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The Fine Structure of the Perineural Endothelium*

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Summary. Fine strands of motor nerves were examined with the electron microscope using thin section as well as freeze-etching techniques. The specimens were taken from frog cutaneous pectoris nerve, rat sciatic nerve, mouse and shrew phrenic nerves and from human skin nerves. The perineural sheath (Henle, Ranvier, Key and Retzius) consists of one to several concentric laminae of endothelial cells; it encases nerve fascicles and eventually individual nerve fibers and terminals. The endothelial cells are extremely thin and fitted together smoothly by overlap and dove-tailing of their border zones. The cell contacts are formed by continuous *zonulae occludentes*, often reinforced by *maculae adhaerentes*, and in depth they comprise 3–15 strands with an average of 5–6 strands per junction. The membranes of endothelial cells are studded with attachment sites and stomata of plasmalemmal vesicles suggesting a high level of pinocytotic activity. This phenomenon is by no means restricted to the external laminae of the endothelial sheath. Each endothelial lamina is vested with basement membranes on both (epineural and endoneural) sides, and the spaces between laminae contain a few collagen fibers and fibroblasts. Occasionally, punctate tight junctions are seen between laminae. Cytological evidence supports the hypothesis that the perineural endothelium provides a relatively tight and highly selective barrier separating the peripheral nerves from surrounding tissue and its extracellular fluid spaces.

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Dedicated to Professor Wolfgang Bargmann, Kiel, on the occasion of his 70th birthday.

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This effect is achieved on the one hand by the sealing of pericellular spaces and on the other hand by a membrane controlled transcellular transport mechanism (pinocytosis), both of which are enhanced by their serial arrangement.

Key words: Perineural endothelium – Ultrastructure – *Maculae adhaerentes* – *Zonulae occludentes* – Plasmalemmal vesicles.

Introduction

The history of the discovery of the perineural sheath dates back to the pioneering studies of the nineteenth century histologists. One of the first descriptions was given by Henle (1841) in his classical treatise entitled: *"Allgemeine Anatomie"*. Decisive progress came with the studies of Ranvier (1871/72), Key and Retzius (1878) and Retzius (1898) whose terminology of epineurium, perineurium and endoneurium is still widely accepted today (see review by Shanta and Bourne, 1968). In fact, light microscopy has since added little to these classical investigations revealing the multilayered concentric arrangement of the perineural cells. Decisive progress was made by the electron microscopists (Robertson, 1956; Pease and Pallie, 1959; Röhlich and Knoop, 1961; Shantaveraappa *et al.*, 1963; Thomas, 1963; Gamble, 1964; Cravioto, 1966; Waggener and Beggs, 1967; Burkel, 1967; Liebermann, 1968; Saito and Zacks, 1970; Babel, Bischoff and Spoendlin, 1970; Gray, 1970; Kerjaschki and Stockinger, 1970; Haller and Low, 1971). These studies emphasized the endothelial nature of the cellular components, their lining with basement membranes and the very close specialized intercellular contacts as well as the high level of pinocytotic activity.

This study is an attempt to provide more information on the fine structure of the perineural sheath by examining *en face* views of membranes and cell contacts with the aid of the freeze-etching technique. A preliminary report was given at the meeting of the Swiss Anatomical Association (Basel) in October 1974 (Akert *et al.*, 1975). When this manuscript was in preparation, a freeze-etch study of perineural sheath in the rabbit sciatic nerve appeared (Reale *et al.*, 1975). The findings of these authors are in good agreement with ours. A special effort was, therefore, made not to duplicate but rather complement their data from our extensive collection of samples and species.

Material and Methods

Fine strands of motor nerve fibers of frog (*Rana esculenta*) cutaneous pectoris muscle as well as from the diaphragm of the mouse (*Mus musculus domesticus*) were used in this study. Phrenic nerves of the shrew (*Suncus etruscus*) and human skin nerves were also examined. The amphibian tissue was kept in frog Ringer solution and fixed with 1% paraformaldehyde (50 mM Na-cacodylate buffer) or 1.25% glutaraldehyde (100 mM Na-cacodylate buffer) at pH 7.2 and an osmolarity of about 270 mosm. In most of these experiments the muscles were fixed with glutaraldehyde at 4° C. After dissecting the tissue into small pieces of about $1 \times 1 \times 0.2$ mm the fixation was continued for 2 hrs at room temperature, or for at least 24 hrs at 4° C. After this the specimens were washed for about 6 hrs in 200 mM Na-cacodylate buffer plus 6.8% sucrose, pH 7.2 and subjected to the freeze-etching procedure (Moor and Mühlethaler, 1963; Moor, 1971). Tiny tissue

blocks were soaked for 30 min at room temperature in 25% glycerol in Ringer solution. They were placed on flat golden tissue holders and rapidly frozen in liquid Freon at −115° C and kept in liquid nitrogen at −196° C before being fractured and etched for 5 min at −110° C in a Balzers BA 360 M vacuum microtome. About 150 successful replicas were examined in Siemens electron microscopes IA and 102.

The phrenic nerve of the shrew was obtained after perfusion fixation of the chest organs with buffered glutaraldehyde (1.5%), osmium tetroxide (1%) and uranyl acetate (0.5%). The other mammalian tissues were obtained by immersion fixation using the same solutions. Sections were made with diamond knives and stained with uranyl acetate and lead citrate. Only a few mouse phrenicus specimens were treated with the freeze fracturing and etching methods (see above); this material was also fixed by immersion in aldehydes.

Results

Thin Sections: Briefly, the perineural sheath is composed of a series of concentric cell layers (Fig. 1) which are extremely thin and separated from each other by an extracellular space. Very small nerve fascicles are encased by a single cell layer, thus resembling a blood capillary except for the fact that the perineural endothelium is vested by a basement membrane not only at the tissue front but also on the "luminal" surface. The extracellular spaces between layers contain small amounts of collagen fibrils running longitudinally and circumferentially (Fig. 4). Occasionally, two layers are in close contact by forming macular tight junctions (Thomas, 1963). Several endothelial cells may be combined to form a cylindrical, sleeve-like layer. Edge-to-edge junctions of adjacent cells are smooth. In the cross-sectional profile one observes both simple and complex (dove-tailing) overlappings (Fig. 3) which are characterized by a series of tight junctions forming a continuous *zonula occludens* (Farquhar and Palade, 1963). However, the real nature of the intercellular contacts can be identified more reliably on the basis of freeze-etch replicas. The subjunctional cytoplasm is characterized by abundant fibrils; it is nearly free of other organelles. The remaining areas of the cells are studded with pinocytotic vesicles (Figs. 3 and 4), which occur throughout all layers of the perineural sheath and by no means selectively in the outer laminae (Fig. 1). Some of them are found in the junctional region.

Freeze-Etch Replicas: Cross-sectional profiles of the perineural sheath (Fig. 2) confirm the information gained by thin sections. Larger membrane faces of endothelial cells expose the two main features: *Cell contacts and pinocytosis.* The former are readily identified as tight junctions. Their fracture patterns according to Wade and Karnovsky (1974) are summarized in Fig. 5 which provides the key for the interpretation of the subsequent electron micrographs. A-faces represent split inner faces of cytoplasmic membrane leaflets. B-faces are the cleaved faces of the external membrane leaflet (Branton, 1966).

The majority of the fracture faces represent patterns 1 or 2 as depicted in Fig. 5 (Figs. 6, 8, 9, 13, 15) in which A-faces contain ridges (Reale *et al.*, 1975) and B-faces are characterized by complementary grooves (Chalcroft and Bullivant, 1970). These formations are relatively distinct because ridges and grooves comprise approximately three quarters of the double diameter. Quite

frequently the ridges are particulate and resemble dashed or dotted lines (Figs. 8, 9). Conversely, the grooves may contain particles which seem to represent the pieces missing from the ridges of the opposite membrane. This is true also for the next patterns (3 and 4, Fig. 5) which are less frequently encountered and consist of the reversal of ridges and grooves with respect to A- and B-faces (Figs. 7, 10, 11). It is to be noted, however, that grooves and ridges in these cases are relatively shallow since their depth or height are equal to only part of a single membrane diameter. This difference is particularly evident when comparing the junctional complexes in Figs. 6 and 9 with those in Figs. 7 and 10.

Patterns 5 and 6 (Fig. 5) which have been postulated by Staehelin (1973) were not found with certainty in the material available. Transitional fracture patterns T_1 and T_2, however, were regularly seen (Figs. 6, 7, 8, 10, 13).

The tight junctions are arranged in a characteristic network of branching and reconverging lines whose main orientation is lengthwise along the overlapping cell borders. This pattern is typical of *zonulae occludentes*. The number of strands seen in the *zonulae occludentes* in the frog material varied between 3 and 15 with a maximum distribution at 5 and 6. Similar figures were obtained in the mouse and shrew phrenic nerves.

In most instances, the meshwork of tight junctions seems to be continuous. Occasionally, one finds limited junctions (not illustrated) which may be interpreted as *maculae occludentes* (McNutt and Weinstein, 1973) and may connect two laminae with each other as suggested by Reale *et al.*, 1975; however, we have been unable to ascertain the exact localization of such discontinuous junctions within the perineural sheath.

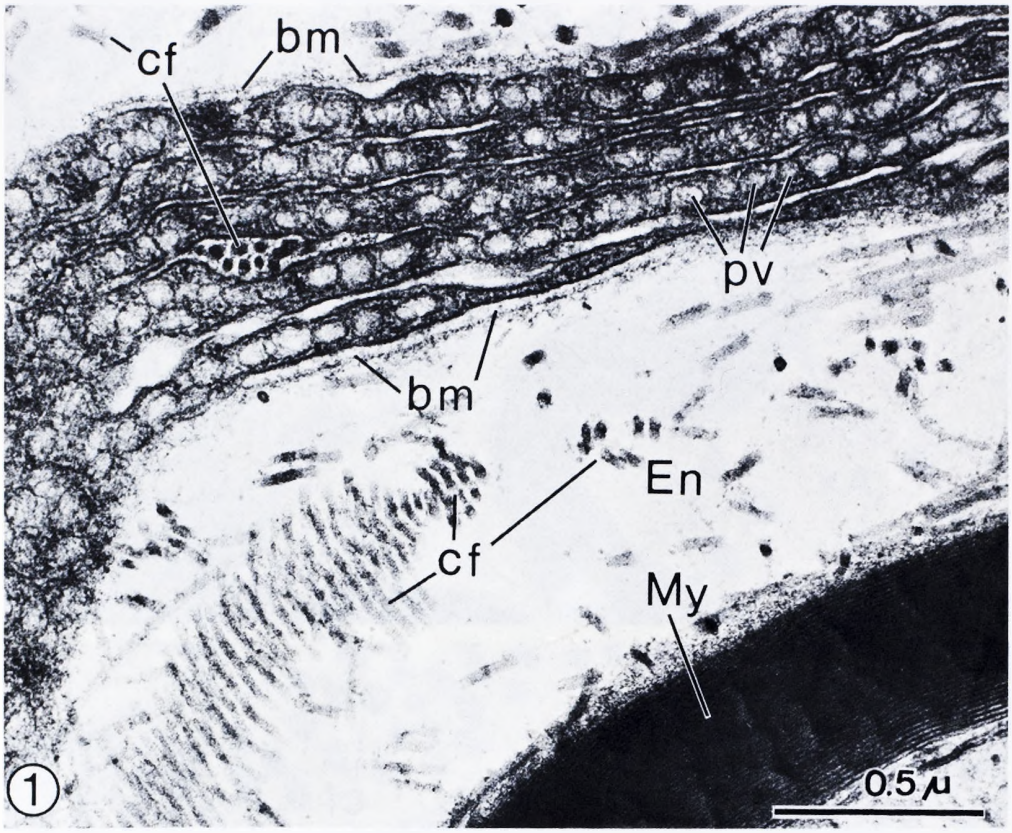
The dividing line between overlapping cells at the level of A-faces is characterized by a central ridge representing a single strand belonging to the occludens junctions (Figs. 6, 7).

The next feature of freeze-etched membrane faces of endothelial cells consists of attachment sites of *plasmalemmal vesicles*. They appear as tiny pits in A-faces and complementary crater-like bumps in B-faces. While these attachment sites are extremely numerous at the endo- and epineural faces on the plasmalemma they are seen somewhat less frequently within the limits of *zonulae occludentes* (Figs. 8–10), and occasionally they are altogether missing in the junctional region (Fig. 6); in some instances (Fig. 11) they were particularly numerous.

Finally, *desmosome*-like attachment plaques are quite frequently encountered (Figs. 10, 12–15). The corresponding membrane region is slightly indented in

Fig. 1. Multilaminated structure of perineural endothelium. Muscle nerve, frog cutaneous pectoris. Glutaraldehyde immersion fixation. *bm* Basement membrane, *cf* collagen fibrils, *En* endoneural space, *My* myelin, *pv* pinocytotic vesicles. Primary magnification × 20,000

Fig. 2. Multilaminated structure of perineural endothelium. Same material as in Fig. 1 prepared with the freeze-etching method. *A* cytoplasmic membrane face of endothelial cell with *zonula occludens* (*zo*). *Fb* fibroblast at the level of the endoneural space. *Encircled arrow heads* in this and the following photographs of replicas indicate the direction of shadow casting. Primary magnification × 8,000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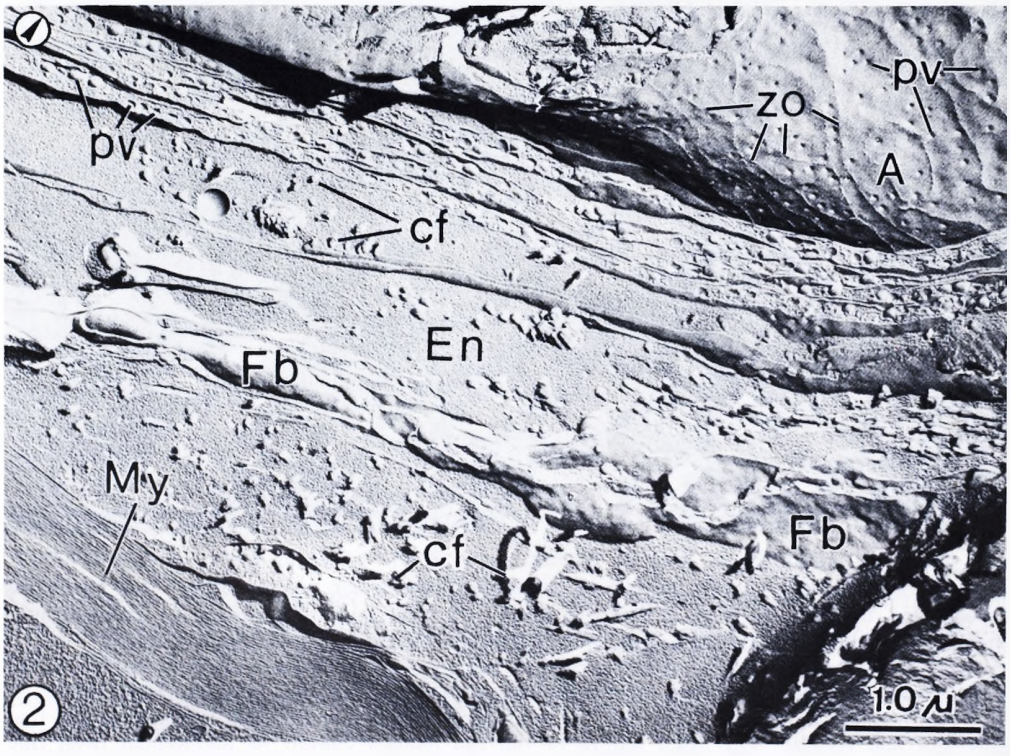
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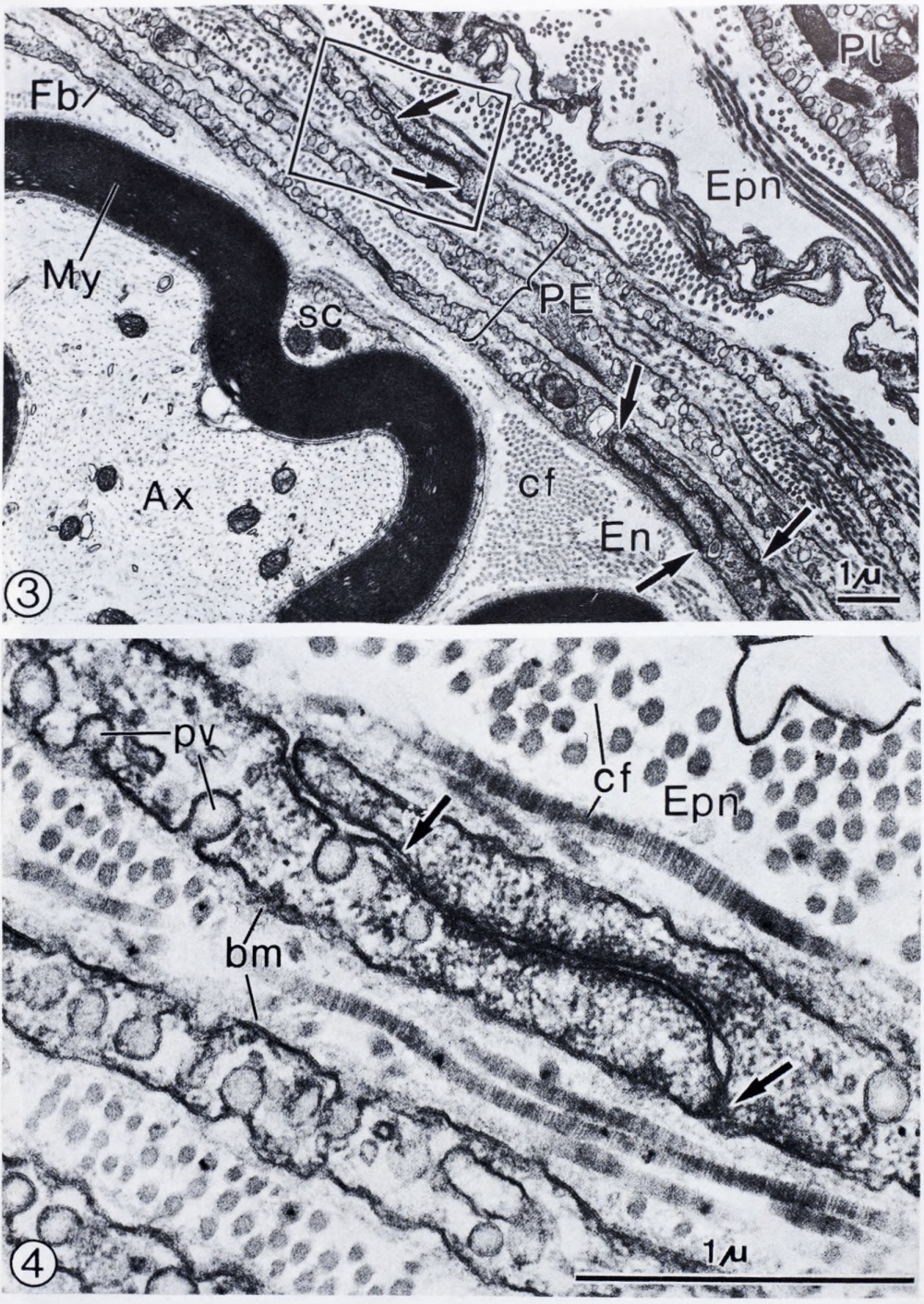


Fig. 3. Perineural endothelium (*PE*) of phrenic nerve in the Etruscan shrew (*Suncus etruscus*). Concentric lamellae consist of a mosaic of endothelial cells which are smoothly fitted by means of specialized junctions (*arrows*). *cf* Collagen fibrils, *Ax* myelinated (*My*) axon, *En* endoneurium, *Epn* epineurium, *Pl* pleural endothelium, *sc* Schwann cell. Primary magnification × 40,000

Fig. 4. Specialized junction of perineural endothelium (*arrows*) from the same specimen shown in Fig. 3 (see *rectangular frame*). Note that the cytoplasm of the subjunctional region contains fine fibrils and few vesicles. The exact nature of the cell contact cannot be determined from this picture. Primary magnification × 80,000

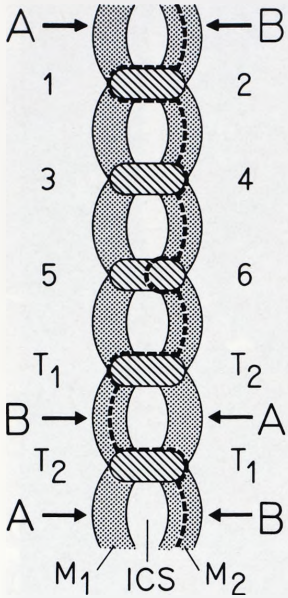


Fig. 5. Summary of fracture planes which (with the exception of 5 and 6) have been encountered in the present study (modified after Wade and Karnovsky, 1974). Two membranes (M_1, M_2) are in multiple contact by tight junctions. *ICS* intercellular space. Even numbers represent fracture faces of the *B*-type (facing the extracellular space), uneven numbers represent the complementary fracture faces of the *A*-type (facing the cytoplasm). In *1 - 6* the fracture remains within the same membrane, in T_1 and T_2 it changes within the tight junction from one membrane to the adjacent membrane. The numbers in this diagram are referred to in the subsequent illustrations. Further explanations, see text

A-faces; the plaques consist of round or oval-shaped aggregations of membrane-associated particles or broken ends of fibrillary structures (Breathnach *et al.*, 1972; McNutt and Weinstein, 1973; Staehelin, 1974). These particles or fibrils are oriented radially with smaller and thinner particulate elements lying near the periphery. The diameter of particles or fibrils may vary between 6 and 12 nm. Desmosomes are found almost exclusively within the limits of *zonulae occludentes*.

Discussion

The present study confirms and extends our knowledge on the remarkable structural organization of the endothelial barrier which separates peripheral nerves from the surrounding tissue and its extracellular fluid spaces. This barrier consists of a multilaminated endothelium, whose layers are formed by extremely thin and smoothly fitted cells. The fitting is achieved by overlap and dove-tailing and the cell borders are sealed tightly by continuous *zonulae occludentes* as

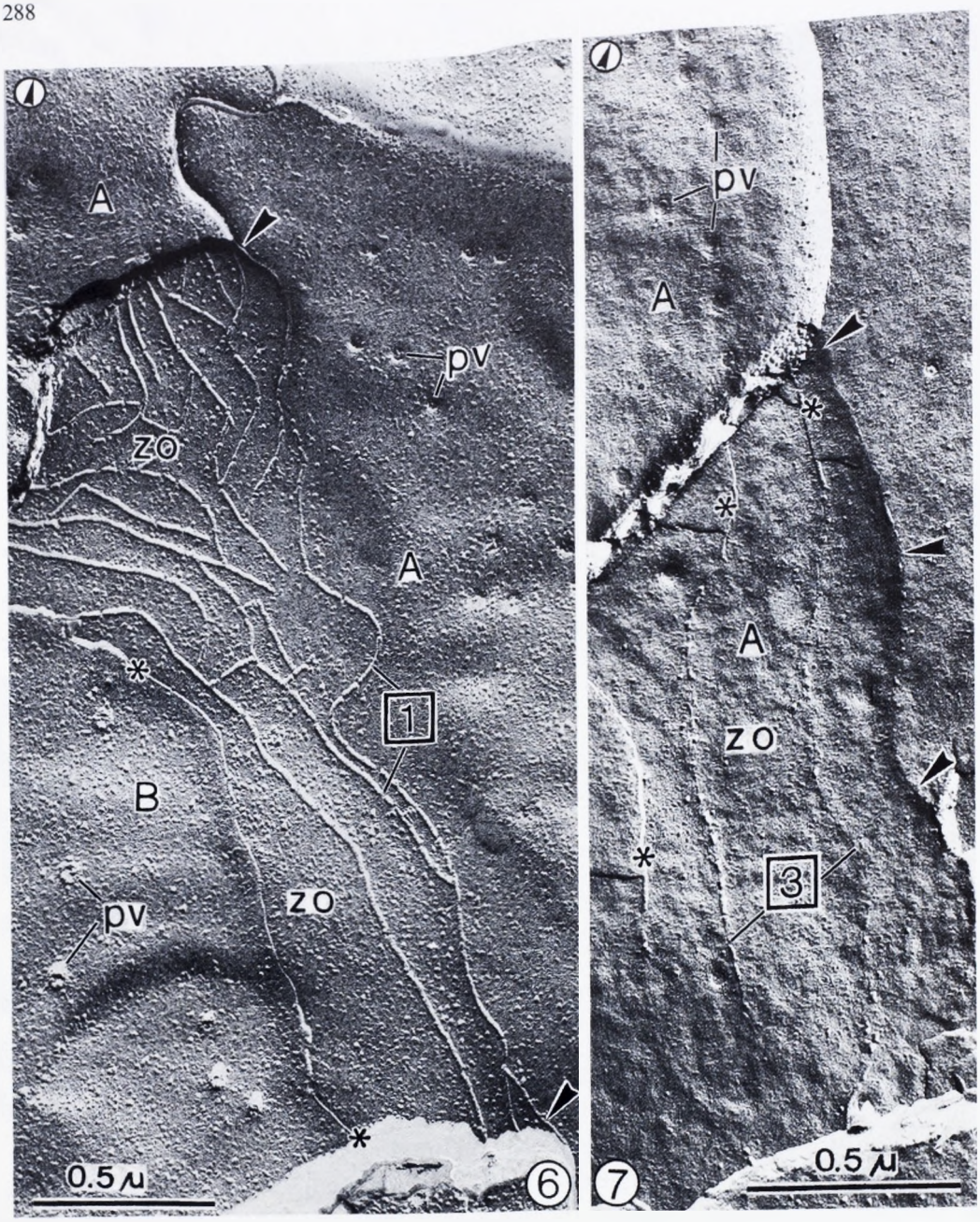


Fig. 6. Region of overlap between two endothelial cells. *Arrow heads* mark the fracture of the overlying sheet, exposing the A-face of the contact zone which consists of branching strands of the *zonula occludens* (*zo*) according to type *1* (see Fig. 5). *Asterisks* mark transitional fracture patterns according to type T_1 or type T_2, where the fracture line follows the ridge of a tight junction. Pinocytotic vesicles (*pv*) are located exclusively beneath the junctional region. Their membrane attachment sites appear as pits in the *A*-face and as bumps in the *B*-face. Phrenic nerve of mouse, glutaraldehyde immersion fixation. Primary magnification ×20,000

Fig. 7. Region of overlap between two endothelial cells. Same situation as in Fig. 6, taken from frog cutaneous pectoris muscle nerve. Note that the fracture pattern conforms to type *3* (see Fig. 5). The ridges are particulate. Note the single stranded central ridge between the folds of the overlapping cells. Glutaraldehyde immersion fixation. Primary magnification ×20,000

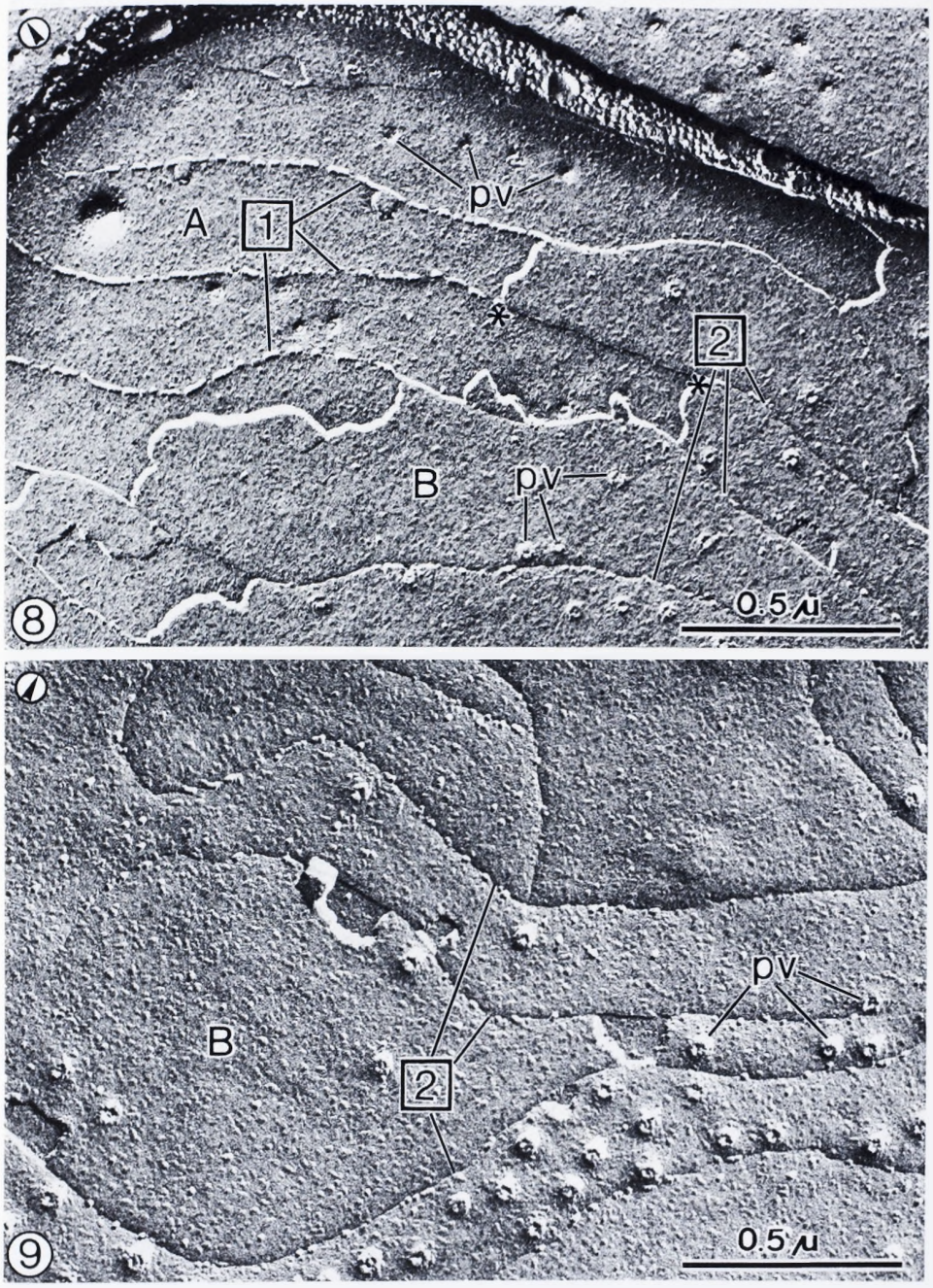
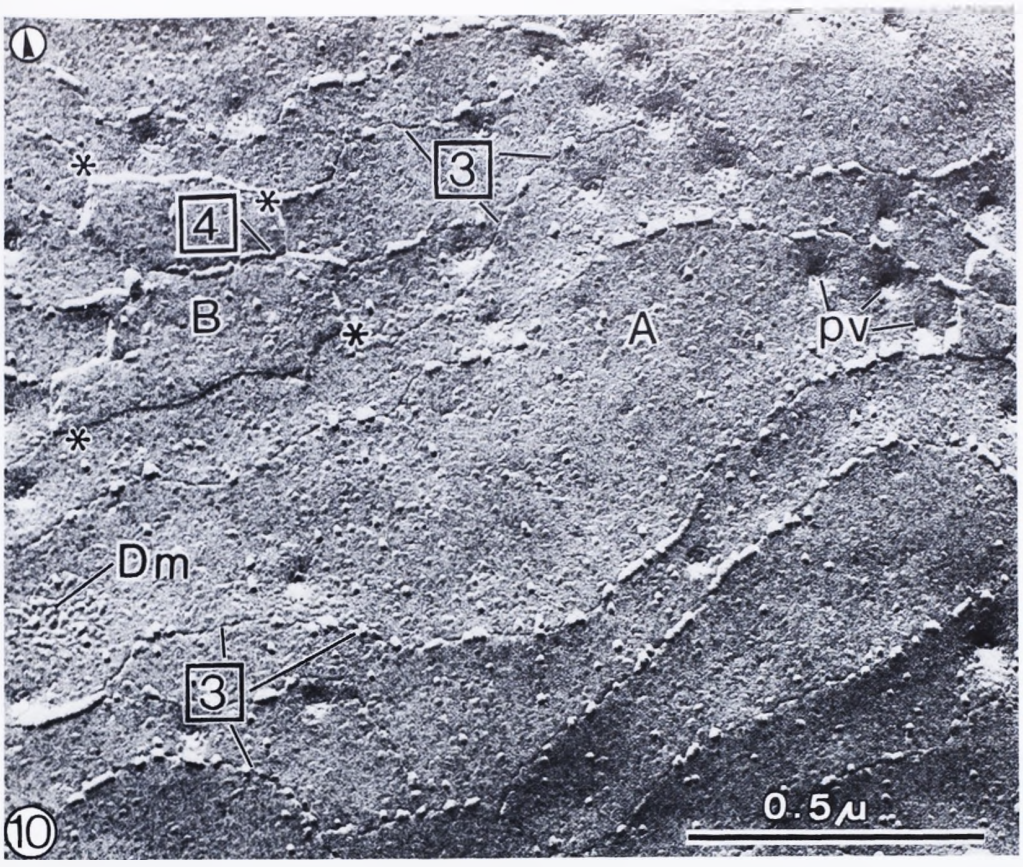


Fig. 8. *Zonula occludens* in perineural endothelium of frog cutaneous pectoris muscle nerve. The tight junctions are fractured according to type *1* and *2* patterns (see Fig. 5). *pv* Pinocytotic vesicles occurring within the junctional district. Glutaraldehyde immersion fixation. Primary magnification × 20,000

Fig. 9. *Zonula occludens* taken from frog cutaneous pectoris muscle nerve. Fracture according to type *2* pattern (Fig. 5). Glutaraldehyde fixation. Primary magnification × 20.000



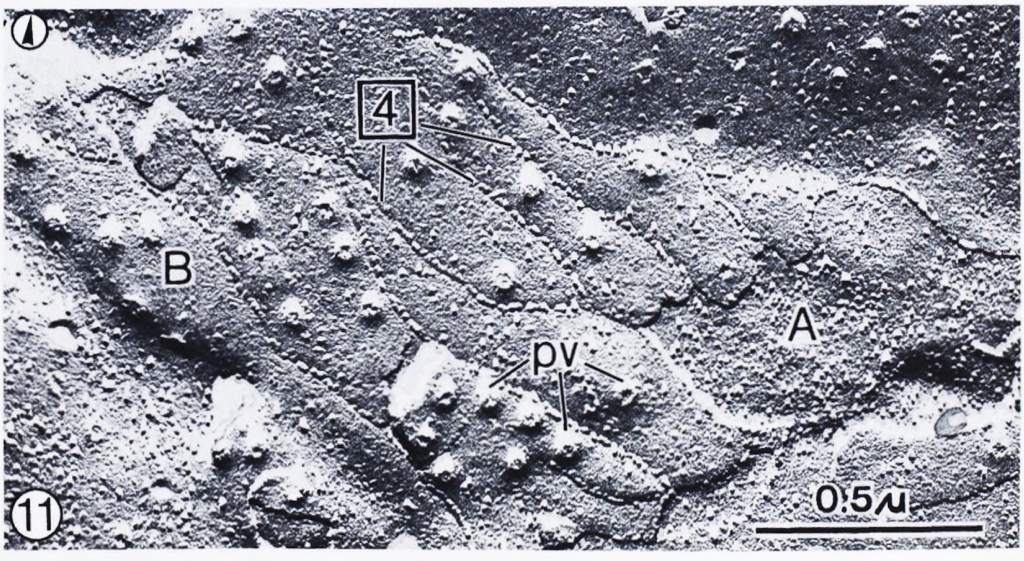
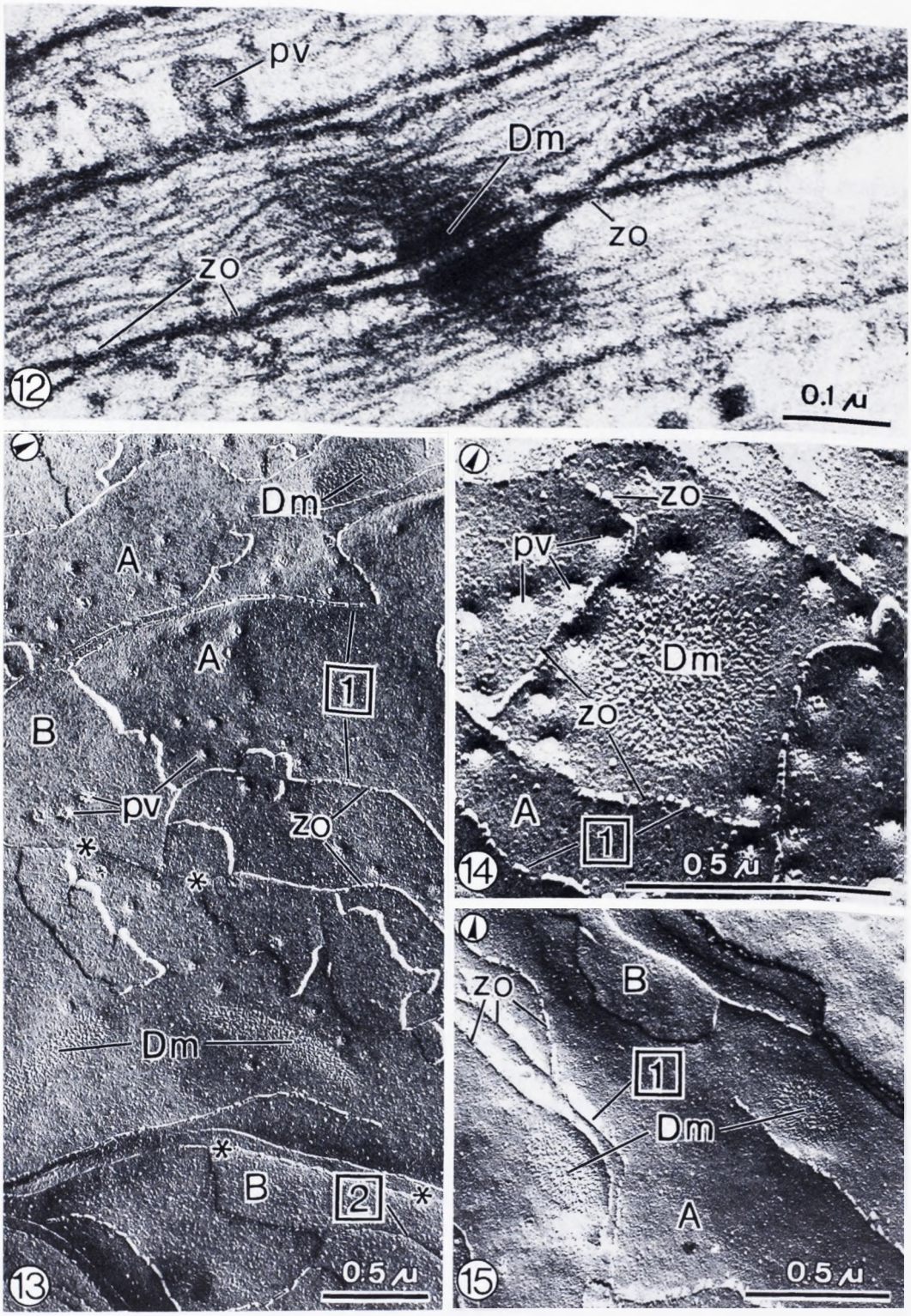


Fig. 10. *Zonula occludens* taken from perineural endothelium of frog cutaneous pectoris muscle nerve. Fracture pattern according to type *3*, *4* as well as T_1 and T_2 (*asterisks*) (see Fig. 5). Note that the grooves in the *A*-face are shallow in comparison with those of the *B*-face illustrated in Fig. 9. *Dm* Desmosome within junctional zone. Glutaraldehyde immersion fixation. Primary magnification × 20.000

Fig. 11. *Zonula occludens* taken from frog cutaneous pectoris muscle nerve. Fracture pattern according to type *4* (see Fig. 5): the *B*-face contains linear ridges that appear particulate. *pv* Pinocytotic vesicles within the zonular district. Glutaraldehyde immersion fixation. Primary magnification × 20.000



Figs. 12–15. Desmosomes (*Dm*) situated in *A*-faces of endothelial plasmalemma in close association with *zonulae occludentes* (*zo*). *Asterisks* indicate T_1 and T_2 fracture patterns of tight junctions. Figs. 12, 13 and 15 are from frog, Fig. 14 from the mouse. Glutaraldehyde immersion fixation. Primary magnification in Fig. 12 ×40,000, in the others ×20,000

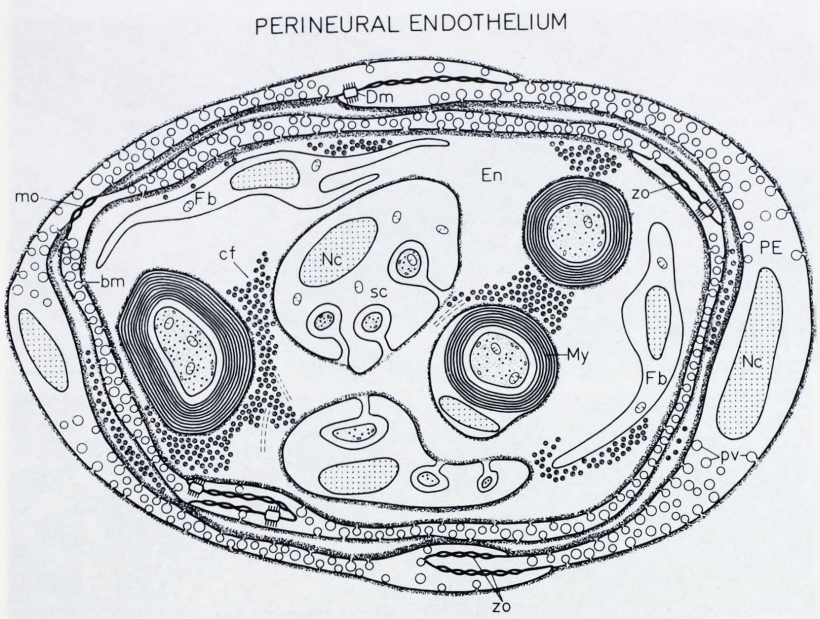


Fig. 16. Summary diagram of perineural endothelium (*PE*). *bm* Basement membrane, *cf* collagen fibrils, *Dm* desmosome, *Fb* fibroblast, *En* endoneurium, *mo* macula occludens, *My* myelin, *Nc* nucleus, *sc* Schwann cell, *zo* zonula occludens (modified after Gray, 1970)

has been demonstrated by Reese and Karnovsky (1967) for the capillary endothelium of mammalian cerebral cortex. Perineural endothelium, however, is equipped with additional features: (1) the concentric arrangement of multiple cylindrical layers, (2) the vestment of these layers with basement membranes both on the epineural and endoneural face, and (3) the presence of collagen fibrils within the spaces between layers (Fig. 16).

The tight junctions found in this study conform structurally to those found in other tissues. The freeze-etch aspect of this striking cell contact has been treated by previous investigators (Kreutziger, 1968; Staehelin *et al.*, 1969; Chalcroft and Bullivant, 1970; Goodenough and Revel, 1970; Friend and Gilula, 1972). It seems noteworthy to point out the fact that our findings can best be interpreted according to the single-fibril model proposed by Wade and Karnovsky (1974) since the overlap between cells at the level of the "suture" line is consistently represented by a *single-stranded* linear ridge between the folds of the bordering membrane (Figs. 6, 7). Several authors have been concerned about variations in the fracture patterns of tight junctions (Dempsey *et al.*, 1973; McNutt and Weinstein, 1973; Staehelin, 1973) and these variations have been related to the effect of varying degrees of aldehyde fixation. We can confirm that more than one type of fracture line was present in our material, and that the ridges of tight junctions were often discontinuous and the corre-

sponding grooves studded with particulate material representing the missing links of the complementary leaflet. These variations may well be accounted for by differences in aldehyde fixation which could not be adequately controlled by means of the immersion procedure which we had to adopt for technical reasons.

Tight junctions in various epithelia have been recently evaluated with respect to morphological criteria of "leakiness" by Claude and Goodenough (1973). These authors succeeded in establishing a reasonably good qualitative correlation between transepithelial permeability and the number and arrangement of strands per *zonula occludens*. When comparing our data with the wide spectrum of morphological and physiological properties tabulated by these authors we have to conclude that the perineural sealing is of the "intermediate" type, *i.e.* ranging about midway between "very leaky" and "very tight" with respect to junctional morphology. This attribute may be appropriate for characterizing the sealing of a single sheath; however, considering the fact that a series of up to 15 endothelial cell layers may be involved in the encasement of nerve fascicles, it seems justified to assume that the multilaminated arrangement results in an additive tightening of the barrier. Permeability studies with tracer molecules are consistent with this conclusion: Protein markers, such as ferritin (Waggener *et al.*, 1965; Hall and Williams, 1971) or horseradish peroxidase (Olsson, 1966; Klemm, 1970; Olsson and Reese, 1971), fail to penetrate the perineural sheath, or at best may reach the most superficial layer (Klemm, 1970). Similar results have been obtained with dye stuffs by Martin (1964) who made a survey of the past literature on this subject and concluded that the endothelial layers were responsible for the barrier effect.

This barrier seems to have two principal leakage points: (1) the region of nerve endings where uptake of relatively large molecules into the axoplasm seems to take place without major obstacles (for review of this literature see Kristensson and Olsson, 1973), and (2) the blood-nerve barrier which seems to be less efficient with regard to both ions (Welch and Davson, 1972) and polar non-electrolytes (Bradbury and Crowder, 1975) than the blood-brain barrier. The physiological significance of the perineural sheath has been the subject of controversy (Krnjevic, 1954), but its powerful role of controlling electrolyte concentration gradients between perineural tissue and endoneural spaces during excitatory and postexcitatory events seems by now well established (Vorontsov, 1962). Our study supports this hypothesis by demonstrating the remarkable structural organization of the perineural endothelium which seems uniquely suited for a barrier mechanism. The latter consists not only in the sealing of an elaborate labyrinth of pericellular spaces, but also by an active control of transport across the endothelial cells by means of pinocytosis. These two mechanisms, which are enhanced by their serial arrangement in the form of concentric lamination, may act as seals and locks for molecules that remain to be specified in future studies.

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