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SUBMICROSCOPIC ALTERATIONS OF MYELIN SHEATH ULTRASTRUCTURE DUE TO LOW TEMPERATURE

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Introduction

The discovery of the fine structure of myelin sheaths of vertebrate nerve fibres is one of the most fascinating chapters of up-to-date ultrastructural research work. As early as 1849, EHRENBERG described the strong double refraction of the sheath of "double contoured nerve fibres", i.e. myelinated nerve fibres [9]. Polarization optical analysis revealed that the myelin sheath consisted of concentrical layers of protein lamellae, associated with lipid molecules arranged in a radial direction [7]. Some years later F. SCHMITT, introducing the small-angle X-ray refraction method as an ultrastructural research tool in biology, suggested that the periodicity of the concentrical protein lamellae measured 170—180 Å [8]. This assumption based on histo-physical methods has been confirmed by electron microscopical investigations. In 1954, BETTY GEREN [3] succeeded in demonstrating that the peculiar concentrical structure of the myelin sheath is a result of a helicoidal winding up of the Schwann cell membrane enveloping the nerve fibre during ontogenetic development. According to ROBERTSON [5], the "mesaxon", i.e. the connective Schwann cell membrane of myelin sheath lamellae, may also be found in the adult state. According to the up-to-date view, the 180 Å thick concentrical (or, even more, helicoidal) lamellar units are bisected (FERNANDEZ-MORAN and FINEAN [2]) by further electron-dense layers. The interspaces are filled out with a light material which, according to SJÖSTRAND and other authorities, is of a lipid character, while the electron-dense layers consist of proteins [1].

Considering that the myelin sheath is formed of the Schwann cell surface membrane, the 180 Å periodicity corresponds to a duplicated cell membrane. According to ROBERTSON, the cell surface membrane consists of a 75 Å thick "unit membrane" [6], associated with an external polysaccharide layer of varying thickness, responsible for the 30 Å difference between 2×75 Å and 180 Å ("difference factor"). The "unit membrane" consists essentially of a bimolecular smectic leaflet of lipids, with their polar groups oriented towards both extremities of the membrane, where they join an external and internal protein layer; the thickness of these protein layers is about 10 Å — which corresponds exactly to the thickness of an alphahelix (PAULING and COREY).

The thickness of the polysaccharide gel associated with the external protein layer varies from cell to cell; in the oocyte, it may be as thick as 1 μ (zona pellucida). Accordingly, investigation of the ultrastructure of myelin sheath might shed new light on the biological properties of the cell surface membrane.

FERNANDEZ-MORAN and FINEAN seem to have been the first to observe the serious electron microscopic alterations of the ultrastructure of myelin sheath in consequence of freezing [2]. Independently of these authors we have succeeded in demonstrating that freezing with dry ice results in a diminution of the birefringence shown by the myelin sheath. Some details of our investigations have been published previously [10]. In the present paper the ultrastructural changes due to freezing as a consequence of alterations in the dynamic state of the molecular membrane structure will be discussed.

Material and methods

The sciatic nerve of a total of 85 albino rats was used. The basic experiment, consisting of the determination of the retardation values of a nerve fibre population was performed as follows.

The rat was killed by decapitation and the sciatic nerve was removed (in some experiments, listed below, the animals were not killed but anaesthesia was induced by intraperitoneally injected urethan). A 5 mm long piece of the nerve was excised in physiological saline and the nerve fibres were completely isolated by means of fine steel needles under a stereomicroscope. A cover-glass was put upon the preparation and it was brought under the polarization microscope equipped with a $\lambda/4$ Senarmont type mica plate compensator and an ocular micrometer. The retardation values of at least 30 nerve fibres were determined. These manipulations were completed in 10 minutes. The retardation values were plotted against the thickness of the nerve fibres in a coordinate system, thus constructing a graph characterizing the retardation of the nerve fibre population investigated. The steepness of this curve was expressed as the tangent of the angle between abscissa and regression curve. In most cases this curve was corrected in order to obtain a straight line instead of the paraboloid resultant.

Freezing of nerve fibres was performed *in vitro* or *in vivo*. Freezing *in vitro* was carried out on the table of the freezing microtome. Freezing *in vivo* was performed under anaesthesia, embedding the carefully isolated sciatic nerve among small pieces of dry ice.

Results

I. *Correlation between thickness of nerve fibres and retardation*

As a rule, thicker nerve fibres exhibit a higher degree of retardation than thinner ones. This is conspicuous from Fig. 1, representing one of the serial determinations. This rule is a statistical one, resulting in a definite spreading of the values. The curve has a convex form and crosses the abscissa at the value of 1.2 micron. The steepness of the corrected regression is $\operatorname{tg} \alpha = 0.82$. This value depends upon the age and size of the animal and varies from 0.8 to 1.0. It is important, therefore, if we want to compare values for different animals, to use animals similar in size and age.

II. *Effect of freezing on the retardation produced by the myelin sheath*

Retardation values of isolated nerve fibres mounted in physiological saline or in Ringer's solution were estimated under the polarization microscope. Without removing the preparation, the whole sample was frozen by dry ice

Thickness in micra	Retardation	
	before freezing	after freezing
8	24	11
6	20	10
5.5	17	9
5.5	18	9
10	29	14
8.5	26	12
7	20	11
6.5	21	12
9.5	30	15
7	22	10
4	11	5

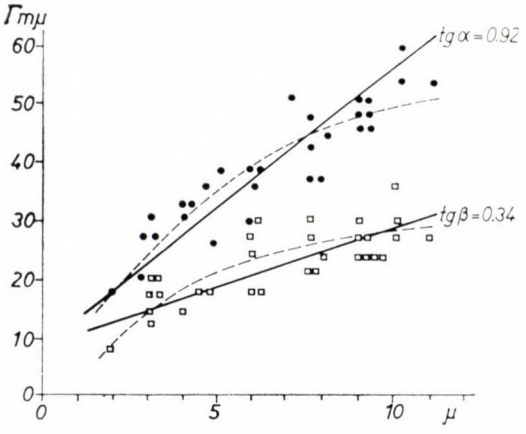

Fig. 1

(replaced if necessary with a new supply) for 5 minutes. Retardation of the same nerve fibres was estimated also after freezing. The values obtained in these experiments were as follows.

All these experiments show that freezing resulted in a marked diminution of the retardation. In the following, it was attempted to compare the retardation values yielded by the myelin sheaths of nerve fibres of the left and the right sciatic of the same animal with the left nerve intact and the right one frozen *in vitro*, i.e. on the table of the freezing microtome. The results of one

of these experiments may be seen on Fig. 1. The marked difference between the regression angles suggests that this method of comparison can be used as a tool of recording the overall situation in a nerve trunk. The difference between the two directional tangents (0.82 and 0.34, respectively) is highly significant.

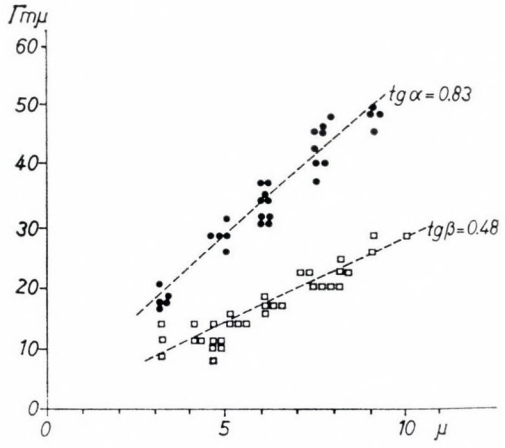

Fig. 2

III. *The effect of freezing* in vivo *on the ultrastructure of myelin sheath*

Freezing of the sciatic nerve with dry ice *in vivo* resulted in a similar diminution of the optical activity of myelin sheaths as observed *in vitro* (Fig. 2). The direction tangent of the retardation values of the population of frozen nerve fibres was 0.4—0.55 in ten experiments, whereas the corresponding values were 0.80—1.00 in the normal (contralateral) sciatic nerves.

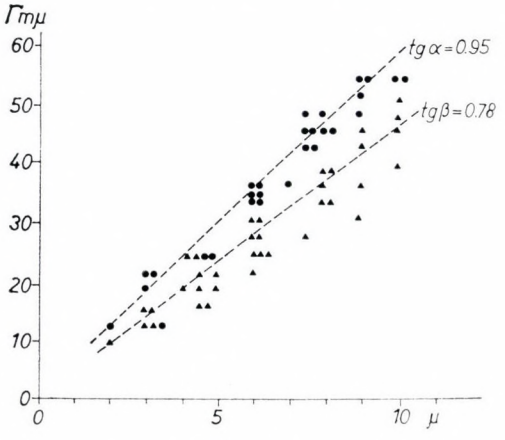

Fig. 3

IV. *Restitution of the optical activity of the myelin sheaths after freezing*

The optical activity of the frozen nerve fibres, if excised and kept under sterile conditions for several hours either at room temperature or at 37° C, remained at the low values characterizing frozen fibres. No signs of restitution of the optical activity were observed.

On the other hand, if the sciatic nerve was frozen *in vivo* and the wound closed, a slow restitution of the original (strong) optical activity was observed. On investigating sciatic nerve fibres at different points of time after freezing it was found that the original (normal) birefringence was attained about

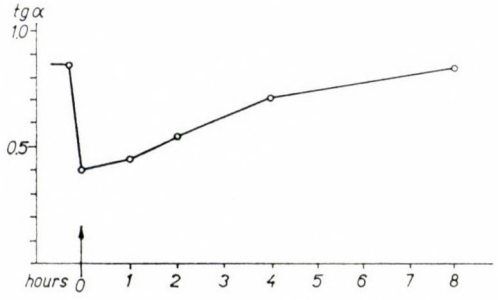

Fig. 4

8 hours after freezing (Fig. 3). In Fig. 4 the directional tangents of the retardation values of nerve fibre populations are demonstrated, plotted against the time interval after freezing (in hours). One hour after freezing practically no alteration could be seen; after two and four hours, the birefringence was slightly enhanced, while eight hours after freezing the optical activity was nearly normal.

V. *The effect of neurotomy on the restitution of the optical activity of myelin sheaths after freezing*

Anaesthesia was induced in rats by means of intraperitoneal urethan. The left sciatic nerve was exposed, frozen by means of dry ice, and transected at the middle of the frozen part. The wound was closed and the animal killed after 8 hours. Three samples were investigated under the polarization microscope: *A)* the proximal part of the frozen (and transected) left sciatic nerve; *B)* the distal part of the same nerve; and *C)* the contralateral nerve (absolute control).

The proximal part of the frozen (and transected) sciatic exhibited complete restitution. The distal part, however, did not show any sign of restitution (Table 1).

VI. *The effect of anoxia on the optical activity of myelin sheaths*

Following median laparotomy, the left common iliac artery was ligated in 4 rats and both sciatic nerves were frozen with dry ice. 8 hours after the operation both sciatic nerves were investigated by polarization. In striking contrast to the right sciatic, the optical activity of the anoxic left sciatic nerve showed the low values characteristic of frozen fibres, although in this experiment no transection was performed. The values for the right sciatic were, 0.95, 0.75, 0.8, 0.86; those for the left one, 0.4, 0.42, 0.5, 0.44. — See also Fig. 5.

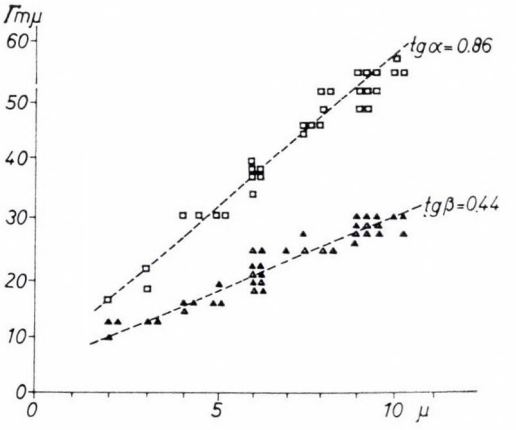

Fig. 5

VII. *The effect of neurotomy on the restitution of the optical activity of frozen nerve fibres at a distance of more than one cm from the site of the transection*

The left sciatic nerve of 4 rats was transected at the middle of the thigh. Both (left and right) tibial nerves were exposed at the ankle and frozen with dry ice. 8 hours after freezing both tibial nerves were investigated.

Both nerves (control and operated side) displayed a similar birefringence (0.7, 0.72, 0.8 for the right, and 0.86, 0.7, 0.67, 0.75 for the left, tibial), indicating a similar degree of restitution in both normal and transected nerves.

Discussion

According to the above results, the degree of retardation produced by the myelin sheath is strictly proportional to the thickness of the nerve fibre. Considering that birefringence is a function of the submicroscopical organization, this result is in good agreement with the fact that in thicker nerve fibres a thicker layer of submicroscopically organized material (i.e. a thicker myelin sheath) is active in producing optical phenomena.

The fact that freezing results in a well-marked diminution of optical activity is in all probability a consequence of the disruption of the submicroscopical lamellae of the myelin sheath. As mentioned above, such a disruption has been observed several years ago by FERNANDEZ-MORAN and FINEAN in electron micrograms of frozen nerve fibres. On the other hand, biochemical investigations suggest that lipoproteids are very sensitive to damage caused by cold. Observations of this kind were published by LOVELOCK [4] and other authors. The destructive effect of freezing was also observed in our previous investigations concerning the potassium content of myelinated nerve fibres. One of us (B. Cs.) has shown that freezing results in serious alterations of the submicroscopical structure on the postsynaptic membrane.

In our opinion, cold-induced alterations of nerve fibre ultrastructure are the basic factors responsible for the temporary conduction blockade induced by freezing, so often used in surgical practice. The fact, however, that the restoration of the ultrastructural organization took a considerable time after the freezing, while conduction returned sooner, suggests that an incomplete submicroscopical organization is sufficient for conduction. The question arises whether or not functional alterations of the nerve fibres (e.g. supramaximal stimulation and several drugs inducing local anaesthesia) result in changes of the sheath ultrastructure.

The difference is remarkable between the restitutive capacity of the proximal and the distal stumps of a transected nerve. In our first experiments this difference was attributed to neurotrophism [10]. Apparently, the proximal stump which had remained in a neurotrophic contact with the nerve cells was only capable of restituting the normal submicroscopical structure, while the distal stump, isolated from the nerve cells, was inert in this respect. Our present findings, however, have shown that this assumption was incorrect. Anoxia of the nerve, like freezing, results in a decrease of birefringence of the myelin sheaths. On the other hand, experiments carried out on transected nerves show that if freezing was performed at a more remote place of the distal stump the restitutive capacity of this neurotrophically isolated segment proved to be just the same as that of the entirely intact control nerve. It seems more plausible, therefore, that the differences observed between the restitutive capacity of the proximal and distal stumps are merely due to the injury of the blood vessels of the nerve, which are known to run parallel with the nerve fibres within the nerve trunk. The molecular organization of the myelin sheath appears to be a dynamic one, depending on the blood supply of the nerve. The other membranes possessing a molecular organization similar to that of the unit membranes constituting the myelin sheath probably suffer similar alterations after freezing or anoxia.

According to our results, the myelin sheath ultrastructure — as revealed by the polarization method — is a sensitive indicator of the state of the nerve

fibres. On the other hand, our findings suggest the molecular structure of the "unit membrane" to be an energy-requiring dynamic state, dependent on the metabolism of the cell. In this respect, the present observations might be looked upon as model experiments concerning the biological capacities of the cell membrane. The answers to several important questions concerning the molecular biology of the cell surface membrane might be revealed by means of similar model experiments performed on the myelin sheath.

SUMMARY

Freezing with dry ice resulted in a significant decrease of the birefringence of the myelin sheath. The birefringence was found to return to normal after several hours if the nerve's blood supply was intact. The molecular organization of the "unit membrane" is a dynamic, energy-requiring state.

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DURCH NIEDRIGE TEMPERATUR VERURSACHTE SUBMIKROSKOPISCHE VERÄNDERUNGEN IN DER ULTRASTRUKTUR DER MYELINSCHEIDE

ILONA TOMITY und B. CSILLIK

Gefrieren mit Kohlensäureschnee verursacht eine signifikante Abnahme der Doppelbrechung der Myelinscheide. Das Maß der normalen Doppelbrechung kehrt *in vivo* in einigen Stunden zurück, vorausgesetzt, daß die Blutversorgung des Nerven intakt bleibt. Die molekulare Organisation der "unit membrane" ist ein dynamischer, energiebedürfender Zustand.

ВЫЗВАННЫЕ НИЗКОЙ ТЕМПЕРАТУРОЙ СУБМИКРОСКОПИЧЕСКИЕ ИЗМЕНЕНИЯ В УЛЬТРАСТРУКТУРЕ МИЕЛИНОВОЙ ОБОЛОЧКИ

И. ТОМИТИ и Б. ЧИЛЛИК

Замораживание углекислотой приводит к достоверному понижению двойного преломления миелиновой оболочки. Нормальная степень двойного преломления in vivo возвращается за несколько часов, если кровоснабжение нерва интактное. Молекулярная организация так наз. «unit membrane» является динамическим, нуждающимся в энергии состоянием.

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