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Freeze-Fracture and Tracer Experiments on the Permeability of the Zonulae occludentes in the Olfactory Mucosa of Vertebrates

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Summary. The junctional belt around the sensory cells in the nasal olfactory mucosa of the frog and in the vomeronasal organ of the mouse appears as a network of interconnected ridges in freeze-fracture replicas. Numerous open-ended ridges were observed and, consequently, open routes from the region below the junctional belt to that above it. Lanthanum nitrate permeates the junctional belt when administered from the surface of the epithelium as well as from the vascular system. When applied at a concentration of 1–3%, the tracer is deposited within the junctional belt forming facets which are visible in tangential sections. These facets correspond to the areas defined by the network or ridges seen in freeze-fracture replicas. Various aspects of these observations are discussed, such as the replacement of cells in the sensory epithelium, the stimulation of extrinsic fibers and the generation of a transepithelial potential.

Key words: Tight junctions — Olfactory mucosa — Lanthanum nitrate — Freeze fracturing.

Introduction

Zonulae occludentes ("tight junctions") were first defined by Farquhar and Palade (1963) to be belts in which the outer leaflets of the membranes of adjoining cells fuse. These belts lie close to the surface in epithelia. As a variation of this type of junction "focal" and net-like fusions were observed in thin sections (Farquhar and Palade, 1963, 1965; Karnovsky, 1967; Trelstad *et al.*, 1967; Brightman and Reese, 1969; Matter *et al.*, 1969; Sanders, 1973). In freeze-fracture replicas in the region of tight junctions investigated so far ridges or furrows appear within the membranes (Staehelin *et al.*, 1969; Goodenough and Revel, 1970; Chalcroft and Bullivant, 1970; Friend and Gilula, 1972; Lorber and Rayns, 1972; Luciano *et al.*, 1973; Pitelka *et al.*, 1973; Staehelin, 1973; Wade and Karnovsky, 1974). While originally it was assumed that tight junctions seal off the intercellular space from the surface (Farquhar and Palade, 1963; Brightman and Reese, 1969) it has been shown that tracer molecules can penetrate tight junctions (Whittembury and Rawlins, 1971; Machen *et al.*, 1972; Martínez-Palomo and Erlij, 1973; Tisher and Yarger, 1973; Claude and Goodenough, 1973). Thus, besides true tight junctions "leaky junctions" have been distinguished (Frömter and Diamond, 1972). It has been assumed that the intramembranous ridges and depressions seen in freeze-fracture replicas might correspond to the meshwork of membrane fusions seen in sections (Friend and Gilula, 1972; Pitelka *et al.*, 1973). The dia-

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grams given by Chalcroft and Bullivant (1970), Staehelin (1973), and Wade and Karnovsky (1974) reflect this interpretation. The observations of Claude and Goodenough (1973) suggest that very tight epithelia have deep zonulae with a complex network of anastomosing ridges, while very leaky epithelia have shallower junctions composed of fewer strands. In our study it is shown in vertebrate olfactory epithelia: (1) that lanthanum penetrates a rather deep belt of "focal tight junctions" from both directions (2) that lanthanum is deposited in facets, which correspond to those facets surrounded by the ridges exposed by freeze-fracturing, and (3) that the intercellular facets are not closed, thus allowing the tracer to penetrate.

We consider the consequences which can be drawn concerning the stimulation of extrinsic fibers within the olfactory epithelium (Graziadei and Gagne, 1973), and the replacement of cells which was recently demonstrated by Graziadei and Metcalf (1971) and Graziadei (1973).

Materials and Methods

Nasal olfactory epithelium of the frog *Rana temporaria* and vomeronasal mucosae of young white mice were subjected to different tracer experiments and to freeze fracture investigation. Tissue was fixed by immersion or perfusion in 6% glutaraldehyde in cacodylate buffer or in Karnovsky's fixative (Karnovsky, 1965). The tissues were washed, postfixed for 90 min in acetate-Veronal-buffered 1% OsO_4 (pH 7.4) and embedded in Durcupan (Fluka, Buchs, Switzerland).

Tracer Experiments with Lanthanum. Solutions with different concentrations of La $(NO_3)_3$ were used: 1 mM, 1% or 3% lanthanum nitrate was added to frog Ringer solution or to 0.05 M Tris-HCl buffer with 4% sucrose added. Drops of the solution (final osmolarity 200–300 milliosmols, pH 7.4) were placed on the surface of the living epithelium or the solution was injected into the carotid vessels of anesthetized animals. After 10–60 minutes the tissue was fixed by local immersion or perfusion, removed, minced and immersed in fixative for 1 hour. In some experiments lanthanum nitrate was added only to the fixative (Revel and Karnovsky, 1967).

Freeze-Fracturing. For freeze-fracturing small pieces of tissue were placed in Karnovsky's fixative (Karnovsky, 1965) for 30 min, then immersed in 20% glycerol in 0.1 M cacodylate buffer, rapidly frozen in liquid Freon, and fractured in a Balzers apparatus (Type BAF 301 Balzers AG, Liechtenstein). In some experiments freeze-fracturing was performed with specimens which had been treated with lanthanum nitrate before fixation, in others unfixed material was used.

Results

The appearance of the junctional belts in the olfactory mucosa as seen in freeze-fracture replica of prefixed material closely resembles the typical pattern observed in zonulae occludentes in other epithelia (Fig. 1). A net of interconnected ridges is associated with the A-faces. Furrows appear on the B-faces. In unfixed preparations the network of ridges appears to consist of fused particles. Nasal and vomeronasal epithelia do not differ, local differences within single epithelia were not observed either. The junctional belts are about 0.35 μm deep and are made up of 5–6 strands. Interestingly, the ridges or furrows constituting the belt are not perfectly connected so as to form a closed net. A number of open ended ridges was seen. One can trace open routes around the ridges from the region below the junctional belt to that above it (Fig. 2). Freeze-fracture replicas or preparations treated with lanthanum before fixation cannot be distinguished from normal untreated specimens.

All experiments with lanthanum yielded essentially the same results. In olfactory mucosae incubated with lanthanum solutions containing 1–3% La $(NO_3)_3$ the tracer is deposited heavily within the junctional belt. In preparations where lower concentrations had been used only smaller granular deposits were found. A similar effect of a variation of tracer concentration has been reported by Tisher and Yarger (1973). When tight junctions permeated with lanthanum were sectioned *en face* the tracer deposits appear in polygonal facets which are separated by light lines connected to a net (Fig. 3). Since the diameter of the dendrites is rather small (Kolnberger, 1971) these regions are of rather limited size and are often seen to turn and approach a plane which runs perpendicular to the plane of section. At these points it can be seen that the light lines are lines of membrane fusions.

The correspondence of the areas defined by the network of ridges seen in freeze-fracture replicas to the facets formed by lanthanum deposits in tangential sections leads to the interpretation that both are equivalent, in the sense that the tracer facets represent a pattern on the outer surface of the membrane which corresponds to the pattern of ridges within the membrane. This conclusion is supported by comparing the size of the facets (Fig. 4). No significant differences can be found. On the contrary, the uniformity of the pattern in different species and in both olfactory organs is revealed.

Further confirmation is given by the fact that in a mucosa which was incubated with lanthanum and then freeze-fractured, the tracer was found only in the sections but not in the freeze-fracture replica. This experiment shows moreover, that the appearance of the junctional structures in freeze-cleave preparations is not influenced by previous lanthanum incubation.

The results of experiments with lanthanum solutions injected into the vascular system were not uniform. Lanthanum was heavily deposited in the capillaries of the mucosa in all cases, but it was found outside these vessels only when used at a concentration of 3% in the perfusion fluid and after at least 35 minutes of perfusion. In these latter cases lanthanum was found in the intercellular clefts, within the junctional belt and on the membranes across this belt as well.

Discussion

In longitudinal sections the membranes of sensory cells and supporting cells appear separated (Thornhill, 1967; Altner *et al.*, 1970; Kolnberger and Altner, 1972; Altner and Altner, 1974) or connected by "focal" tight junctions (Reese and Brightman, 1970; Theisen, 1972), which have been thought to form continuous belts around supporting and bipolar cells. The latter view has been proposed by Reese and Brightman (1970), since horseradish peroxidase injected into the circulation one hour before fixation did not reach the surface of the epithelium. Our experiments with lanthanum show clearly that the tracer moves across the apical junctional complex into the intercellular clefts of the epithelium, as was observed in earlier experiments with horseradish peroxidase administered from the surface (Kolnberger, 1971; Kolnberger and Altner, 1972). Interestingly, lanthanum not only penetrates if dissolved in Ringer solution or a buffer placed on the epithelium before fixation (Machen *et al.*, 1972; Luciano *et al.*, 1973; Neaves, 1973). It is also

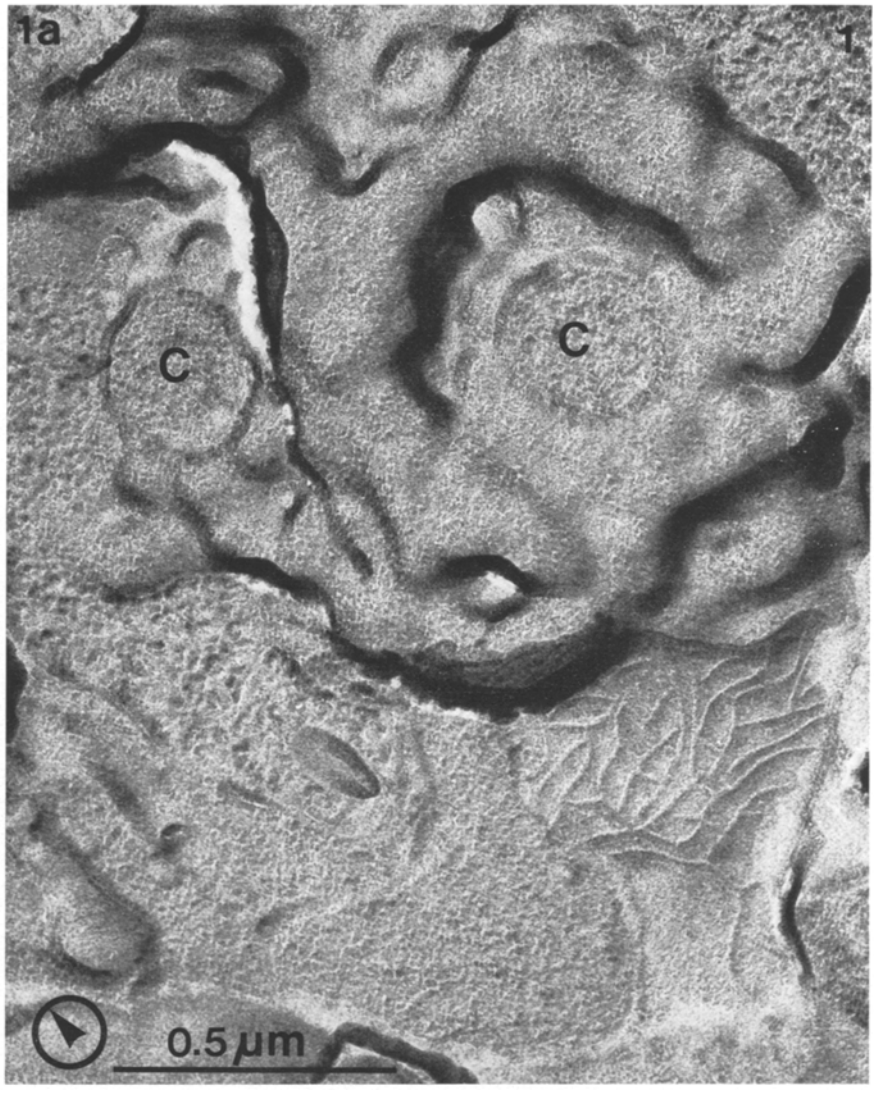
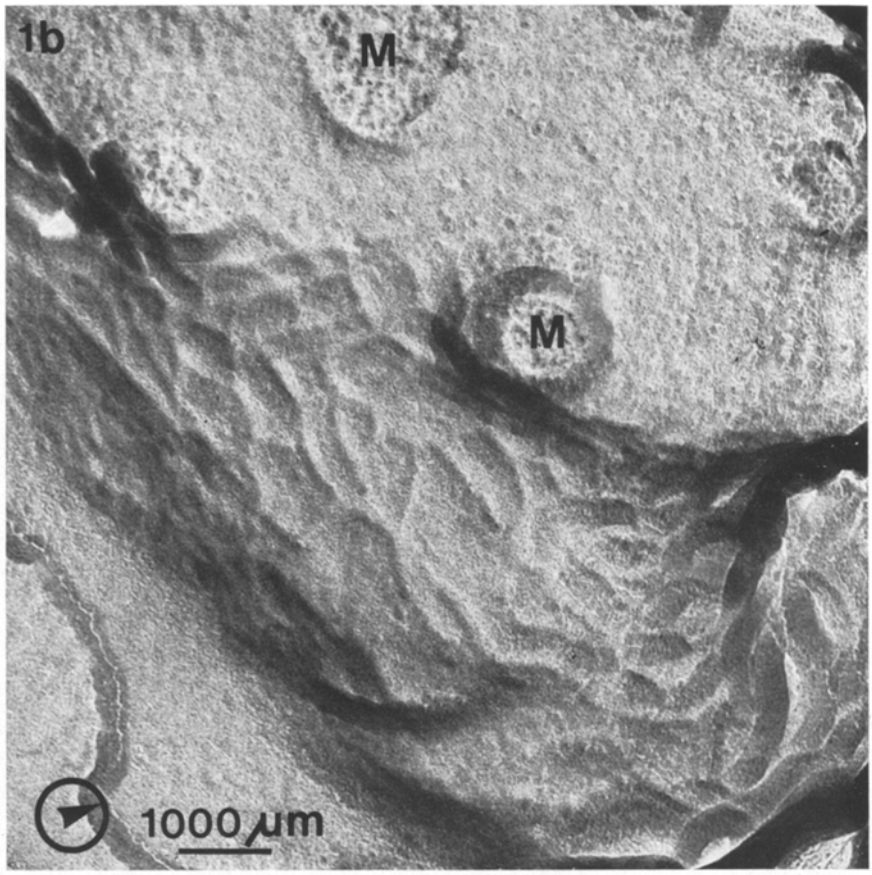


Fig. 1a and b. Freeze-fracture replica of the apical portion of a sensory cell in the olfactory nasal mucosa of a frog (a) and in the vomeronasal mucosa of a mouse (b). A protrusion which bears cilia (*C*) or microvilli (*M*), respectively, projects into the overlying mucus. The network of ridges on the A-face of the membrane around the neck of the cell represents the junctional belt. *Circled arrowhead* indicates shadowing direction in this figure and figure 2. a $\times 75000$, b $\times 120000$

found in the intercellular spaces of the epithelium when it is added to the fixing fluid, as was observed in the kidney tubule of the rat (Martínez-Palomo and Erlij, 1973; Tisher and Yarger, 1973). Of special interest is the pattern of tracer deposition in tangential sections. A similar pattern has been observed only in



Sertoli cell tight junction following efferent ductule ligation (Neaves, 1973), and in the bile canaliculus following treatment with acetone, by whose action the zonulae occludentes are said to become leaky (Goodenough and Revel, 1970). Hüttner *et al.* (1973) observed a similar pattern in lanthanum-permeated coronary artery endothelium in the rat.

Careful comparison of their location, arrangement and size shows close correspondence between the facets formed by tracer deposition and those exposed in the junctional belt by freeze-fracturing (Fig. 4). Since many loose-ended ridges are seen in our freeze-fracture replicas and routes can be traced around them across the whole belt, it is suggested that tracer particles can penetrate the junction on the assumption that the ridges correspond to the fusion lines (Friend and Gilula, 1972; Pitelka *et al.*, 1973; Staehelin, 1973). A mechanism of this kind has been proposed by several authors (Matter *et al.*, 1969; Friend and Gilula, 1972; Pitelka *et al.*, 1973; Neaves, 1973).

On the other hand, it has been stressed that the ridges must be considered to consist of rows of closely spaced particles which can be seen in freeze-fracture replicas of unfixed material (Staehelin, 1969, 1973). Such rows of particles were

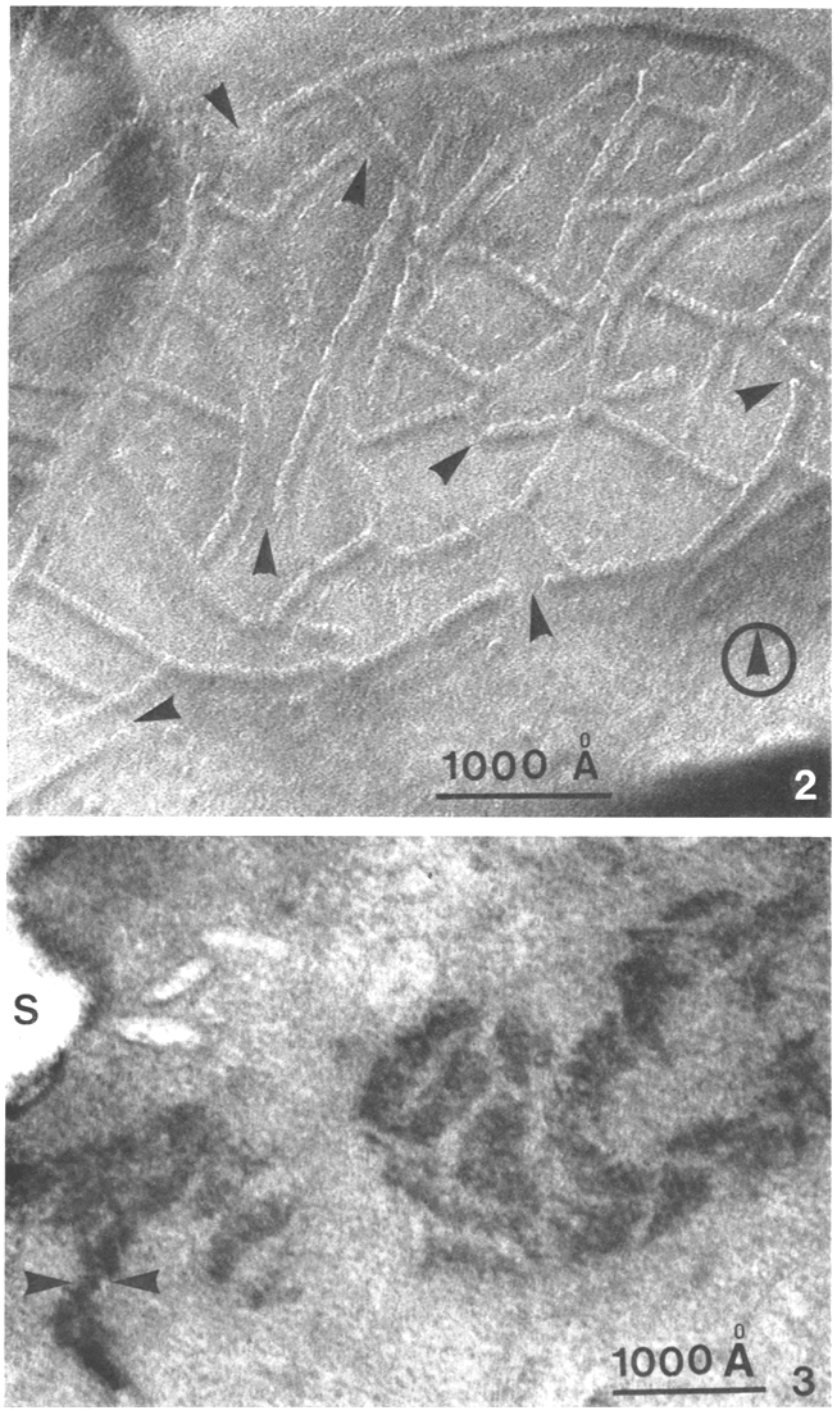


Fig. 2. Part of a junctional belt from a replica of a sensory cell in the olfactory nasal mucosa of a frog. Several free-ending ridges are seen (*arrows*). ×240000

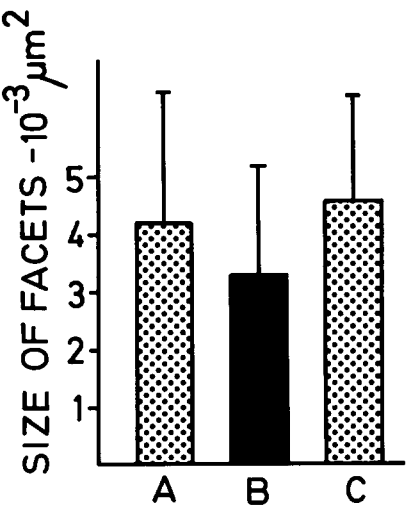


Fig. 4. Comparison of mean size of facets in the junctional belts in olfactory epithelia exposed by freeze-fracturing (*stippled columns*) and formed by lanthanum deposits in tangential sections (*solid column*): *A*, *B* frog nasal olfactory mucosa, *C* mouse vomeronasal olfactory mucosa. A total number of 188 facets was measured by planimetry. Bars indicate one standard deviation above the mean

also found in our material when submitted to freeze-cleaving without fixation. This finding suggests that the leakiness of a junction might be due to the passage of tracer in between the particles (Luciano *et al.*, 1973). In this case the arrangement of ridges would seem to have no effect on diffusion. Then the open-ended ridge segments can be thought of as places "where tight junction seals are being newly formed or extended" (Staehelin, 1973). According to Claude and Goodenough (1973) the leakiness could be related to the extent of the junctional belt. A comparison of our measurements with the data collected by these authors shows, that the olfactory epithelia would then probably belong to epithelia of intermediate permeability.

In olfactory epithelia a turnover of receptors and supporting cells has been shown by autoradiography (Graziadei and Metcalf, 1971; Graziadei, 1973). The regular replacement of cells should lead to a loosening and restoration of junctions. Since those cases in which morphological symptoms of degeneration have been found in thin sections seem to be exceptions (Andres, 1969; Kolnberger, 1971) we have no clear-cut criteria for identifying such cells in freeze-fracture replicas. Until now we have not found local differences in the appearance of the network of ridges in olfactory epithelia which could be thought of as places of junctional development. Changes in junctional structure however, need not be accompanied

Fig. 3. When lanthanum-filled junctions are exposed by *en face*-sectioning, the tracer deposits form electron-dense facets. On the left side an intercellular cleft turns from the sectioning plane to a plane perpendicular to it (*arrowheads*). Olfactory mucosa of the frog; *S* surface of the epithelium. ×200000

by changes in leakiness of the junction. The junctions in the olfactory epithelium seem to be leaky under the experimental conditions used in our experiments. Thus the leakiness does not reflect a special physiological state such as was observed in the mammary gland (Linzell and Peaker, 1972; Pitelka *et al.*, 1973). The hypothesis that the permeability of the zonulae occludentes could be due to a hyperergic reaction of the mucosa (Themann *et al.*, 1971) is not confirmed by our results. The routes of passage as revealed by lanthanum could well be channels for stimuli to reach the membranes of the extrinsic innervation of the olfactory epithelium (Graziadei and Gagne, 1973). On the other hand, the generation of a transepithelial potential as proposed by Thurm (1970, 1972) postulates a barrier between the intercellular clefts and the surface, a view which does not agree with our results. In this respect it must be borne in mind that the experiments with lanthanum do not necessarily represent the diffusion of physiologically important ions (Matter *et al.*, 1969) and that leakiness and tightness for ions are not necessarily visible in the electron microscope (Frömter and Diamond, 1972).

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