Department of Anatomy, Histology and Embryology of the Medical University, Szeged
(Director : Prof. A. GELLÉRT)

THE EFFECT OF DENERVATION ON THE CHOLINESTERASE ACTIVITY OF MOTOR END PLATES

GY. SÁVAY and B. CSILLIK

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Biochemical investigations had long ago revealed the high cholinesterase activity of the myoneural junction. GOMORI's ingenious first method [9] for the histochemical demonstration of this enzymic activity had given rise to extensive experimental work. The three essential methods developed for this purpose differ in the chromogenic substrates (acetlythiocholine [11, 4, 8] ; naphtylacetate [5, 14] ; indoxylacetate [1, 10]) used for visualising the cholinesterase activity.

In our previous experiments carried out by means of the naphtylacetate method [15] it has been established that the motor end plates of striated muscle exhibit a cholinesterase activity even 28 days after their nerves had been cut. However, in view of the lipoid solubility of the azo dye (the end product of the naphtylacetate method), these preliminary investigations did not reveal the cytochemical details. Hence by using other histochemical methods and extending the duration of the experiment it seemed justified to re-examine this problem of both theoretical and practical (e. g. myopathy, neural dystrophy, etc.) importance.

The naphtylacetate, indoxylacetate and acetylthiocholine methods reproduce the cholinesterase activity of sound motor end plates in a quite different manner (Figs. 1, 2, 3). The naphtylacetate method shows enzymic activity in the form of a homogeneous patch, obviously due to diffusion of the azo dye in the lipoid substances. Thus the lipoid solubility of the end product seems to be a serious handicap of the naphtylacetate method. On the other hand, both the indoxylacetate and acetylthiocholine methods produce a strucurally detailed picture of the subneural plate. The modification of the indoxylacetate method employed by us* allows for a considerably better localisation, than

*Fixation in 8 per cent neutral formaldehyde. Frozen sections incubated for 10 minutes at 56° C in the following solution (modified after Holt, 10) :

Indoxylacetate, 0,01 g.
Acetone, 0,5 ml
2M NaCl solution, 25 ml
Barbiturate buffer, p_H 8,6, 10 ml
M/100 potassium ferricyanide solution, 1 ml
M/100 potassium ferrocyanide solution, 1 ml
Distilled water, 12,5 ml

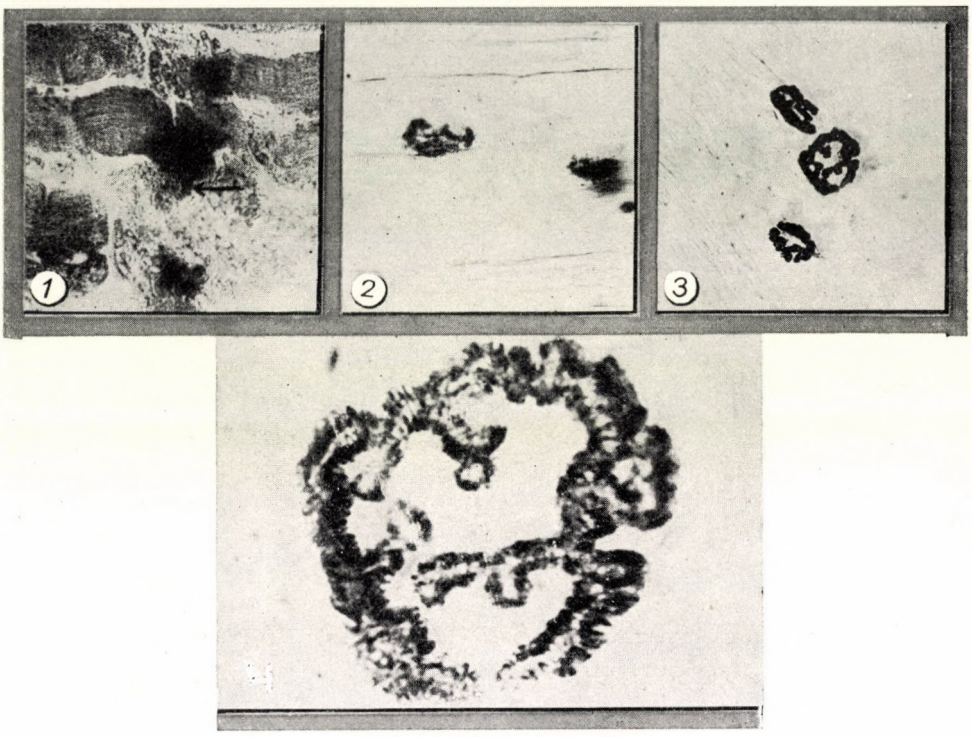


Fig. 1. Cholinesterase activity of sound motor end plates. Naphtylacetate technique. Magn. ×240 *Fig. 2.* As Fig. 1 but using indoxylacetate method. Magn. ×240. *Fig. 3.* As Fig. 1 but using acetylthiocholine method. Magn. ×240. *Fig. 4.* The same end plate as in Fig. 3, but with Magn. ×1500. Note the enzymic activity of the palisade-like structures

BARNETT and SELIGMAN's [1] original procedure, although it does not reveal structural details as truly as the acetylthiocholine method does. HOLT [10] succeeded in achieving even finer localisation by means of diindoxyl diacetate unfortunately a substance not available to us. Hence acetylthiocholine remained the substrate most suitable for the cytochemical demonstration of cholinesterase activity of denervated motor end plates.

Material and methods

The experiments were performed on 22 albino rats of both sexes, weighing 140 to 180 g each. The right sciatic nerve was severed in ether anaesthesia and the wound was closed under sterile conditions. The animals were sacrificed by decapitation 1 to 200 day after the operation.

Small pieces of both the right and left gastrocnemius muscles weres fixed in chilled 8 per cent neutral formaldehyde for 5 hours. Frozen sections 30—50 μ thick were made and fixed again in 8 per cent neutral formaldehyde for 30 minutes, then washed twice in distilled water and subsequently incubated at pH 6,2. The method employed differed from that described by GEREBTZOFF [8] only in that incubation was performed in two steps, i. e. the sections were incubated first at 37 °C for 10 minutes, then at 56 °C for 5 minutes. After the reaction had been completed the sections were dehydrated in an alcohol series, cleared in xylene and toluene and mounted in balsam.

Microscopical findings

Normal subneural apparatuses

Sound subneural plates exhibited an intensive cholinesterase activity; muscle fibres and nerve fibres did not show any reaction. In accordance with the data reported by COUTEAUX and TAXI concerning the motor end plates of the frog [4], and with those by GEREBTZOFF concerning the motor end plates of the rat [8], we also found that the enzymic acitivity formed a trough-like structure surrounding the terminal arborisations of the nerve fibre. Owing to the gradual rise in temperature in the course of incubation it could be established that this trough was composed of numerous palisade-like small rodlets (mitochondria?) lying vertically to the axis of the fibre (Fig. 4.), a fact previously observed on the end plates of amphibians [4]. In some motor end plates it could be seen that the double-contoured pattern of the subneural apparatus remained open at the entrance of the nerve fibre (Fig. 6.), while at the places corresponding to the endings of the fibre it ended blindly, as if it would enclose the trough. The apparatuses exhibited a great variety in both form and size, but they were all roundish and the distance between the two borderlines of the plate was uniform. Their tension was normal.

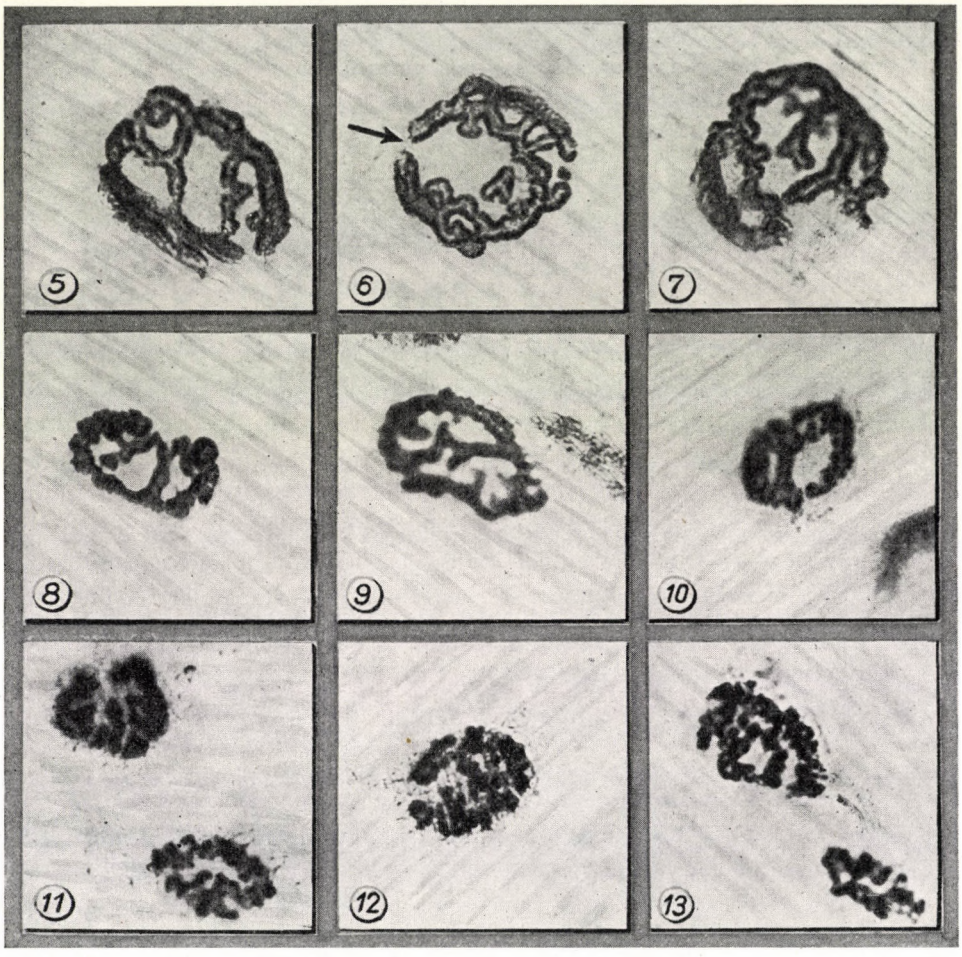
Denervated subneural apparatuses

1 to 10 days after the denervation the subneural apparatuses had not yet shown any conspicuous change.

The first sings of a change appeared on the 10th to 14th postoperative day. The originally parallel course of the two cholinesterase-active borderlines became irregular, the two lines approached each other on certain points and diverged on others. (State of hypersegmentation, Figs. 8, 9, 10). On the other hand, the subneural plates became angular, their tension broke down. It was hardly possible to determine the behaviour of the palisade-like structure composing the borderline, its order of magnitude being at the limits of the resolving capacity of the method, but it was felt that they had become gradually rounded, displaying a string-of-pearls pattern instead of the previous palisade-like one.

The changes described do not appear in every end plate to the same degree. Even two weeks after denervation there were many subneural apparatuses with a normal, or practically normal cholinesterase activity.

On the 28th to 30th postoperative days the changes became graver. No end plate displaying a completely normal histochemical structure was found at this period. Some of them showed signs of fragmentation; the angular



Figs. 5—13. Gradual destruction of enzymic activity of the subneural apparatuses after denervation. Acetylthiocholine method. Magn. ×800.

Fig. 5. Sound motor end plate. *Fig. 6.* Sound motor end plate. Arrow pointing to entrance of nerve fibre. *Fig. 7.* Sound motor end plate. *Fig. 8.* 10 days after denervation. *Fig. 9.* 12 days after denervation. *Fig. 10.* 14 days after denervation. *Fig. 11.* 28 days after denervation. *Fig. 12.* 28 days after denervation. *Fig. 13.* 32 days after denervation

lobuli of the end plates, though of unchanged cholinesterase activity, became separated from one another (Figs. 11, 12, 13).

Nerve fibres in muscles with intact innervation did not show any sign of reaction (Fig. 22), while 28 to 30 days after denervation the degenerating nerve fibres exhibited cholinesterase activity. As seen in Figs. 23. and 24., every nerve fibre proceeding towards the subneural apparatuses stained intensely in this stage, exhibiting a picture similar to that of silver impregnated specimens.

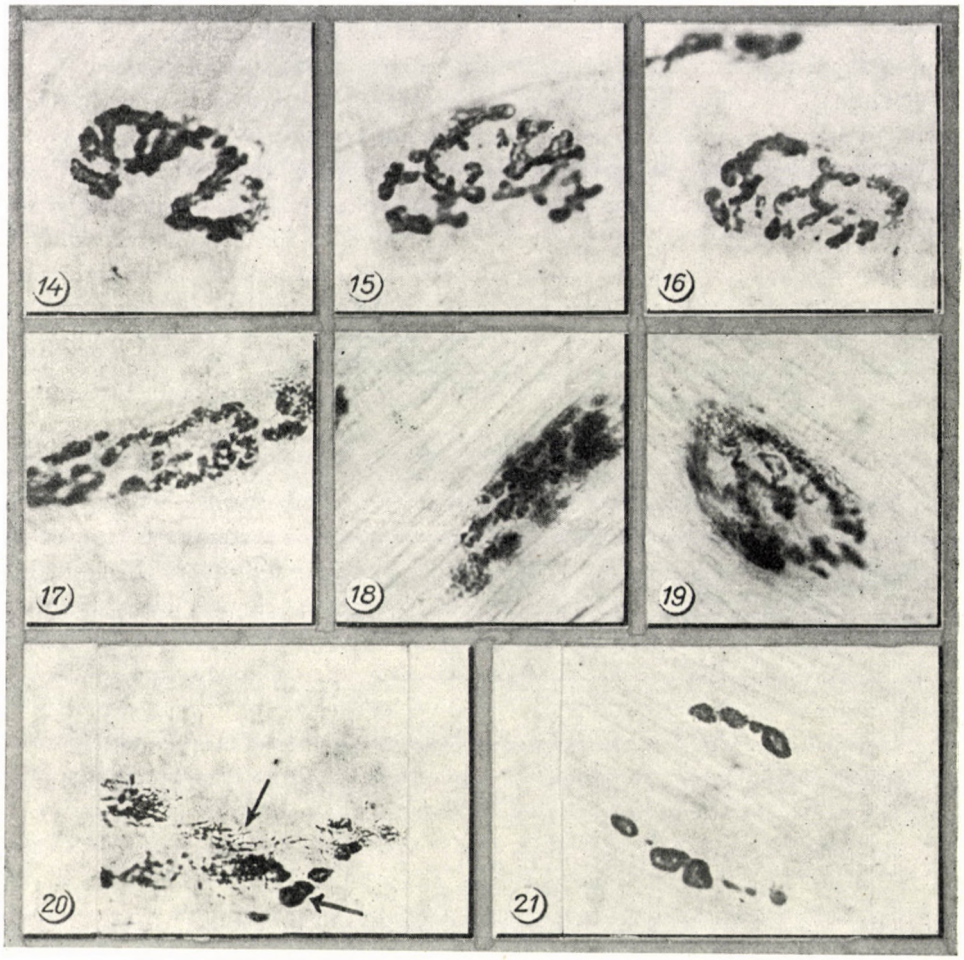


Fig. 14. 63 days after denervation. *Fig. 15.* 70 days after denervation. *Fig. 16.* 70 days after denervation. *Fig. 17.* 150 days after denervation. *Fig. 18.* 170 days after denervation. *Fig. 19.* 184 days after denervation. *Fig. 20.* Cholinesterase activity of regenerating motor end plates, 184 days after denervation. The upper arrow = remnants of the late end plate. The lower arrow = first signs of a regenerating end plate. Magn. ×800. *Fig. 21.* Cholinesterase activity of regenerating end plates, 184 days after denervation. Magn. ×800

The activity of the degenerating nerve fibres disappeared gradually in the third month after denervation.

Two months after the nerve had been severed all subneural plates were in a state of fragmentation ; their cholinesterase activity, however, did not decrease significantly (Figs. 14, 15, 16). A considerable diminution of enzymic activity took place in the course of the fourth month only. At the same time, the border-lines broke up and only a granular enzmyic activity located more or less to the site of the late end plate could be found. One half year after denervation every subneural apparatus was in this granular state (Figs. 17, 18, 19).

Moreover, here and there even this structure become disorganized, with only some scattered granules indicating the remnants of the end plates.

Though it is beyond the scope of the present investigation, it is briefly mentioned that in the last stage regenerative phenomena could also be detected. As the first sign of regeneration, three, four or even more small rings or loops, 4 to 5 μ in diameter, exhibiting a very intensive cholinesterase activity appeared in groups (Figs. 20, 21). They were often located in the neighbourhood, or in the very territory of granular enzymic activity of the destroyed end plates (Fig. 20). Somewhat later the rings joined, forming primitive, non-segmentated, subneural apparatuses with a very intensive enzymic activity, resembling the simple apparatuses described by GEREBTZOFF in young animals [8].

Discussion

The present experiments indicate that the cholinesterase activity of the myoneural junction persists for a long time after the nervous structure of the telodendrion had been destroyed. This had been assumed on the basis of biochemical analysis [2, 3, 7, 12] and has been established previously in histochemical investigations performed by means of the naphtylacetate technique [15]. In addition, the present findings indicate that the structure exhibiting enzymic activity, NACHMANSOHN's so-called «post-synaptic membrane» [13], suffers gradual destruction after denervation. The process of this gradual destruction, which could not be demonstrated by the naphtylacetate technique, may be divided into the stages of hypersegmentation, fragmentation and granulation.

As stated by VULPIAN and PHILIPPEAUX as early as 1863, the denervated striated muscle contracts on stimulation of its vasodilator nerve [17]. Considering that this effect is enhanced by eserine and abolished by atropine, it was supposed that it is related to alterations in the acetylcholine-cholinesterase system of the denervated end plate. Since biochemical determinations did not reveal a considerable loss of cholinesterase activity in denervated muscles, in a recent paper [2]. BROOKS holds the opinion that cholinesterase activity has no role in the phenomenon. However, in view of the above described cytochemical changes in the denervated subneural plates, some correlation not detectable by summative biochemical methods seems to exist between cholinesterase activity and Vulpian-phenomenon.

As mentioned above, degenerating nerve fibres exhibited cholinesterase activity, while sound nerve fibres did not react to thiocholine. It is well known that by biochemical methods cholinesterase activity has been revealed in sound myelinated nerve fibres [16, 18]. This enzymic activity may be demonstrated also by means of histochemical methods, if lipoid-soluble substrates (naphtylacetate [5], indoxylacetate) penetrating the myelin sheath are employed.*

*Unpublished results from our laboratory.

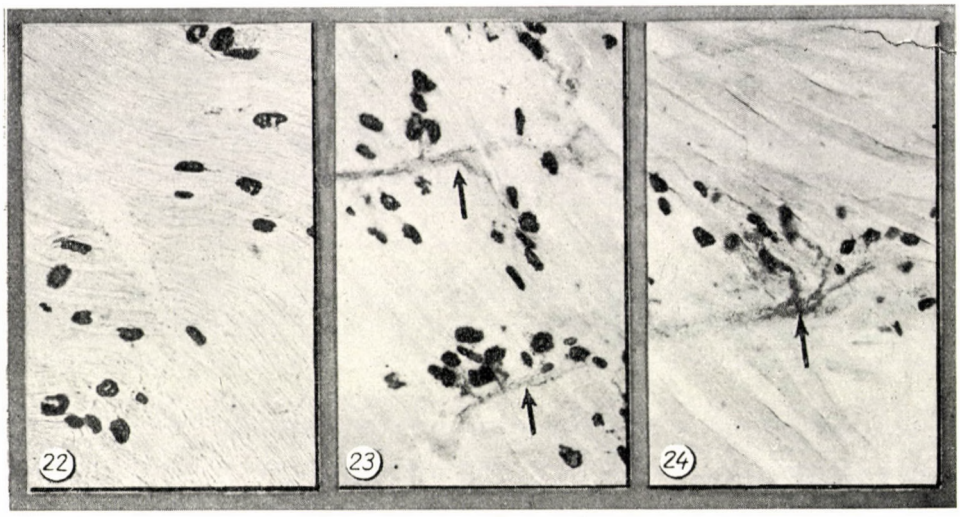


Fig. 22. Cholinesterase activity in motor end plates of a sound muscle. Nerve fibres do not react. Magn. ×100. *Fig. 23.* Cholinesterase activity in a denervated muscle. 28th postoperative day. Nerve fibres show cholinesterase activity. Magn. ×100. *Fig. 24.* As Fig. 23 ; 32nd postoperative day. Magn. ×100

Acetylcholine, a compound insoluble in lipoid, does not penetrate the sound myelin sheath, hence no enzymic activity may be detected by its use. When, however, the fibre degenerates the destructed myelin sheath becomes permeable for acetylthiocholine, resulting in the staining of degenerating nerve fibres.

The question may arise whether the cholinesterase reaction in degenerating nerve fibres is correlated with a hypothetical increase in their enzymic activity. On the basis of biochemical investigations indicating that the pseudo-cholinesterase activity of degenerating nerve fibres remains unaltered while their true cholinesterase activity decreases [16], the hypothesis may be rejected.

As it has been stated on the basis of our previous investigations [6], the cholinesterase activity of the sensory end apparatuses is also bound to a structure independent of the terminal arborisation of the sensory nerve fibres. It could be established that these «pre-synaptic membranes» retain their enzymic activity even when their nerve fibres had been degenerated. Thus the behaviour of the denervated motor end plate is analogous to that of the denervated receptor apparatus.

The cholinesterase activity of regenerating motor endplates will be discussed in a following paper. It is, however, presumed that a correlation exists between the cholinesterase activity of the degenerating and regenerating neural plates. It seems obvious that the activity of the degenerating end plates may influence the direction of the regenerating nerve fibres.

We are indebted to Prof. A. GELLÉRT for his interest in the work and his constant encouragement, to Dr. S. J. HOLT (London) and to Dr. K. B. AUGUSTINSSON (Stockholm) for supplying the acetylthiocholine iodide used in the investigations, and to undergraduate I. SCHNEIDER for technical assistance.

Summary

The histochemical methods for demonstrating cholinesterase activity in motor end plates have been compared and the characteristic differences between the pictures obtained by the naphtylacetate, indoxylacetate and acetylthiocholine methods have been described and discussed. The behaviour of denervated subneural apparatuses of the rat has been investigated histochemically by means of the acetylthiocholine technique. In agreement with our previous findings it has been found that the cholinesterase activity of the subneural apparatuses persists for 180 days after denervation. The cytochemical changes in the structure exhibiting cholinesterase activity occur in three stages, with hypersegmentation, fragmentation, resp. granulation of the denervated subneural apparatuses.

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ДЕЙСТВИЕ ДЕНЕРВАЦИИ НА АКТИВНОСТЬ ХОЛИНЕСТЕРАЗЫ ДВИГАТЕЛЬНЫХ ПЛАСТИНОК

ДЬ. ШАВАИ и Б. ЧИЛЛИК

Авторы сравнивают методики, основанные на применении нафтилацетата, индоксилацетата и тиохолина, служащие для микроскопического выявления холинестеразной активности двигательных пластинок. Они исследовали на крысах при помощи тиохолиновой методики поведение субневральных аппаратов в связи с денервацией и установили в полном согласии со своими прежними исследованиями, что активность холинестеразы субневральных пластинок сохраняется еще в течение 180 дней после денервации. При этом, обладающая ферментативной активностью структура подвергается цитохимическим изменениям (гиперсегментация, фрагментация, грануляризация).

EINFLUSS DER DENERVATION AUF DIE CHOLINESTERASEAKTIVITÄT DER MOTORISCHEN ENDPLATTEN

Gy. Sávay und B. Csillik

Es werden die zum mikroskopischen Nachweis der Cholinesteraseaktivität der motorischen Endplatten dienenden Naphthylacetat-, Indoxylacetat- und Thiocholin-Methoden verglichen. Mit der Thiocholin-Methode wird das Verhalten der subneuralen Apparate von Ratten im Zusammenhang mit der Denervation untersucht. Im Einklang mit ihren früheren Untersuchungen stellen die Autoren fest, dass die Cholinesteraseaktivität der subneuralen Endplatten noch 180 Tage nach der Denervation weiter fortbesteht. Dabei wird die eine enzymatische Aktivität aufweisende Struktur zytochemischen Veränderungen unterworfen (Hypersegmentation, Fragmentation, Granularisation).

Dr. Gyula Sávay
Dr. Bertalan Csillik
Szeged, Kossuth L. s. u. 40. Hungary