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In vivo and in vitro Formation of the Junctional Complex in Choroid Epithelium

A Freeze-Etching Study

R. Dermietzel, K. Meller, W. Tetzlaff, and M. Waelsch

Institut für Anatomie, Universitätsklinikum Essen; Institut für Anatomie, Arbeitsgruppe Experimentelle Cytologie, Ruhr-Universität Bochum, Bundesrepublik Deutschland

Summary. The junctional complex of choroid epithelial cells was studied during in vivo formation, disaggregation after trypsin treatment, and in vitro reaggregation. The in vivo formation begins with the occurrence of amorphous patches of particles followed by the formation of small particulate rows and polygonal-ordered particle assemblies. Further arrangement of the zonula occludens continues with the confluence of particles and smooth contoured ridges. At the 9th day stage a fully developed zonula occludens has developed. In a subsequent step nexus become integrated within the tight junction formation. Disaggregation after trypsination results in fragmentation of the zonulae occludentes. Parts of the disassembling aggregates become incorporated in vacuoles indicating an endocytotic mode of "digestion". The in vitro reconstruction of the zonula occludens proceeds from remnants of the former zonula occludens. On the 3rd to 4th day of cultivation mature tight junctions are visible. In vitro integrations of nexus were observed during a later phase. On the 7th day, cultivated choroid epithelial cells reveal well differentiated junctional complexes consisting of continuous zonulae occludentes and integrated gap junctions.

Key words: Chick embryo – Choroid epithelium – Junctional complex – Development – Freeze-etching.

Introduction

The freeze-etching technique has provided extensive information about the structural and macromolecular composition of membrane contacts. As shown by

Send offprint requests to: P.D. Dr. R. Dermietzel, Institut für Anatomie, Universitätsklinikum der GHS, Hufelandstr. 55, 4300 Essen, Federal Republic of Germany; *or:* Prof. Dr. Karl Meller, Institut für Anatomie, Arbeitsgruppe Experimentelle Cytologie, Ruhr-Universität Bochum, Universitätsstr. 150, 4630 Bochum, Federal Republic of Germany

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the general review of Staehelin (1974) on tight junctions and that of McNutt and Weinstein (1973) on gap junction formation, two aspects appear of prime importance: (1) characterization of biochemical components of gap junctions (Goodenough and Stoeckenius, 1972; Goodenough, 1974; Goodenough et al., 1974; Gilula, 1974), and (2) the process of development of membrane contacts and the mechanisms which regulate this development (Yee, 1972; Revel et al., 1973; Albertini and Anderson, 1974; Decker and Friend, 1974; Johnson et al., 1974; Decker, 1975; Elias and Friend, 1976; Decker, 1976).

The developmental mechanisms of contact during neurulation have been studied by Revel et al. (1973) and Decker and Friend (1974). However, these studies are limited to the formation of neuroepithelial contacts during the early phase of embryonal development. Moreover, during this phase it is not possible to differentiate between the newly built structures of the neuroepithelial contacts and those structures which are the result of a degradation process during further differentiation of the neuroepithelium.

In the present paper, the freeze-etching technique was employed in order to study in vivo and in vitro the development and the differentiation of the neuroepithelial junctions in the plexus epithelium. The embryonic plexus epithelium provides a simple model for studying the formation of neuroepithelial junctions (Meller and Wechsler, 1965), since its junctional complex does not undergo such dramatic degradation mechanisms as other neuroepithelial areas. Moreover, it retains its in situ specialization and polarization after trypsination and cultivation in vitro (Meller and Wagner, 1968; Meller et al., 1969).

Material and Methods

Cell Cultures: Choroid plexus from 11 day-old chick embryos were dissected and transferred to a 10 ml solution of Ca^{2+}- and Mg^{2+}-free saline (CMF). The dissociation medium (0.05% trypsin, Difco 1:250 in CMF saline) was preheated to 37° C. The plexus were washed in CMF saline and transferred to 5 ml of prewarmed trypsin and then stirred gently with a Teflon-covered magnetic stirring bar. After 8 min of agitation, the supernatant, containing dissociated cells, was removed, filtered through silk, and placed in a 50 ml centrifuge tube with 20 ml nutrient medium (Eagle's minimum essential medium, 10% horse serum, 6 mg/ml glucose). Fresh prewarmed trypsin was then added to the choroid plexus material, followed by stirring. After 8 min the cells were again collected. This procedure was repeated five times. The collected cell suspension was centrifuged and the cell pellet resuspended in fresh nutrient medium. The cell suspensions were cultivated in rotating flasks in a CO_2-enriched incubator. Cultivation lasted from 2 to 7 days.

Freeze-etching: For freeze-etching all choroid plexus cell cultures, embryonic choroid plexus from the 5th, 6th, 7th, 9th, and 11th day, and plexus from three day-old chicken were fixed (2–4 h) in phosphate buffered 2.5% glutaraldehyde. After fixation the plexus were washed in the same buffer and immersed in 20% glycerol/tyrode solution for a minimum of 30 min. The specimens were mounted on Balzers' gold disks, rapidly frozen in liquid nitrogen cooled Freon 22, and stored in liquid nitrogen. All freeze-fracturing and etching was performed in a Balzers' (BA 360 M) apparatus at −115° C. The etching time varied between 30 and 60 sec. The etched specimens were shadowed with platinum-carbon using an electron beam source (EVM 052). Replicas were cleaned with 40% chromic acid for 24 h, washed in distilled water, and mounted on Formvar coated copper grids. The replicas were examined with Philips EM 200 or EM 400 electron microscopes at an accelerating voltage of 80 kV.

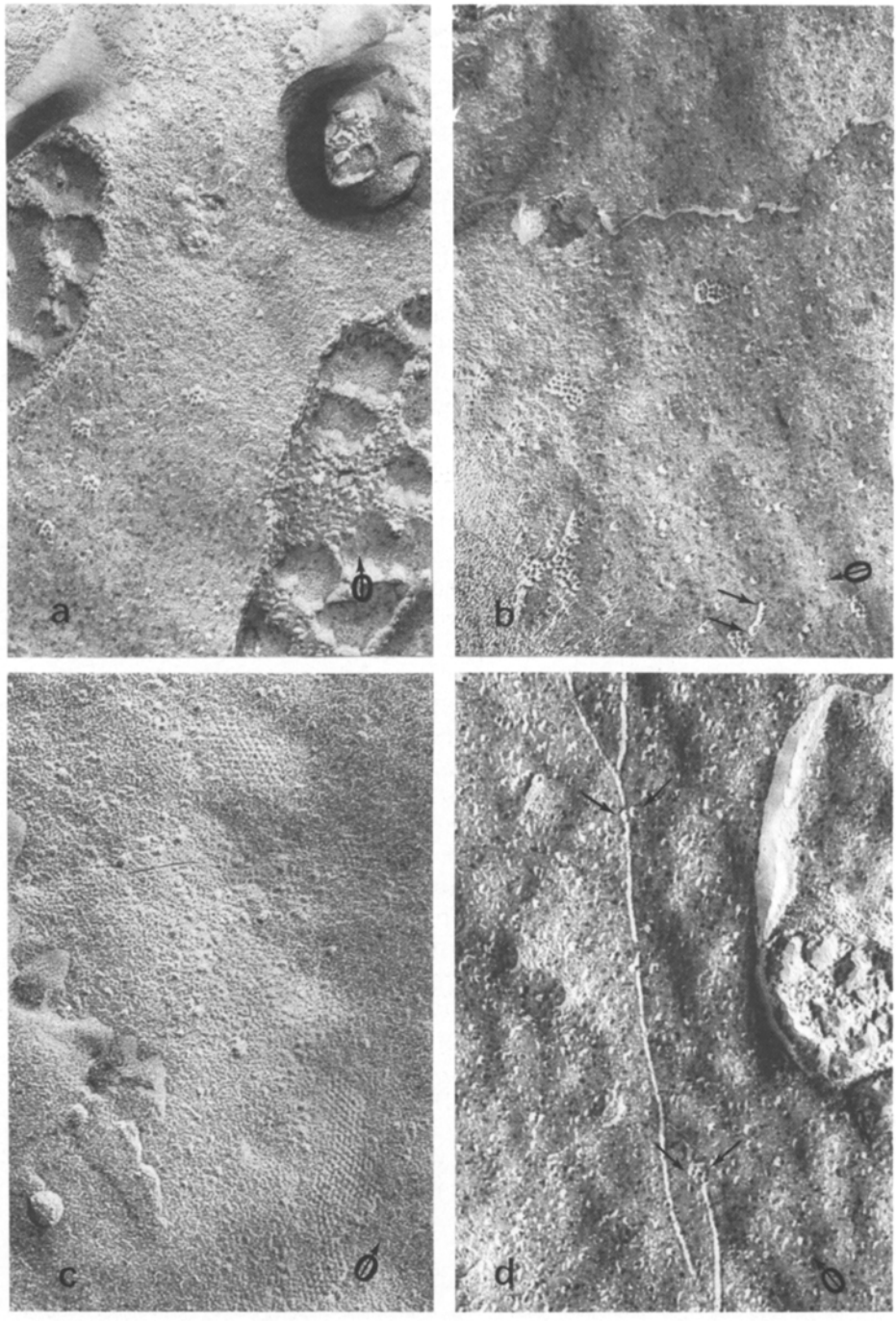


Fig. 1. a Plexus epithelium of five day-old chick embryo. The first differentiation in the presumptive junctional zone is the occurrence of small amorphous particle clusters. × 110,000. **b** In a further stage particle assemblies ordered in a polygonal pattern and short particulate row (*arrow*) become visible. × 110,000. **c** E-face aspects of early nexus formation. × 136,000. **d** Smooth contoured strands with small patches of particles in close relationship (*arrow*). The arrow (Ø) in the lower right corner indicates the direction of shadowing. × 110,000

Results

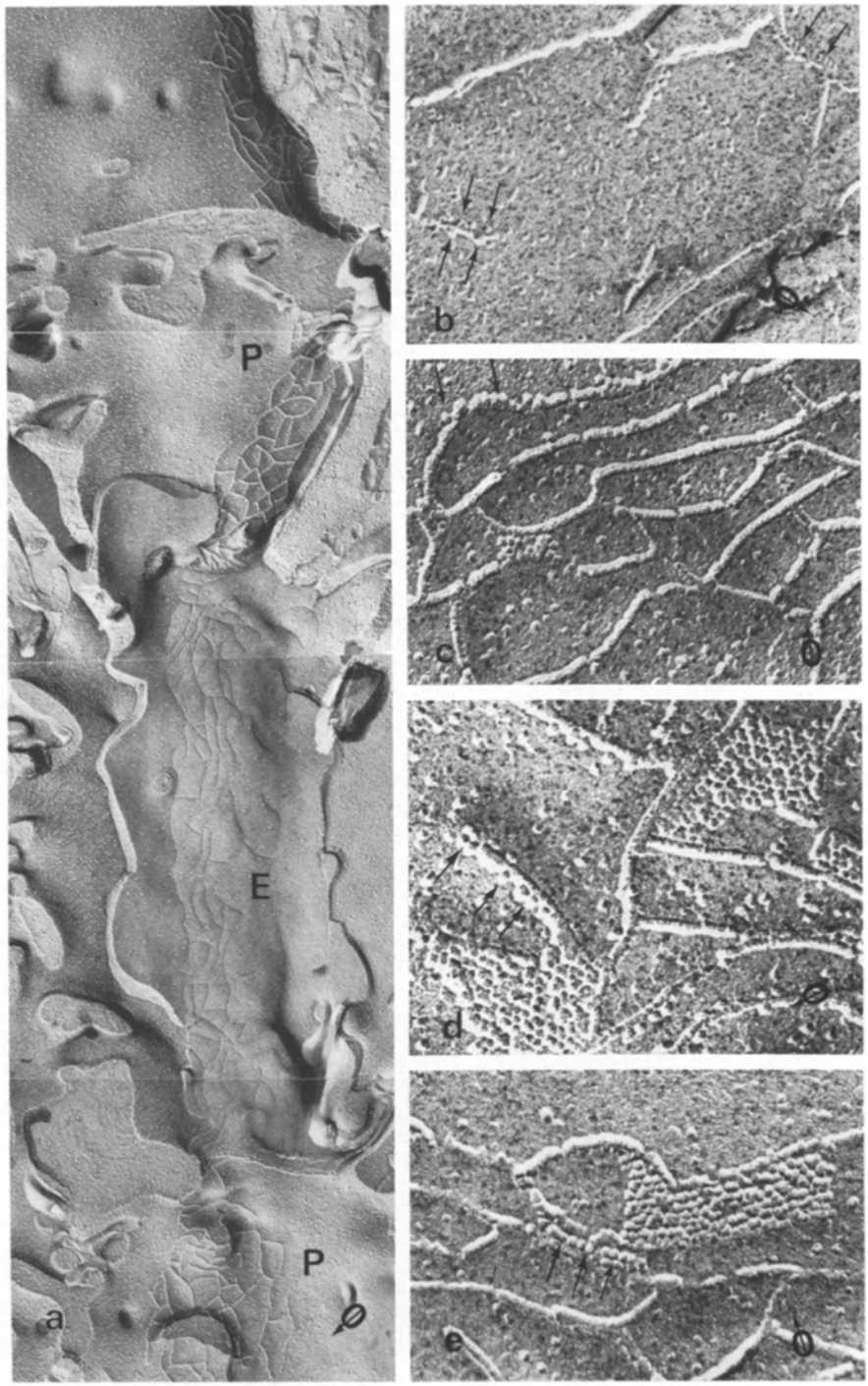
In vivo Formation of Choroid Junctional Complex

The first choroidal *anlage* appears in the ventricles of 5 to 6 day-old chick embryos. The apical surfaces of epithelial plexus cells are characterized by balloon-like microvilli. Freeze-fracture replicas of face-P membrane aspects of 5 day-old epithelial cells show a remarkable paucity of membrane-associated particles. The first visible signs of differentiation on fracture face-P is the occurrence of amorphous patches of particles (Fig. 1 a). As few as six to ten particles make up these assemblies. A further step in the development of the junctional complex is the occurrence of short, single chains of closely apposed particles, which may interconnect with the small aggregates. Simultaneously, particle plaques become visible which reveal a polygonal arrangement (Fig. 1 b). This highly ordered arrangement is documented by complementary pits on face-E membrane surfaces (Fig. 1 c). These plaques are assumed to be gap junctions.

Subsequently, the short particulate chains are elongated by the apposition of single globules. The particulate subunits of the chains fuse and smooth contoured fibrils become apparent (Fig. 1 d). In the apical membrane zones these segments of smooth ridges are oriented vertically to the axis of the cell. On the 6th day outgrowth and branching of the fibrils result in the construction of a junctional network. Smooth contoured ridges, however, are still interconnected by islets of small particles during this phase of development. Cells from the 9th day stage have developed a more or less complete zonula occludens. The basal portion of the junctional band frequently exhibits open-end fibrillar segments (Fig. 2a). Arrays of loosely arranged particles are in close relationship with the open-end strands. Between the 9th and 11th day an additional formation of nexus proceeds within the more or less fully developed zonula occludens. Particles 10 nm in diameter abut against tight junctional ridges. Subsequently, the particles form rows along the strands. These rows are predominantly associated with the most apical ridge of the zonula occludens (Fig. 2 b–e). In other areas of the zonula the nexus assembly occurs initially at the crossing points of the tight junctional strands. The "mature" junctional complex of the choroid epithelium has developed at the 11th day.

The junction itself is a band extending 0.1 to 0.5 μm in an apico-basal direction. The interconnected fibrils on face-P and the complementary furrows on E-face aspects form a loosely arranged network with remarkably wide facets. A preferred parallel orientation of the strands which was found in tight epithelia (Claude and

Fig. 2. a Zonula occludens at 9th day stage. The zonula occludens is fractured along the apical portion of three neighboring cells. In the basal area of the zonula open-end strands, or furrows, are frequent. Note that no nexus are integrated within the junctional band. *P* P-face; *E* E-face aspects. ×28,500. Freeze-cleaved replicas of the integration process of gap junctions within the zonula occludens (b–e). **b** and **c** Small fragments (b) of the continuous strands (c) of the zonula act as nucleation sites for the insertion of particles (*arrows*). b ×112,100; c ×190,000. **d** and **e** In addition to fully developed gap junctions typical particle rows along the tight junctional strands (*arrows*) are still visible. d ×213,750; e ×213,750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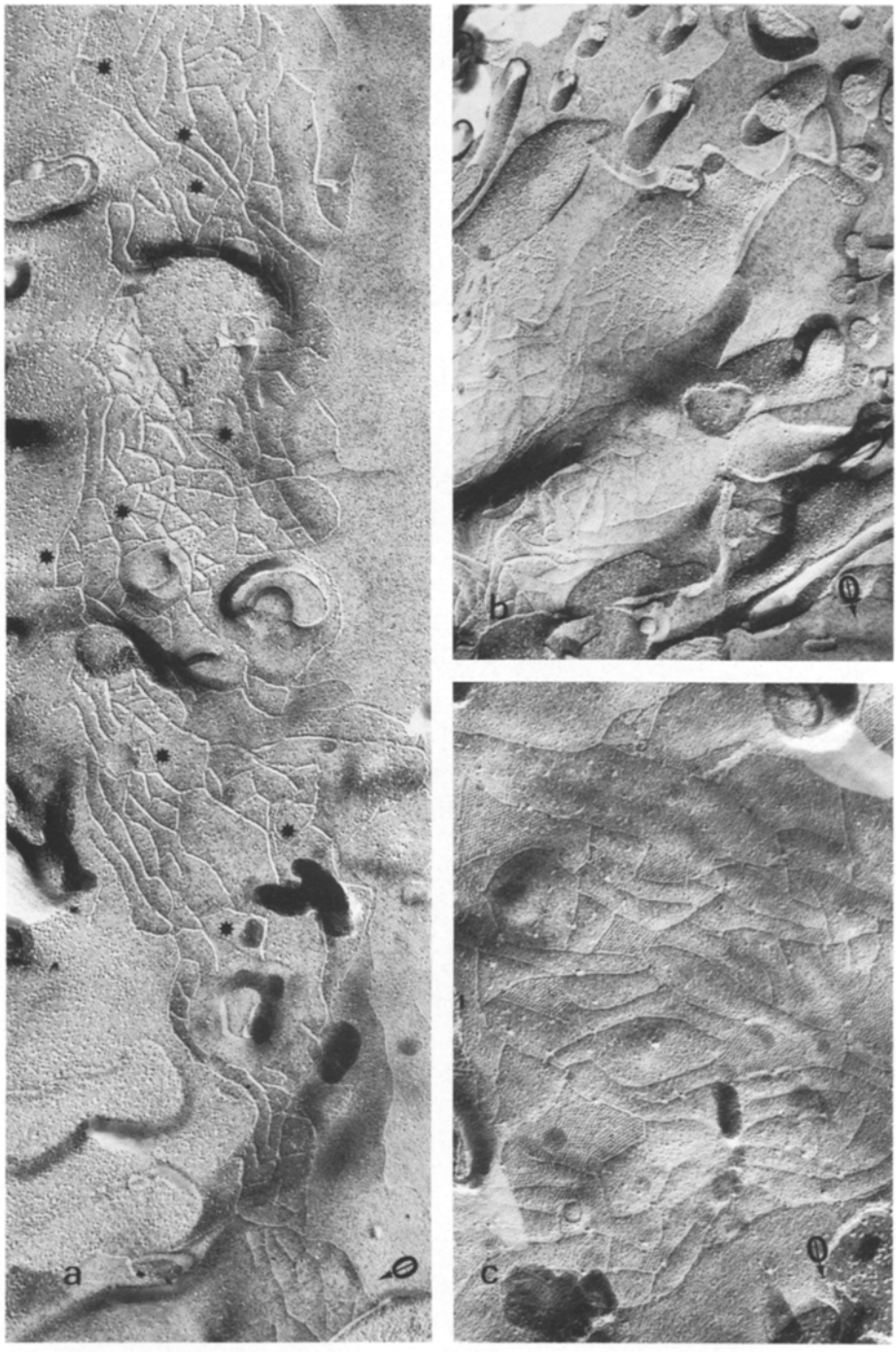

Fig. 3. a "Mature" zonula occludens on the 11th day. The junctional complex consists of a continuous band of strands on the P-face aspects. Several gap junctions are integrated within the facets of the zonula occludens (*asterisks*). **b** Apical portion of 11 day choroid epithelial cell. Nexus are located above the zonula occludens reaching the bases of microvilli. ×34,200. **c** E-face aspect of 11 day choroid epithelial cell. Note the high amount of integrated nexus. ×70,680

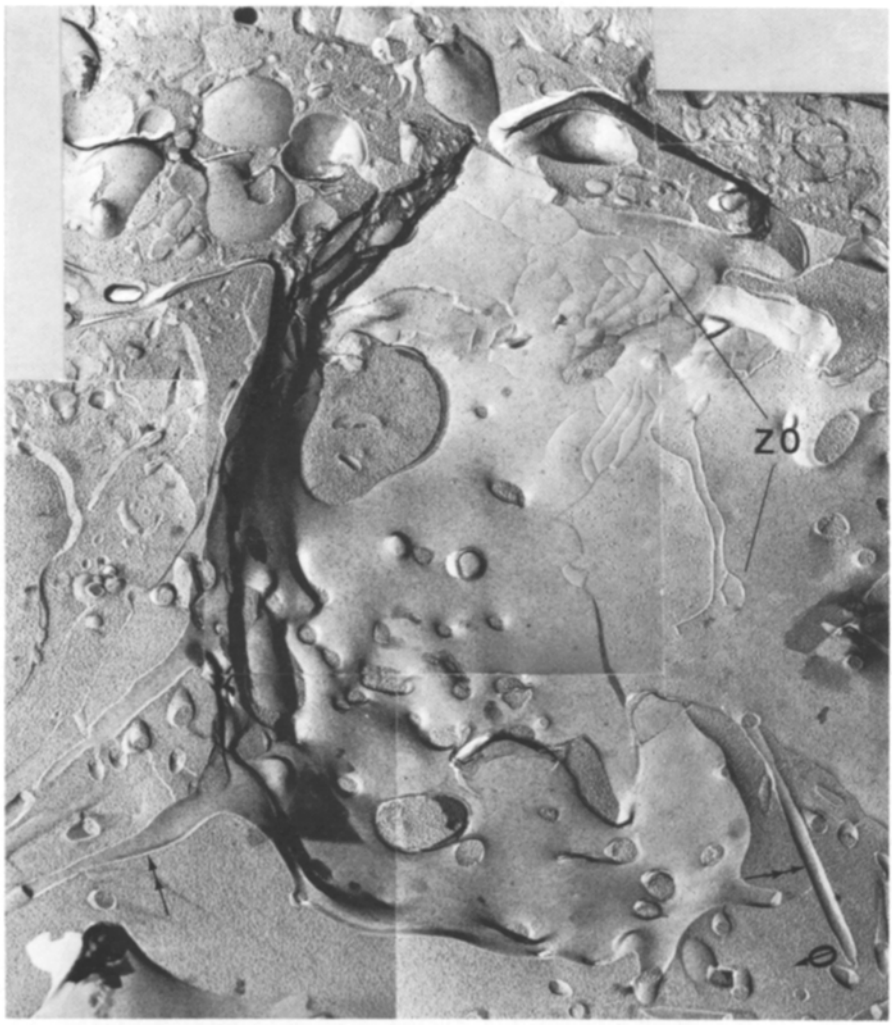

Fig. 4. A single trypsinized plexus epithelial cell. Cilia are found at the apical pole of the cell (*double arrows*). The upper lateral portion of the cell is still in contact with a neighboring cell. The zonula occludens (*ZO*) is fragmented. × 14,500

Goodenough, 1973) is not prevalent (Fig. 3a). Nexus of different size are integrated within the zonula occludens (Fig. 3b, c). The most enlarged gap junctions are in the apical position above the tight junctional band. They extend up to the base of the microvilli (Fig. 3b). Their basal demarcation, however, is regularly formed by the first apical junctional strand.

Disaggregation

Trypsination of the choroid epithelium induces a degradation of the zonula occludens and a loss of the integrated gap junctions. The trypsinized cells exhibit

Fig. 5. a Characteristic pattern of a trypsinized zonula occludens. Most of the strands are "digested". ×42,750. **b** High magnification micrograph of a trypsinized zonula occludens. A peculiar diagonal substructure of the strands on face-P is visible (*arrows*). ×165,110. **c** The "base" of the junctional strands (*arrows*) after trypsinization appears as a flat membrane elevation. ×275,500

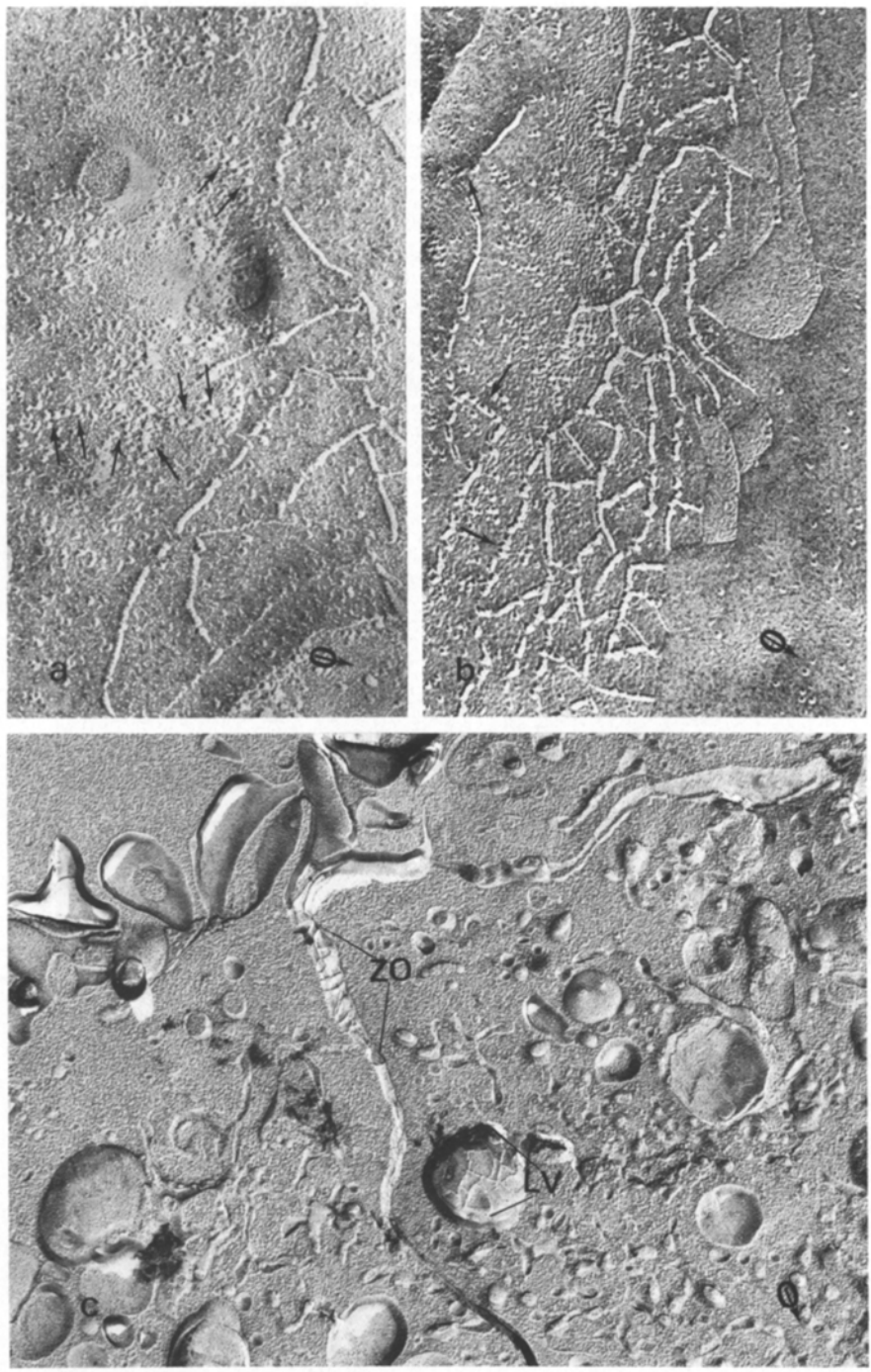

Fig. 6. a On the second day of cultivation particle rows (*arrows*) are visible in relation to fragments of the former zonula occludens. × 117,800. **b** On the 3rd day the strands have increased in continuity. The remodeling mechanism by particle apposition (*arrows*) is clearly visible. × 62,700. **c** Epithelial cells on 3rd day of cultivation. Fragments of tight junctions appear in a lysosomal (?) vacuole (*LV*). *ZO* zonula occludens. × 25,935

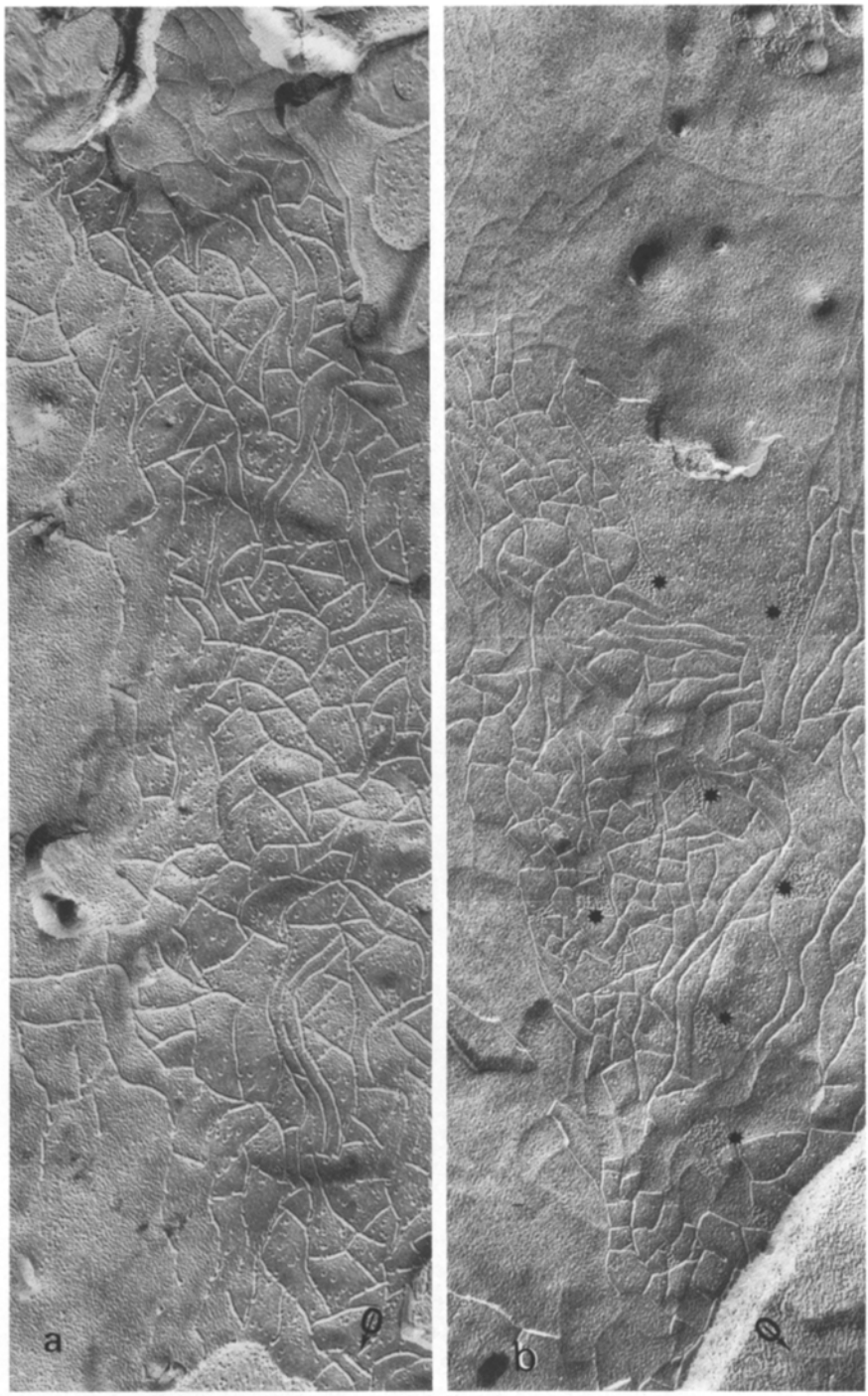

Fig. 7. a The strands of the zonula occludens on 4th day of cultivation are smooth contoured and continuous. Note that within the compartments of the zonula several particles occur. ×48,450. **b** Seven day cultivated cell. The junctional complex at this reaggregation phase is most similar to the in vivo arrangement on the 11th day. Nexus of different sizes are encompassed by tight junctional strands (*asterisks*). ×40,375

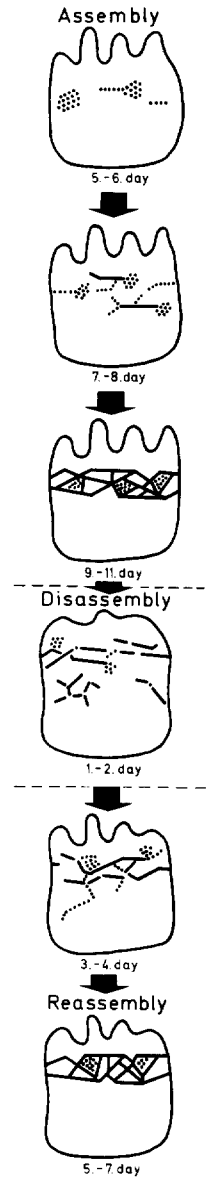

Fig. 8. Diagrammatic representation of the in vivo assembly, disassembly, and in vitro reassembly of the junctional complex in the choroid epithelium. It should be emphasized that the diagram depicts only the principal steps in the developmental mechanisms. The time scale is of more statistical value; it does not allow a precise temporal determination of particular developmental stages

a circular contour. The lateral portion of the cell surface is frequently delineated by segments of tight junctions, some of which are attached to cytoplasmic protrusions derived from neighboring cells. Transitional zones from P-faces containing fibrillar junctional segments of E-faces with corresponding furrows are frequently encountered (Fig. 4). The former apical portion of the cell surface can be easily identified by its microvilli. The degradation process of the zonula occludens most likely takes place in two phases.

The first phase is induced by the digestive effect of trypsin. It results in a fragmentation of the junctional strands. Preferentially, the short, vertical intersecting ridges of the zonula occludens are subjected to this lytic process (Fig. 5a). High magnification micrographs of the fragmented junctional strands reveal that, in continuity with the fibrillar segments, flat membrane elevations are prevalent which reveal no particulate elements (Fig. 5b, c). It is believed that these flat membrane elevations constitute part of the basis of the "digested" ridges of the tight junctions. Furthermore, a peculiar substructure becomes apparent by this trypsin-induced lytic process. It consists of a diagonally running striation of the flat membrane elevations (Fig. 5b).

The second phase of degradation includes the incorporation of tight junctional remnants into intracellular vacuoles. This process becomes apparent when the trypsin-treated cells are cultivated and will be discussed below.

Reaggregation of Cultivated Cells

The cultivated cells form typical rosette-like configurations. On the 2nd day of cultivation a resynthesis of the zonula occludens in the apical zone of the epithelial cells develops. The in vitro reconstruction of the tight junction includes fragments of the former zonula occludens. These fragments of former tight junctions probably act as nucleation sites from which the resynthesis proceeds (Fig. 6a). Chains and small assemblies of particles merge with smooth contoured fibrillar segments. At this stage of rearrangement no gap junctions are visible within the newly forming zonula occludens.

Fragments of tight junctions were often observed apart from the reconstituting junctional complex. These maculae occludentes appear as single fibrils or in groups. They frequently exhibit a ring-like configuration. The fragments in part become incorporated into membranes of lysosomes (Fig. 6c). This endocytotic mode of digestion was encountered during the 2nd to 4th day of cultivation. The reconstruction of the zonula occludens in vitro takes place within a time-course of a few days. At the 3rd to 4th day of cultivation most of the replicas across the apical zones show well developed tight junctions (Fig. 6b).

A new formation of nexus starts around the 5th day of cultivation. This remodeling process of gap junctions is almost identical with the mechanisms described in the in vivo development of this type of junction (Fig. 7a, b).

Discussion

1. The Formation of Tight and Gap Junctions in vivo

Recently, different models have been put forward to explain the development of tight junctions (Montesano et al., 1975; Gilula et al., 1976; Revel and Brown, 1976). According to these reports, this process appears to take place in the following sequence: a) The development begins with small clusters of particles within the presumptive junctional region. b) Subsequently, single particles or groups of

particles fuse to form segments of fibrils. c) The next step involves the outgrowth and branching of the ridges which become smooth contoured during proliferation. d) Finally, the fibrils reside on the apical pole of the corresponding epithelial cells in the form of typical band-like arrangements. In the present report this sequence has been observed during the formation of the membrane contacts in the developing plexus epithelium. The plexus epithelium, however, represents a specific pattern of junctional arrangement (Brightman et al., 1975; Dermietzel, 1976), namely the very close topographical relationship between tight and gap junctions. This intimate spatial and temporal relationship raises the question whether the developmental mechanisms of these membrane contacts are independent or if a mutual synergistic effect exists between both structures. Revel et al. (1973) as well as Decker and Friend (1974) reported that the appearance of isolated particles followed by particle clusters is the first step in the formation of membrane contacts in the developing neuroepithelium. Montesano et al. (1975) described a similar mechanism for the development of tight junctions in the fetal rat liver. Our findings on plexus epithelium indicate that a common particle pool for both kinds of membrane contacts presumably exists. The differentiation of these particles in gap and tight junctional subunits may depend on a further process of particle arrangement. The finding of Decker and Friend (1974) that gap and tight junctions arise simultaneously is supported by our investigation, however, only in the early stages of development. The small nexus in the early stages of neuroepithelium formation are probably essential for its intercellular communication. The fate of the "early" small gap junctions, however, is unclear at present.

During the later stages, the formation of tight and gap junctions does not run synchronously. The tight junctions are first fully developed, then followed by a final integration of gap junctions. It seems that the completely formed zonula occludens serves as a guideline for the formation of fully developed gap junctions in plexus epithelial cells.

2. Degradation and Formation of Membrane Contacts in the in vitro System

The breakdown of tight junctions after trypsination is not comparable with the in vivo degradation mechanisms described by Revel et al. (1973), Decker and Friend (1974) as well as Elias and Friend (1976). In the in vitro system, employed in the present report, one can differentiate between two disintegration mechanisms. The first might be a direct effect of trypsin on the protein complexes of the cell membrane, or an additional effect of trypsin on the cell coat, which results in the partial breakdown of the tight junctions. This breakdown appears to affect mainly the short segments of the tight junctions. The appearance of a structural component after the action of trypsin in the region of tight junctions is of particular interest. This component is characterized by a diagonal orientation of subunit structures. Similar structures on P-face membrane aspects were found to be present in endothelial cell contacts (Yee and Revel, 1975; Montesano, 1975; Simionescu et al., 1975; Dermietzel et al., 1977). These contacts were termed "abortive" tight junctions by Yee and Revel (1975). The question whether the

subunit structure, appearing after trypsination, is identical with the structures observed in vivo as an independent cell contact unit still remains unsettled. Secondly, further degradation of tight junctions is carried out by lysosomes. A similar process has been reported in vivo for intestinal epithelium and for isolated pancreatic cells (Staehelin, 1973, 1974). Preferentially, tight junctional remnants outside the re-forming zonula occludens are subjected to this endocytotic mode of digestion. An intriguing feature observed during disaggregation was the disappearance of gap junctions after trypsination. This finding, however, conflicts with results of Goodenough and Revel (1971), who did not find a dissolution of gap junctions after pronase treatment of isolated gap junctions.

The synthesis of the zonula occludens in vitro is a hemiconservative process. Remnants of the former zonula serve as "condensation points" from which the remodeling mechanism starts. It therefore seems most likely that the membrane area where the re-forming process takes place is identical with the former apical portion of the cell. The different phases of in vivo and in vitro formation of the junctional complex of the choroid plexus epithelium are diagrammatically represented in Figure 8.

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