methylene blue (9).

OBSERVATIONS

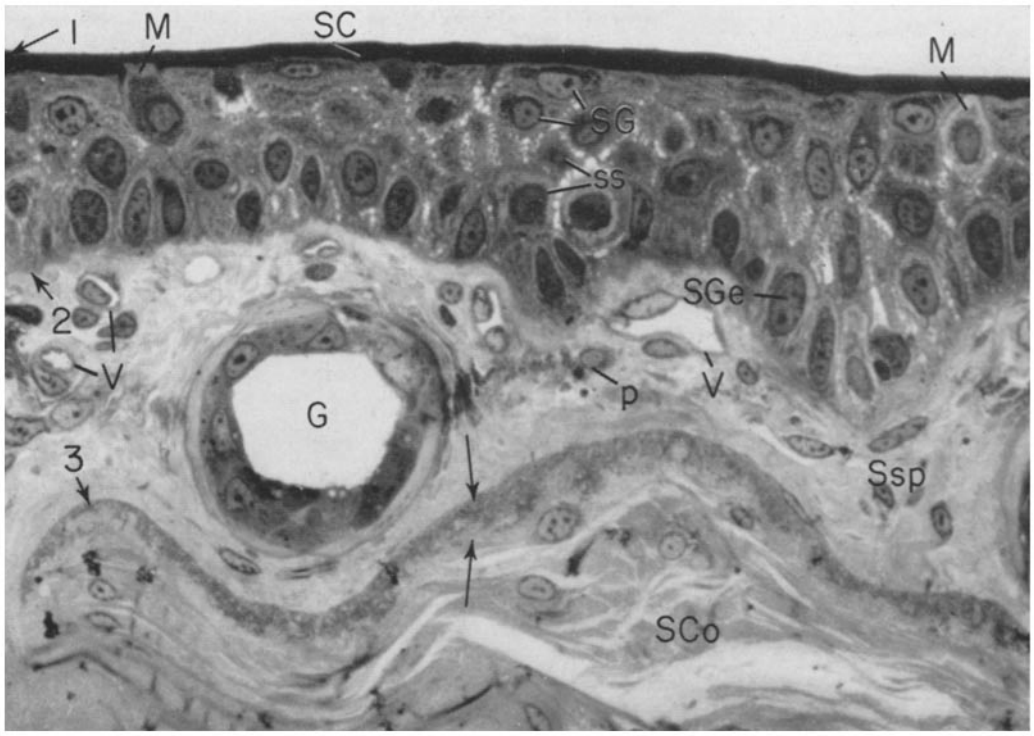
The observations presented are limited primarily to cell junctions and associated structures (*i.e.*, cell membranes, intercellular spaces, etc.) of the frog epidermis. Other aspects, such as differentiation and keratinization of the epidermis and the structure of the dermis, are not treated in any detail. However, for the sake of orientation, a brief description of the general organization of the skin is included. The bulk of the observations pertain to the skin of the frog; observations on toad and larval skin are mentioned where they are appropriate or instructive.

General Histology and Cytology of Frog Epidermis

The frog skin consists of an epidermis composed of 5 to 7 layers of epithelial cells, and an underlying dermis containing blood vessels, glands, and

[1] Preliminary reports of these findings have already been published (4, 5).

[2] This is an adaptation, for animal tissue, of the procedure of Kellenberger *et al.* (7). Specimens fixed in OsO_4 or glutaraldehyde followed by OsO_4 are treated for 2 hours at room temperature with 0.5 per cent uranyl acetate in Michaelis buffer. The final pH of the solution is 5.0. Since neither calcium nor salt is necessary for staining, the procedure can be simplified by using acetate-Veronal buffer without any additives as a diluent for both the OsO_4 and uranyl acetate. If the tissue is fixed in OsO_4 or glutaraldehyde followed by OsO_4 in phosphate buffer, the blocks must be washed (2 to 3 changes each of 15 minutes) before transfer to the staining solution in order to avoid precipitation of uranyl as uranyl phosphate. This technique is particularly effective in demonstrating the stratification of all cellular and intracellular membranes. A certain amount of extraction seems to be incurred by the specimens in the uranyl solution; it is more noticeable after fixation in OsO_4 in acetate-Veronal than after the other procedures mentioned. Contrast in sections prepared from blocks processed according to this technique can be further increased by staining.



General Abbreviations

SC: stratum corneum
SG: stratum granulosum
SS: stratum spinosum
SGe: stratum germinativum
B: basement membrane
Co: collagen fibril
D: dermis
Is: intercellular space
Zo: zonula occludens (occluding zonule)
cm: cell membrane
d: desmosome
cd: composite desmosome
md: modified desmosome
dm: dense material in intercellular space
er: endoplasmic reticulum (rough surfaced)
f: cytoplasmic filaments
fl: fusion line
g: Golgi complex
i: intermediate line in desmosome
il: inner leaflet of the cell membrane
ol: outer leaflet of the cell membrane
m: mitochondria
mc: mucous coat
n: nucleus
r: ribosomes
sg: small secretion granule
lg: large secretion granule

FIGURE 1 Photomicrograph showing the general organization of the abdominal skin of the frog (*Rana pipiens*). The epidermis (between arrows *1* and *2*) is a stratified epithelium with 1 to 2 layers of partially cornified, squamous cells in the *stratum corneum* (*SC*), 1 to 3 layers of cuboidal or polyhedral cells in the *stratum granulosum* (*SG*) and *stratum spinosum* (*SS*), and a basal layer of cuboidal or columnar cells in the *stratum germinativum* (*SGe*). The dermis is divided into a loose layer, the *stratum spongiosum* (*Ssp*) (between arrows *2* and *3*) which adjoins the epidermis, and a deeper more compact layer, the *stratum compactum* (*SCo*) (arrow *3* to bottom of field). The *s. spongiosum* contains blood vessels (*V*), bodies of glands (*G*), pigment cells (*p*), and various fibrillar and cellular connective tissue elements. The *s. compactum* (*SCo*) consists of a series of undulating layers, each composed of bundles of collagen fibrils in parallel array. From layer to layer the direction of the bundles changes by ~90°. A discontinuous layer of extracellular, material, which stains red with this procedure, forms a series of arches (unnumbered arrows) between the two dermal strata.

Near the top of the epidermis at either edge of the field are two mitochondria-rich cells (*M*) which have a fusiform shape and extend from the middle layers to the *s. corneum*. The one on the right has a relatively pale cytoplasm.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH ' and embedded in Araldite. One-micron section stained with azure II–methylene blue. × 900.

various fibrillar or cellular connective tissue elements (Fig. 1). The epidermis contains one or two outer layers of partially cornified, squamous cells (*stratum corneum*), 3 to 4 intermediate layers of cuboidal or polyhedral cells (*stratum granulosum* and *stratum spinosum*), and a basal layer of cuboidal or columnar cells (*stratum basale* or *germinativum*) (Fig. 1).

Stratum germinativum

The cells of the *s. germinativum* (Fig. 2)[3] have the usual set of subcellular components in relatively small quantities: a few mitochondria and rough-surfaced elements of the endoplasmic reticulum, some free ribosomes, a relatively rudimentary Golgi complex with one or two small flattened cisternae and associated vesicles, and variable numbers of small (pinocytic?) vesicles found especially near the lateral cell membranes. In addition, there is a well developed system of cytoplasmic filaments (formerly called tonofilaments (*cf.* 10)) grouped in bundles and anchored in either desmosomal or "basal" plates (11, 12) (Fig. 18). The basal surface of these cells rests on a basement membrane which forms a continuous layer, 500 to 700 A thick, at the dermal-epidermal boundary (Figs. 2 and 18).

Stratum spinosum and Stratum granulosum

As the cells of the epidermis differentiate and move from the germinal layer out to the *s. spinosum* and *s. granulosum*, they flatten and their bundles of cytoplasmic filaments become more abundant and more tightly packed (Figs. 3 and 6). At the same time, their rough-surfaced endoplasmic reticulum becomes more voluminous and their Golgi complexes more elaborate, with the number and size of the cisternae increasing. Multivesicular bodies also increase in number, and the cells acquire dense bodies of irregular size and shape with a heterogeneous content. Morphologically, these elements represent lysosomal derivatives, *i.e.*, residual bodies, and lytic and autolytic vacuoles (13). Moreover, they give a positive reaction for acid phosphatase in specimens fixed in glutaraldehyde, incubated in Gomori medium and examined in the electron microscope following the general procedures given in reference 6. The same applies to similar bodies found in the *s. corneum*. The frequency of all these bodies increases decidedly in the *s. granulosum* (Fig. 6) in which many autolytic vacuoles contain small vesicles, of the type seen in multivesicular bodies, as well as recognizable remnants of cell components, mostly ribosomes (Fig. 6, inset). This finding suggests that there is extensive fusion of multivesicular bodies with autolytic vacuoles as cell differentiation progresses.

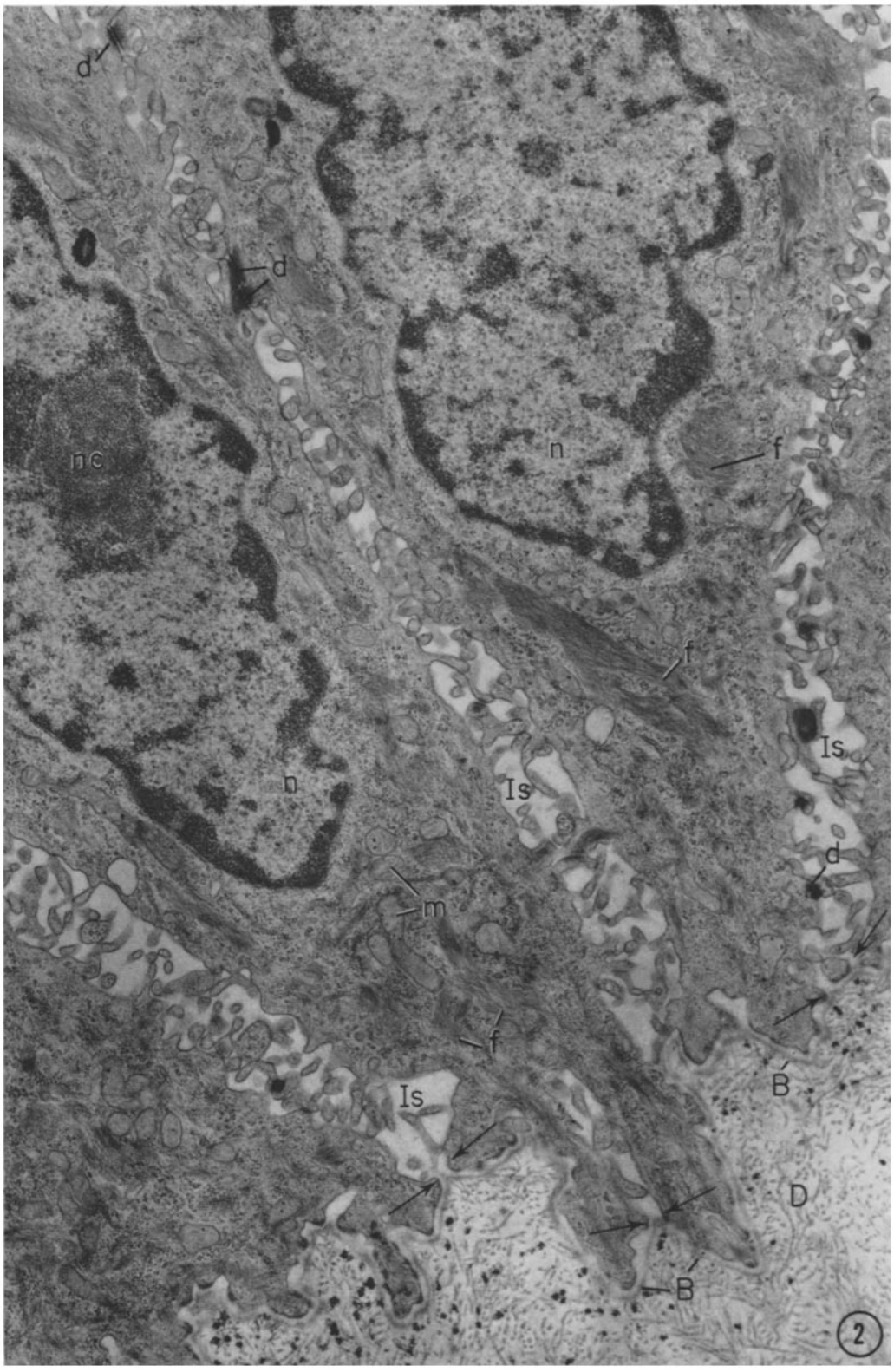
The cells of these strata contain cytoplasmic granules of two distinct types: one type is relatively small (100 to 150 mμ) in size; is spherical, ovoid or tear-like in shape; has a finely particulate, moderately dense content; and has a distinct limiting membrane. Granules of this type seem to be formed within Golgi cisternae (Fig. 4). Mature granules first appear randomly distributed

[3] Fig. 2 is from the skin of the toad, but the basic organization is the same as in the frog.

FIGURE 2 Base of the epidermis in toad skin, showing portions of four large cells of the *s. germinativum*, a highly indented dermal-epidermal junction, and part of the dermis (*D*) which forms a series of micropapillae containing mostly collagen fibrils. The basement membrane (*B*) forms a continuous (~500 A) layer which closely parallels the basal cell membranes of the germinative cells and separates dermal and epidermal elements.

The intercellular spaces (*Is*) between the basal cells are expanded and of complicated geometry due to extensive interdigitation between microvilli and other processes of adjoining cells. The spaces are open toward the basement membrane since the apposed cell membranes remain separated by a distinct (200 to 400 A) gap (arrows). Note the low frequency of the desmosomes (*d*) on the lateral cell aspects. The cytoplasm of the basal cell contains prominent bundles of filaments (*f*) as well as mitochondria (*m*) and numerous free ribosomes. The nuclei (*n*) show large nucleoli (*nc*) and dense, peripheral chromatin masses.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Araldite. Section doubly stained with uranyl and lead. × 14,000.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throughout the cytoplasm of cells immediately distal[4] to the *s. germinativum*; they are particularly numerous in the cells just beneath the outermost granular layer, where they accumulate along the distal front of the cell (Figs. 3 and 4); and are usually absent from the outermost granular layer.[5] In this connection, it should be mentioned that whereas the intercellular spaces in the *s. germinativum*, *spinosum*, and *granulosum* appear "empty" (*i.e.*, they are occupied by a material of low density), a substance similar in density and texture to the granule content fills the intercellular spaces between the *s. granulosum* and *s. corneum* and within the latter (Figs. 3, 5, and 6). The orientation of the granules along the distal cell surface, and their disappearance from the cytoplasm at a level where a similar material appears in the intercellular spaces suggest that the granule content is discharged into, and fills the intercellular spaces. Images suggesting granule discharge by membrane fusion have been occasionally encountered.

A second type of cytoplasmic granule, also membrane-limited, is much larger in size (300 to 900 mμ), more variable in shape, has a content of higher density and finer texture, and occurs primarily in the perinuclear cytoplasm (Figs. 3 and 4). These larger granules are found predominantly in the two layers located just beneath the *s. corneum*, and, in contrast to the granules of the first type, are most numerous in the outer granular layer. There is some suggestive evidence that they are formed within Golgi vacuoles (Fig. 4), but their ultimate fate is not altogether clear. They decrease in number as the cells keratinize, and some, at least, are retained in such cells and finally included and apparently destroyed in autolytic vacuoles.

Stratum corneum

Cornified elements occur only distal to the intercellular spaces filled with electron-opaque material. By implication, keratinization of a cell moving into the *s. corneum* begins between the discharge of its own small granules and the discharge of the same granules from the cell moving behind it into the *s. granulosum*. Cornification occurs gradually, for all stages from partially to fully keratinized elements can be found in this layer. Fully keratinized cells appear very dense (Figs. 3, 5 to 7), are completely occupied by cytoplasmic filaments, and contain only remnants of cell organelles which are sometimes included in autolytic vacuoles. Partially cornified cells have a cytoplasmic matrix of a variable but generally lower density, and their organelles are more numerous and less altered (Fig. 6). Some of these cells still have recognizable ribosomes, usually clumped in the remaining spaces between filaments and cell organelle remnants (Fig. 6). Ribosomal particles are absent or not recognizable in

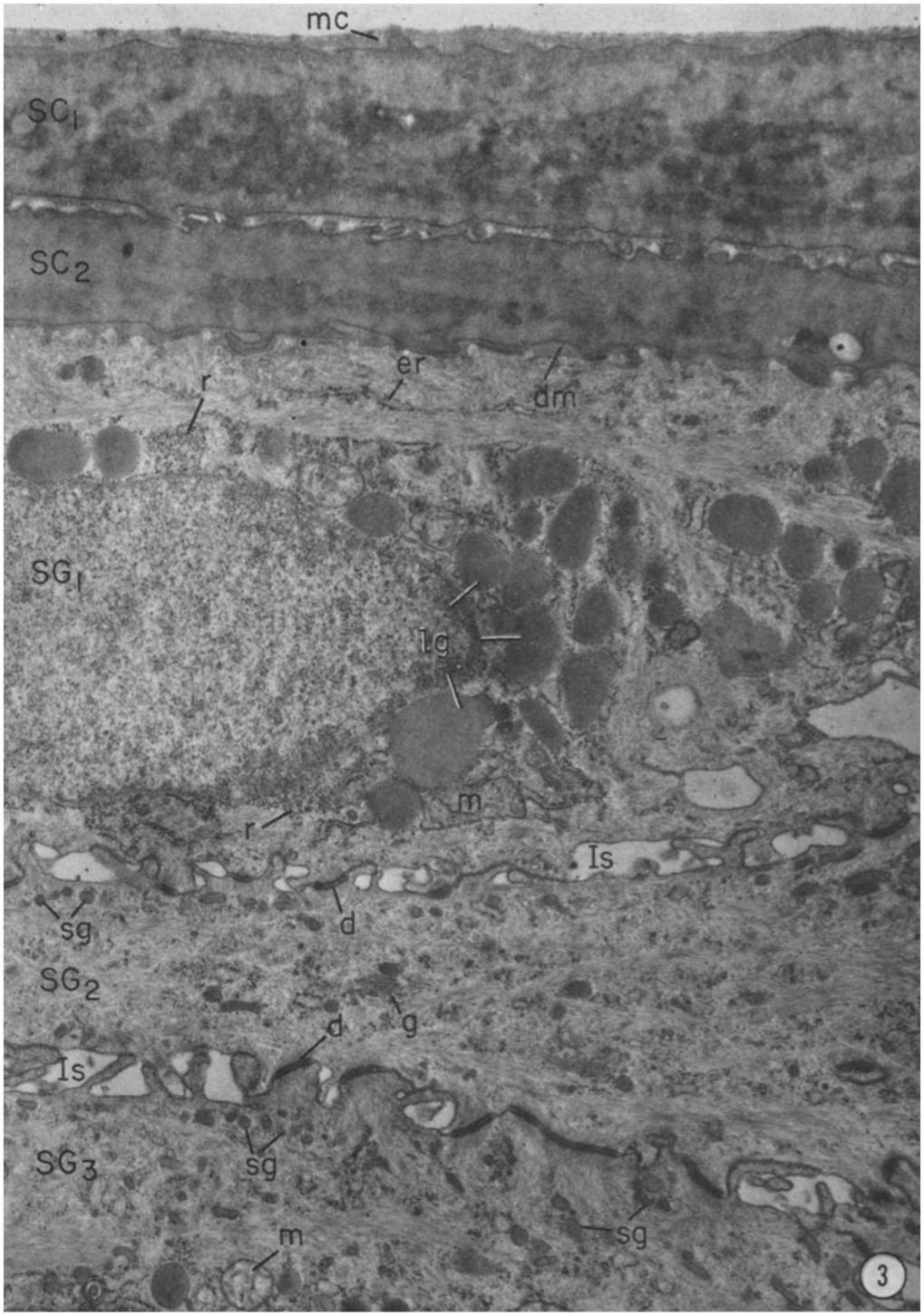
[4] The terms "distal" and "proximal" are used throughout the text to refer to the position of epidermal structures in relation to the basement membrane.

[5] They occasionally occur in small numbers along the distal front of these cells.

FIGURE 3 Outer front of the frog epidermis showing several cornified (SC_1 and SC_2) and granular (SG_1 to SG_3) layers. A dense filamentous layer (*mc*) or "mucous coat" is seen along the outer skin surface. The cytoplasm of the cornified cells appears dense and without clearly recognizable cytoplasmic organelles. The granular cells contain numerous clusters of ribosomes (*r*), a few mitochondria (*m*), and occasional cisternae of the endoplasmic reticulum (*er*) and of the Golgi complex (*g*), concentrated mostly near the nucleus. In addition, they contain numerous dense granules of two distinct types: one type (*lg*) is relatively large (300 to 900 mμ), and is found in greatest numbers in the perinuclear cytoplasm of the outermost granular cell (SG_1). The other type of granule (*sg*) is smaller (100 to 150 mμ); is absent from the outer granular cell; and is concentrated near the distal cell membranes of the second and third granular layers (SG_2 and SG_3).

Numerous desmosomes (*d*) can be made out between cells of the lower layers. The intercellular spaces (*Is*) contain a material of low density (in all probability, just embedding plastic), except between the outer granular and inner cornified cells where they are occupied by a material (*dm*) of high density.

Specimen and section preparation as for Fig. 2. × 17,000.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fully keratinized cells. When cells move from the *s. granulosum* to the *s. corneum*, they acquire a shell of dense cytoplasmic material along the cell membrane (*vide infra*) and the latter becomes thicker, as already noted by Rhodin and Reith (14) in mammalian keratinizing epithelia.

Special Cell Types within the Epidermis (Mitochondria-Rich Cells)

As is well known, various specialized cell types occur scattered throughout the epidermis. In the frog, occasional lymphocytes, macrophages, and pigment cells can be recognized, particularly in the deep strata. In addition, we have regularly encountered another type of cell, located predominantly in the *s. spinosum* and *s. granulosum*. Such cells are typically shaped like an elongated flask with a perikaryon in the *s. spinosum* and a thick main process extending up to the *s. corneum* where it sometimes branches into a number of secondary processes. Cytologically, the most distinctive features of these cells are a relatively light matrix and the presence of large numbers of mitochondria, especially in the main process. We refer to these elements as "mitochondria-rich cells," as done by Choi (15) for similar cells found in the toad bladder (15, 16). They contain the usual set of cell organelles, but usually no secretion granules, and are attached to neighboring epidermal cells by desmosomes which are similar to, but noticeably less frequent than, those between usual epidermal cells at this level.

Cell Membranes

The cell membranes of all except the cornified elements are of the thin, asymmetrical type, with a total thickness of about 70 to 80 A, and an outer leaflet thinner and less dense than the inner one. As pointed out elsewhere (1), the outer leaflets of membranes of this type are difficult to see in OsO_4-fixed specimens, but can be regularly demonstrated after fixation or staining in $KMnO_4$ (compare Figs. 13 and 18, which were not stained in $KMnO_4$, with Figs. 14, 15, and 17 which were). The outer leaflets are especially well demonstrated after staining in block with uranyl acetate (Figs. 16, 19, and 20). In tissues fixed in glutaraldehyde followed by OsO_4, the usual asymmetry is reversed by this procedure (see Figs. 16 and 19).

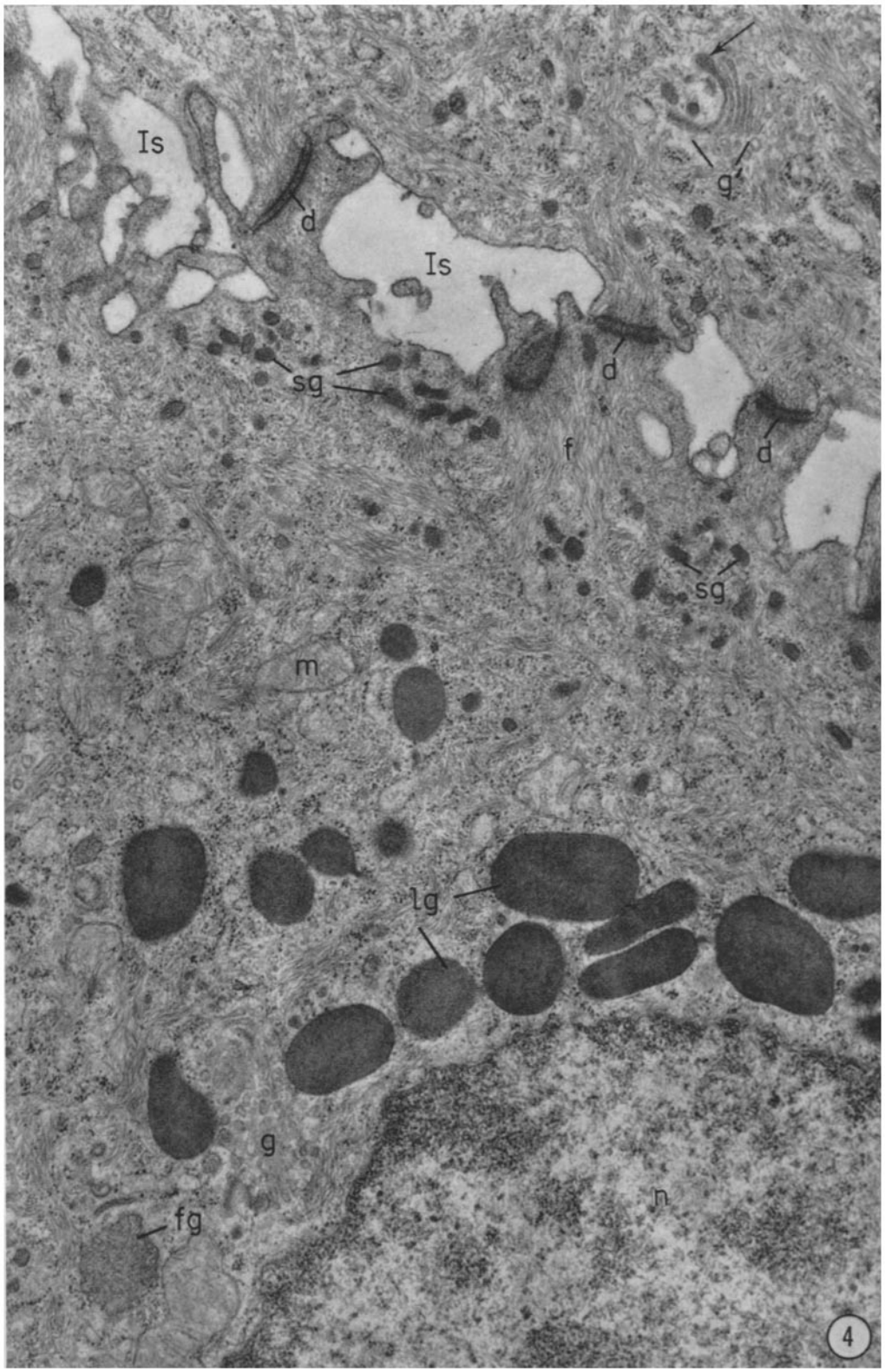
The entire cell membrane of the cornified cell is of the thick (100 A), nearly symmetrical type (*cf.* 1). The dense outer leaflet is clearly visible in OsO_4- or glutaraldehyde-fixed tissue stained only with lead (Figs. 7 to 9 and 11). The light layer stands out sharply because of the generally high density of the cornified cell cytoplasm (Figs. 8 and 9). This thick membrane of the cornified cells is backed all around the cell surface by a continuous (200 to 300 A thick) shell of dense material located in the cytoplasm immediately adjacent to the inner leaflet (Figs. 7, 10 to 13).

The surface membrane of the cells of the outer cornified layer is covered by a dense fibrillar coating (Figs. 5, 7 to 9, and 11) which varies in

FIGURE 4 Cells from the *s. granulosum* of the frog epidermis, to illustrate the two different granule populations found in the cells of the middle strata. One type (*lg*) is large in size, membrane-bounded, variable in shape, and occurs primarily in the perinuclear cytoplasm. The other type (*sg*), also membrane-limited, is much smaller in size, ovoid or tear-shaped, and accumulates along the cell membrane at the distal cell front, facing the expanded intercellular spaces (*ls*).

Numerous free ribosomes, bundles of cytoplasmic filaments, and mitochondria (*m*) occupy the remaining cytoplasm. Some of the filaments (*f*) converge on desmosomes (*d*) at the cell junctions. Part of a Golgi complex is seen at *g* on the lower left. Near it there is an irregularly shaped body (*fg*) with a moderately dense content which is believed to represent a "condensing vacuole," *i.e.*, a granule in formation within the Golgi complex. Part of another Golgi complex (*g'*), consisting of a pile of 5 to 6 flattened cisternae, is seen in an adjoining cell on the upper right. A small granule appears to be forming by "budding" from the tip of the innermost Golgi cisterna (arrow).

Specimen and section preparation as for Fig. 2. × 28,000.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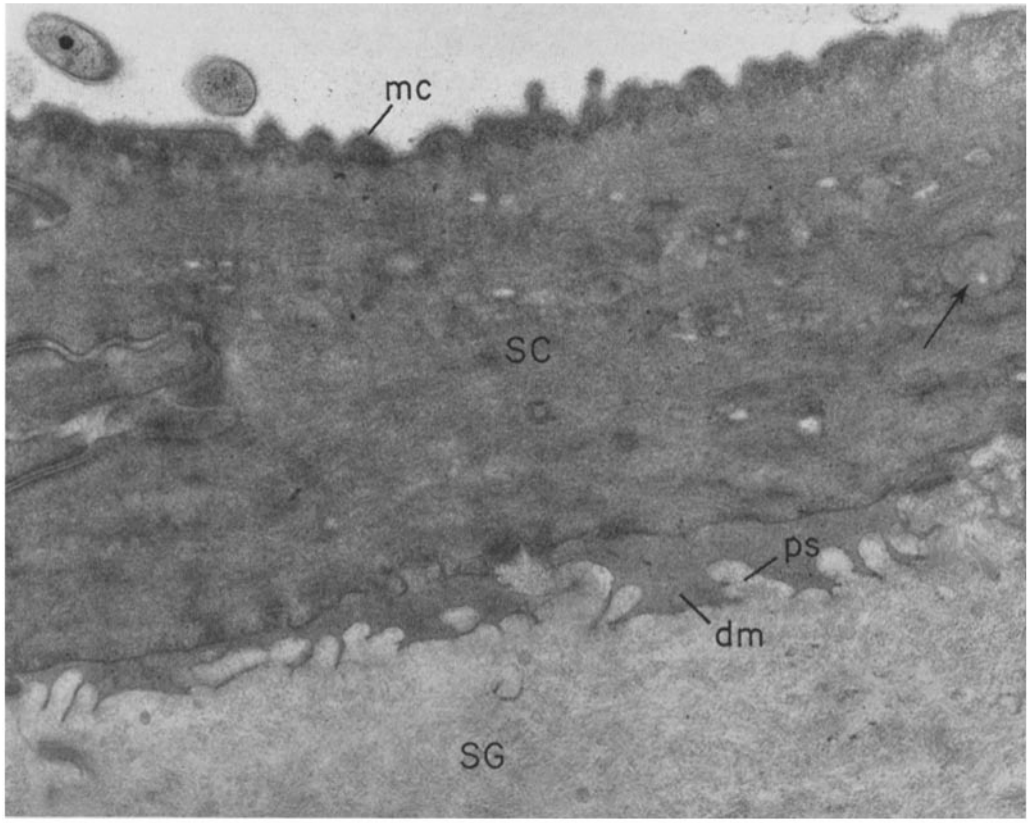


FIGURE 5 Outer front of the frog epidermis showing portions of a cornified (*SC*) and of a granular cell (*SG*). The intercellular spaces between the two layers are occupied by a material (*dm*) of high density and relatively fine texture which completely fills these spaces and outlines their connections. The contours of the spaces are more elaborate on the *s. granulosum* side owing to the presence of numerous pseudopodia (*ps*) projecting from the cell surface. Remnants of cytoplasmic organelles (arrow) are barely visible in the cytoplasm of the cornified cells. A fluffy mucous coat (*mc*) can be made out along the outer membrane of the same cells. Several bacteria occur in close association with the outer skin surface.

Specimen fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6) with sucrose, dehydrated in alcohol, stained with PTA, and embedded in Araldite. Section doubly stained with uranyl and lead. × 20,000.

thickness and appearance according to the method of fixation and staining: it appears particularly thick and well preserved after fixation in phosphate-buffered OsO_4 or in glutaraldehyde followed by OsO_4, but is less abundant after fixation in acetate-Veronal–buffered OsO_4. Its fibrillar texture is particularly evident after PTA staining. This layer presumably corresponds to the "mucous coats" previously described in the epithelium of the toad bladder (15, 16), gastric (17) and intestinal (18) mucosa.

Cell Junctions

IN THE STRATUM CORNEUM: On the outer front of the epidermis, near the point at which the membranes of two adjacent cells turn from the distal to the lateral cell surface, the outer leaflets of the converging membranes come together and fuse into a single dense band ~30 to 40 A thick (Figs. 7 to 9). The two fused membranes measure 170 to 180 A across. The extraneous fibrillar coating of each membrane stops at, or shortly before, the line of mergence. Usually, the band of fusion runs

uninterrupted for 0.1 to 0.3μ in the distal to proximal direction, although occasionally one or several focal splittings occur within the junction (Fig. 8). At the proximal end of the junction, the outer leaflets separate again, leaving in between an intercellular space of 250 to 300 A which is retained, except for focal dilatations, along most of the remaining parallel, zig-zagging course of the lateral cell membranes (Fig. 7). Such areas of membrane fusion are found in the location described wherever the plane of sectioning approaches normal to the plane of the intercellular boundary, and, in favorable sections, are seen to run for considerable distances along the apical perimeter of the cells. Hence, it can be assumed that they are continuous around each cell and throughout the *s. corneum*, like their counterparts already described in various columnar epithelia (1). As such they can be properly described, according to the nomenclature we introduced, as *zonulae occludentes*, *i.e.*, closing belts.

The occluding zonule is followed by a whole series of elements (Figs. 7, 10, and 12) which resemble desmosomes in that they are characterized by strict parallelism of the apposing cell membranes, the presence of laminar densities in the intercellular space, and the occurrence of concentrations of dense amorphous and fibrillar material in the subjacent cytoplasmic matrix. They differ, however, in several respects: the plate of intercellular material is usually more dense; there is no distinct cytoplasmic plaque, but rather a continuous shell (described above) of condensed cytoplasmic matrix around the entire cell; and the bundles of filaments associated with this shell are rather indistinct. (For further details see legend for Fig. 12). These junctional elements are commonly more curved than desmosomes (Figs. 7 and 12) and occasionally show sharp bends. In addition, the fraction of the lateral cell surface occupied by such elements is much greater than that occupied by ordinary desmosomes elsewhere in the epidermis (see Fig. 7).

These modified desmosomal elements are interrupted at intervals by focally expanded intercellular spaces and occasionally by small areas of membrane fusion; the latter are irregularly distributed and shallow in depth and hence are considered to be discontinuous structures, *i.e.*, maculae or fasciae (spots or bands), rather than zonulae (belts).

When more than one layer of cornified cells is present (generally there are *two* such layers), occluding zonules are found in a similar (distal) location along the lateral aspects of the deeper layer (Fig. 10).

Along the proximal surface of the innermost cornified layer, across the intercellular spaces characterized by a dense content, there are bipartite or composite desmosomes (Fig. 13). Their distal half has the structure of the modified desmosomes of the cornified cells (*i.e.*, thicker cell membrane, continuous cytoplasmic shell instead of a distinct plaque) and their proximal half that of the ordinary desmosomes of the deeper layers. These composite junctional elements thus reflect the distinctive features of the involved cell surfaces.

IN THE STRATUM GRANULOSUM: Occluding zonules are found in yet another location, *i.e.*, between cells of the outer layer of the *s. granulosum* which face the intercellular spaces filled with an electron-opaque substance (Fig. 14). Like those in the keratinizing layers, these zonules are located where the cell membranes turn from the distal to the lateral cell surface and are found in most normal sections. In this case, however, the total thickness of the junction is less (only 120 to 140 A), as expected from the smaller dimensions of the component cell membranes. Typical desmosomes (*vide infra*) are found along the remainder of the perimeter of these cells, except for their distal aspect which, as described above, is provided with bipartite or composite desmosomes.

IN THE OTHER STRATA: Typical desmosomes or adhering maculae form the predominant type of junctional element found in the remaining strata. They are least numerous and complex in the *s. germinativum* and reach their fullest development and highest frequency in the *s. granulosum*. The structure of desmosomes is by now well known[6] (Fig. 16): they consist of areas of strict parallelism of the cell membranes; an intercellular space occupied by a plug or disc of dense material bisected by a denser central layer; dense cytoplasmic

[6] There is still some lingering confusion (see reference 19) concerning the interpretation of the "intermediate dense layer" (20) or "lateral dense line" (19). According to Odland (20) and more recently to Roth and Clark (19), this layer is an extracellular structure, whereas our evidence indicates that in the epidermis, as in many other epithelia studied (21, 1), it is the outer leaflet of the cell membrane (see Fig. 16).

plaques backing the inner membrane leaflets; and profuse bundles of cytoplasmic filaments converging on the inner aspect of each plaque.

Small areas of membrane fusion also occur throughout the epidermis (Figs. 15 and 17). Based on their limited dimensions and irregular distribution, they are believed to be discontinuous structures, *i.e.*, maculae or fasciae rather than zonulae.

Intercellular Spaces

Except for the spaces between the *s. corneum* and *s. granulosum*, which contain the electron-opaque amorphous material described above (see Figs. 3 and 5), intercellular spaces throughout the epidermis are occupied by a material of low density with only occasional clumps of flocculent precipitate (Figs. 2 to 4 and 6). The geometry and volume of these spaces vary considerably from layer to layer: they reach their greatest volume in the *s. spinosum* in which they are frequently expanded into so called "intercellular lakes." Indeed it is the firm adherence of the cells at the desmosomes, combined with the marked dilation of the intercellular spaces in between, that creates the typical "prickle-cell" appearance of this stratum. The spaces are least voluminous in the *s. granulosum* and are most tortuous and complex in the *s. germinativum* owing to extensive interdigitation between cells (Figs. 2 and 18).

Arrangements at the basal ends of the intercellular spaces, where germinal cells meet the basement membrane, are somewhat variable: most often there is the usual 200-A gap between adjoining cells (Figs. 2 and 18); sometimes the gap is wider or is bisected by an intermediate line similar to that present in the urinary slits of glomerular capillaries; occasionally it is bridged by a desmosome (Fig. 19); and only rarely areas of membrane apposition rather than fusion (Fig. 20) are encountered in this location. Hence, the intercellular spaces at this level are apparently patent or open towards the dermis. This situation contrasts markedly with that described above at the surface of the epidermis.

Dermo-epidermal Junction

At the base of the *s. germinativum*, where the epidermis meets the dermis, there are two distinct extracellular layers: (1) a thin (500 to 700 A)

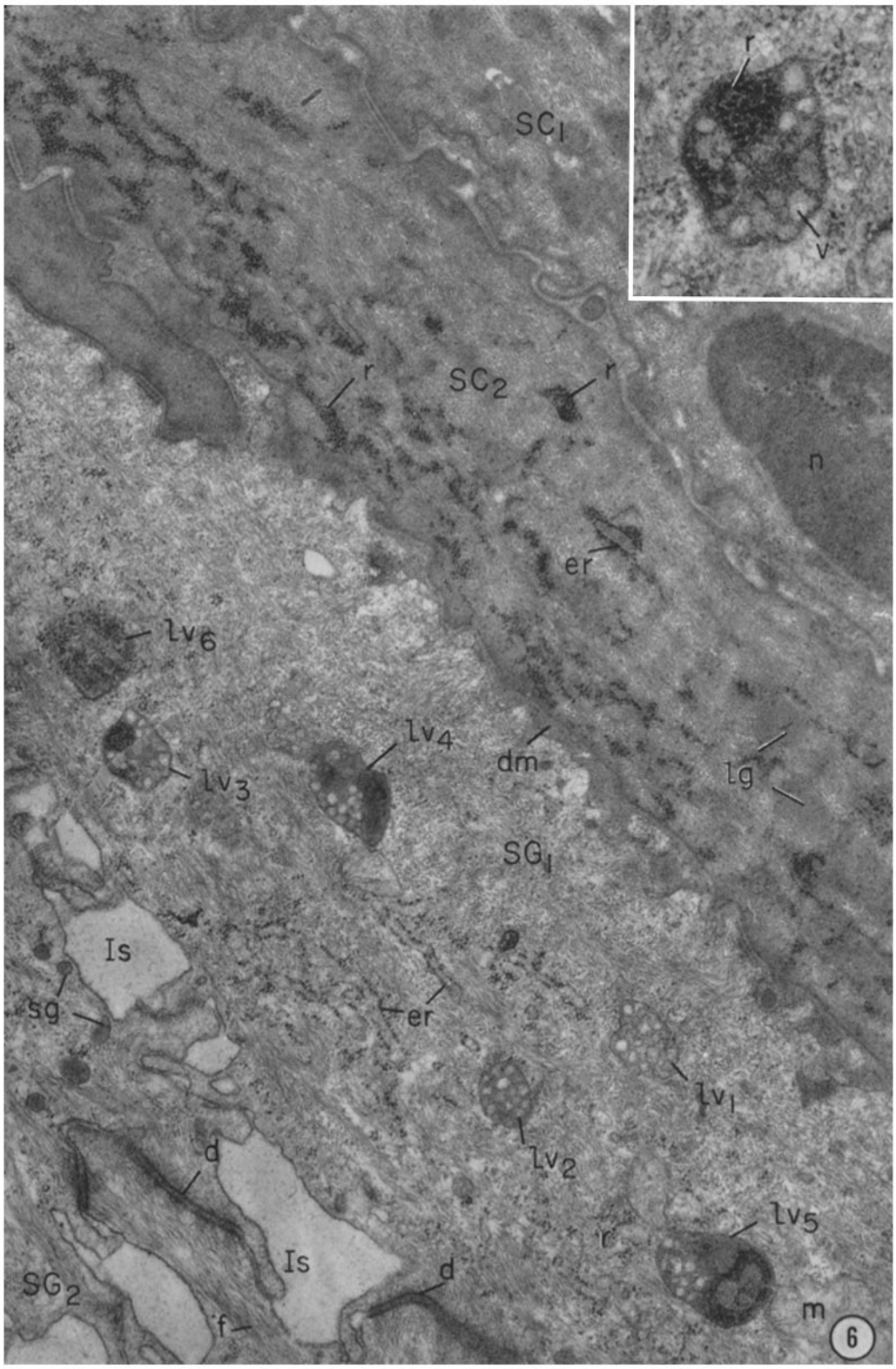
FIGURE 6 Field near the outer front of frog epidermis showing parts of cells from two cornified (SC_1, SC_2) and two granular (SG_1, SG_2) layers. The cytoplasm of the granular cells contains numerous cytoplasmic filaments (some of which (*f*) are seen to insert on the desmosomes (*d*)), a few profiles of rough-surfaced endoplasmic reticulum (*er*), free ribosomes (*r*), mitochondria (*m*), and a number of bodies (lv_1 to lv_6) which represent lysosomal derivatives. Several of the latter (lv_1 and lv_2) contain small vesicles embedded in a matrix of high density, and correspond to multivesicular bodies. Others (lv_3 to lv_6), which also contain ribosomal aggregates, correspond to autolytic vacuoles. One of these is enlarged in the inset. The presence of vesicles of the type found in multivesicular bodies along with recognizable remnants of cell organelles suggests that multivesicular bodies and autolytic vacuoles merge.

As the cells keratinize and move outward, they become increasingly more dense and compact, more devoid of recognizable cell organelles, and more filled with cytoplasmic fibrils. The cell of the second cornified layer (SC_2) is only partially keratinized and still contains recognizable organelles: large clusters of ribosomes (*r*), several large granules (*lg*), and elements of the rough-surfaced endoplasmic reticulum (*er*). The outermost cornified cell is fully keratinized; both its nucleus (*n*) and cytoplasm appear very dense, with the latter occupied almost completely by tightly packed bundles of filaments.

Note that the intercellular spaces between the granular and cornified cells are filled with dense material (*dm*), whereas those between granular cells (*Is*) appear "empty." Several granules of the small variety (*sg*) are seen in the cytoplasm near one of these spaces.

The inset shows an autolytic vacuole with its distinct limiting membrane, dense matrix, and content of ribosome aggregates (*r*) and membrane-limited vesicles (*v*) with light content.

Specimen and sections prepared as for Fig. 2. × 30,000. Inset, × 66,000.


SC1
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finely fibrillar layer, which closely parallels the basal cell membranes of the *s. germinativum* and corresponds to basement membranes found elsewhere wherever epithelium adjoins connective tissue; and (2) a thicker (5 to 7 μ) frequently discontinuous layer, which consists of plies of collagen fibrils whose orientation changes by $\sim$90° from one ply to another. This last layer has an organization similar to that of the basement lamella of larval amphibian epidermis (11, 12, 22). It is visible by light microscopy, and has been called the "basement membrane" or glassy layer of the skin (*cf.* 23, 24). In this study, however, we shall use the term "basement membrane" to refer to the first, thin (500 to 700 A) layer which lies in close association with the germinal cells, since this is the type of structure to which the term is generally applied in the electron microscope literature, including that concerning the epidermis (*cf.* 25).[7]

[7] For other reasons than possible confusion in the description of skin layers, "basement membrane" has been criticized by Fawcett (27) and Coggeshall and Fawcett (26) who proposed "basement lamina" and "basal lamina," respectively, as more adequate terms. Admittedly, "basement membrane" is not accurately descriptive in many situations, but we prefer to retain it because it is widely used and generally understood to designate the structure with which we are concerned. Attempts to establish a new nomenclature based on the chemistry rather than morphology of this structure (see Bennett (28) "glycocalyx") have inherent merits, but may be premature (*cf.* 29, 30).

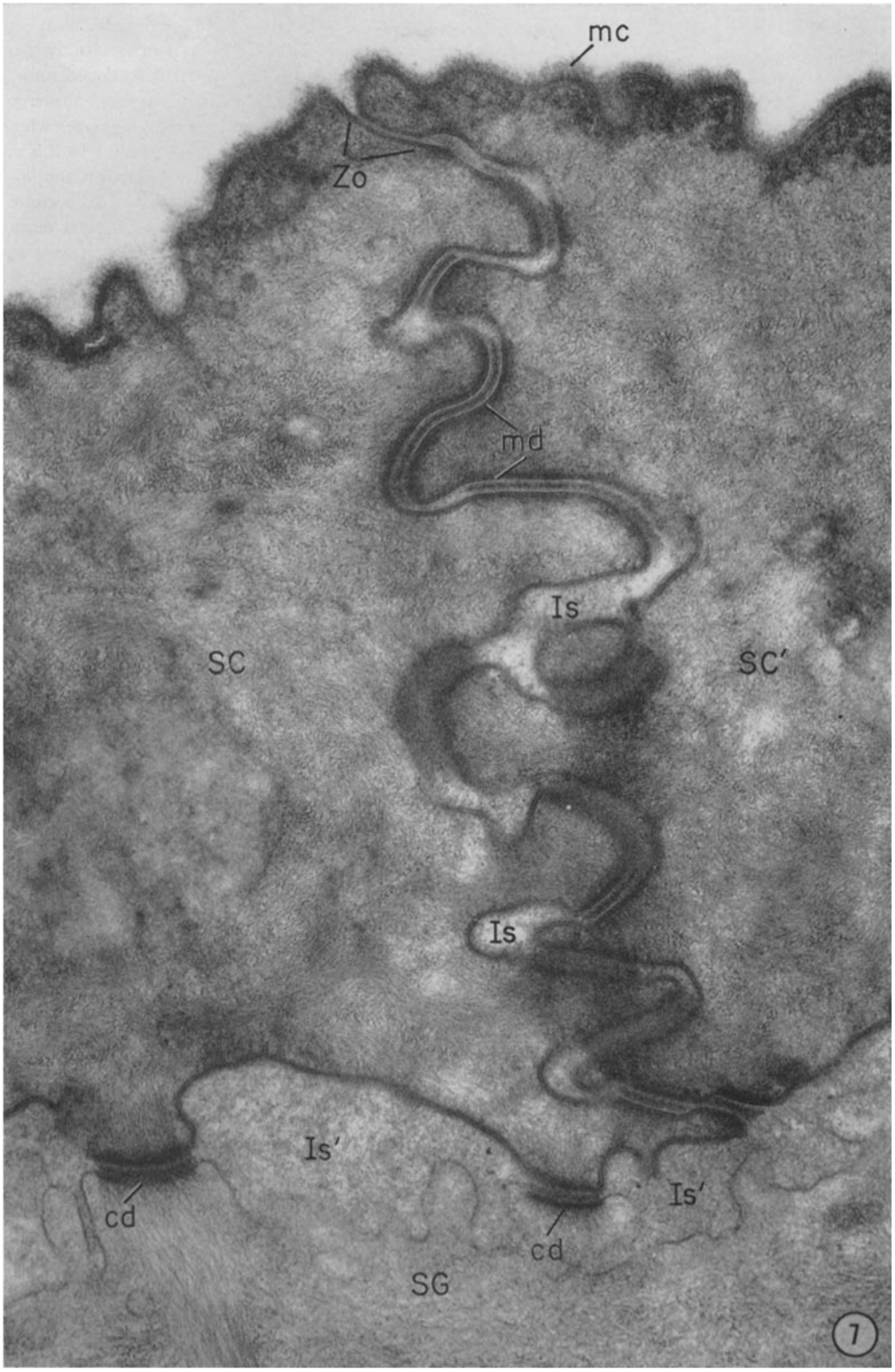
The dermal front of the epidermis and the basement membrane which courses strictly parallel to it have a highly irregular, scalloped surface (Fig. 18). Between the scallops are numerous dermal micropapillae, 0.3 to 0.5 μ wide, which are part of a relatively wide space (1 to 2.5 μ), located between the basement membrane and the plies of collagen fibrils. This space is occupied by a variety of fibrillar elements disposed mostly at random (Fig. 18). Some of these elements are typical collagen fibrils. Others are finer fibrils, $\sim$100 A in diameter, of a type found in many other locations in the connective tissue (*cf.* 31). Finally, others appear to be a characteristic component of this part of the dermis (Fig. 18) and are described in detail in a subsequent paper (32).

Junctions in Toad Skin

In the number and arrangement of its layers, the toad epidermis is quite similar to that of the frog, except that cell stratification is less regular and transition between non-cornified (granular) and cornified cells is less abrupt. As far as junctional arrangements are concerned, the situation is identical: the cells of the 1 or 2 outermost cornified layers are joined at the distal end of the corresponding intercellular spaces by occluding zonules (Fig. 9); a second (or third) network of similar zonules occurs in the outer granular layer; modified, composite, and regular desmosomes are found in the usual locations; occluding maculae or fasciae (Figs. 15 and 17) occur scattered

FIGURE 7 Junction line between two cornified cells (*SC* and *SC'*) on the outer front of the frog epidermis, showing the parallel zig-zagging course of the closely apposed, lateral cell membranes. Near the point where the cell membranes turn from the superficial to the lateral cell surface, there is a *zonula occludens* (*Zo*) which measures about 1700 A in depth. It is formed by the fusion of the outer leaflets of the converging membranes with resultant obliteration of the intercellular space at this level. At the proximal end of the occluding zonule, the outer leaflets separate again leaving between an intercellular space of 250 to 300 A which is retained, except for focal dilatations (*Is*), along most of the remaining tortuous course of the lateral cell membranes. Below the occluding zonule, there is a whole series of modified desmosomal elements (*md*) with their characteristic dense plugs in the intercellular spaces. (See also Figs. 10 and 12.) Bends occur along some of these elements. Two of the composite desmosomes (*cd*), characteristically found along this cell surface, appear at the base of the cornified cells facing the *s. granulosum* (*SG*). (See Fig. 13.) In this location the intercellular spaces (*Is'*) are occupied by a granular material of moderate density. Note that junctional elements occupy a greater proportion of the lateral than of the proximal cell surface. A fluffy, finely filamentous layer or "mucous coat" (*mc*) covers the outer surface of the epidermis.

Specimen and section preparation as for Fig. 5. × 55,000.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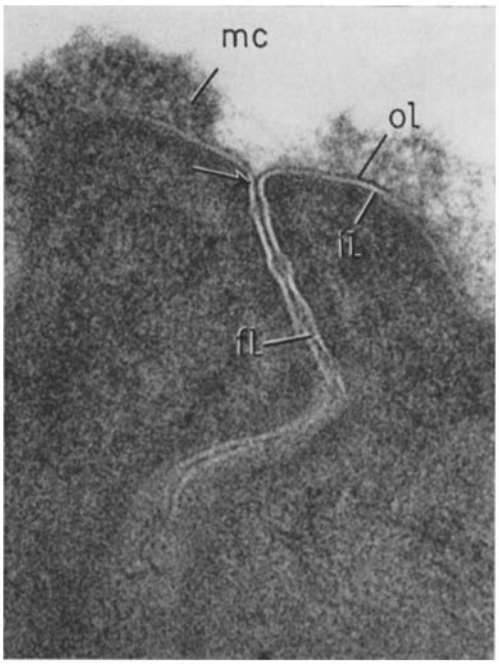


FIGURE 8 Detailed structure of an occluding zonule between two cornified cells of the frog epidermis, showing fusion of the adjoining cell membranes (arrow) where the outer leaflets (*ol*) converge. The total width of the junction is ~180 A. Within the junction there are several focal splittings of the fusion line (*fl*) followed by refusion. Note that the cell membrane of the cornified cells is of the thick (~100 A), nearly symmetrical type with the outer leaflet (*ol*) appearing as thick and as dense as the inner one (*il*). The light layer stands out sharply due to the great density of the background cytoplasm of the cornified cells. A thick layer of fluffy, finely filamentous material (*mc*) coats the outer surface of the cell membrane.

Specimen fixed in glutaraldehyde in phosphate buffer (pH 7.4), postfixed in OsO_4, and embedded in Araldite. Section doubly stained in uranyl and lead. × 165,000.

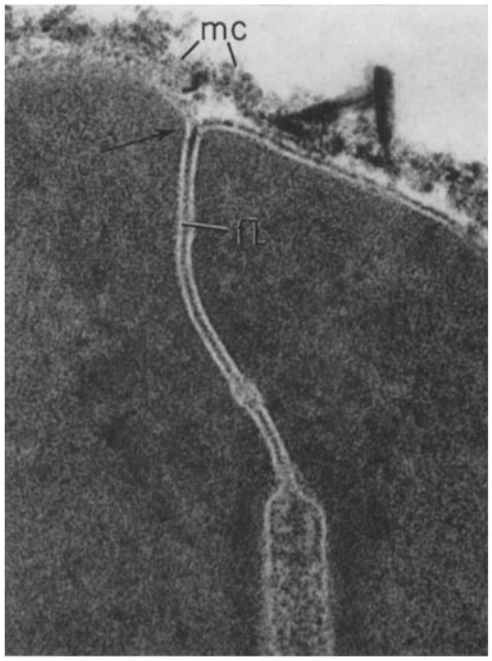


FIGURE 9 Occluding zonule between two cornified cells of the toad epidermis, similar to Fig. 8 (frog), showing the point of mergence of the adjoining cell membranes (arrow) and the fusion line (*fl*) of the junction. Specimen and section preparation as for Fig. 2. × 165,000.

through all layers; and the intercellular spaces of the *s. germinativum* generally remain opened towards the dermis by "gaps" which measure ~200 A or more (Fig. 2).

In other respects, however, the cells of the toad epidermis differ from those of the frog. In each layer, their cytoplasm is much more heavily endowed with organelles than in the corresponding layers in the frog epidermis. Cells of the *s. germinativum* contain predominantly free ribosomes and bundles of filaments in large quantities (Fig. 2). As the cells differentiate and move toward the distal strata, cell organelles increase in number and variety so that the cells of the *s. granulosum* are literally packed with free ribosomes, filament bundles, mitochondria, numerous granules of several distinct types, and smaller quantities of rough-surfaced endoplasmic reticulum and Golgi elements. In addition, the cells of the *s. spinosum* and *s. granulosum* contain large numbers of irregularly shaped dense bodies with a heterogeneous content, probably residual bodies of lytic or autolytic vacuoles.

Junctions in Larval Epidermis

In *Amblystoma* larvae approaching metamorphosis, the epidermis has a more regular (less scalloped) dermal front, is only 4 or 5 cell layers thick, and is less clearly stratified than in the adult amphibians examined; however, a *s. corneum* and a *s. granulosum*, each one cell layer thick, are clearly recognizable. The system of cell junctions

is generally similar[8] to that described in the adult frog. It consists of typical occluding zonules and modified desmosomes in the *s. corneum*, composite desmosomes between the *s. corneum* and *s. granulosum*, a more or less complete set of occluding zonules immediately behind the outer front of the *s. granulosum*, and regular desmosomes throughout the rest of the epidermis. As in the frog, the intercellular spaces are closed towards the outer front of the epidermis and open, frequently through gaps larger than 200 A, towards the dermis; an intercellular subcompartment, filled with discharged secretion, is found between the *s. granulosum* and *s. corneum*.

In young *Xenopus* larvae (stage 55) the epidermis is only two cell layers thick. The cells of the outer layer are joined to one another by typical junctional complexes composed of an occluding zonule in the usual location, followed proximally by an adhering zonule of variable depth, and, in places, by one or more small desmosomes. The complex is, therefore, similar to that described previously in columnar epithelia (1). The cells of the inner layer are joined by usual desmosomes.

No frog larvae have been examined in this study, but a note recently published by Lanzavecchia (33) clearly demonstrates the existence of typical occluding zonules in the outer cell layer of the epidermis in *Rana esculenta* larvae.

DISCUSSION

General Organization of the Epidermis

Our findings demonstrate the regular occurrence of occluding zonules, *i.e.*, belts of membrane fusion, between the cells of the outermost layer of the frog epidermis. These zonules bind together the superficial cells into an uninterrupted sheet which constitutes a structurally continuous barrier throughout the entire epidermis.

Although the amphibian epidermis is a stratified squamous epithelium, the junctional arrangement is basically similar to that encountered in the simple columnar epithelia studied previously, in that the intercellular spaces are closed near the surface facing the external medium and open toward the subepithelial spaces.

The arrangement differs, however, in other respects: the complete junctional complex characteristic of lumen-lining epithelia (*i.e.*, a *zonula occludens*, followed by a *zonula adhaerens*, and a *macula adhaerens* or desmosome) is found only in the distal (outermost) epidermal layer of early larvae. In the corresponding layer of adults and larvae approaching metamorphosis, the occluding zonule is followed directly by a series of modified desmosomes. A second set of occluding zonules occurs in the proximal cornified layer (when such a layer is present), and a second or third set is usually encountered in the distal layer of the *s. granulosum*. Throughout the rest of the epidermis, junctional elements—irrespective of type (*i.e.*, *maculae occludentes* and conventional desmosomes)—are discontinuous structures. As a result of this arrangement, the epidermal cells are firmly bound together; yet most intercellular spaces form a continuous network, a common labyrinthine compartment, which is closed towards the external medium and opened toward the interstitia of the dermis through the numerous, patent intercellular gaps of the *s. germinativum*. This main compartment may not include the intercellular spaces of the *s. corneum*, which apparently form one or two isolated subcompartments: one located within the *s. corneum*, and the other between the latter and the *s. granulosum*. Both seem to be closed by occluding zonules on each side, and usually contain a dense material, presumably discharged secretion.

The cells of the epidermis, joined by numerous junctional elements, form a network complementary to that of the extracellular spaces. Structurally, this network is divided into distinct cell territories, but functionally it may behave as a continuous compartment (*vide infra*).

Structural Aspects

JUNCTIONAL ELEMENTS: A rather extensive literature already exists on various aspects of the fine structure of the integument in a variety of species (*cf.* 25, 34, 35). As far as intercellular junctions are concerned, previous studies have dealt primarily with the structure of desmosomes (20, 36), the predominant junctional element of the epidermis. Several investigators (37, 38) have noted that the desmosomes found along the

[8] In some places, however, where keratinization is less advanced, the occluding zonule is followed by an element reminiscent of an adhering zonule and by one or more desmosomes within the cornified layer. In such cases, composite desmosomes are absent and the existence of a distinct intercellular subcompartment between the *s. corneum* and *s. granulosum* is questionable.

lateral aspects of fully or partially cornified cells differ from those in the other strata. These modified desmosomes appear in micrographs published in many studies of the epidermis (*e.g.*, 39, 25), but their special features have not been commented upon. Our observations show that such junctions occupy a much larger proportion of the lateral cell surface in the *s. corneum* than do conventional desmosomes in the deeper layers of the epidermis. It follows that prior to, or during keratinization interdesmosomal membrane must be withdrawn into the cell, or that the cell must produce and discharge into the extracellular space the material needed for the formation of additional intercellular plates.

The composite desmosomes along the proximal aspect of the innermost cornified layer have not been described before.

"Quintuple-layered cell interconnections," probably corresponding to our *maculae occludentes*, were found by Karrer (21) in the human cervical epithelium, and a similar junctional element was mentioned by Brody (40) in psoriatic human epidermis. Recently Dewey and Barr (41) described, under the name of "nexus," the same type of junction in the deeper strata of the frog epidermis. They indicate that such junctional elements are more frequent after fixation in $KMnO_4$ than in OsO_4. In our experience, the difficulty in recognizing *maculae occludentes* in OsO_4-fixed tissues is due primarily to inadequate contrast in the outer leaflets of the cell membranes. With our staining procedures, we have found such junctions throughout all the strata of the frog epidermis after OsO_4-fixation.

SECRETORY GRANULES: Our study demonstrates the occurrence of two distinct types of granules in amphibian epidermal cells; both are membrane limited, have a relatively homogeneous, dense content, and seem to originate in the Golgi complex.

One type, the smaller (100 to 150 mμ) and coarser in texture, appears to be discharged into the intercellular subcompartment located between the *s. corneum* and the *s. granulosum*. Granules which correspond in size and location to this first type have already been described in the mammalian epidermis (human (42) and mouse (43, 14)), as well as in the epithelia of the oral (14, 44) and esophageal (14) mucosae. Some of these granules differ in the organization of their content (*cf.* 43, 44), but all seem to have a similar fate: discharge into the intercellular space between the *s. corneum* and the *s. granulosum*, as originally suggested by Frei and Sheldon (43).

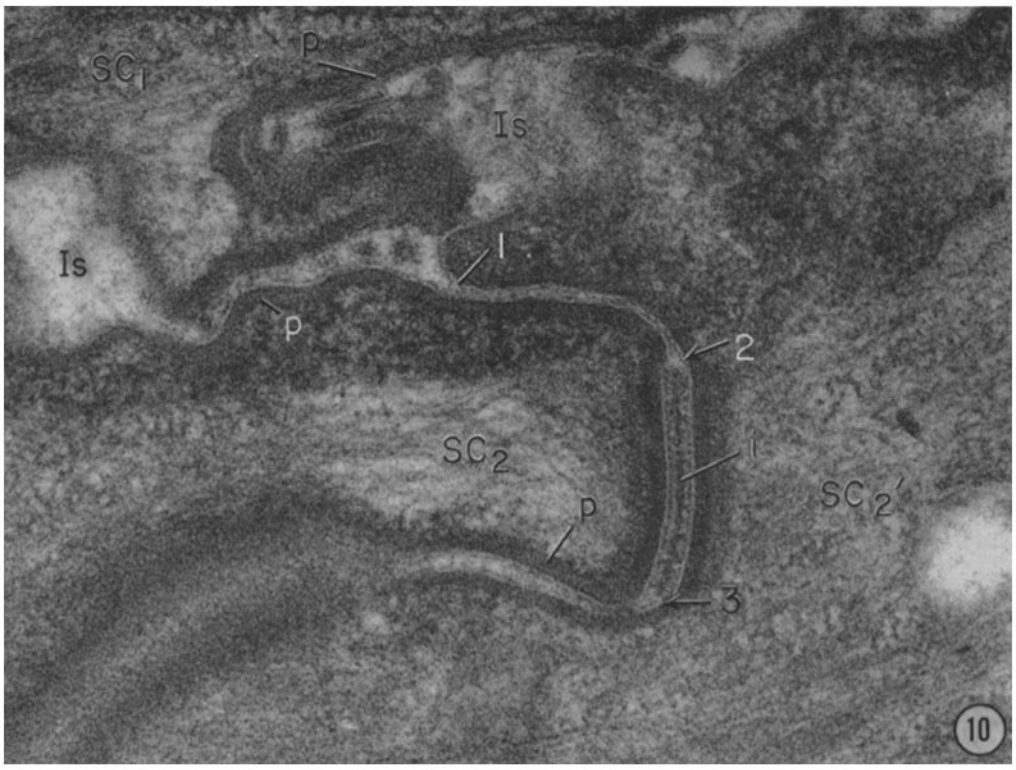
The granules of the second type are much larger (300 to 900 mμ).[9] Their exact fate is un-

[9] The large granules are visible with the light microscope (*cf.* Fig. 1); hence their presence justifies the recognition of a *s. granulosum* in amphibian epidermis. It should be pointed out, however, that they do not consist of keratohyalin (*cf.* 46) like the granules found in a typical *s. granulosum* of mammalian skin.

FIGURE 10 Junction between two cells of the second cornified layer in the frog epidermis. Part of the outer cornified layer (SC_1) is seen above. The cells of the second layer (SC_2 and SC_2') are joined by a *zonula occludens* located along the lateral intercellular spaces (between arrows 1 and 2). Occluding zonules presumably form in this location in anticipation of the desquamation of the outer layer. Immediately below the occluding zonule is a modified desmosomal element (arrows 2 to 3) with its characteristic dense plug of intercellular material bisected by a distinct intermediate line (*i*). The three layers (two dense and one light) of the cell membrane can be followed throughout the modified desmosome. Note that the entire cell membrane of the cornified cells is backed by a continuous shell of condensed cytoplasm (*p*) and the intercellular spaces (*Is*) between the cornified layers contain a granular material of moderate density. × 110,000.

FIGURE 11 Surface of a cornified cell (*SC*) in the frog skin, showing the fibrillar, presumably "mucous" coat (*mc*) adhering to the outer leaflet of the cell membrane and the continuous shell or plaque (*p*) of condensed cytoplasm found along the cytoplasmic side of the membrane. Numerous filaments (*f*), some of which converge on this dense cortical layer, make up the cytoplasmic matrix.

Specimens fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6) with sucrose and embedded in Araldite. Sections doubly stained with uranyl and lead. × 105,000.


SC1
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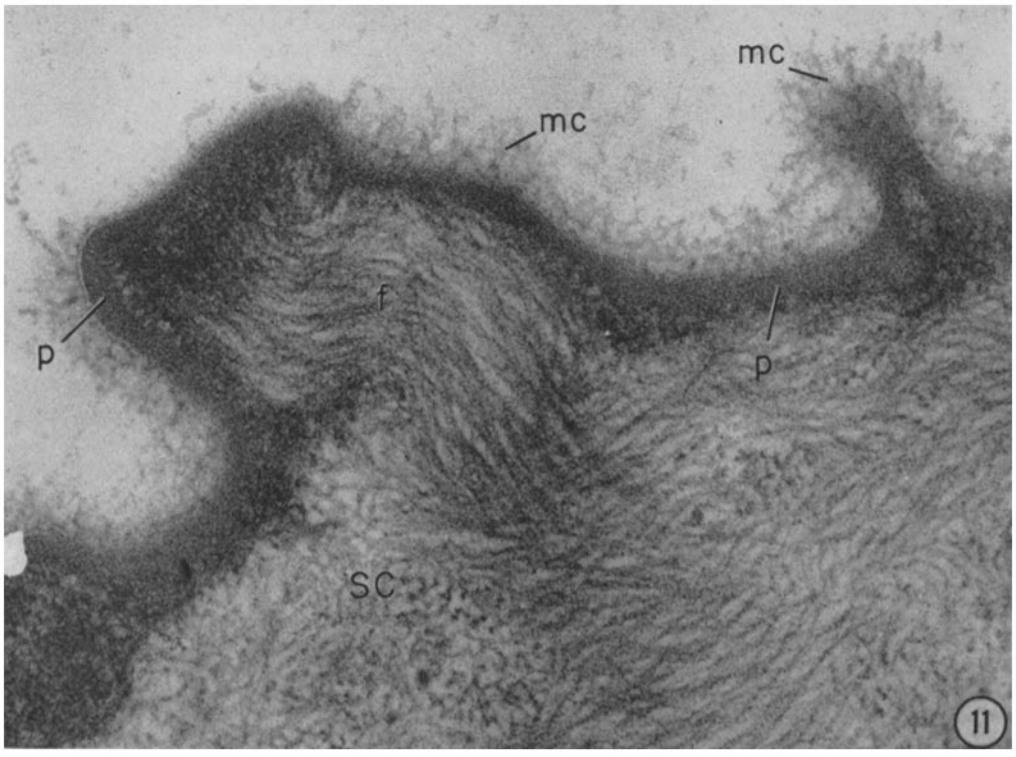
mc
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11

known, but they persist in the outer granular layer. Although they decrease in number as the cells keratinize, some remain intact in the cytoplasm while the cells move from the *s. granulosum* to the *s. corneum* and eventually appear to undergo degradation within autolytic vacuoles. These large granules apparently have no counterpart in most other keratinizing epithelia, but have been illustrated previously in the frog epidermis by Voute (45) and by Parakkal and Matoltsy (46). The latter have pointed out that mucus is formed in the amphibian epidermis at the stage at which mammalian epidermis produces keratohyalin granules. They did not distinguish two different granule populations, and apparently assume that in the frog all epidermal granules contain mucus. Our findings demonstrate the presence of two distinct granular types in this same species; raise the possibility that their content is chemically different; and suggest that one type, the smallest, is concerned with the production of the dense material that fills the intercellular spaces between the *s. granulosum* and *s. corneum*.

Functional Implications

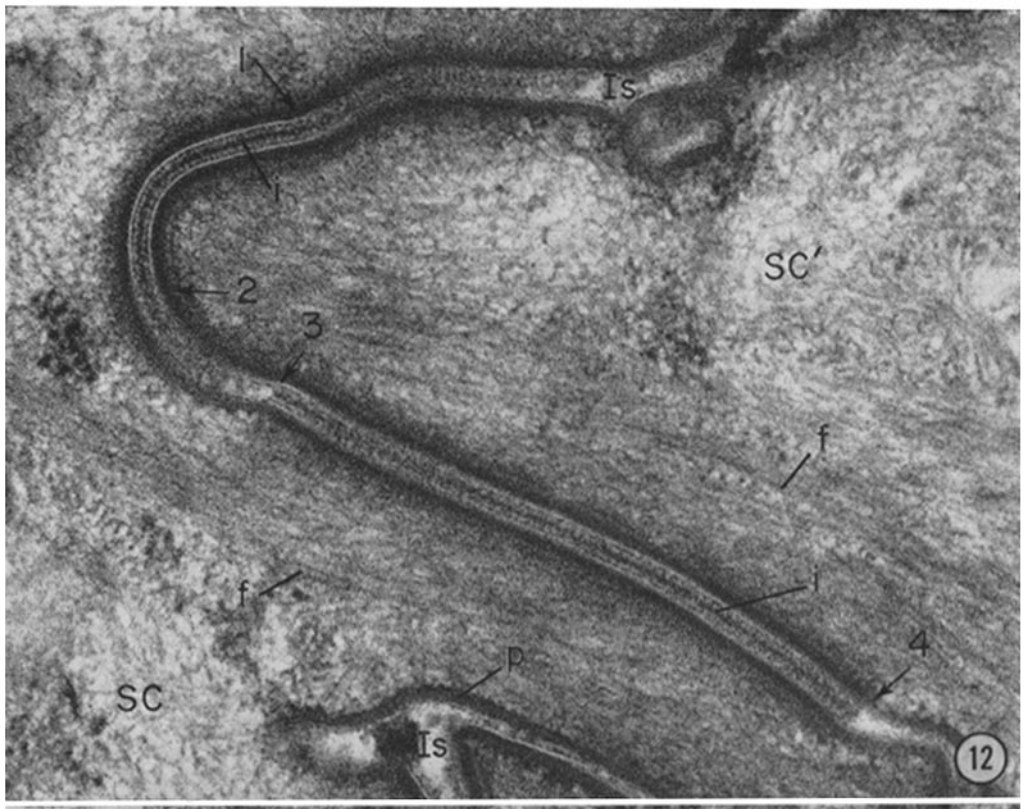
OSMOTIC RESPONSE: Cell membranes fused by *zonulae occludentes* on a succession of fronts form the only structurally continuous barriers in the frog epidermis. The first barrier is located at the outer front of the *s. corneum* and has a well established continuity. It is followed at a distance of 3 to 4 μ by one or two additional continuous barriers: one of these is present only in places in which the *s. corneum* is composed of more than one cell layer; the other is usually recognized at the distal front of the *s. granulosum*. These additional barriers are probably formed in anticipation of desquamation. Their existence creates the extracellular subcompartments already mentioned, which could function as a buffer space between the external and the internal medium of the frog, and as a closed space in which the mucous coat material is accumulated and the extensive plugs of the modified desmosomes characteristic of the *s. corneum* are elaborated.

The fact that the intercellular spaces of the frog epidermis are closed toward the external medium and open toward the dermis provides the neces-

FIGURE 12 Two modified desmosomes (arrows 1 to 2 and 3 to 4), along the lateral surfaces of two cornified cells (*SC* and *SC'*) in frog skin. These elements resemble desmosomes in the strict parallelism of their apposing cell membranes, the presence of dense plugs bisected by an intermediate line (*i*) in the intercellular space, and the occurrence of concentrations of dense, amorphous, and fibrillar material (*f*) in the subjacent cytoplasmic matrix. However, the plate of intercellular material is more dense; the associated concentration of cytoplasmic material takes the form of a continuous shell around the entire cell (well shown at *p*); and the bundles of filaments are somewhat less distinct owing to aggregations of amorphous material in the cytoplasm near the junction. These elements also occupy a much greater proportion of the lateral cell surface than do ordinary desmosomes in the deeper layers. × 85,000.

FIGURE 13 Typical "composite" desmosome found between cornified (*SC*) and granular (*SG*) cells at the base of the innermost cornified layer. The cell membranes can be followed throughout the junction. The membrane of the cornified cell is of the thicker (~100 A), nearly symmetrical variety, while that of the granular cells is of the thinner (~80 A), asymmetrical variety with the outer leaflet (*ol*) thinner and less dense than the inner one (*il*). The cell membrane of the cornified cell is backed by a condensed shell (*p*) of cytoplasmic material around the entire cell surface, whereas a dense cytoplasmic plaque occurs only along the desmosome in the granular cell. Thus the composite desmoso have a bipartite or composite structure due to the distinctive properties of the involved cell surfaces. Cytoplasmic filaments (*f*) converge on the desmosomal plates on either side of the junction; they stand out more sharply in the granular cell against its less dense cytoplasmic matrix. In the intercellular space (*Is*) there is an unidentified round, dense mass (arrow); it may represent the residue of a discharged large granule or a grazing section through a blunt pseudopodium of the cornified cell.

Specimens and sections prepared as for Fig. 11. × 135,000.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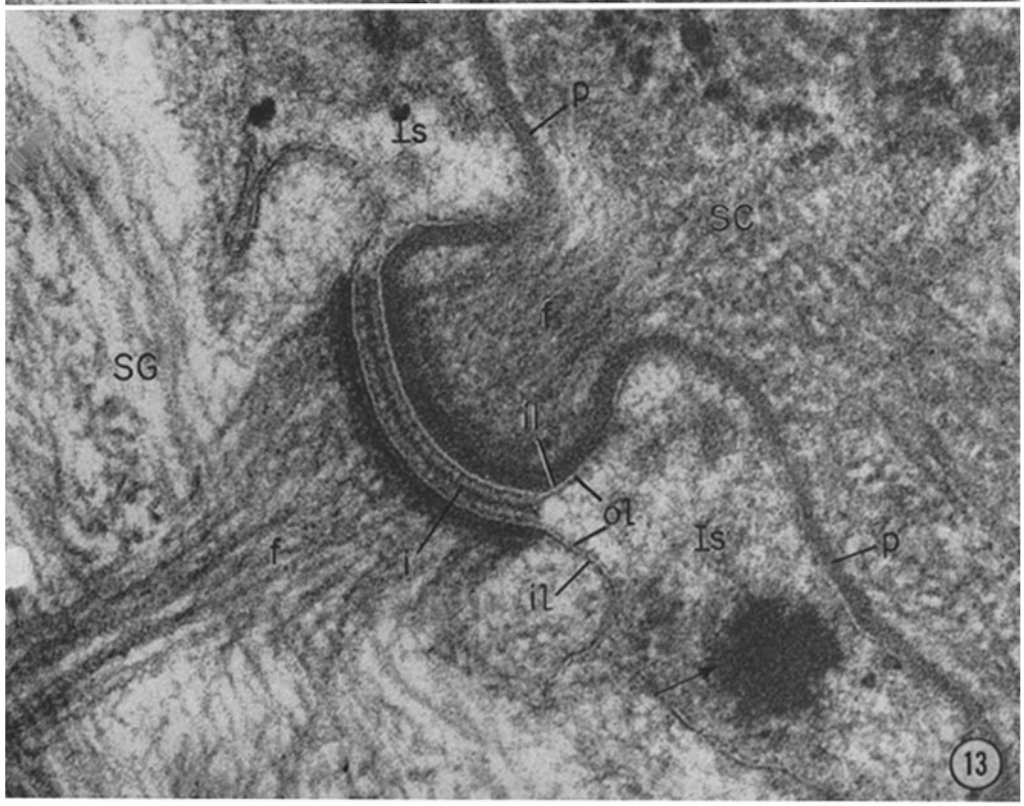
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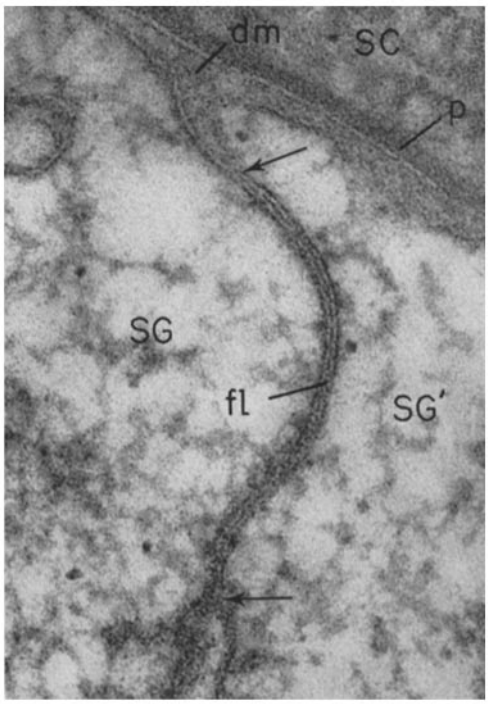


FIGURE 14 Junction between two cells (*SG* and *SG'*) of the distal granular layer (frog epidermis), showing an occluding zonule (between arrows) which characteristically occurs in this location. The fusion line (*fl*) of the junction is clearly visible, but the point of mergence (upper arrow) of the converging cell membranes cannot be made out owing to oblique sectioning of the membranes at this level. The total thickness of the junction is less (~120 A) than that of occluding zonules in the cornified layers (Figs. 8 and 9) owing to the thinner nature of the constituent cell membranes.

Specimen fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6) with sucrose, dehydrated in acetone, stained in block with $KMnO_4$, embedded in Araldite. Section doubly stained with uranyl and lead. × 165,000.

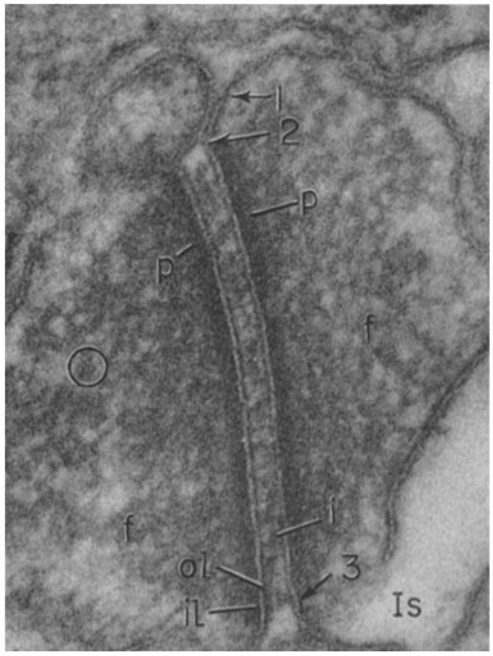


FIGURE 15 Desmosome (arrows 2 to 3) and an area of membrane fusion (*macula occludens*) (arrows 1 to 2) between two cells of the *s. spinosum* of toad epidermis. The depth of the area of membrane fusion is very shallow (~400 A). A number of the characteristic features of desmosomes are clearly shown: the dense intercellular plug, with its intermediate line (*i*) (in three dimensions a plate); cytoplasmic plaques (*p*) along the inner leaflet of the cell membrane; and the converging bundles of cytoplasmic filaments (*f*) seen here cut mostly in cross-section. Many of these filaments appear tubular (circled). Note that the inner (*il*) and outer (*ol*) leaflets of the cell membranes are visible throughout the desmosome, and on the right the outer leaflet can be followed from the fusion line of the tight junction (at *2*) into the "intermediate dense layer" (20) or "lateral dense line" (19) of the desmosome.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6), dehydrated in acetone, stained in block with $KMnO_4$, and embedded in Araldite. Section doubly stained with uranyl and lead. × 165,000.

sary structural explanation for the asymmetric osmotic response observed by MacRobbie and Ussing (3), who found that the epidermis swells when hypotonic solutions[10] are placed on the inner surface of the skin, but not when they are brought into contact with the outer surface. Taken together, these morphological and physiological findings indicate that the *zonulae occludentes* of the frog epidermis are impermeable to water, certain ions, and small, water-soluble molecules. By analogy it may be assumed that the same degree of impermeability characterizes occluding zonules in general, but this point remains to be established by future work.

[10] The clearest results were obtained by using graded dilutions of a Ringer solution prepared with an anion to which cell membranes are impermeable (SO_4^{--}). The osmotic response is more complex with anions and cations to which the cell membranes are permeable.

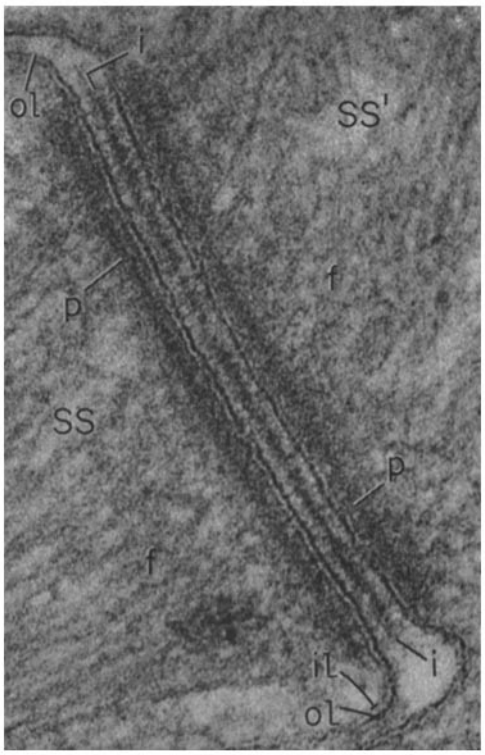


FIGURE 16 Desmosome between two cells (*SS*, *SS'*) in the *s. spinosum* of the frog epidermis. It consists of two plaques (*p*) of dense material disposed parallel to the inner leaflets of the plasma membranes and separated therefrom by a lighter space. The intercellular space at this level measures ~250 A across and is occupied by a disc of dense material bisected by a denser central layer (*i*) referred to as intercellular contact layer (20) or median stratum (21). Bundles of cytoplasmic filaments (*f*) converge on the inner aspect of each plaque. In this type of preparation the trilaminar structure of the cell membrane is clearly visible but the usual asymmetry is reversed, with the outer leaflet appearing denser than the inner one. Along the cell below, the outer leaflet can be clearly followed into the desmosome where it constitutes the intermediate dense layer (20) or lateral dense line (19).

Specimen fixed in glutaraldehyde in cacodylate buffer (pH 7.4), postfixed in OsO_4, stained in block in uranyl acetate, and embedded in Araldite. Section doubly stained with uranyl and lead. × 165,000.

SKIN POTENTIAL: Our results are more difficult to reconcile with available data on the frog skin potential. The existence of structurally continuous barriers at or near the outer front of the epidermis implies that the site of the skin potential is there and favors a potential profile with a few steps clustered near the surface. Recently, Ussing and Windhager (47), using penetrating microelectrodes, have recorded a multistep potential profile in the majority of cases. Yet, Engbaek and Hoshiko (48) and Whittembury (49) have found a two-step potential profile in the frog and toad skin, respectively, and have concluded that these steps are located at the outer and inner surface of the *s. germinativum*.[11]

In an earlier work, Ottoson *et al.* (50) recorded one-step potential profiles and placed this step at the level of the basement membrane, which appeared to them at the time to be the only structurally continuous barrier in the epidermis. This view has not been supported by more recent research (47–49, 5); moreover, evidence obtained from work on blood capillaries indicates that basement membranes are highly permeable to small molecules and also allow, to a certain extent, the passage of macromolecules (51, 52). Finally, Scheer and Mumback (53) have described a two-step potential profile, placing one-step at the level of the epithelium and the other at that of the *tela subcutanea*. (See, however, Franz and Van Bruggen (54)). Our preliminary observations suggest that the *tela* is the wall of lymphatic sacs.[12]

The wide disagreement on the location of the skin potential and the variation in results from one skin to the next may be related to technical difficulties inherent in microelectrode techniques, as well as to the existence of an extensive and pervasive extracellular compartment within the epithelium. The potential profile may be affected more by the intra- or extracellular location of the recording microelectrode than by the position of the latter in the depth of the epidermis, a possibility that is also considered by Ussing and Windhager (47).

[11] Back diffusion (leaks) along the intercellular spaces, and means to prevent it, were not considered in these interpretations.

[12] The wall includes a phagocytic endothelial layer, a continuous basement membrane, and an adventitia containing blood vessels, nerves, crystal-bearing cells, and other fibrillar and cellular connective tissue elements. The endothelial cells do not appear to be joined by *zonulae occludentes*. Moreover, the lymphatic sacs are interrupted by septa connecting the dermis to the fasciae of the underlying muscles. Hence there does not appear to be a continuous barrier at this level.

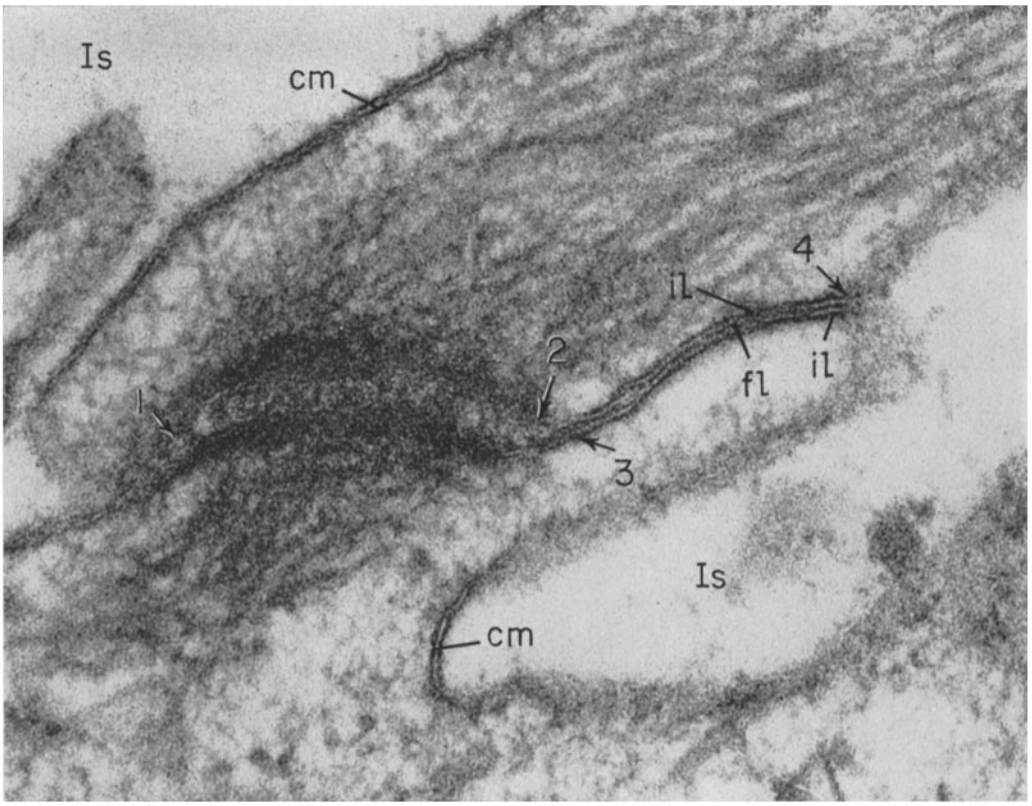


FIGURE 17 Junction between two cells of the *s. spinosum* in the toad epidermis, showing a desmosome (arrows *1* to *2*) cut obliquely, and an area of membrane fusion (between arrows *3* and *4*). The points of convergence of the adjoining cell membranes at either end of the latter cannot be distinguished because of oblique sectioning of the membranes, but the inner membrane leaflets (*il*) and fusion line (*fl*) can be clearly seen throughout the junction. The triple-layered structure of the cell membrane (*cm*) is visible on other aspects of the cell surface. The area of membrane fusion is unusually extensive for this location in the epidermis, and is attributed to the fact that the section cuts lengthwise through an occluding fascia. Similar occluding maculae (*cf.* Fig. 15) and fasciae occur in the frog epidermis.

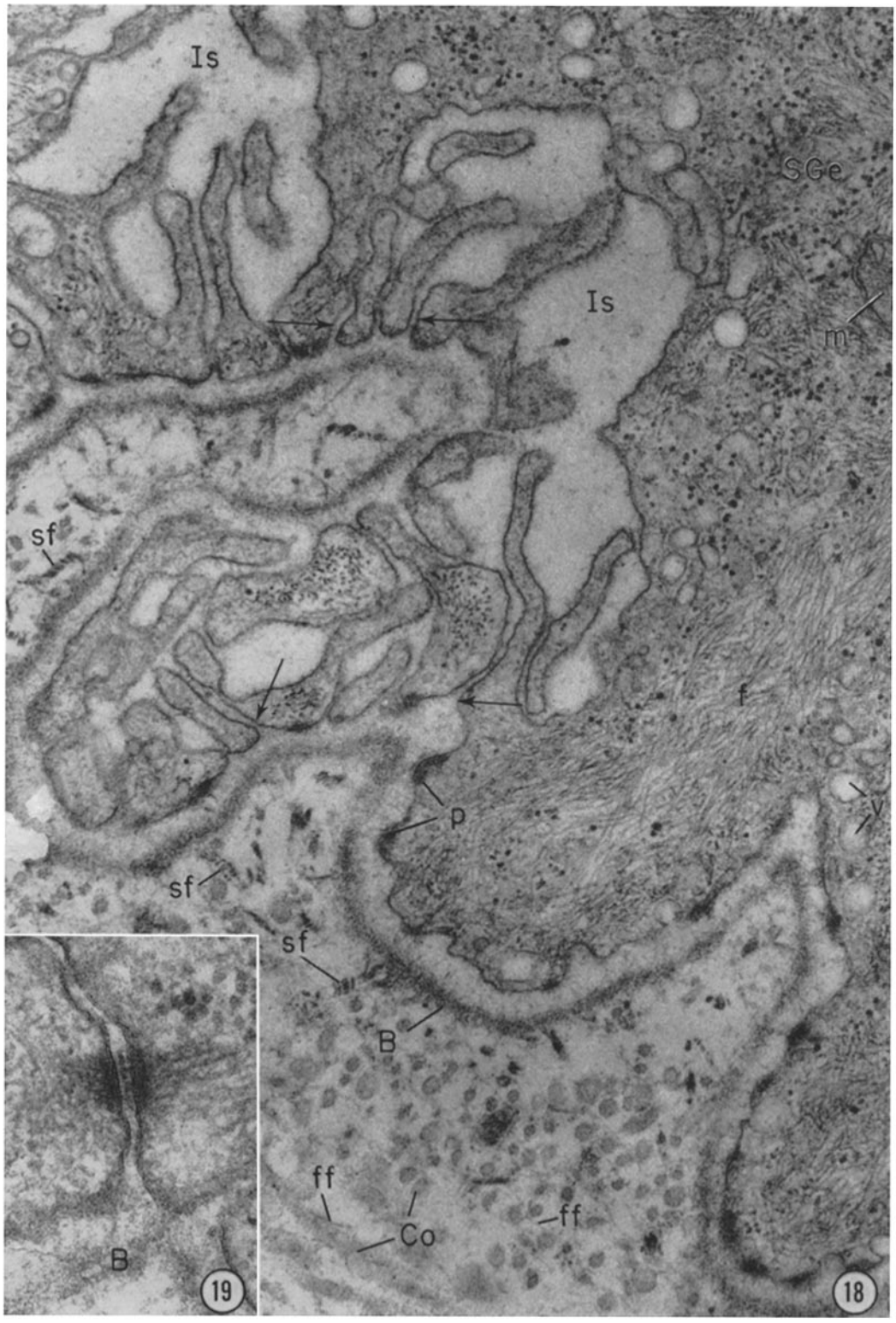
Section and specimen preparation as for Fig. 15. × 180,000.

FIGURE 18 Dermo-epidermal junction in the frog epidermis. The adjoining cells of the *s. germinativum* are elaborately interdigitated along their lateral surface forming a number of club-like processes. The intercellular spaces (*Is*) between the processes are expanded and of complicated geometry; they appear to be patent or "open" toward the basement membrane (*B*), for the cell membranes of most processes (arrows) remain separated by a distinct gap of 200 to 300 A. The cytoplasm of the basal cell (*SGe*) on the right is occupied by conspicuous bundles of filaments (*f*) and also contains a few mitochondria (*m*), small (pinocytic?) vesicles (*v*), and numerous dense particles, some of which are probably ribosomes and others glycogen. Along the basal cell membrane there are periodic areas of cytoplasmic densification (*p*) corresponding to so called "basal plates" or "bobbins" (*cf.* 11, 12). Note the dense, broad-banded fibrils (*sf*) present in the micropapillae and the fine fibrils (*ff*) and collagen fibrils (*Co*) seen throughout the rest of the dermis.

Specimen and section preparation as for Fig. 5. × 48,000.

FIGURE 19 Another intercellular space between two cells of the *s. germinativum* at the base of the frog epidermis, showing a desmosome which occasionally occurs in this location.

Specimen and section preparation as for Fig. 16. × 100,000.


Is
Is
SGe
m
sf
f
p
V
sf
sf
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ff
Co
ff
18
B
19

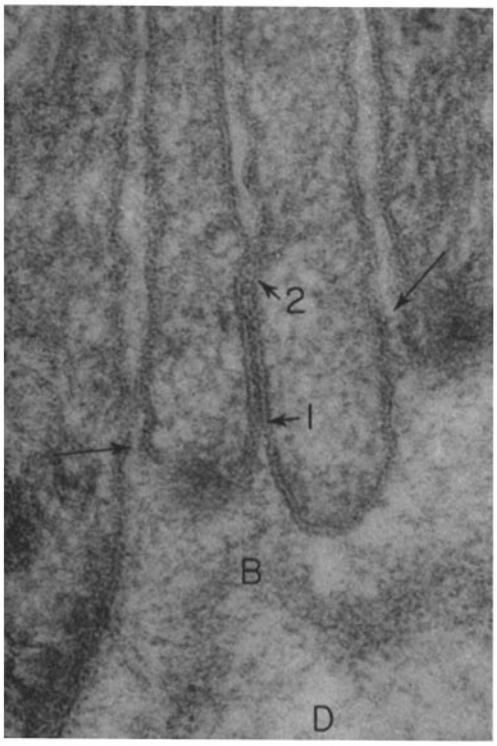

FIGURE 20 Interdigitations between germinal cells at the base of the frog epidermis. Most of the interspaces at this level are separated by a gap of 200 Å or more (unlabeled arrows). Occasionally the space is narrower, and areas of close approximation of the adjoining cell membranes are seen (between arrows *1* and *2*). Note, however, that the outer membrane leaflets, while closely apposed, remain separate, *i.e.*, they are not fused into a single line as in the case of the *zonulae occludentes*.

Specimen and section preparation as for Fig. 16. × 180,000.

FUNCTIONAL SPECIALIZATION AMONG JUNCTIONAL ELEMENTS: A certain degree of functional specialization is apparent among various junctional elements (*cf.* 1). One of the functions of the *zonulae occludentes* is to restrict or prevent exchanges along the intercellular spaces. We have previously shown (1) that these zonules are impermeable to macromolecules (hemoglobin, pancreatic enzymes), and our present observations imply that they are also impermeable to water, ions, and small, water-soluble molecules. It follows, as postulated, that they can play a passive role in the maintenance of chemical and electrochemical potential gradients across an epithelium by sealing its intercellular spaces. Preliminary experiments (*cf.* 1) indicate that the occluding zonules also function in cell to cell attachment, since they are the last elements of the junctional complex to break under tension.

The desmosomes and related structures (composite and modified desmosomes) are involved primarily in cell to cell attachment. In the epidermis they appear to be solid, relatively rigid structures which serve to anchor an extensive system of cytoplasmic filaments. The result is the transformation of a population of apparently independent cells into a mechanically continuous structure.

The *maculae* and *fasciae occludentes* scattered throughout the epidermis deserve special attention. Such junctions, characterized by focal obliteration of the intercellular space, may represent areas of low resistance coupling which could facilitate conduction from one cell to another (*cf.* 41). This assumption is supported by the demonstration that various electrotonic junctions, such as electrical synapses (55–57, 41) and connections between the cells of smooth muscle (58, 41) and myocardium (59, 41), consist of similar areas of membrane fusion. In the amphibian epidermis, these *maculae occludentes*, together with the *zonulae occludentes* of the *s. corneum* and *s. granulosum*, may represent permeable regions which allow ion movement from cell to cell and thereby permit rapid equilibration of Na^+ and K^+ concentrations throughout the cells of the entire epidermis, thus transforming its cell population into a functionally continuous compartment. The existence of a continuous cellular compartment within the epidermis has also been considered recently by Ussing and Windhager (47) and demonstrated in the case of K^+ by the results of Koefoed-Johnsen (60). The former assumed, however, that the desmosomes are the areas of rapid cell to cell diffusion.

A functionally similar situation has been described in the salivary gland of *Drosophila flavorepleta* larvae by Kanno and Loewenstein, who have demonstrated a low-resistance coupling between the cells of this glandular epithelium (61, 62) and rapid diffusion of a relatively large ion (fluorescein) throughout the epithelium without leakage into the extracellular space, after its injection into a single cell (62, 63). Wiener, Spiro, and Loewenstein (64) have concluded that in this epithelium the site of low resistance cell-to-cell coupling as

well as of high resistance along the intercellular spaces is a "septate junction." The latter, like the "septate desmosomes" (65, 66), appears to be a characteristic junctional element of epithelia in invertebrates.

The bearing of our findings and interpretations on current physiological models of the frog skin were briefly treated in a previous paper (5) and will be discussed in detail in a paper to follow (67).

Note Added in Proof: In a recently published paper (*J. Cell Biol.*, 1965, 24, 297), Matoltsy and Parakkal suggest that the small granules present in the middle layers of the epidermis contain a special membrane-coating substance which, upon discharge into the intercellular spaces, renders the cell membranes resistant to keratinolytic agents (0.1 N NaOH). This interpretation differs from their previous views quoted on page 282 of this paper. It is not clear, however, from their new evidence, whether the resistant material represents a transformed cell membrane, the subjacent shell of dense cytoplasm characteristic of cornified cells, or a combination of these structures.

This research was supported by research grants AM-09090, and IIE-05648, from the United States Public Health Service, and by Public Health Service research career program award 1-K3-GM-25, 109.

We gratefully acknowledge the technical assistance of Mrs. Karin Taylor, Miss Agneta Stromblad, Mrs. Lee Adinolfi, and Mrs. Sarah Benton.

Received for publication, November 6, 1964.

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