LOCALIZATION AND PROPERTIES OF THE
CHOLINESTERASE IN CRUSTACEAN MUSCLE

NEIL I. SPIELHOLZ and WILLIAM G. VAN DER KLOOT

From the Department of Physiology and Biophysics, New York University School of Medicine, New York 10016. Dr. Spielholz's current address is Institute of Rehabilitation Medicine, New York University Medical Center, New York 10016. Dr. Van der Kloot's current address is Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11790.

ABSTRACT
Cholinesterase (ChE) activity is present in crustacean muscle extracts. However, since acetylcholine (ACh) is not a neuromuscular transmitter in these animals, the role and exact localization of ChE was unknown.

The histochemical localization of the enzyme was studied in whole muscle and in the sarcoplasmic reticulum fraction of the extract. 50-µm frozen sections of glutaraldehyde-fixed crayfish tail flexor muscle were incubated with acetylthiocholine (ATC) as substrate, and examined under the electron microscope. After some modifications in published techniques, dense deposits were found associated with the sarcolemma, sarcolemmal invaginations, and transverse tubules. No deposits were found in 10^{-4} M eserine, or if butyrylthiocholine (BTC) was substituted for ATC. The vesicles in the sarcoplasmic reticulum fraction which demonstrate the activity must represent minced bits of these membranes.

Using a spectrophotometric method, the kinetics of the crustacean muscle enzyme was compared to the acetylcholinesterase (AChE) on mammalian red blood cells and in the lobster ventral nerve cord. Surprisingly, and contrary to previous reports, the crustacean muscle enzyme did not demonstrate substrate inhibition. While a number of similarities to AChE were found, this lack of substrate inhibition makes questionable an unequivocal similarity with classical AChE.

INTRODUCTION

In the last decade a number of studies have been made of the histochemical localization of acetylcholinesterase (AChE) in vertebrate muscles. There is general agreement that most of the enzyme is found at the end plate, but AChE also is reported in T tubules (Ulbrecht and Kruckenberg, 1965; Miledi, 1964; Teräväinen, 1969), in longitudinal tubules of the sarcoplasmic reticulum (Karnovsky, 1964; Miledi, 1964), or in the A band (Karnovsky, 1964). Obviously it is difficult to decide whether the properties of the enzyme found away from the end plate are the same as those of the enzyme at the junction. It is also possible that the AChE is synthesized at some site in the cell and then moves to the end plate where it is incorporated into the membrane; in this case, the enzyme that appears at other sites in the fiber might simply be in transit, and serve no functional role except at the end plate.

On the other hand, most crustacean muscles do
not respond to acetylcholine (ACh), to anticholinesterases, or to cholinergic blocking agents (Katz, 1936; Bacq, 1936, 1937; Ellis et al., 1942). Apparently the usual neuromuscular transmitter in crustacea is glutamate (van Harreveld and Mendelson, 1958; Takeuchi and Takeuchi, 1964). Recently, Futamachi (1972) has shown that the tonic flexor muscles of the crayfish abdomen are probably cholinergic. Although in the great majority of crayfish and lobster muscles ACh is almost surely not the transmitter, it has been known since the work of Bacq and Nachmansohn (1937) that a cholinesterase (ChE) is present in extracts made from crustacean muscle. ChE has been found in the microsomal, or sarcoplasmic reticulum fraction of lobster muscle (Van der Kloot, 1966). This enzyme has many of the characteristics of true- or AChE (E.C.3.1.1.7). However, some caution is required before ascribing the localization of this enzyme to the muscle fibers because tissue homogenates inevitably include muscle sense organs and nerve, both known to contain AChE (Maynard and Maynard, 1960; de Lorenzo et al., 1969).

We decided, therefore, to localize the enzyme histochemically, to see how it is distributed in these noncholinergic muscles. We also studied the localization of the enzyme in the sarcoplasmic reticulum fractions. In addition, the characteristics of the enzyme were studied and compared with the AChE found in lobster nerve cord and on mammalian red blood cell membranes. A preliminary report of this study was given (Spieholz and Van der Kloot, 1972).

METHODS

Muscle Histochemistry

Enzyme localization was studied on deep flexor muscles from crayfish tails (Procambarus clarkii). Tails were quickly separated from the thorax by cutting with cold scissors. The relatively soft cuticle covering the ventral surface was then removed as completely as possible, including amputation of the swimmerettes. The superficial flexor muscles remain attached to the inner surface of the cuticle, leaving the deep flexors exposed. To prevent shortening of the deep flexor muscles during the first fixation, a deep dish was partly filled with wax. A channel was cut out of the center of the wax which was now filled with fixative. The tail was placed with its exposed ventral surface down into the groove, the sides of the remaining exoskeleton making contact with the edges of the groove maintained the tail in an extended position. The remainder of the dish was then filled with fixative, covering the entire structure.

Fixation was carried out with cold 3% glutaraldehyde in 0.05 M Tris-maleate buffer, pH 7.4. Phosphate buffer was not used to avoid precipitation of phosphate salts by the high divalent cation concentrations in crustacean saline. Time in this fixative was 20 min. Preliminary studies had shown that fixation for 2 h inhibited the histochemical activity.

The tails were then washed three times for 5 min in cold buffer. Small cylinders (about 5 mm long and 3 mm wide) of the superficial fibers were cut out under a dissecting microscope. These cylinders were placed in cold buffer, changed three times during the day, and placed overnight in the refrigerator.

The following day, 30-μm frozen sections were cut from these cylinders in a cryostat. The cylinders were oriented on the cutting block so that sectioning produced either longitudinal or cross sections.

Enzyme incubations were carried out according to Karnovsky (1964). Stock solutions were: sodium citrate, 100 mM; copper sulfate, 30 mM; potassium ferricyanide, 5 mM; and 0.05 M Tris-maleate buffer, pH 6.0. These solutions were usually made up the preceding day and kept in the refrigerator until use. The final incubation media were prepared just before use as follows: 5 mg of acetylthiocholine (ATC), or 6 mg of butyrylthiocholine (BTC), were dissolved in 6.5 ml of the Tris-maleate buffer, pH 6.0. The following were then added in sequence, with constant mixing on a magnetic stirrer: sodium citrate, 0.5 ml; copper sulfate, 1.0 ml; H2O, 1.0 ml (or 1.0 ml of 10-3 M eserine for inhibitor study); potassium ferricyanide, 1.0 ml; and sucrose, 1.5 g. The media were prepared in 20-ml beakers. At first, incubations were performed with the beakers in ice. However, it was found that success was only achieved by incubating the sections at room temperature for 1 h. Those sections which were to be exposed to both inhibitor and substrate were precubated for about 15 min in 10-4 M eserine, in the same buffer. At the end of the required time in the substrate media, the sections were washed once with cold buffer, pH 7.4, and reixed in 1% osmium tetroxide (same buffer, pH 7.4) for 1 h. The specimens were then washed four times with cold 0.44 M sucrose, dehydrated through a graded series of ethanol solutions, cleared in propylene oxide, and embedded in Epon 812. Thin sections were cut using glass knives on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.), and taken up on 200- or 300-mesh copper grids. The sections were stained with 4% uranyl acetate, counterstained with lead citrate, and examined with a Zeiss 9-A electron microscope.

Sarcoelastic Reticulum Fraction Histochemistry

A live lobster (Homarus americanus) was placed in an ice bucket for 15–20 min. The tail was amputated using cold scissors. Care was taken not to include the...
digestive tract which runs longitudinally through the tail. This precaution was necessary to avoid contamination with digestive proteolytic enzymes. This separation was easily accomplished by holding the animal in one hand by the thorax, letting the tail hang down. The exoskeleton, cuticle, and muscle connecting the tail with the thorax were then cut. As this was performed, gravity gently helped separate the tail and its contents from the thorax, exposing the digestive tract. When all connections, except the digestive tract, were sectioned, gentle manual traction on the tail completed the procedure, the digestive tract slipped free and remained with the thorax.

The ventral nerve cord was cut away and the muscle within the tail quickly scooped out and weighed in a prechilled beaker containing a small amount of cold 0.05 M Tris-maleate buffer, pH 7.4. For each gram of muscle, 4-5 ml of cold buffer were added and the mixture put through a cold meat grinder. The ground muscle was homogenized for 2 min in a cold blender, and then spun in the cold at 1,000 g for 20 min. The supernate was filtered through cold glass wool, and the filtrate spun at 8,000 g for another 20 min. This supernate was then spun in a Beckman ultracentrifuge, no. 30 rotor (Beckman Instruments, Inc., Spincro Div., Palo Alto, Calif.), at 17,500 rpm for 1 h. The resultant pink pellet is the microsomal, vesicular, or sarcoplasmic reticulum preparation.

The supernate was drained or suctioned off, and the pellet covered with 2-3 ml of cold 3% glutaraldehyde in 0.05 M Tris-maleate buffer, pH 7.4, for 20 min. While in this fixative, the pellet was broken up into smaller pieces with the sharp edge of a broken wooden applicator stick. The bits of pellet were then processed for histochemical study as in the foregoing section.

Biochemical Assay

ChE activity was studied using the spectrophotometric method of Ellman et al. (1961). The buffer was 0.05 M Tris-maleate, pH 7.4. Substrates were either ATC or BTC. The thiocholine released by hydrolysis reacts with 5, 5-dithiobis-2-nitrobenzoate to produce yellow 5-thio-2-nitrobenzoate anion. Inhibitors were either eserine, quinidine, or 1, 4-bis(4-allyl dimethyl ammonium phenyl)pentane-3-1-dibromide (BW284C51); this was a gift from the Burroughs Welcome Co., Inc., Tuckahoe, N. Y.

SarcoPlasmic Reticulum Fraction: This fraction was prepared as outlined above, up to the point of obtaining the pellet. These pellets were then resuspended on 3.0 ml of cold buffer and ground slowly in a cold Potter-Elvehjem homogenizer. This solution was diluted 10- to 50-fold and assayed.

Lobster Ventral Nerve Cord: Live lobsters were chilled and the tail and ventral cuticle removed. The ventral nerve cord was carefully dissected free of muscle and nerve trunks. Essentially the entire cord was freed from its surroundings, and placed in cold buffer. Stumps of nerve trunks and adhering connective tissue or muscle were cut away under a dissecting microscope. The cleaned cord was placed in a small volume of fresh, cold buffer, minced with a cold scalpel blade, and the suspension ground in a cold homogenizer. The suspension was then centrifuged at 1,000 g for 20 min, and the supernate suctioned off and kept for further study.

Human and Rabbit Red Blood Cell Ghosts: 2 ml of fresh whole blood were drawn into heparinized syringes, either from the ear vein of a rabbit or from the antecubital veins of two human volunteers. A spin at 2,000 rpm for 10 min separated the formed elements from the plasma. The plasma was carefully suctioned off. Ghosts were obtained by mixing the packed cells with distilled water, centrifuging at 8,000 g for 20 min, and discarding the red supernate. This procedure was repeated and the colorless precipitate resuspended in 3.0 ml or more of cold buffer.

RESULTS

We initially fixed crustacean muscle for 2 h in 3% glutaraldehyde followed the next day by cold incubation in Karnovsky's medium (1964) for 1 h. This procedure was uniformly unsuccessful. Extensive trial and error showed that two major changes were required: (a) Fixation in 3% glutaraldehyde for 2 h apparently resulted in complete inhibition of the crustacean muscle enzyme. Chemical assays of homogenates showed some of the ChE activity remains after 20 min in this fixative, so this fixation time was adopted; (b) Deposits were not seen if incubation was performed in the cold, as in the original method. If performed at room temperature, however, the sections fixed for 20 min had substantial deposits. At the beginning of incubation the medium was a clear blue-green. By the end of the hour it was a clear light brown. This color change occurred in the cold or at room temperature.

Figs. 1-6 are representative samples of sections incubated with the revised technique. Black, electron-dense deposits are clearly visible in each field. These deposits are found in close association with the sarcolemma, sarcolemmal invaginations, and the transverse tubular system which in crustacean muscle runs both transversely and longitudinally between adjacent myofibrils (Brandt et al., 1965; Selverston, 1967). In many sections, substantial lengths of the above membranes can be followed, while in others the deposits appear patchy.
Figure 1  Crayfish muscle, longitudinal section, incubated for ChE. Deposit (d) lines tubules between adjacent myofibrils and the sarcolemma of two adjacent fibers. Connective tissue (ct) separates the fibers. Part of a subsarcolemmal nucleus (N) is seen. × 24,000.

Figure 2  Crayfish muscle, longitudinal section, incubated for ChE. Deposit (d) is associated with the sarcolemma beneath connective tissue (ct) fibers. (N) is part of a subsarcolemmal nucleus. × 36,000.
Figure 8  Crayfish muscle, longitudinal section, incubated for ChE. Deposit (d) lines a long length of a longitudinally oriented transverse tubule. × 52,000.

Figure 4  Crayfish muscle, cross section, incubated for ChE. Deposit (d) is present along parts of transverse tubules and sarcolemmal invaginations (S.I.). × 35,000.
FIGURE 5  Crayfish muscle, cross section, incubated for ChE. Deposit (d) lines the membrane of a sarcolemmal invagination (S. I.) and the transverse tubule (t) which branches from it. × 71,000.

FIGURE 6  Crayfish muscle, cross section, incubated for ChE. Deposit (d) lines a long length of transverse tubule. × 50,000.
FIGURE 7  Crayfish muscle, longitudinal section, incubated with $10^{-4}$ M eserine. No deposit. The small black dots randomly dispersed among the filaments were occasionally seen even in preparations with no substrate. $\times 53,000$.

FIGURE 8  Crayfish muscle, cross section, incubated with $10^{-4}$ M eserine. No deposit. $\times 28,000$. 
Deposits are not found within the myofibrils themselves. Neither are there deposits near mitochondria nor nuclei.

The deposits did not appear in sections incubated in the presence of $10^{-4}$ M eserine (Figs. 7 and 8). This shows the activity is enzymatic in nature and not due to spontaneous hydrolysis followed by fortuitous, selective deposition. Sections incubated with BTC as substrate showed no deposits.

The micrographs show another feature which may be pertinent to the discussion. The extracellular space which borders a muscle fiber, or which separates two fibers from one another, is filled with tightly packed connective tissue fibers. A structural barrier exists, therefore, between the surface of a muscle fiber and the extracellular fluid bathing these fibers. This enveloping connective tissue shell may act as a diffusion barrier to prevent, or retard, externally applied substances from reaching the surface membrane of the fibers. The existence of this barrier was demonstrated by Orentlicher and Reuben (1971) who found that there was an appreciable delay between an abrupt elevation in the extracellular $K^+$ and the depolarization of the cell membrane.

One of the reasons for undertaking this study was the detection of ChE activity in preparations of sarcoplasmic reticulum. Therefore, we were somewhat surprised to find that in the muscle the enzyme activity appeared to be associated solely with the sarcolemma, its invaginations, and with the transverse tubular system. Consequently, we applied the same histochemical techniques to the study of the microsomal preparation.

The most striking observation made on the microsomal preparation is that, on the ultrastructural level, it is not homogenous. This is shown in Fig. 9 which is from a sample not incubated for ChE. At least three different configurations were found: (a) Elliptical-circular shapes, occasionally appearing with an attached "tail." Some of these structures appear clear on their insides, others appear grayish, while still others contain both clear and gray zones. Measurements of longest axis of these shapes give a range from 50 to 350 nm. The width of the tails is much less variable, ranging from 20 to 30 nm. (b) Oblong shapes, primarily gray in color. The width of these structures, regardless of their length, varies from 20 to 30 nm. The similarity in size and gross appearance suggests that the oblong shapes and the tails are parts of the same structure. (c) Very thin, threadlike shapes, with poorly defined lateral boundaries.
FIGURE 10  Lobster muscle, sarcoplasmic reticulum fraction, incubated for ChE. Large, circular, electron-dense deposits are present outside the elliptical-circular profiles. $\times$ 27,000.

FIGURE 11  Same as Fig. 10 but higher magnification. $\times$ 54,000.

Their length is frequently up to, and even greater than, 1 $\mu$m. The width of these structures is estimated to vary between 0.005 and 0.015 nm.

Roughly spherical, electron-dense objects, measuring between 20 and 60 nm are frequently seen. Their relationship to the three general shapes previously described is that they are almost exclusively associated with the elliptical-circular...
profiles. Some of these dense objects apparently lie freely inside the elliptical-circular profiles. On other occasions, the objects appear to be in contact with the membrane of the profile. Two types of contact are seen. Most frequently, the object is on the inner surface of the membrane, but in a number of instances they appear to lie partly in and partly out of the profile. These objects have not been observed associated with either the tails, the oblong structures, or the thin threads.

Figs. 10 and 11 are electron micrographs taken of a sarcoplasmic reticulum preparation which had been incubated for ChE activity. Many circular, electron-dense figures are seen throughout the fields. The majority of these figures lie in contact with the outer surface of the elliptical-circular profiles.

Measurements of these dense figures give diameters ranging from 40 to 120 nm. These dense figures differ, therefore, in three main respects from the dense objects seen in preparations which had not been incubated for ChE: (a) the dense figures in the incubated preparations tend to be larger than the dense objects in the unincubated preparations; (b) there are many more dense figures in the incubated preparations; and (c) the dense figures in the incubated preparations are primarily associated with the outer surfaces of the vesicle's limiting membrane. The smaller dense objects noted in the unincubated preparations are usually inside the vesicles.

When $10^{-4}$ M eserine is included in the incubation solution, the large dense figures are not seen. Figs. 12 and 13 are examples of the complete inhibition achieved by this anti-ChE. Similar lack of figures was noted in preparations incubated without the substrate ATC, or if BTC was substituted. The large dense figures are, therefore, almost surely deposits of copper ferrocyanide accumulating as the end result of the reaction sequence initiated by the enzymatic hydrolysis of ATC. If the precipitates form and grow in the vicinity of the enzyme, then the enzyme is closely associated with regions of the outer surfaces of the membranes of the elliptical-circular profiles.

**Properties of the ChE**

The purpose of these experiments was to determine whether the crustacean muscle enzyme's characteristics were like those of a specific AChE (E.C.3.1.1.7) or like those of the nonspecific ChE (E.C.3.1.1.8). One way to make this distinction is by the use of inhibitors. The crustacean muscle enzyme is 100% inhibited by $10^{-4}$ M BW284C51, which has been shown by Austin and Berry (1953) and Copp (1953) to be a competitive, selective inhibitor of AChE. The crustacean muscle enzyme was not inhibited by $10^{-4}$ M quinidine, an effective inhibitor of nonspecific ChE (Wright and Sabine, 1948).

The apparent $K_m$ for the crustacean muscle enzyme is $3 \times 10^{-4}$ M. In the same buffer system, the $K_m$ of the AChE from red blood cell membranes was found to be $7 \times 10^{-5}$ M, and the $K_m$ in the lobster nerve cord was $5 \times 10^{-5}$ M. By these criteria, the crustacean muscle enzyme appears to be a typical AChE.

Although the histochemical studies showed no deposits when BTC was used as substrate, homogenates of crustacean muscle slowly hydrolyze BTC, at about 7% of the rate of ATC hydrolysis. This might be because of the presence of a small amount of nonspecific ChE in the fibers. However, the crustacean muscle enzyme does not show substrate inhibition (Fig. 14). This was an unexpected finding, since substrate inhibition of the crustacean muscle enzyme was reported by both Walop and Boot (1950) and Van der Kloot (1970). Therefore we repeated this experiment five times, with different preparations, and convinced ourselves that with the same reagents we could obtain clear substrate inhibition in preparations from crustacean nerve cord and red blood cell membranes (Fig. 15), but not with the muscle. We conclude that the crustacean muscle enzyme resembles AChE in its reaction to inhibitors, but is quite unlike the classical AChE's in that it does not show substrate inhibition. Until the enzyme is purified, we cannot say whether a single enzyme is responsible for hydrolyzing both ATC and BTC.

**DISCUSSION**

In crustacean muscle, ChE activity is associated with the sarcolemma, and its continuations into the fiber as sarcolemmal invaginations and the transverse tubular system. The findings of ChE in transverse tubules is similar to the results of Ulbrecht and Kruckenberg (1965) and Teräväinen (1969) for mammalian muscle. However, neither of these investigators found activity associated with the sarcolemma. This discrepancy may come from differences in technique. We incubated 50-µm frozen sections to facilitate diffusion of substrate (and other reactants).
to the enzyme. The connective tissue layer found around the muscle fibers may be a diffusion barrier. Neither of the investigators of mammalian muscle used frozen sections. Ulbrect and Krucken-berg used whole muscle fibers, while Teraväinen used "... pieces about 0.5 mm thick."

Nyberg-Hansen et al. (1969) attempted to circumvent the diffusion problem, while working with the whole mammalian muscle, by employing a noncharged analogue of ATC as substrate. This compound, 3,3-dimethylbuthylthioacetate (DMETA), contains a quaternary carbon instead.
of the quaternary nitrogen of the thiocholine moiety. Since the quaternary carbon is electrically neutral, DMBTA should penetrate biological membranes more easily than ATC. However, they found no deposits outside of the neuromuscular junction.

Table I summarizes a number of previous studies concerning ChE localization in muscle fibers. It is apparent that a number of discrepancies exist. It remains to be answered whether these discrepancies represent true species differences or instead reflect differences in technique.

We have been unable to find any similar studies concerning crustacean muscle before this investigation. Crustacean axons, however, have been investigated. de Lorenzo et al. (1969) studied single giant axons from walking-leg nerves of lobsters, minced into small blocks, and exposed to ATC. Activity was found near or on the surface of the axolemma, i.e., in the Schwann cell-axon inter-

| Investigator       | Muscle           | Substrate          | Technique                          | Deposit at                        |
|--------------------|------------------|--------------------|------------------------------------|-----------------------------------|
| Ulbrecht and       | Rabbit psoas     | ATC                | Whole muscle                       | T tubules                         |
| Kruckenberg        | (1965)           |                    |                                    |                                   |
| Teräväinen         | Rat extracocular | ATC                | Finely cut muscle                  | T tubules                         |
| (1969)             |                  |                    |                                    |                                   |
| Nyberg-Hansen      | Rat diaphragm    | DMBTA              | Whole muscle                       | Neuromuscular junction only       |
| et al. (1969)      |                  |                    |                                    | Neuromuscular junction; terminal  |
| Barnett (1962)     | Rat diaphragm    | Thiolacetic acid   | Whole muscle                       | axon membrane; synaptic vesicles   |
| Davis and Koelle   | Mouse intercostal| Gold-thiocholine;  | Frozen sections                    | Neuromuscular junction            |
| (1967)             |                  | gold-thiolacetic    |                                    |                                   |
| Miledi (1964)      | Frog sartorius   | ATC                | Whole muscle                       | T tubules; some longitudinal tubules; neuromuscular junction |
| Karnovsky (1964)   | Rat ventricle    | ATC                | Frozen sections                    | Longitudinal tubules; A band       |

Several points emerge from our histochemical studies concerning CrH localization in muscle fibers. The majority of these investigators conclude that the enzymatic activity in muscle and nerve is due to AChE. Our biochemical studies indicate, however, that while the crustacean muscle enzyme has many of the characteristics of AChE, one important feature, i.e., substrate inhibition, is missing. It is possible then, that we are dealing with an isozyme of the AChE that is present in the lobster ventral nerve cord.

Table 1
Summary of Previous Histochemical Studies

418 The Journal of Cell Biology · Volume 59, 1973
studies: (a) In crustacean muscle, ChE activity is present along membranes concerned with depolarization and the inward spread of depolarization. Thus, the membranes of the sarcolemma and the membranes forming the sarcomemmal invaginations and transverse tubules demonstrate this activity: (b) The presence of this enzyme at specific sites in crustacean muscle suggests that the activity in extracts is mostly from muscle and not from other tissue contaminating the preparation. The difference between the kinetic properties of the muscle enzyme and the nerve enzyme strengthen this conclusion. The unusual properties of the lobster muscle ChE should interest comparative enzymologists. (c) Since the enzyme apparently does not function in neuromuscular transmission in the muscles studied, it seems likely that the subcellular locations represent sites of use rather than sites of synthesis or product accumulation. (d) Since the histochemical studies show that the ChE-activity in crustacean muscle is limited to the sarcolemma and transverse tubules, more can be said concerning the enzyme activity in the sarcoplasmatic reticulum fraction. The vesicles in this preparation which demonstrate the activity must represent minced bits of the sarcolemma or its prolongations which have rescaled in the shape of elliptical-circular profiles. This enzyme, therefore, can serve as a marker in attempts to differentiate these membranes from other subcellular particles.

The physiological role of the enzyme remains an intriguing question. Since glutamate and γ-amino butyric acid, not ACh, are the usual transmitters in these animals, what function could the enzyme serve? Its presence along the excitable membranes of crustacean muscle supports Nachmansohn's (1959, 1966) thesis of the universal role of AChE in impulse conduction. There is, however, considerable evidence to the contrary (Podolsky and Costantin, 1966; Woddin and Wieneke, 1970; Hoskin, 1971). We point out also that the membranes responsible for impulse conduction are also the membranes which separate the intracellular from extracellular fluids. Therefore, the presence of an enzyme in these membranes is not unequivocal evidence for its role. Instead, the enzyme may serve a long-term metabolic role and not function in moment-to-moment phenomenon.

We are grateful to Dr. Chandler A. Stetson for his advise and assistance.

This work was supported in part by grant NB-04874 from the Public Health Service, and, in part, grant GM-00920 from the Institutes of General Medical Sciences of the National Institutes of Health.

Received for publication 11 April 1973, and in revised form 20 July 1973.

REFERENCES

AUSTIN, L., and W. K. BERRY. 1953. Two selective inhibitors of cholinesterase. Biochem. J. 54:695.

BARQ, Z. M. 1936. Demonstrations sur l'acetylcholine et la cholines esterase chez les Invertebres. Ann. Physiol. Physiochim. Biol. 12:661.

BARQ, Z. M. 1937. Cholinergic nerves in invertebrates. Proc. R. Soc. Lond. B Biol. Sci. 123:418.

BARQ, Z. M., and D. NACHMANSOHN. 1937. Cholinesterase in invertebrate muscle. J. Physiol. (Lond.). 89:368.

BARNETT, R. J. 1962. The fine structural localization of acetylcholinesterase at the myoneural junction. J. Cell Biol. 12:247.

BRANDT, F. W., J. P. REUBEN, L. GRADIER, and H. GRUNDFEST. 1963. Correlated morphological and physiological studies on isolated single muscle fibers. I. Fine structure of the crayfish muscle fiber. J. Cell Biol. 25:233.

BRZIN, M. 1966. The localization of acetylcholinesterase in axonal membranes of frog nerve fibers. Proc. Natl. Acad. Sci. U. S. A. 56:1560.

COPP, F. C. 1953. Diacid bases. I. Compounds related to 1:5-diphenyl-pentane-pp'-bia (trialkylammonium) salts as anticholinesterases. J. Chem. Soc. (Lond.). 3:3116.

DAVIS, R., and G. B. KOELLE. 1967. Electron microscopic localization of acetylcholinesterase and nonspecific cholinesterase at the neuromuscular junction by the gold-thiocholine and gold-thiolacetic acid methods. J. Cell Biol. 34:157.

DE LORENZO, A. J. D., W. D. DETTBARN, and M. BRZIN. 1969. Fine structural localization of acetylcholinesterase in single axons. J. Ultrasttruct. Res. 28:27.

ELLIS, C. H., C. H. THIENES, and C. A. C. WIERSMA. 1942. The influence of certain drugs on the crustacean nerve-muscle system. Biol. Bull. (Woods Hole). 83:334.

ELLMAN, G. L., K. D. COURTNEY, V. ANDRES, JR., and R. M. FEATHERSTONE. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88.

FUTAMACHI, K. J. 1972. Acetylcholine: Possible neuromuscular transmitter in crustacea. Science (Wash. D.C.). 175:1373.

HOSKIN, F. C. G. 1971. Diisopropylphosphofluoridate and tabun: Enzymatic hydrolysis and nerve function. Science (Wash. D.C.). 172:1243.
Karnovsky, M. 1964. The localization of cholinesterase activity in rat cardiac muscle by electron microscopy. J. Cell Biol. 23:217.

Katz, B. 1936. Neuromuscular transmission in crabs. J. Physiol. (Lond.). 87:199.

Matsura, H., and K. Fujita. 1968. The ultrastructural localization of acetylcholinesterase in the trigeminal ganglion of the rat. Histochem. J. 1:176.

Maynard, E. A., and D. M. Maynard. 1960. Cholinesterase in the crustacean muscle receptor organ. J. Histochem. Cytochem. 8:376.

Miledi, R. 1964. Electron-microscopical localization of products from histochemical reactions used to detect cholinesterase in muscle. Nature (Lond.). 204:293.

Nachmansohn, D. 1959. Chemical and Molecular Basis of Nerve Activity. Academic Press, Inc., New York.

Nachmansohn, D. 1966. Chemical control of the permeability cycle in excitable membranes during electrical activity. Ann. N. Y. Acad. Sci. 137:377.

Nyberg-Hansen, R., E. Kinvik, P. Aarskog, and J. A. B. Barstad. 1969. Electron microscopic localization of cholinesterase at the neuromuscular junction by a quaternary carbon analogue of acetylthiocholine as substrate. Histochem. 20:40.

Orentlicher, M., and J. Reuben. 1971. Localization of ionic conductances in crayfish muscle fibers. J. Membrane Biol. 4:209.

Podolsky, R. J., and L. L. Costantin. 1966. The internal membrane system and muscle activation. Ann. N. Y. Acad. Sci. 137:1039.

Selverston, A. 1967. Structure and function of the transverse tubular system in crustacean muscle fibers. Am. Zool. 7:515.

Spielholz, N. I., and W. G. Van der Kloot. 1972. Histochemical localization of cholinesterase in crustacean muscle. Fed. Proc. 31(2):350.

Takeuchi, A., and N. Takeuchi. 1964. The effect on crayfish muscle of iontophoretically applied glutamate. J. Physiol. 170:296.

Teravainen, H. 1969. Distribution of acetylcholinesterase in extraocular muscle fibers of the rat. Histochem. 18:174.

Ulbrecht, G., and P. Kruckenber. 1965. Acetylcholinesterase in the sarcoplasmic reticulum of skeletal muscle. Nature (Lond.). 206:305.

Van der Kloot, W. G. 1966. Inhibitors of active Ca2+ uptake by fragments of sarcoplasmic reticulum of lobster muscle. Comp. Biochem. Physiol. 17:76.

Van der Kloot, W. G. 1970. The effects of potentiators and anticholinesterases on the kinetics of calcium uptake by isolated sarcoplasmic reticulum from the lobster and the rat. Comp. Gen. Pharmacol. 1:209.

Van Harreveld, A., and M. Mendelson. 1959. Glutamate-induced contractions in crustacean muscle. J. Cell. Comp. Physiol. 54:35.

Walop, J. N., and L. M. Boot. 1950. Studies on cholinesterase in Carcinus maenas. Biochim. Biophys. Acta. 4:566.

Woodin, A. M., and A. A. Wieneke. 1970. Action of DFP on the leucocyte and the axon. Nature (Lond.). 227:460.

Wright, C. I., and J. C. Sabine. 1948. Cholinesterase of human erythrocytes and plasma and their inhibition by antimalarial drugs. J. Pharmacol. Exp. Ther. 93:230.