CHOLINESTERASE ACTIVITY OF NODAL AND INTERNODAL REGIONS OF MYELINATED NERVE FIBERS OF FROG

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ABSTRACT

The distribution of cholinesterase (Ch-esterase) in isolated myelinated fibers of the frog has been investigated. Quantitative microgasometric measurements have confirmed the previous histochemical observations. Both approaches indicate that in frog nerve fibers acetylcholinesterase (ACh-esterase) is the only or the predominant enzyme when selective inhibitors and different substrates are used: acetylcholine (ACh), butyrylcholine, and acetyl-B-methylcholine (Mecholyl). By means of the microgasometric technique, a significant difference in ACh-esterase activity between axons isolated from ventral (37.2 ± 1.7 μmole $\times 10^{-5}$ ACh/mm^2/hr) and dorsal roots (2.0 ± 0.9 μmole $\times 10^{-5}$ ACh/mm^2/hr) was found. In the region of the node of Ranvier the enzyme activity (50.4 ± 4.4 μmole $\times 10^{-5}$ ACh/mm^2/hr) appears to be considerably higher than in the internodal area (36.6 ± 2.1 μmole $\times 10^{-5}$ ACh/mm^2/hr). The findings are discussed in relation to the theory of saltatory conduction and the ACh system.

INTRODUCTION

The theory of saltatory conduction along myelinated axons rests on the assumption that the myelin sheath is an insulator which forces the circulating currents to leave and enter at the nodes of Ranvier the regions free of myelin. The myelin sheath is interrupted at these areas which are 1–2 mm apart, depending on fiber size. If the assumption is correct and excitation jumps from node to node, the system involved in the mechanism of excitation should be concentrated in the area of the nodes of Ranvier. If we assume that the action of acetylcholine (ACh) is essential for the permeability change that takes place during the conduction of an action potential (1), the ACh system including the enzyme inactivating ACh, ACh-esterase, should be more concentrated in the region of the nodes. It has been known for a long time that ACh-esterase is present in various types of nerve fibers (2–4). According to reported observations based on histochemical staining techniques, this enzyme is present in the cytoplasm, at the axon hillock, and at the surface of the nerve fiber itself (5–7). More intense staining of nodes of Ranvier was reported previously (8–10), and the significance of this finding connected with the mechanism of saltatory conduction by Gerebtzoff (8,9). Because most quantitative techniques are not sensitive enough to detect ACh-esterase activity in single fibers, whole nerve bundles were used. For a more precise localization, histochemical techniques and the Cartesian diver procedure (3) were applied. Recently, a convenient micro-

TABLE I

Cholinesterase Activity of Desheathed Frog Sciatic Nerve Bundles

Determined with the colorimetric technique (22) and expressed in μmoles of ACh hydrolyzed per gram (fresh tissue) per hour

Intact nerve	Homogenized nerve
33.2	34.9
35.6	38.9
20.4	30.9
25.0	36.7
14.2	16.8
21.7	19.3
24.5	29.2
20.0	28.3
11.5	13.0
9.6	11.6
Mean 21.6 ± 2.7	26.0 ± 2.9

technique has been developed using a magnetic diver (11, 12) with a sensitivity of detecting 1 μmole × 10^{-5}/mm²/hr ACh hydrolyzed by ACh-esterase (13). This enables us to investigate the distribution of the ACh-esterase along a single myelinated nerve fiber. We especially were interested to see whether there was an increase of enzyme concentration in the region of the Ranvier node compared to that of the internodes. Furthermore, it has been reported that there was a difference in ACh-esterase concentration in motor and sensory fibers in mammals (1, 14). Since there are physiological differences between these fibers (15–19), it seemed worthwhile to investigate this problem more closely in isolated frog fibers. Using the Koelle-Friedenwald (5) and Karnovsky (20) procedure, we were able to confirm previous findings of cholinesterase localization at the nodes of Ranvier (8–10). In the present work, the histochemical findings were confirmed by the quantitative method.

METHODS

Measurement of Enzyme Activity

For the magnetic diver measurements, myelinated fibers of frogs' sciatic nerve, dorsal roots, and ventral roots were isolated according to Staempfli (21) and then broken into nodal (80–200 μ) and internodal parts (750–1000 μ), with a dark-field dissecting microscope. The diameter of the fiber and the length of each isolated part was measured by means of a calibrated eye piece. 8–15 fragments of the internodal and nodal regions, respectively, were pooled together for each individual cholinesterase (ChE) measurement. The samples, together with the conventional substrate-bicarbonate solution, were introduced into the ampulla, and their activity was measured as previously described (13).

TABLE II

Cholinesterase Activity of Intact Desheathed Frog Sciatic Nerve Bundles

Expressed in μmoles of ACh hydrolyzed per gram (fresh tissue) per hour

Control nerve	Triton-treated nerve
16.0	27.0
19.5	20.5
11.3	28.0
15.7	16.3
Mean 15.6 ± 1.68	22.9 ± 2.7

The colorimetric technique of Hestrin (22) was used for ACh-esterase determination of the roots and the whole nerve. After isolation, nerve bundles were desheathed under a dissecting microscope. The nerve taken from one side of the animal was homogenized with a hand homogenizer, while that of the other side was used intact. The substrates tested were ACh (5×10^{-3} M), acetyl-B-methylcholine (10^{-2} M), and butyrylcholine (10^{-2} M). These substrates were dissolved in frog Ringer's solution of the following composition (mM/liter): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8. The substrate solutions were buffered with 10^{-1} M Tris. In some experiments nerves were pretreated with Triton 100 ×, a surface active agent, and then assayed for ACh-esterase activity. A few isolated fibers were used for a histochemical analysis of ACh-esterase activity.

Histochemistry

For the histochemical examination small bundles or single isolated fibers were incubated in the histochemical media. Compared to microtome sections of nerves, the fiber bundles used by us, did not have a uniform thickness and, therefore, were not suitable for examination under high magnification. High magnification was used only on isolated nerve fibers. Prior to histochemical treatment the samples were fixed in the cold (0–2°C) glutaraldehyde for 10–15 min and rinsed in Ringer's solution for 2 hr. Triton 100 × was added to the rinsing solution, (final concentration, 0.2%).

The copper-ferrocyanide procedure according to Karnovsky (20) was employed for the histochemical localization of ChE. The pH of the solutions was 6. Single isolated nerve fibers or small bundles were

TABLE III

Cholinesterase Activity at the Nodes of Ranvier and of Internodal Segments of Sciatic Nerve Fibers

Expressed in μmoles of ACh hydrolyzed per hour per gram fresh tissue or in μmole × 10^{-5} per mm^2 of surface area. The enzyme activity was determined with the microgasometric technique, using the magnetic diver (6).

Nodal sections		Internodal sections	
μmoles/g/hr	*μmole* × $10^{-5}/mm^2/hr$	*μmoles/g/hr*	*μmole* × $10^{-5}/mm^2/hr$
34.0	6.9	18.0	3.6
31.0	6.3	23.0	4.6
19.0	3.8	19.0	3.8
28.0	5.7	16.0	3.2
23.0	4.7		
Mean 27.0 ± 2.2	5.4 ± 0.4	19.0 ± 1.5	3.6 ± 1.2

incubated in the normal or control histochemical media for various lengths of time (1–20 hr) at 0–2°C.

The substrates tested were acetylthiocholine-iodide (AThCh), 5×10^{-3} M, and butyrylthiocholine-iodide (BuThCh), 1×10^{-2} M. Both substrates were used freshly synthesized or were recrystallized before use.

All solutions were cooled to 0°C before mixing. Koelle-Friedenwald's medium prepared in this way was stable for more than 24 hr, whereas the histochemical medium according to Karnovsky started to show a slight turbidity after 6–8 hr.

The media, in which the tissue was incubated, were stable during the experiment, provided there was a large excess of media compared to the amount of tissue used. In our experiments, 0.4–0.8 mg of nerve fibers was incubated in 2–3 ml of media.

Iso-OMPA (tetraisopropyl pyrophosphortetramide) (2×10^{-5} M) and BW 284 C 51 (1,5-bis[4-allyldimethylammoniumphenyl] pentan-3-one dibromide) (2×10^{-5} M) were employed. 1×10^{-5} M eserine was used as a general inhibitor of ChE. It has been recently suggested (7) that the apparent histochemical localization of ChE at the nodes of Ranvier is an artefact due to the binding of copper at this site. To test this objection, necessary control experiments were performed.

Samples prepared for normal histochemistry were incubated for 1–20 hr in (*a*) 0.1 M copper sulfate, (*b*) copper glycinate 0.1 M in regard to copper sulfate, (*c*) Koelle's or Karnovsky's histochemical medium without substrate, and (*d*) normal media to which the above listed inhibitors were added.

RESULTS

Since the enzyme activity varies markedly from frog to frog, homogenized and intact nerves were taken from the same animal. Table I shows the cholinesterase activity of intact and homogenized sciatic nerves. The values in the table are matched so that each line shows the enzymatic activity for nerves from the same animal. The differences between homogenized and intact nerves vary from specimen to specimen: showing sometimes only small, sometimes larger differences, indicating that substrate can reach the enzyme quite readily in intact desheathed fibers. In order to check the thoroughness of the homogenization, part of a sample was sonicated and then assayed for enzyme activity. It was found that sonication decreases, to a certain extent, the cholinesterase activity of the homogenate from 12.1 ± 3 μmoles/g/hr to 9.5 ± 0.8 μmoles/g/hr. When intact nerves were treated with a detergent, Triton 100 X, which is known to increase the permeability of nervous structure, the cholinesterase activity of intact nerves was increased (Table II). Table III summarizes the results obtained with the magnetic diver technique, which used isolated myelinated fibers taken from sciatic nerve without separating them into sensory or motor fibers. The enzyme activity of the nodal area is about 30% higher than that of the internodal area, whether calculated on a weight or surface-area basis. It should be kept in mind that it is difficult to free the nodal area completely from internodal parts, so that probably this difference is actually even larger when only the nodal area is compared with the internodal section (an area of about 60–120 μ of the fiber with the node in the middle was used as nodal area). Since it was difficult to distinguish, from the total population of fibers of the sciatic nerve, between the contributions of the sensory fibers and those of the motor

fibers, it was important to use uniform populations of either of them. The best sources for motor fibers are the ventral roots and for sensory fibers the dorsal roots. As is well known, the motor roots have a rather homogeneous histological appearance compared with that of the sensory roots. This is due to the large mass of fibers with a diameter of about 14 μ with the number of fibers decreasing on either side, the largest fiber having a diameter of 20 μ. The range of the diameters of fibers in the dorsal roots is about the same, the largest fiber having a diameter of 20 μ. But there is a much smaller number of fibers with a 14 μ diameter in the dorsal roots than in the motor roots. The peak in numbers comes at 7 μ. The number of fibers smaller than 11 μ is greater than that of fibers larger than 11 μ (15, 24). Table IV shows the cholinesterase activity of single fibers taken from the ventral root. The nodal area shows almost 30% higher activity than the internodal section or the total nerve fiber. Table V shows the same type of experiments performed on the sensory fibers.

TABLE IV

Distribution of Cholinesterase Activity in Fibers of Ventral Roots of Frog

The activity is expressed in μmole $\times$ 10^{-5} of ACh hydrolyzed per mm^2 surface area per hour. The enzyme activity was determined with the microgasometric technique (6).

	Total axon	Internodal parts	Nodal parts
	32.3		
	41.6		
	38.2		
	37.0		
		36.2	57.8
		33.0	42.5
		40.5	51.3
Mean	37.2 ± 1.7	36.6 ± 2.1	50.4 ± 4.4

TABLE V

Distribution of Cholinesterase Activity in Fibers of Dorsal Roots of Frog

Determined with the microgasometric technique (6) and expressed in μmole $\times$ 10^{-5} of ACh hydrolyzed per mm^2 surface area per hr.

	Total axon	Internodal parts	Nodal parts
	1.2		
	3.0		
	1.8		
		2.2	2.1
		1.3	3.1
		2.1	1.6
		1.0	
Mean	2.0 ± 0.9	1.65 ± 0.20	2.2 ± 0.4

Histochemically treated axons show a sharply localized ChE reaction at the nodal region (Figs. 1 and 2).

The incubation time required in our experiments for a demonstration of ChE at the node was 6 hr. When incubation time was prolonged to 10–

TABLE VI

Cholinesterase activity of ventral and dorsal roots with different substrates.

The activity was measured with the colorimetric technique (22) and is expressed in μmoles of substrate hydrolyzed per gram fresh tissue per hour.

	Substrates		
	ACh	Acetyl-B-methylcholine	Butyrylcholine
	5×10^{-3} M	10^{-2} M	10^{-2} M
Ventral root	130.4 ± 33.9	39.5 ± 2.5	4.5 ± 0.25
Dorsal root	21.9 ± 9.6	13.9 ± 5.8	8.0 ± 2.0

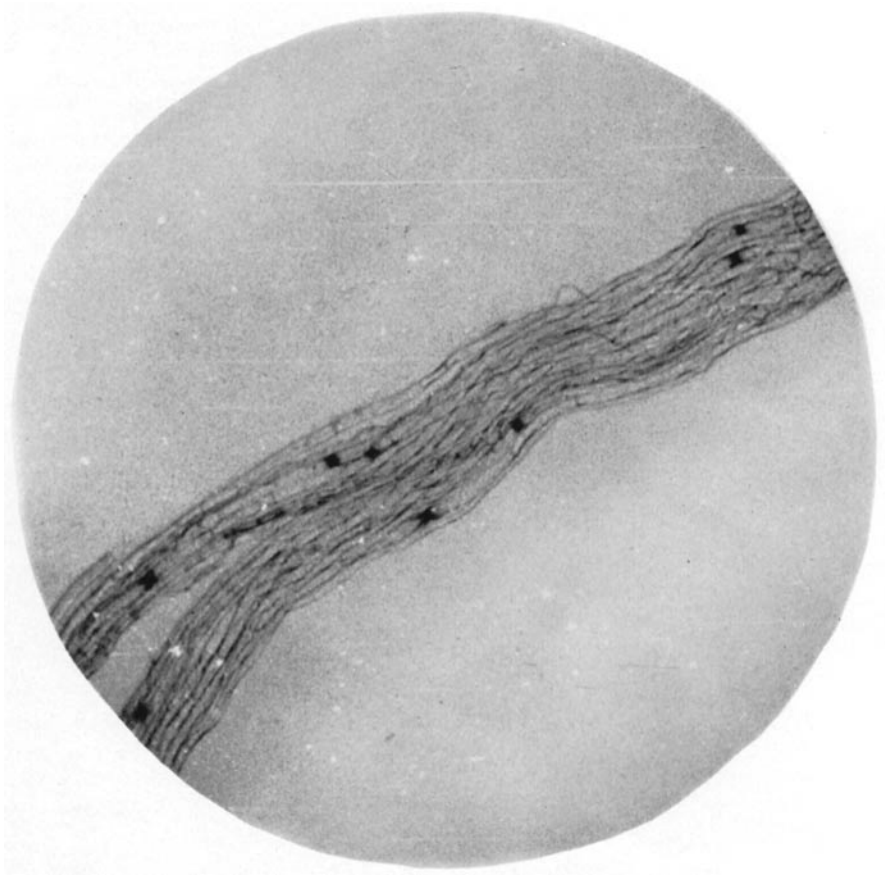

FIGURE 1 Fiber bundle of the sciatic nerve; Karnovsky's procedure; incubation time, 7 hr. Dark spots are the deposits of the ChE reaction product at the nodes of Ranvier. × 140.

12 hr, the deposits of the reaction product became more pronounced.

No staining of the nodes was detected in the medium with AThCh as substrate to which BW 2×10^{-5} M or eserine 1×10^{-5} M was added. Negative results were obtained also with BuThCh as substrate, and in experiments when samples were incubated in the usual medium from which the substrate was omitted. Even after incubation for 20 hr in Karnovsky's medium with BuThCh or with AThCh and BW or eserine, there was no localized staining of the nodal area in spite of the progressed decomposition of the medium. The precipitate formed in the medium during such a long period of incubation was distributed uniformly on the surface of the samples.

In all control experiments performed in the standard media with the usual copper concentration ($CuSO_4$, 0.03 M; copper as glycinate or citrate) no staining was observed even after prolonged incubation (up to 20 hr).

In agreement with Zenker (23), a pronounced staining of the nodes was obtained when nerve fibers were incubated in 0.1 M $CuSO_4$, and only an occasional, slight staining in 0.03 $CuSO_4$ in the absence of glycine.

DISCUSSION

The present investigations, as others before, have demonstrated the presence of ACh-esterase all along the peripheral myelinated nerve (1–5). A new finding is the apparently, considerably higher concentration in the nodal area than in the internodal area. This is more significant when only motor fibers are used. Moreover, a 15–20 times higher (microgasometric technique) and a six

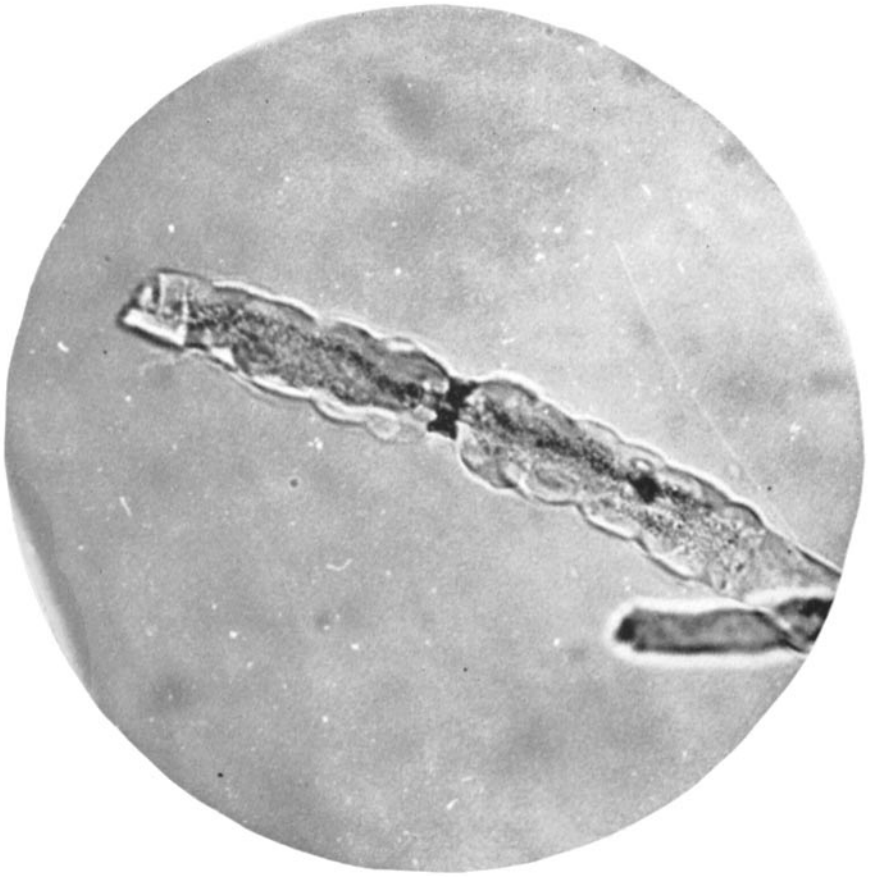

FIGURE 2 Single nerve fiber preparation taken from the ventral root; Karnovsky's procedure; incubation time, 7 hr. Heavy deposits of the ChE reaction product at the node of Ranvier; much lighter deposits at the internodal parts of the axon. × 1200.

times higher (Hestrin technique) ACh-esterase activity is found in motor fibers than in sensory fibers. Two quantitative procedures employed in this work show a different ratio of ChE activity between motor and sensory fibers (Tables IV–VI). This discrepancy may be due to the higher proportion of nonmyelinated fibers incorporated into the dorsal roots used for the Hestrin ChE determination. On the other hand, fibers isolated for microgasometric measurements always were myelinated. Observations based on electron microscopic histochemistry (6) indicate that in fact nonmyelinated axons exhibit a higher ChE activity. The experiments with whole sciatic nerve, therefore, do not represent a true picture since these nerves are mixed and contain almost equal numbers of motor and sensory fibers. Our experiments show that the Ch-esterase present in frog nerve is only or mostly of the ACh-esterase type, which can be concluded from the slow rate of the butyrylcholine hydrolysis as measured with histochemical and biochemical techniques. Higher ACh-esterase activity at the nodal region is consistent with the assumption that the excitable process takes place there. Gerebtzoff demonstrated a localization of ACh-esterase at the nodes of peripheral myelinated fibers of the rat and related these findings to the theory of saltatory conduction. He concluded from his experiment that the conditions of ACh and ACh-esterase coupling as postulated by Nachmansohn (1) are present at this level (8). However, the considerable ACh-esterase activity of internodal sections as measured by microgasometry and demonstrated by histochemistry still requires an explanation as to its possible functional significance. No explanation

for the difference in ACh-esterase activity between motor and sensory fibers can be given at present, but it should be kept in mind that these two types of fibers also show marked differences in various electric parameters. Several investigations have shown differences in the physiological properties of medullated nerve fibers (12–15). It seems that myelinated nerve fibers are subject to a greater degree of variation than had been accepted previously As was pointed out by Gasser (19), the properties of nerve fibers are determined not only by their size. Two kinds of nerve fibers of the same size and accordingly the same rate of conduction may have different properties in several other respects. Sensory fibers in general have a lower threshold, accommodate less, and show easier repetitiousness. Stimulating currents of long duration in sensory fibers cause easier repetitive firing than in motor fibers, and the positive after-potential in relation to the magnitude of the negative one is larger in sensory fibers. Some of these differences might have a relationship to the marked differences in ACh-esterase concentration; however, it would be premature at present to relate specific features and a lower threshold and facilitated repetitiousness to the lower enzyme concentration. The investigation will be extended to the presence and distribution of the other components of the ACh system, ACh, and choline acetylase, as well as other chemical factors which may be involved.

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