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STUDIES ON THE HISTOLOGY AND PERMEABILITY OF THE PERIPHERAL NERVOUS BARRIER

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It has been shown in a previous paper (*Weiss* and *Röhlich*, 1954), that India ink, administered perineurally does not penetrate the sciatic nerve of the rat even in high concentrations and after long periods of time. At the time this interesting fact could only be interpreted by assuming a barrier interposed between the connective tissue and the nerve fibres, which prevented free diffusion of colloidal solutions. Therefore it was decided to investigate the problem of this diffusion barrier more thoroughly.

The peripheral nervous barrier has been dealt with by several authors. The concept of the barrier has been introduced by *Feng* and *Gerard* (1930), as well as *Feng* and *Liu* (1949, 1950), who have shown that the connective tissue sheath of the frog's sciatic nerve acts as a diffusion barrier. The rate of action of most of the various solutions (measured in action potential and time of depolarisation) is greater after desheathing or splitting the nerve.

Experiments referring to such function of the connective tissue have been known since long (*Overton*, 1904 ; *Rice* and *Davis*, 1928). *Overton* had demonstrated, that the nerve reacts much later to toxic agents than the muscle. According to that author this difference is due to the diffusion-inhibiting action of the perineurium.

The validity of *Feng* and *Gerard*'s, and *Feng* and *Liu*'s experiments has been strongly questioned by *Lorente de Nó* (1947, 1950, 1952), who found *Feng* and *Liu*'s technique inadequate, for various reasons, viz. (i) Any attempt at ascertaining the interval between the application of the test solution to the nerve and the establishment of a total conduction block is out of place, «since total conduction block cannot be established until the test substance has produced a profound change in the properties of those fibres which are least sensitive to its action, which may occur long after the test substance has reached full concentration at the axis of the nerve». (ii) Removal of the sheath enlarges the interfibrillar spaces, thus increasing the possibility of diffusion. (iii) Removal or splitting of the sheath modifies the function of the nerve fibres both quantitatively and qualitatively. (iv) «The epineurium respresents only a small part of the connective tissue sheath ; the main part of the sheath is the endo-

neurium», which cannot be removed at all. Further, «the connective tissue sheath is freely permeable to solutes, be they ionized or not» and the existence of the connective tissue sheath may be ignored in neurophysiological experiments.

The results of a great number of experiments, however, appear to contradict this theoretically acceptable criticism. *Crescitelli* (1951), in developing a suitable method for desheathing and resheathing found that after pulling back the sheath («resheathing»), the action of antihistamines on the nerve was again a belated one, i. e., the barrier properties returned unchanged. The barrier effect was equally demonstrated on the isolated sheath ; *Keynes* and *Stämpfli* (1949) filled a bag prepared of the connective tissue sheath of the frog's sciatic nerve with Ringer's solution labelled with K^{42} ions. Outward diffusion was very slow unless Ringer's solution saturated with chloroform was used. In similar experiments, *Shanes* (1954) has investigated the exchange of Na^{22} across the perineurium. Observations made on isolated nerve fibres *(Huxley* and *Stämpfli*, 1951 ; *Stämpfli*, 1952) indicate that a change in the ionic composition of the medium acts on the nerve fibre in a time of the order of one second, while on the whole nerve in several hours. Noteworthy is the work of *Krnjević* (1954 a), who on perfusing the frog's sciatic nerve through the aorta found that the effect on the perfused nerves of the various solutions developed considerably sooner than on perineural application.

The question as to whether or not the sheath has a role in the barrier is still being discussed. Mostly physiologists are engaged in this discussion, their experiments are principally of a neurophysiological character and have been performed on amphibians. The purpose of the present study is to give the assumed barrier a morphological basis, or rather to find the very element of the sheath, that acts as a barrier. In addition, we have endeavoured to demonstrate histochemically the barrier action of this element.

Terminology

Since various terms have been used differently by various authors so that a comparison of literary data has become cumbersome, we propose to apply the nomenclature of *Key* and *Retzius* (1876) i. e., to call the fine loose connective tissue among the nerve fibres, endoneurium ; the lamellar connective tissue surrounding the nerve trunk, perineurium ; and finally the loose connective tissue (with fat-cells) around it, epineurium (see Fig. 8, 10).

I. Morphology of the barrier

Material and methods

Our observations were carried out mainly on the sciatic nerve of the adult white rat, but in some instances they were extended to some other nerves of the same animal and to the sciatic nerve in the newborn rat, guinea pig, frog and newt.

The following histological methods were used. Aoyama silver method for Golgi material ; impregnation of the intercellular cement by *Ranvier ;* several connective tissue stainings (Mallory, Azan, Giemsa). Demonstration of the cell borders (intercellular cement) was made according to Ranvier (0,5 per cent silver nitrate solution for 10 min., reduction by sunlight), the whole nerve was mounted in glycerol, covered and observed. Isolated membranes were prepared by placing freshly excised nerves or impregnated nerves (Ranvier) into Ringers's solution on a depression slide. Connective tissue fibres of the epi- and perineurium were then stripped with the aid of two dissecting needles under a dissecting microscope. The remaining few fibres were then removed under higher power. A silky membrane with a surface quite smooth remained on the nerve trunk (showing double cell border contours on impregnated nerves). After splitting the membrane longitudinally, the nerve fibres were carefully removed, the membrane was spread out, fixed by osmic vapours, stained with nuclear stains (safranin, iron-haematoxylin) and, after dehydration and clearing, mounted in balsam. Isolation of the membrane is a lengthy procedure requiring patience.

Observations

According to our own report of earlier experiments, the granules of perineurally injected India ink were found only among the fibres of the perineurium, but never among the nerve fibres. In the same report mention was made of a membrane that might be brought into causal relation with the above finding. This membrane has been further investigated with the Aoyama silver method and has been found to be a homogeneous black line between the perineurium and the nerve fibres (Fig. 2), to be continuous and surrounding, as a separate sheath, the bundle of the nerve fibres at the inner surface of the perineurium. It has also been found to follow the branchings of the nerve trunk and to cover the smaller branches. Its thickness was 1 to 2 μ. On simultaneous Azan staining, the membrane could easily be distinguished from the collagen fibres of the epi- and perineurium. For this membrane, we venture to suggest the name perilemma.

On longitudinal sections, even simple haematoxylin-eosin staining permits of detecting the perilemma (Fig. 3). If twisted, it appears to correspond in width exactly to the thickness of the section. It contains great flattened, pale, irregularly shaped or frequently oval nuclei. They are sometimes hardly detectable because of their paleness. The chromatin structure is very fine and evenly granular after common fixatives (formol, Bouin, Zenker, Carnoy). The nuclei sometimes overlap, indicating that the perilemma consists of at least two cellular layers. The same is seen on isolated perilemma preparations. (Fig. 4).

The cell borders can be demonstrated by silver impregnation *(Ranvier)* (Fig. 5 and 6). The cells are polygonal, their mean diameter averages 90 μ. The lines of the cell borders run straight, are not wavy like those of endothelial cells. Two easily descernible cellular layers lie one above the other. Sometimes it appears as if there would be a third layer too, but this cannot be stated definitely. That the cellular membrane is not between the perineural lamellae, is clear — among others — from the observation that after removing the perineural fibres, the cell border contours remain unchanged.

The two cellular layers are so very close to each other as to be practically adhering, no cleft is found between them under normal circumstances. It is possible that the above mentioned homogeneous membrane (Aoyama method) is a basement membrane interposed between the cellular membranes. Further studies are required to prove this hypothesis.

The cytoplasm of the cells stains poorly; with Giemsa's dye it stains a pale violet.

Roughly the same results were obtained on examining the sciatic nerve of the newborn rat and the guinea pig.

II. Investigation of the barrier permeability with the Prussian blue method

Material and methods

The experiments were made using sciatic nerves from 30 adult white rats.

Ferric ion diffusion was chosen to test barrier permeability because these ions are demonstrated by the Prussian blue method very readily and accurately. The reaction is sensitive, the blue precipitate produced is well dispersed, stable, insoluble in water, as well as in acids and lipid solvents and, accordingly suitable for microscopic technic. In order to approach physiological conditions as much as possible, the experiments were carried out *in vivo*. The manipulation was the following. Without disturbing the nerve, a 2 cm long portion of the sciatic nerve with its chief ramification was set out carefully. Isotonic (cca 150 mM) ferric chloride solution was injected perineurally; the solution covered the sciatic nerve entirely, its level attaining the edges of the wound. The cavity was filled up from time to time. At definite intervals after the application of the ferric chloride solution, a solution of potassium ferrocyanide was applied around the nerve for 10 min. A 2 cm long nerve segment was then excised and kept in a potassium ferrocyanide solution for ½ to 1 hour in order that the reagent penetrating the nerve from both ends should not fail to demonstrate the ferric ions inside the nerve trunk. After short washing, the nerve was fixed in Bouin's solution and embedded in paraffin. Longitudinal and transverse sections were prepared and stained with Rawitz' carmine, azocarmine or acid fuchsin.

A ferric chloride solution saturated with ether was tried to break through the barrier. The ferric solution was shaken with ether, then drawn off with a separatory funnel. The solution was used as above.

Intraneural ferric chloride injections were also given to investigate the outward diffusion. The finest hypodermic needle, specially sharpened, and a tuberculin syringe were used. Slight pressure was applied in administering the injection to the most proximal portion of the sciatic nerve. A small peristaltic wave of the nerve indicated the effectiveness of the injection (the amount of which was usually 0,01 to 0,02 ml). Thereafter, the method as described above was applied, involving the application of potassium ferrocyanide solution *in vivo* then *in vitro*, then of Bouin fixation, embedding in paraffin, etc.

Observations

1. Perineural injections. Potassium ferrocyanide solution was applied at various periods of time following administration of ferric chloride solution. On the 2 to 3 minute preparations, the whole epi- and perineurium was found to be blue; this means that the ferric ions penetrated the whole epi- and perineurium in that short time. The blue reaction ended abruptly by producing a sharp line on the inner surface of the perineurium. It can be shown with the Aoyama method that this line corresponds to the outer surface of the perilemma.

The same was seen on the 10, 15, 30, 60, 90, 120, 150 and 180-minute preparations (Fig. 8). Consequently even within 180 min. the ferric ions could

not penetrate the nerve trunk in any demonstrable concentration. It was remarkable that the greater part of the neighbouring muscle fibres stained blue.

Between the 3rd and 4th hours, the picture was fairly the same, but this time the Prussian blue appeared among the nerve fibres beside the perilemma sharply demarcating the contours of the nerve fibres (Fig. 9). The whole nerve was blue in the 6-hour preparations.

It was interesting to find, that initially, the Prussian blue reaction among the nerve fibres was positive in the smaller nerve branches only, while the larger branches were left intact. In longitudinal sections, the ferric chloride in the nerve yielded, at the sites of the Ranvier nodes, cross-shaped figures like those produced by the Ranvier silver nitrate method (Fig. 7).

2. Diffusion of ferric chloride solution saturated with ether. It was investigated whether the lipid-solvent ether had any effect on the barrier. The ferric chloride solution saturated with ether was administered perineurally and the diffusion into the nerve was being observed.

On inspection in the 5th, 10th, 15th minutes, there was no Prussian blue to be found in the nerve. In the 20 and 25 minute preparations, the blue stain appeared among the nerve fibres on the periphery and in the 30—35 min. preparations, the core of the nerve was quite blue.

3. Intraneural injections. The Prussian-blue reaction was positive inside the nerve with a sharp line of demarcation showing between it and the perineurium. This line corresponded to the inner surface of the perilemma as shown by the Aoyama method. The same was seen in the 15, 150, 210 and 330-minute preparations (Fig. 10), proving that even within these periods the ferric ions were unable to pass into the connective tissue in any demonstrable concentration. No experiments extending over longer periods were carried out.

In most Prussian blue preparations, the perilemma was sharply outlined and stained intensely blue (Fig. 8).

Discussion

The epi- and perineurium having become saturated with ferric ions in as little as 2-to 3 minutes, the connective tissue sheath obviously meant no essential hindrance to the movement of these ions. On the other hand, diffusion was brought to a sudden standstill for a period of 3 hours on the inner surface of the connective tissue sheath, i. e. on the perilemma. On comparing the time of diffusion within the connective tissue (2 min.) with that of diffusion from the connective tissue sheath into the nerve (3—4 hours), the conclusion may be drawn, that a barrier effect does in fact exist. This is an additional evidence against *Lorente de Nó*'s statement.

The experiments described above have shown that the ferric ions penetrate the whole thickness of the epi- and perineurium in a short time, and that

thus neither the epineurium nor the perineurium are essential parts of the peripheral nervous barrier. This view appears to contradict the opinion of several authors. *Causey* and *Palmer* (1953) have shown in vitro experiments that soaking nerves in P^{32} labelled Ringer's solution, nerves stripped of their epineurium contained more P^{32}. It may be assumed, however, that it was the desheathing that had given rise to some minor lesions in the perilemma and this was the reason of the increased activity in such nerves. Our results are incompatible with the hypothesis of *Feng* and *Liu* (1949) who held that the barrier was represented by a sieve composed of connective tissue fibres. *Benoit*, *Stahl*, *Cotte* and *Seite* (1953) have found the perineurium to give a positive McManus-Hotchkiss reaction and therefore suggested that a polysaccharide was responsible for the barrier effect. The action of hyaluronidase was investigated by *Nordquist* (1952) in connection with the conduction block produced by procaine in peripheral nerves. He found that application of hyaluronidase shortened the blocking time and attributed this fact to the action of hyaluronidase on connective tissue. It is noteworthy that only the highest concentration of hyaluronidase produced a significant difference which, however, was far less than the great difference produced by desheathing. (In *Feng* and *Liu*'s experiments the relation of times required to produce complete conduction block by applying 15 mM cocaine, before and after desheathing was 210 min. to 4 min., while in *Nordquist*'s experiment with 11 mM procaine it averaged 100 min. to 50 min.) *Hallén* (1949) obtained negative results with hyaluronidase. Nor have *Eckenhoff* and *Kirby* (1951), using hyaluronidase in their patients, achieved positive results in regional nerve block.

Our peri- and intraneural experiments have shown that diffusion into the nerve and out of the nerve stops on the inner surface of the perineurium for a considerable time. In a previous paper (*Weiss* and *Röhlich*, 1954), a double cellular layer at the inner surface of the connective tissue sheath had been mentioned as being responsible for the barrier action. This membrane was further investigated in this study and was given the name perilemma ; it consists of two cellular layers and perhaps of a basement membrane. From the present histological findings it is evident that it is this well-defined membrane which is responsible for the barrier effect. Limited space prevents us from dealing in detail with the remarkable observations of *Key* and *Retzius* (1876) and *Ranvier* (1878). With cell border impregnation of human nerves they had obtained the same results as we did. They had correctly concluded that the nerve trunk is covered by endothelium-like cells. This had led them to assume that the perineurium is a system composed of lamellae, each covered by endothelial cells, and clefts, the latter communicating with the subarachnoid space. Yet, there are only two cellular layers to be seen one above the other in their diagrams and not more whereas, — according to their own hypothesis — at least 10 to 40 ought to be visible. Nor did we find more than two of them, or at the utmost three. More-

over, our isolation experiments have shown that the whole epi- and perineurium can be pulled off without destroying the outlines of the overlying cellular layers, meaning that the double layer — the perilemma — remained intact on the nerve trunk. Obviously then, at least in the white rat and the guinea pig, there exists a covering around the sciatic nerve which is not a constitutional element of the connective tissue sheath. Thus the sheath of the peripheral nerve consists of 1. the perilemma, 2. the perineurium and 3. the epineurium (Fig. 1). The detailed microscopic anatomy of the perilemma will be treated of in a future publication.

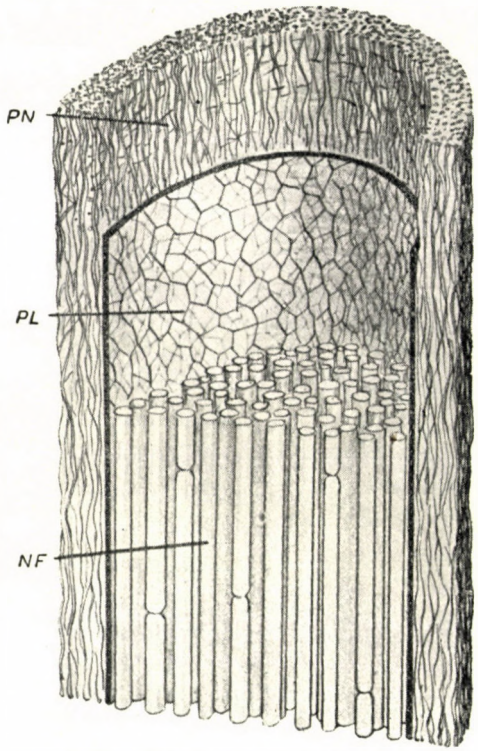

Fig. 1. Three-dimensional diagram of the sheaths of the peripheral nerve. The epineurium is not illustrated. PN: perineurium, PL: perilemma, NF: nerve fibres

Most recently, *Krnjević* (1954 b) studying the frog's perineurium, has described — independently — a similar cellular membrane. Mostly on the basis of theoretical considerations, he arrived at the same conclusion as we have, namely that this cellular membrane was the cause of the barrier effect. In connection with the barrier, others (*Overton*, 1904; *Rashbass* and *Rushton*, 1949; *Hodgkin* 1951; *Huxley* and *Stämpfli* 1951; *Shanes* 1954) too have taken into consideration the role of the endothelial cells described by *Key* and *Retzius*, and by *Ranvier*, but have failed in furnishing evidence in favour of this theory.

Ferric chloride solution saturated with ether passes the perineurium and the perilemma in considerably less time (20 to 25 min.) than a non-saturated solution (3 to 4 hours). Our pertaining experiments have brought additional evidence in support of the decisive role played by the perilemma in the peripheral nervous barrier. It is well known, that lipid solvents increase the permeability of the cell- and basement membranes rich in lipids. Our results seem to agree well with the literary data viz. that chloroform reduces the barrier action for the passage of K^{42}ions (*Keynes* and *Stämpfli*, 1949) ; that the sheath is much more permeable for lipid soluble substances (ethanol, acetone) (*Overton*, 1904 ; *Feng* and *Liu*, 1949 ; *Krnjević*, 1954 a).

What is the possible role and significance of the barrier? Presumably to maintain the special internal environment of the nerve by preventing free diffusion of substances into it. A stable internal environment is probably an important condition of normal nervous action. Even in pathological processes the barrier may be of significance ; intact nerves have been demonstrated by *Kurucz* (1954) in entirely pathological (inflammatory, anthracotic) surroundings in the lung. The inflammatory cells infiltrated the connective tissue, but the nerve fibres remained intact. Probably, the barrier cannot be neglected in therapy either.

The electrical role of the barrier remains to be discussed briefly. As well known, the nerve has a significant transverse resistance and capacity which decreases almost to disappearence after the sheath has been removed. (*Bishop*, *Erlanger* and *Gasser*, 1926 ; *Schmitz* and *Schäfer*, 1933 ; *Cole* and *Curtis*, 1936 ; *Rössel*, 1943 ; *Rashbass* and *Rhuston*, 1949 ; *Lundberg*, 1951). This electrical «barrier» produces distortion of the action potential as recorded from the nerve surface, profoundly affects irritability and electrotonus. This highly polarizable resistance also renders ionic displacements difficult. It seems at hand to identify the polarizable structure with the perilemma.

As to the histogenesis of the perilemma, no experimental data are available to the authors, who can but subscribe to the view of *Krnjević* concerning the possibility of an ektodermic origin of the cellular membrane. One of their future aims is to investigate into this problem. They consider the origin of the perilemma to be analogous to that of the leptomeninx in the central nervous system, while the perineurium may be compared with the dura mater.

Summary

A membrane consisting of two layers of flattened cells and perhaps of a basement membrane has been described on the inner surface of the perineurium of the rat's sciatic nerve. For this structure, the name perilemma is being suggested. The perilemma can be impregnated and isolated.

Diffusion of isotonic $FeCl_3$ solution injected perineurally has been investigated with the Prussian blue method and found to be stagnating on the outer surface of the perilemma for about 3 hours.

Diffusion of the $FeCl_3$ solution applied intraneurally stopped on the inner surface of the perilemma for several hours ; the solute did not pass it in $5\frac{1}{2}$ hours.

$FeCl_3$ solution satured with ether penetrated the perilemma in 20 minutes, when applied perineurally.

The conclusion is that the peripheral nervous barrier may be identified with the perilemma.

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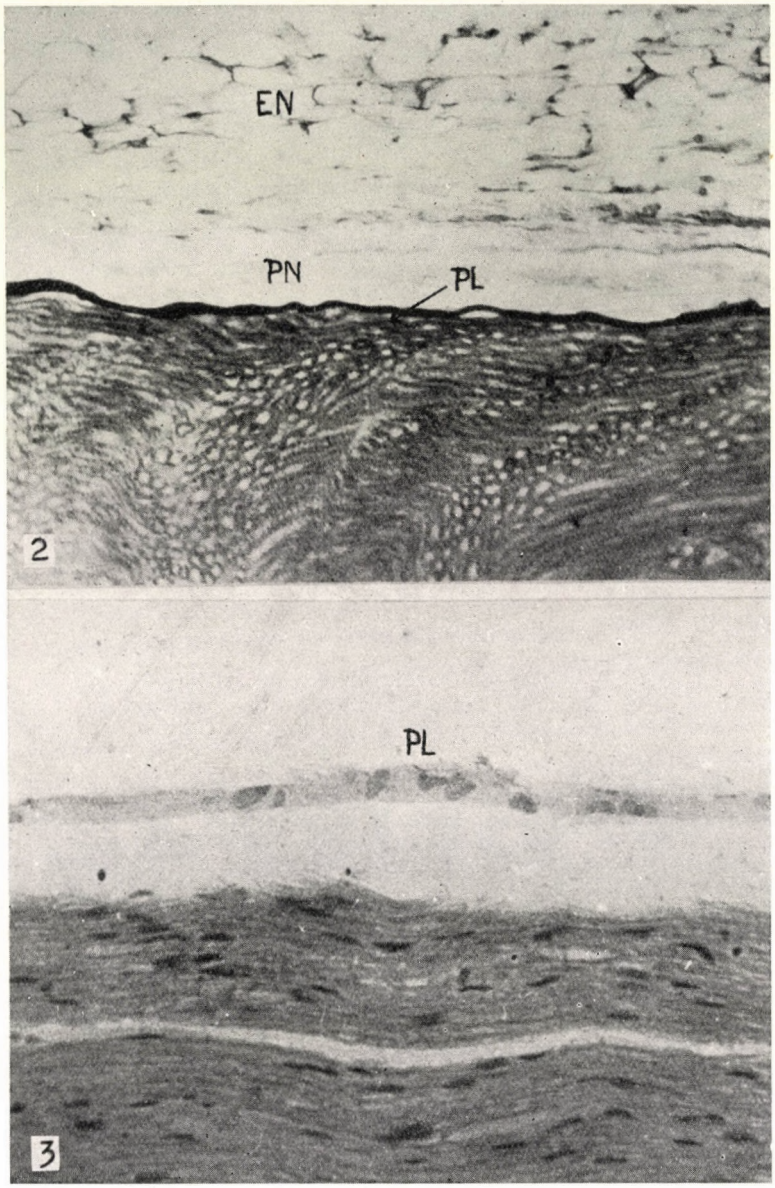


Fig. 2. The perilemma. Aoyama silver method. EN : epineurium, PN : perineurium, PL : perilemma

Fig. 3. The perilemma twisted. Note the pale oval nuclei. Epi- and perineurium are not seen. Haematoxylin-eosin stain. PL : perilemma

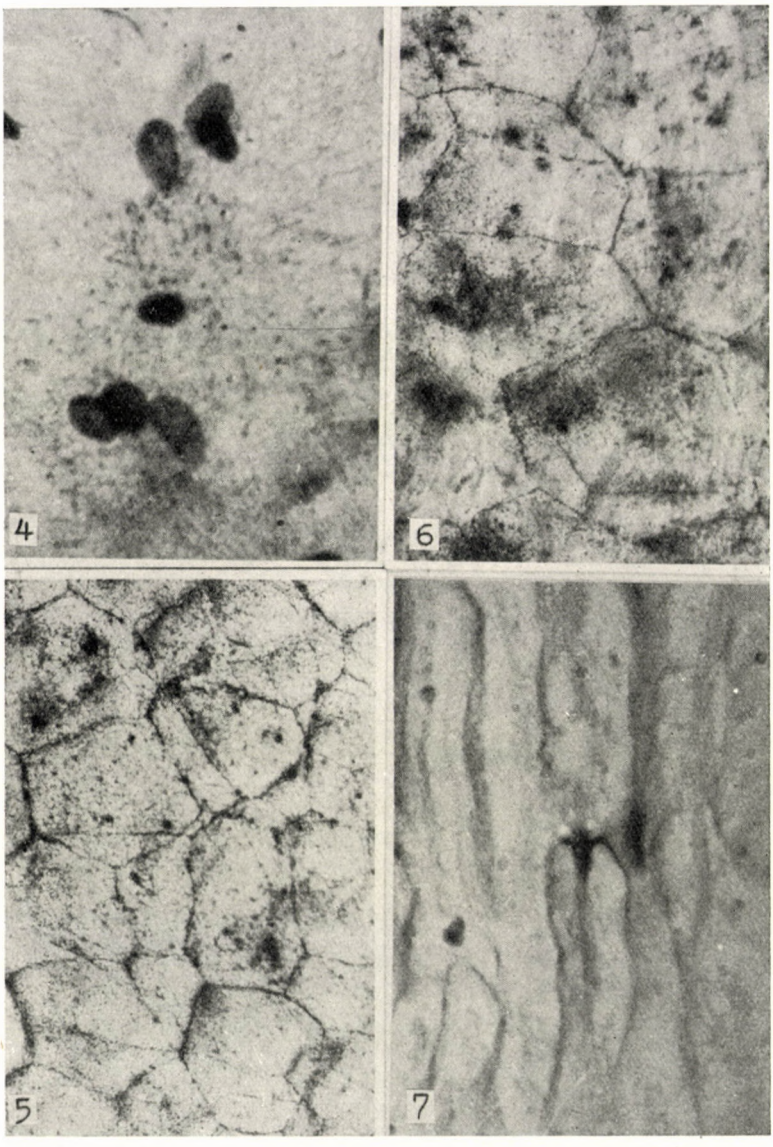
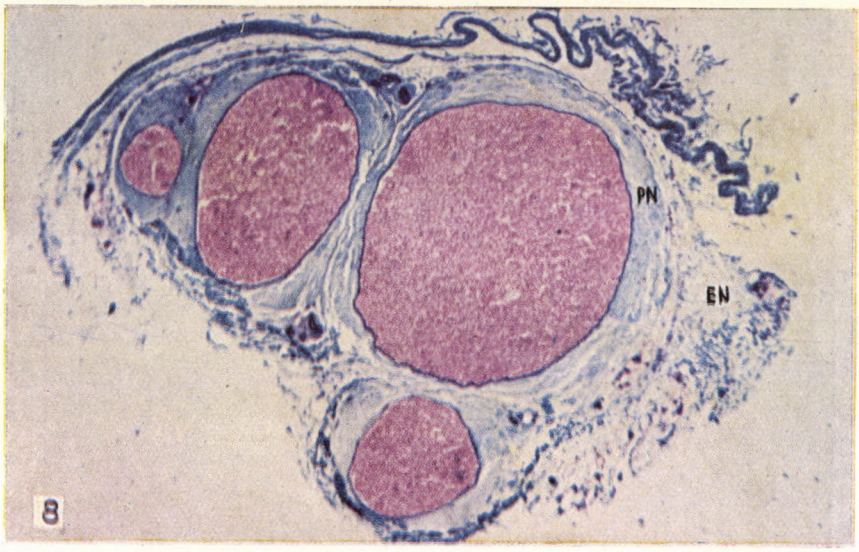


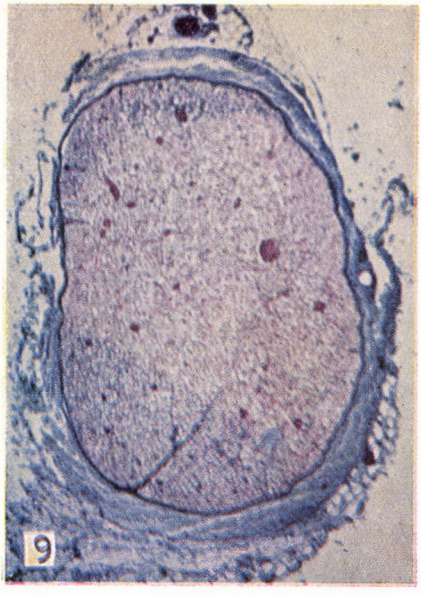
Fig. 4. Nuclei of the perilemma shown on an isolated preparation. Note overlying nuclei. Osmic fixation, safranin staining

Fig. 5. Cell borders of the perilemma demonstrated by Ranvier's silver method. Isolated preparation

Fig. 6. Cell border contours of the perilemma of a smaller nerve. The whole nerve is mounted in glycerol. Ranvier's silver method

Fig. 7. Cross-shaped figure at the node of Ranvier. See text. Six hour Prussian blue preparation





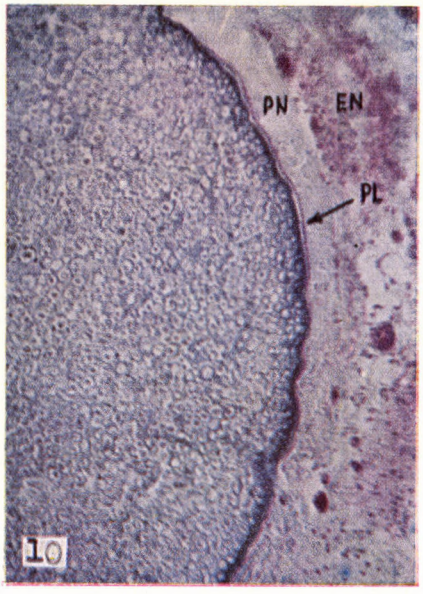


Fig. 8. A 3 hour Prussian blue preparation. Note the diffuse blue reaction in the connective tissue sheath indicating presence of ferricl ions. The blue colour ends with a sharp line towards the nerve fibres, at the surface of the periemma. Acid fuchsin stain. PN : perineurium, EN : epineurium

Fig. 9. 4 hour Prussian blue preparation. Observe the blue reaction among the nerve fibres. Acid fuchsin stain

Fig. 10. A 330 min. Prussian blue preparation after intraneural injection of ferric ions. Blue reaction among the nerve fibres with a sharp line of demarcation at the inner surface of the perilemma. Acid fuchsin stain. EN : epineurium, PN : perineurium, PL : perilemma

ИССЛЕДОВАНИЯ ПО ГИСТОЛОГИИ И ПРОНИЦАЕМОСТИ НА ПЕРИФЕРИЧЕСКОМ НЕРВНОМ БАРЬЕРЕ

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Диффузионный барьер у периферических нервов, за последнее время часто является весьма спорным вопросом. Диффузионный барьер означает, что в процессе обмена ионами между внутренней частью периферических нервов и их средой имеются препятствия. Исходя из результатов своих прежних опытов, авторы старались доказать существование барьеры и выяснить его микроскопическую анатомию. Эксперименты были проведены, главным образом, на белых крысах.

1. В первой части статьи авторы описывают под названием «перилемма» клеточную оболочку, находящиеся на внутренней поверхности соединительнотканной оболочки нерва. Перилемма состоит из двух совершенно плоских клеточных слоев, а, возможно, и из базальной мембраны. Ее можно выявить методом импрегнации по Аояма, импрегнацией клеточной оболочки и другими гистологическими методами.

2. Во второй части своих опытов авторы исследовали диффузию ионов железа. Они выявили ионы железа при помощи раствора ферроцианида калия. Полученная берлинская лазурь представляет собой тонкий, нерастворимый осадок; реакция является крайне чувствительной. Авторы впрыскивали крысам изотонический раствор хлористого железа вокруг седалищного нерва, а затем после определенного промежутка времени вводили животным раствор ферроцианида калия. Диффузия ионов железа приостановилась резкой линией на внешней поверхности перилеммы и диффузия в пространство между нервными волокнами началась на подопытных животных только после четырех часов. Насыщенный эфиром раствор прорвал барьер уже после 25 минут. Диффузия введенного внутриневрально раствора железа препятствуется перилеммой на 5 часов.

Авторы пришли на основе своих опытов к тому выводу, что диффузионный барьер действительно существует, и что он представлен клеточной оболочкой, названной перилеммой. Авторы в данной статье излагают значение барьера.

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