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The Permeability Barrier in the Epidermis of the Grass Snake During the Resting Stage of the Sloughing Cycle

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Summary. Tracer and freeze-fracture replication techniques show that there are two morphologically and topographically distinct permeability barriers in the epidermis of the grass snake. Tight junctions interconnect the apico-lateral plasma membranes of the uppermost living cells, establishing an ionic or osmotic gradient between the stratum germinativum and alpha layer. The second barrier is formed by intercellular lipid sheets in the overlying mesos layer, which are very similar to the barrier found in the stratum corneum of mammals.

Key words: Snake epidermis – Permeability barrier – Electron microscopy, tracer – Freeze fracture.

The epidermis of higher vertebrates is characterized by the formation of a thin protective layer – a stratum corneum which reflects adaptation to a terrestrial environment by providing protection against harmful external influences, and by establishing a barrier against cutaneous water loss.

Recent reports modify previous understanding of the mammalian permeability barrier. While, until recently, the bulk of the stratum corneum was considered to form the barrier (Scheuplein and Blank 1971), in the last few years the role of the intercellular space – and especially the lamellar lipid material in it originating from membrane-coating granules – has attracted more attention (Elias and Friend 1975; Elias et al. 1979). Both concepts postulate a continuous regeneration of the barrier by differentiation of new keratinocytes and the desquamation of horny flakes from the surface. In squamate reptiles however, which shed their epidermis periodically, the permeability barrier has to be built up again with the formation of a new epidermal generation.

The epidermis of lizards and snakes has been extensively studied at both the light and electron microscopical levels (see Maderson et al. 1972; Landmann 1979). Squamate epidermis is unique in displaying four distinct keratinized layers above

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the stratum germinativum, namely the alpha-, mesos- and beta-layers and the *Oberhäutchen*. While the latter two consist of feather- (beta-)type keratin, the others contain the alpha-type. The *Oberhäutchen* and beta-layer form a homogenous keratin complex lacking an intercellular space, while the mesos- and alpha layers show distinct keratinized cells roughly comparable to the stratum corneum of other vertebrates.

Numerous physiological and ecological studies deal with the protective function of the reptilian integument against cutaneous water loss (see Bentley 1976; Shoemaker and Nagy 1977). Very little, however, is known about the structure and location of the permeability barrier. Based on stripping experiments, Maderson et al. (1978) conclude that the impermeability of the epidermis depends on the thickness of the cornified material. They suggest the barrier to be localized mainly in the alpha-layer. Electron microscopic studies (Landmann 1979, 1980a), however, indicate that two different mechanisms contribute to the establishment of the barrier, namely lipid lamellae in the intercellular space of the mesos layer, and tight junctions at the apico-lateral membrane of the uppermost stratum germinativum cells. This view is backed by Roberts and Lillywhyte (1980) who show that in squamate epidermis a complex mixture of polar and neutral lipids localized in the mesos layer, contributes much more to the establishment of the barrier than keratin does.

The aim of this study is to more precisely identify and localize the barrier sites by extending previous thin section studies to tracer experiments, freeze-fracture replication, and scanning electron microscopy.

Materials and Methods

Skin biopsies were taken from the back of the grass snake *Natrix natrix* (L.) during the resting stage of the sloughing cycle (for a description of the cycle see Maderson 1965a; Maderson and Licht 1967; Landmann 1979). Outer scale as well as hinge regions (Maderson 1965b; Roth and Jones 1967) were examined.

Electron Microscopy

Samples were fixed at room temperature by immersion in cacodylate-buffered 2% glutaraldehyde-2% formaldehyde (Karnovsky 1965) for 4 h at constant agitation, followed by postfixation in cold 1.3% s-collidine-buffered osmium tetroxide (Bennett and Luft 1959) for 2 h. Some specimens were postfixed in Karnovsky's (1971) osmium-potassium ferrocyanide. Subsequently they were rapidly dehydrated in graded acetones and embedded in Epon 812. Semithin sections stained with paraphenylene-diamine (Estable-Puig et al. 1965) were used to determine the stage of the sloughing cycle and to select appropriate areas for thin sectioning. Silver-gray sections were cut on a Reichert OmU2 microtome, mounted on formvar coated copper grids, stained with uranyl acetate and lead citrate, stabilized with carbon and examined in Philips EM 301 and Jeol 100 CX electron microscopes.

Tracer Methods

For tracer studies 15 mM $LaCl_3$ in saline was injected subcutaneously 45 min before biopsy. In order to improve penetration of the tracer, in some samples lanthanum was additionally added in the same concentration to all processing agents up to acetone 90%. Stained and unstained sections were examined.

Disruption of Tight Junctions. In order to enable the tracer to penetrate beyond the level of the tight junctions, samples were incubated for $^1/_2$ h in a strongly hypertonic sucrose solution (20% w/v)

containing 15 mM $LaCl_3$. Such a solution is known to disrupt tight junctions by osmotic forces (Goodenough and Gilula 1974; Wade and Karnovsky 1974). Subsequently the samples were fixed with aldehydes containing lanthanum and processed as described above.

Solvent Extraction of Lipids. After aldehyde fixation, lipids were extracted by pyridine or chloroform-methanol (1 : 3) containing 15 mM $LaCl_3$ for 2 h at room temperature and 24 h at 60° C. Samples with or without prior sucrose treatment were used. Further procedure followed the method described above.

Freeze-fracture Technique

Samples were fixed in aldehydes as described above, rinsed in buffer and cryoprotected with 10% and 25% (w/w) glycerol in buffer for 1 h each. Fragments of scales were then placed in hollow copper rivets, rapidly frozen in melting propane and fractured at −136° C in an apparatus of our own design (Stolinsky 1975). After replication with platinum-carbon, samples were allowed to thaw slowly in the final glycerol solution, and transferred to 5% sodium-hypochlorite for 48 h. After gradually increasing the concentration of sodium-hypochlorite up to 10%, samples were kept at 37°C for 24 h. The replicas were repeatedly washed, the remainder of tissue was finally digested in gradually increasing concentrations of nitric acid, and heated to 60° C for $^1/_2$ h. Finally, replicas were washed thoroughly in distilled water and mounted on copper grids.

Scanning Electron Microscopy

Samples were fixed in aldehydes as described above. In some specimens, whole scales were split with fine tweezers thus separating the *Oberhäutchen* and beta layer from the alpha layer and stratum germinativum at the level of the mesos layer. Other scales were cross-sectioned with a fine razor blade. All samples were rapidly dehydrated in graded acetones, critically point-dried with liquid CO_2 and sputtered with gold for examination in a Jeol 100 CX scanning attachment. Other samples were obtained by stripping off the scales with double sided adhesive tape. Some scales attached to the tape were stripped again in order to reveal mesos layer surfaces while others were cross-sectioned. These last samples were sputtered without prior fixation or dehydration.

Results

An Overview of Snake Epidermis

The body of squamate repitles is covered by overlapping scales whose number and arrangement is species specific and therefore widely used as a taxonomic criterion. The scale is an elevation of the dermis and all epidermal layers of each scale are continuous with the next one. The scales are divided into outer and inner surfaces and a flexible hinge region (Maderson 1965b).

The complex stratified epidermis is periodically regenerated and shed. A few days after shedding, in the perfect resting condition (Maderson and Licht 1967), the epidermis consists of the following layers (Figs. 1–3): The innermost is the stratum germinativum, consisting of a stratum basale and one or several layers of undifferentiated cells which resemble basal and spinous mammalian keratinocytes. Above the stratum germinativum is found the alpha layer, the lowermost keratinized layer. Like the mammalian stratum corneum it is composed of distinct horny cells. There is a gradual transition to the next layer, the mesos layer. Within this layer, specimens tend to split easily during preparation (Fig. 1). Its cells are extremely flat and thin. The uppermost layer is the *Oberhäutchen*-beta-layer complex, a homogenous stratum of beta-keratin. Its surface shows a microornamentation which is believed to reduce wear and friction, to produce interference colours, and which is used as a taxonomic criterion.

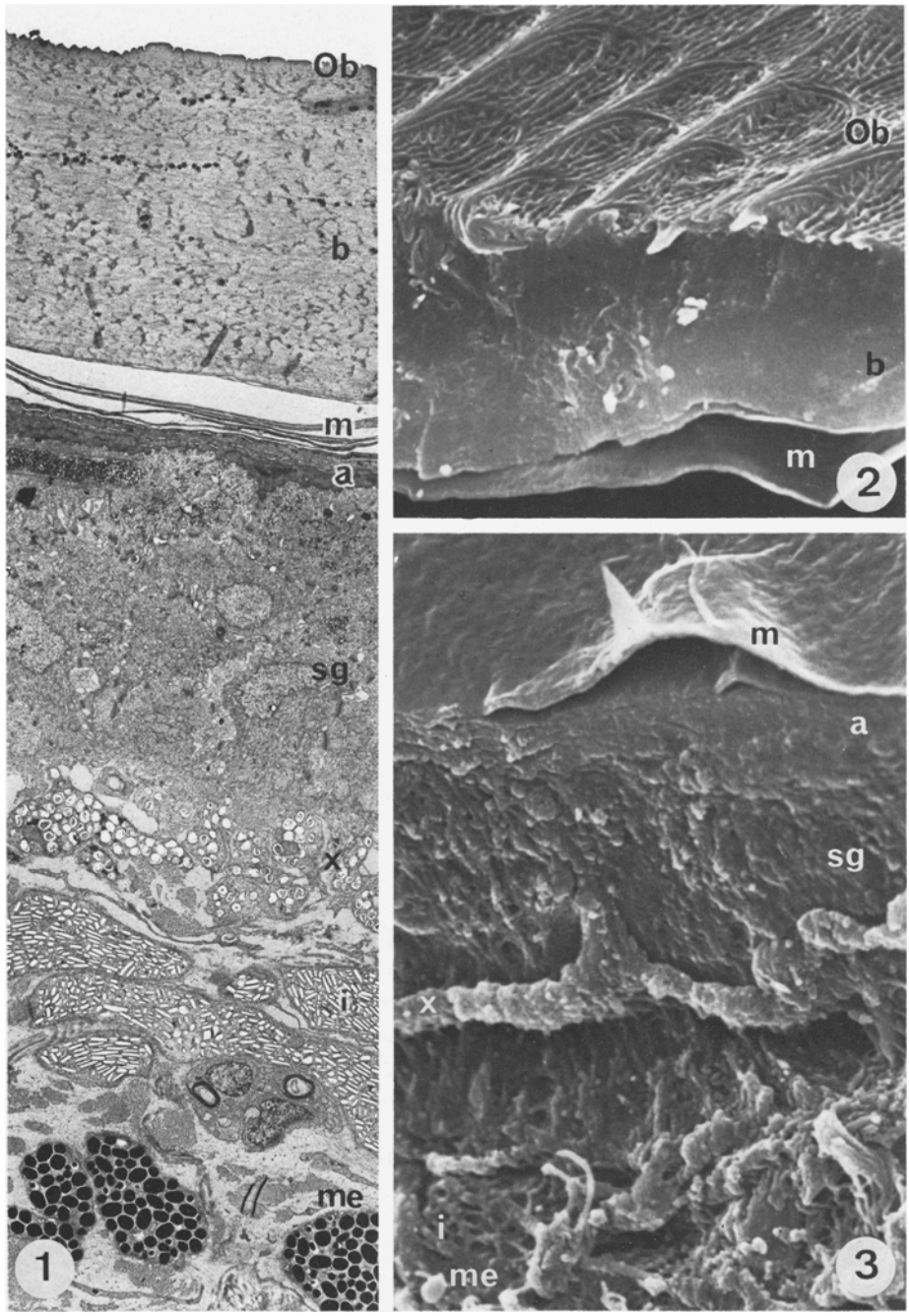


Fig. 1. Thin section of the outer scale surface. *Oberhäutchen* (*Ob*) and beta layers (*b*) cannot be distinguished in the fully keratinized condition. Both together form a homogenous stratum of beta keratin. The mesos layer (*m*) consisting of flat cells split during preparation. Alpha layer (*a*) and stratum germinativum (*sg*) resemble mammalian stratum corneum, spinosum and basale. In the dermis xanthophores (*x*), iridophores (*i*), and melanophores (*me*) can be identified. ×3,200

Fig. 2. SEM of superficial cornified layers of the outer scale surface. The surface view of the *Oberhäutchen* (*Ob*) displays a characteristic microornamentation. Splitting from deeper layers occured at the level of the mesos layer (*m*) a few thin cells of which can be seen adhering to the beta layer (*b*). ×5,000

Fig. 3. SEM of the lower epidermal layers and subjacent dermis. Note surface view of mesos layer (*m*), alpha layer (*a*) and stratum germinativum (*sg*). In the dermis can be seen xanthophores (*x*), iridophores (*i*), and melanophores (*me*). ×8,000

In the subsequent renewal phase, a new epidermal generation is formed and matures. Finally shedding occurs and the cycle is repeated (see Maderson 1965a; Maderson and Licht 1967; Roth and Jones 1970; Landmann 1979).

In the dermis three layers of chromatophores are found: the xanthophores, the iridophores and the melanophores (Alexander and Fahrenbach 1969; Taylor and Hadley 1970) (Figs. 1 and 3).

Structural Features of Barrier Sites

In the uppermost layer of stratum germinativum cells, as previously described (Landmann 1979; 1980a) the distal portions of the lateral plasma membranes are closely apposed and connected by tight junction fusion points. These are clearly demonstrable in freeze-fracture replicas (Figs. 4, 5). From their extent it is clear that they form continuous zonulae occludentes. Within one single tight junction two different patterns can be identified: a "loosely interconnected network" (Hull and Staehelin 1976) consists of 5–6 strands and has a depth of about 0.2 μm (Fig. 5, right) while an "evenly cross-linked" network (Fig. 5, left) shows up to 10 strands and is about 0.5 μm deep.

The mesos layer consists of extremely flattened flake-like cells. These cells show features similar to mammalian cornified cells. In the intercellular space between the cells lamellar material is found (Fig. 6). 4–5 (varying from 1–8) continuous sheets run parallel to the cell surface. Occasionally electron-dense material is interspersed between the sheets. The width of the intercellular space varies from 20–75 nm.

Replicas of the mesos layer reveal smooth fracture faces of mesos cell plasma membranes which are characterized by desmosomes (Fig. 7). The fracture-plane frequently runs through the intercellular space revealing particle-free faces of the lamellar sheets. A maximum of six steps (Fig. 8) can be counted in these regions being in good correspondence with the thin-section pictures of 4 or 5 striations (cf. Fig. 6).

Tracer Studies

After subcutaneous injection, lanthanum percolates freely upward from the dermis, penetrates the basal lamina and fills and labels the intercellular space of the stratum germinativum. Outward flow is abruptly interrupted at a level just beneath the alpha layer which is the lowermost keratinized layer (Figs. 9 and 10). This barrier coincides with the tight junctions between the latero-apical plasma membranes of the uppermost living cells (Fig. 11). Since no tracer leaks into the intercellular space between stratum germinativum and alpha layer, it is clear that the tight junctions are arranged in the form of belt-like zonulae occludentes as indicated by the freeze-fracture observations. These features are present in all regions of the scale.

Application of tracer with solutions does not alter the results except for penetration of the tracer for a few μm in the intercellular space of the alpha layer at the lateral cut surface of the samples.

Disruption of the tight junctions by hypertonic sucrose concentration allows tracer to penetrate further upward and to label the intercellular space of the alpha layer too (Fig. 12). Preservation of the viable stratum germinativum cells is obviously poor in these preparations. Labelling in the alpha layer is not so continuous as in the stratum germinativum. Tracer is never found beyond the level

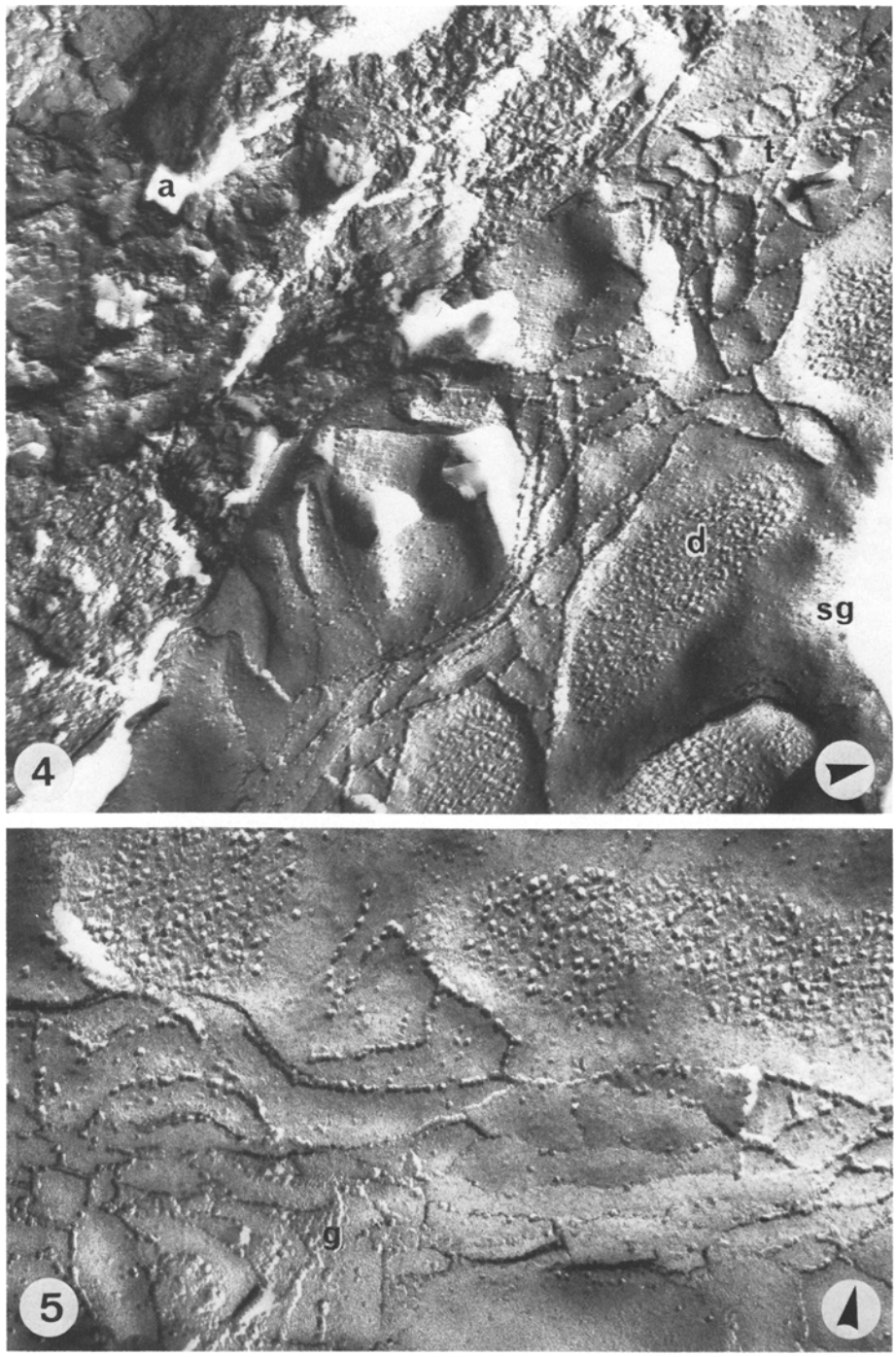


Fig. 4. Freeze-fracture replica of the stratum germinativum alpha layer transition zone. Cross fractured alpha layer cell (*a*), P-face of uppermost stratum germinativum cell (*sg*) exhibiting desmosomes (*d*), and portion of tight-junctional belt (*t*) characterized by typical strands. × 70,000

Fig. 5. Higher magnification micrograph of tight junction. The alpha layer to be found at the bottom of the picture. P-face of stratum germinativum cell is characterized by particle-ridges while the E-face has complementary grooves occupied by isolated particles. Note probable gap junction (*g*). × 112,000

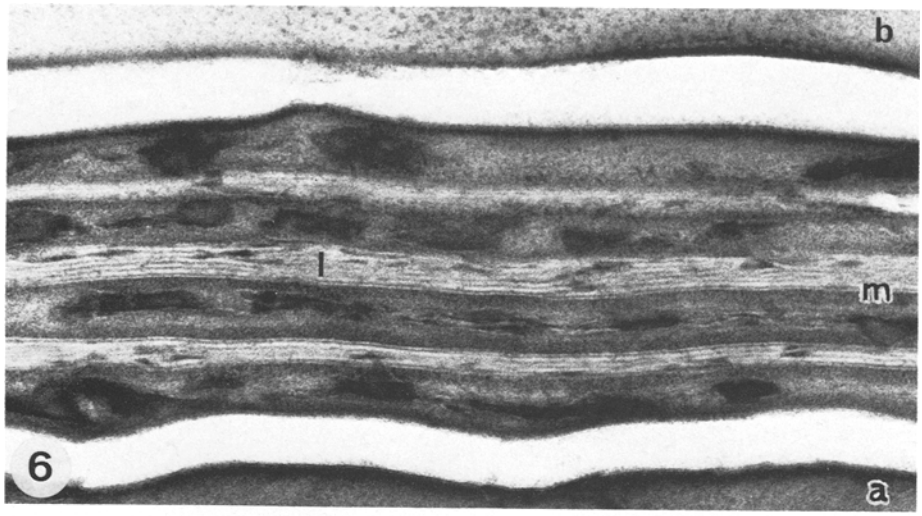


Fig. 6. The mesos layer (*m*) which is sandwiched between the alpha layer (*a*) and the superficial beta layer (*b*) consists of flat cornified cells. Its intercellular space is filled with lamellar sheets (*l*). Osmium-potassium ferrocyanide postfixation. ×100,000

Fig. 7. Replica of mesos layer. The P-face of the plasma membrane (*P*) exhibits desmosomal particles (*d*) many of which lie in depressions. Where the fracture plane has traversed the intercellular space, it reveals lamellar sheets (*l*) outlined by characteristic steps (see also Fig. 8). ×37,000

Fig. 8. Replica showing lamellar sheets and steps from the intercellular interval of the mesos layer. Note double steps (*arrows*) indicating preference for fracturing at the revealed levels. ×70,000

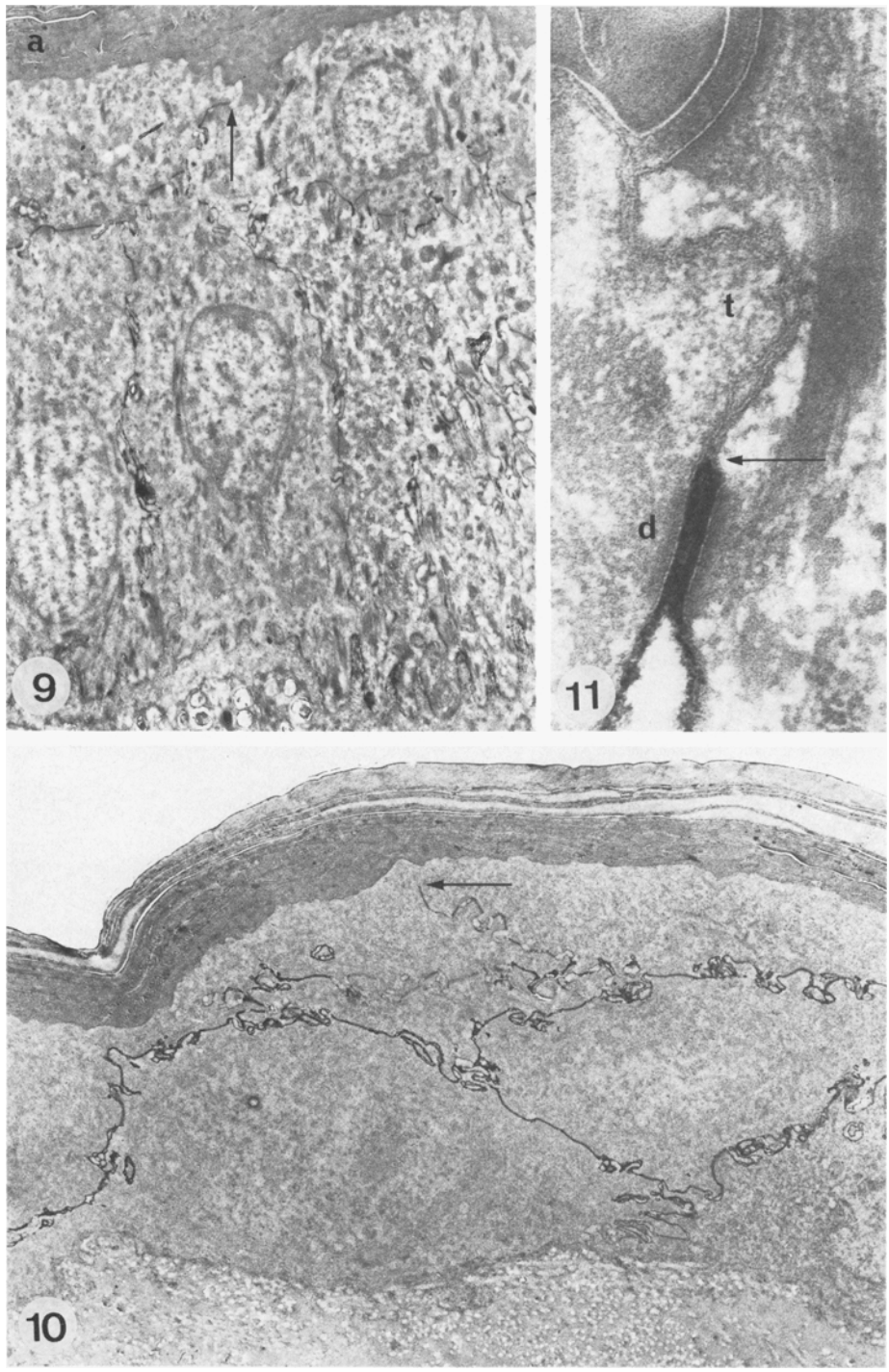
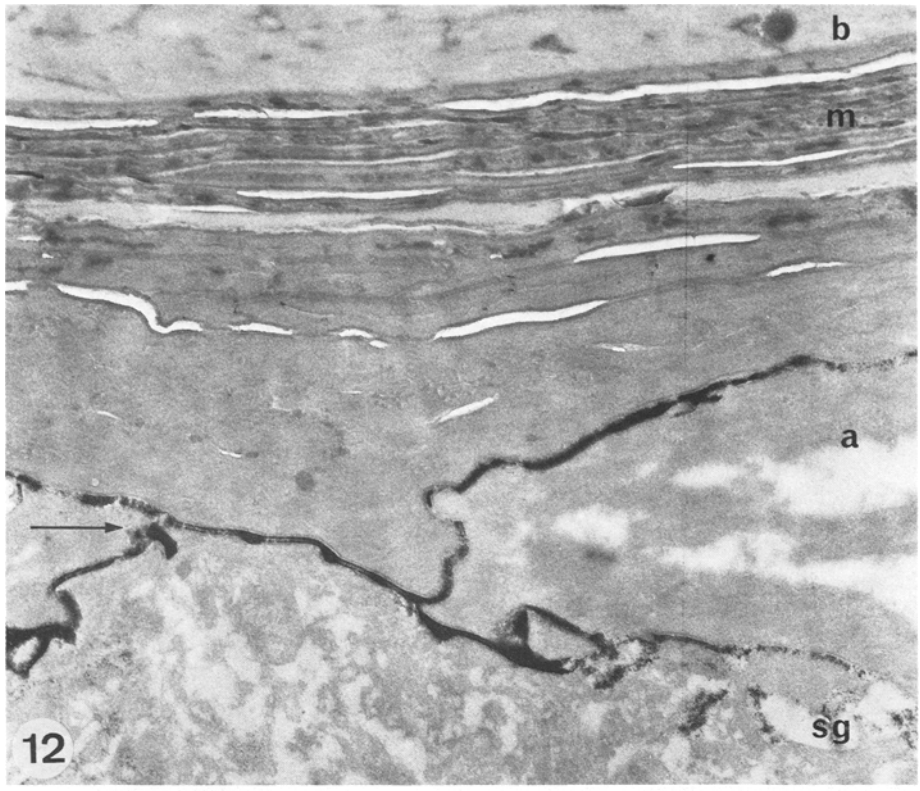


Fig. 9. Lanthanum-filled intercellular space of stratum germinativum of an outer scale surface epidermis. *Arrow* indicates stop of outward flow of tracer just beneath the alpha layer (*a*). Unstained section. × 6,200

Fig. 10. Despite different arrangement of stratum germinativum cells, lanthanum penetrates to the same level (*arrow*) in the hinge region epidermis. Unstained section, × 7,900

Fig. 11. Barrier to outward flow of tracer (*arrow*) is established by tight junctions (*t*) between the apicolateral plasma membranes of the uppermost stratum germinativum cells. Note desmosome (*d*) just beneath tight junction. Unstained section, × 120,000



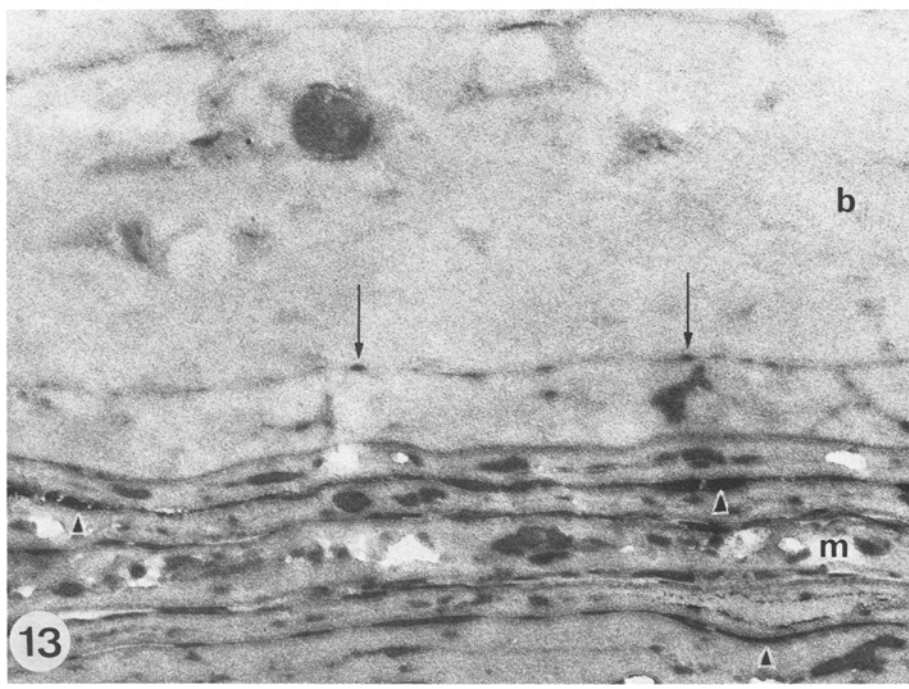


Fig. 12. Disruption of the tight junctions (*arrow*) of the stratum germinativum (*sg*) admits tracer to the intercellular space of the alpha layer (*a*). Note absence of lanthanum in both mesos (*m*) and beta (*b*) layers. Unstained section, × 22,500

Fig. 13. Extraction of the intercellular sheets of the mesos layer (*m*) by lipid solvents allows lanthanum (*arrowheads*) to penetrate this layer also. *Arrows* point to patches of tracer in the intercellular space present in the lowermost beta layer (*b*). Unstained section, × 54,000

of transition between alpha layer and mesos layer. This indicates that the intercellular space of the mesos layer is sealed by a barrier mechanism which is not disrupted by hypertonic solutions. Further evidence for the existence of a barrier is the observation that tracer contained in fixatives does not penetrate the intercellular space of the mesos layer from the lateral cut surface of the samples. But this is true only if the mesos layer is mechanically not distrubed. The structure responsible for this barrier consists of the intercellular lamellar sheets of the mesos layer.

In order to proove the barrier nature of the sheets in the intercellular space of the mesos layer, the material which is known to contain a considerable amount of lipids (Landmann 1980b) was extracted with lipid solvents. Such treatment of the samples permits labeling of the mesos layer intercellular spaces with lanthanum (Fig. 13). This labeling is almost continuous and can be followed up to the intercellular space present in the lowermost part of the beta layer (see Landmann 1979). The rest of the beta layer – *Oberhäutchen* complex is not penetrated by tracer under any circumstances.

Discussion

The present findings indicate that there are two structurally and topographically different barrier mechanisms in *Natrix* epidermis during the resting stage of the sloughing cycle: tight junctions between the uppermost cells of the stratum germinativum and lamellar lipid sheets in the intercellular space of the mesos layer. These two barriers have inherently different permeability properties:

Tight Junctions

The thin sections, freeze-fracture and tracer results clearly demonstrate the presence of tight junctions, in the form of belt-like zonulae occludentes between the uppermost living cells, capable of stopping outward flow of lanthanum through the intercellular space.

According to Hull and Staehelin (1976) loosely interconnected networks can stretch or compress extensively under tension while evenly cross-linked networks retain their basic morphology under normal stress conditions. The combination of both patterns within one single tight junction has never previously been reported. Due to the fragmentation of the replicas during digestion it could not be determined whether the tight junctions belong to the outer scale surface, or to the hinge region where stretch is to be expected and where the presence of loosely interconnected networks may reflect an adaptation to stress changes in the epidermis.

Tight junctions are known to allow the existence of steep ionic and osmotic gradients by preventing or reducing the diffusion of ions and molecules through the intercellular space (Staehelin 1974). The tightness of the seal can be roughly indicated by the depth of the junction and the number of strands therein (Claude and Goodenough 1973): Junctions with a depth of 0.2 μm and containing 5–6 strands belong to the intermediate type, those with a depth of 0,5 μm and consisting of up to 10 strands to the very tight one. This seal blocks only the paracellular flow, while the transcellular one – the other component of total flow – is not effected. This means that epidermal tight junctions in themselves do not protect an organism from

desiccation, because transcellular flow can still be operative. This is well illustrated by amphibian epidermis which has been extensively studied both morphologically and physiologically. Tight junctions occupying a similar position just beneath the stratum corneum (Farquhar and Palade 1965) are known to be practically impermeable to the passage of ions (Ussing and Windhager 1964; Martinez-Palomo et al. 1971). Nevertheless, frog epidermis shows high rates of cutaneous water loss as well as large water influx through osmosis (Shoemaker and Nagy 1977). By analogy therefore, it seems possible that in snake epidermis the intercellular space of the stratum germinativum is sealed off efficienty against ionic flow, but not against water flow into the overlying keratinized layers; the alpha layer is not completely dehydrated. Its content of ions and solutes is controlled by the apical cell membrane of the uppermost stratum germinativum cells. As shown here by tracer studies, there is no intercellular water barrier in the alpha layer. The barrier therefore must lie at a more superficial level.

Mesos Layer Lipid Sheets

The present experiments show that the lamellar sheets occupying the intercellular space of the mesos layer prevent outward flow of tracer. If lanthanum is allowed to penetrate the intercellular space of the alpha layer by disrupting the tight junctions, it is stopped at the level of the mesos layer provided that the latter is mechanically undisturbed during the processing of specimens. Since these sheets are known to consist mainly of lipids (Landmann 1979, 1980b), they can be removed by treatment of the samples with lipid solvents. In such samples, tracer is able to percolate the mesos layer and to label the intercellular space completely. In the lowermost beta layer further egress is impeded by the absence of an intercellular space.

The sheets originate from mesos granules present in immature mesos cells (Landmann 1979; 1980b). The same mechanism is found in mammalian epidermis where the content of membrane-coating granules is extruded into the intercellular space and rearranged into hydrophobic broad sheets (Matoltsy 1966; Martinez and Peters 1971; Lavker 1976; Elias et al. 1977a, 1979). The sheets have been demonstrated to act as a barrier against tracer flow (Schreiner and Wolf 1969; Squier 1973; Elias and Friend 1975), and their role in the establishment of the permeability barrier has been proved eyperimentally (Elias and Brown 1978). The similarity between membrane-coating granules and mesos granules has been shown by morphological and cytochemical methods (Landmann 1980b). The similarity of the lipid sheets of the *Natrix* mesos layer to lamellar intercellular material of mammalian stratum corneum is indicated by their freeze-fracture appearance (Breathnach et al. 1973; Elias et al. 1977b).

Lipid lamellar sheets are thus demonstrated to prevent tracer flux through the epidermis of both mammals and snakes. Due to their arrangement, enveloping the cells from all sides and filling the intercellular space, they are able to prevent both the paracellular as well as the transcellular pathway for diffusion. It can be concluded therefore that the lipid sheets in the mesos layer act as the main permeability barrier against transcutaneous water loss and that they represent the main source of the "lipid barrier" which, after extraction, has been shown to lead to as much as a 15-fold increase in cutaneous water loss (Roberts and Lillywhite 1980).

Unlike mammalian epidermis, where this system is continuously renewed by the differentiation of new keratinocytes, in snakes it is formed once during a sloughing cycle by the differentiation and maturation of a new mesos layer.

In his review of cutaneous water loss in reptiles, Bentley (1976) draws attention to the fact that squamate species occupying desert areas have smaller rates of loss than those from moister and more temperature habitats. There are only a few studies dealing with the thickness of the mesos layer, but the available data of iguanid epidermis (Alexander 1970; Maderson et al. 1970) show, that the thickness of the mesos layer is proportional to the aridity of the habitat. This would also back the assumption that the mesos layer acts as the main barrier against transcutaneous water loss.

Beta Layer and Oberhäutchen

The *Oberhäutchen* – beta layer complex shows no intercellular space and is not penetrable to tracer. The possibility that it takes some part in the establishment of the barrier has to be considered however. Indirect proof that this is not the case stems from observations on scaleless mutant snakes (Licht and Bennett 1972; Bennett and Licht 1975) which lack the thick beta layer normally present at the outer scale surface. Histological examination of such mutants shows an extremely thin layer of beta keratin presumably consisting only of the one cell thick *Oberhäutchen*. Nevertheless, these animals show the same rates of cutaneous water loss as normal animals. Therefore it can be concluded that the function of the *Oberhäutchen* – beta layer complex is mainly mechanical; this means it protects the subjacent keratinized and viable epidermal layers against abrasion and injury. Since the delicate mesos layer is regenerated only once during the sloughing cycle, a protection against abrasion is of eminent importance.

In studying the histological and physiological effects of the removal of superficial cornified material, Maderson et al. (1978) advanced the idea that the barrier is localized within the alpha layer. This would seem to disagree with the present observation that the intercellular space of the alpha layer is penetrated by tracer after disruption of the tight junctions of the stratum germinativum. The main point made by these authors is that there is a sudden rise in cutaneous water loss after removing cornified material by adhesive tape stripping, and a decline over the next two weeks, while the cornified material is being replaced by a tissue with the histological characteristics of a normal alpha layer. The disagreement however, is not as wide as might appear: (1) Stripping will affect the mesos layer even more than the alpha layer due to its more superficial position, and (2) since alpha and mesos layers are modifications of the same alpha keratin synthesizing keratinocytes (Maderson et al. 1972), there may be mesos granules and intercellular lipid sheets in the regenerated material. But clearly such a hypothesis needs further examination at the ultrastructural level.

In a simplified manner one could regard snake epidermis as consisting of a basal part containing both stratum germinativum and an alpha layer, the whole of which is comparable to amphibian epidermis. This basal part would prevent the loss of solutes but would not block cutaneous water loss. Superimposed is an effective permeability barrier consisting of the mesos layer. This barrier is similar to that of

the mammalian epidermis. In order to protect this delicate barrier against mechanical injury an additional layer is necessary. The resistant *Oberhäutchen* – beta layer complex provides this. Due to the fact that the individual cornified layers are differentiated independently one after the other – thus forming the complex stratification of the epidermis – they can not be regenerated continuously. Each and all of them have to be renewed periodically by completely new formation and maturation of cells, and sloughing thus becomes a necessity. This is an essential difference between squamate and other vertebrate epidermis which is reflected in their different morphologies.

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