CHOLINESTERASE ACTIVITY PER UNIT SURFACE AREA OF CONDUCTING MEMBRANES

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ABSTRACT

According to theory, the action of acetylcholine (ACh) and ACh-esterase is essential for the permeability changes of excitable membranes during activity. It is, therefore, pertinent to know the activity of ACh-esterase per unit axonal surface area instead of per gram nerve, as it has been measured in the past. Such information has now been obtained with the newly developed microgasometric technique using a magnetic diver. (1) The cholinesterase (Ch-esterase) activity per mm^2 surface of sensory axons of the walking leg of lobster is 1.2×10^{-3} μM/hr. ($\sigma = \pm 0.3 \times 10^{-3}$; SE $= 0.17 \times 10^{-3}$); the corresponding value for the motor axons is slightly higher: 1.93×10^{-3} μM/hr. ($\sigma = \pm 0.41 \times 10^{-3}$; SE $= \pm 0.14 \times 10^{-3}$). Referred to gram nerve, the Ch-esterase activity of the sensory axons is much higher than that of the motor axons: 741 μM/hr. ($\sigma = \pm 73.5$; SE $= \pm 32.6$) *versus* 111.6 μM/hr. ($\sigma = \pm 28.3$; SE $= \pm 10$). (2) The enzyme activity in the small fibers of the stellar nerve of squid is 3.2×10^{-4} $\mu M/mm^2$/hr. ($\sigma = \pm 0.96 \times 10^{-4}$; SE $= \pm 0.4 \times 10^{-4}$). (3) The Ch-esterase activity per mm^2 surface of squid giant axon is 9.5×10^{-5} μM/hr. ($\sigma = \pm 1.55 \times 10^{-5}$; SE $= \pm 0.38 \times 10^{-5}$). The value was obtained with small pieces of carefully cleaned axons after removal of the axoplasm and exposure to sonic disintegration. Without the latter treatment the figure was 3.85×10^{-5} $\mu M/mm^2$/hr. ($\sigma = \pm 3.24 \times 10^{-5}$; SE $= \pm 0.93 \times 10^{-5}$). The experiments indicate the existence of permeability barriers in the cell wall surrounding part of the enzyme, since the substrate cannot reach all the enzyme even when small fragments of the cell wall are used without disintegration. (4) On the basis of the data obtained, some tentative approximations are made of the ratio of ACh released to Na ions entering the squid giant axon per cm^2 per impulse.

INTRODUCTION

It has been proposed that during activity of excitable membranes the action of acetylcholine (ACh) on a receptor triggers the chain of events which change the ionic permeability (17, 18, 19). Acetylcholinesterase (ACh-esterase) removes ACh, thereby restoring the barrier to the flow of ions. One of the necessary prerequisites for this theory is the presence of an adequate concentration of ACh-esterase in excitable membranes. It was established nearly thirty years ago that relatively high concentrations of the enzyme are indeed present in a great variety of different types of fibers throughout the animal kingdom (17). Most of the fibers are capable of hydrolyzing 30 to 300 μmoles of ACh per gram nerve (fresh weight) per hour, although some have markedly higher or lower activities. However, it was apparent from the onset of the investigations that the enzyme is

not evenly distributed in the cell (3, 20), and indirect evidence indicated a strong concentration in the region of the excitable membrane. More direct evidence for the localization of the enzyme at or near the membrane was recently obtained by a combination of electron microscopy with staining techniques (2) or by separating cell membranes by differential centrifugation (1, 12, 26). Using discontinuous density gradients, Karlin (12) separated subcellular structures of the electroplax of *Electrophorus electricus* and found in the membrane-rich fraction a much greater specific activity of cholinesterase (Ch-esterase). The evidence that the action of ACh is inseparably associated with electrical activity further supports the assumption of a localization of most of the enzyme at or near the cell wall.

It is apparent that referring the activity of Ch-esterase to gram nerve (or other excitable tissue) does not adequately express the actual enzyme concentration. It is pertinent to obtain a quantitative evaluation of Ch-esterase activity per unit surface area of conducting fibers rather than per gram nerve.

Axons from the squid stellar nerve and the walking leg of lobster were used in this study, since the electrical characteristics, concentrations of Ch-esterase, and effects of compounds acting on the ACh system have been extensively studied in these preparations (6–8, 11, 21, 22). In addition, the nerve fibers of the walking leg of lobster are of special interest since their axons are sensitive to ACh. The ester depolarizes and blocks electrical activity. The concentration of Ch-esterase in homogenized nerve is very high; about 900 to 1000 μmoles of ACh are split per gram wet weight fiber per hour.

The development of ultramicro methods for measuring the enzyme activity of extremely small samples of tissue permits working with cellular units or even subcellular structures. In this communication we present data obtained with the magnetic diver gasometric technique developed by Brzin *et al.* (4, 5), which allows us to estimate Ch-esterase activity of axons per unit surface area rather than per gram tissue.

MATERIALS AND METHODS

The Measurement of Enzyme Activity

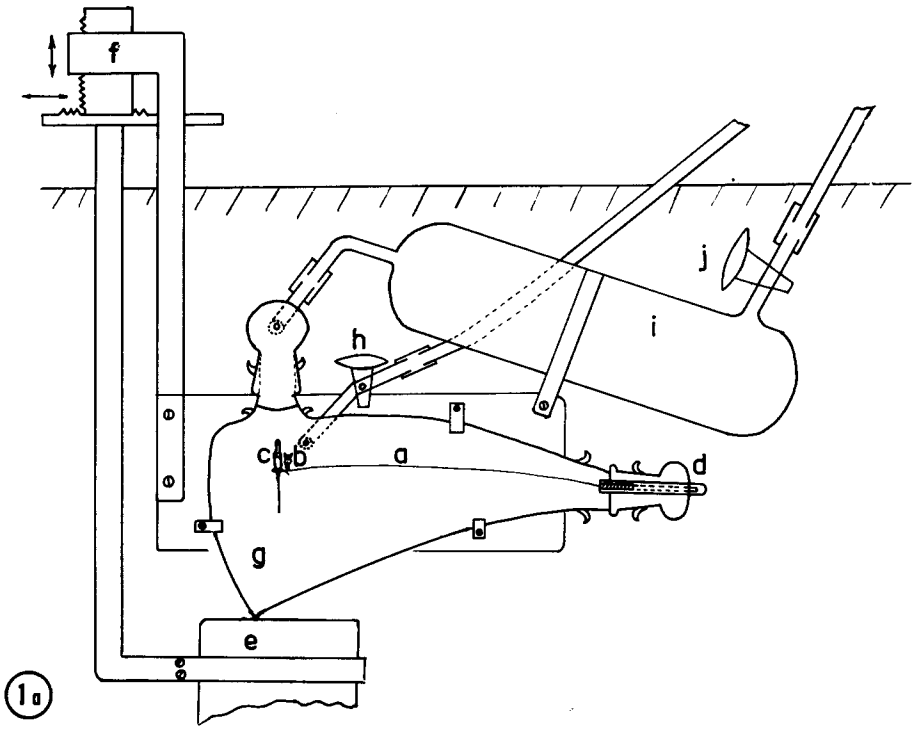
The hydrolysis of ACh was measured microgasometrically using the recently developed magnetic diver technique (4, 5). The shape of the gasometric chamber as well as the procedure for filling the ampulla with gas mixture, sample, and substrate are similar to those in the Cartesian ampulla diver method for measurement of cholinesterase activity (27). The buoyancy of the Cartesian diver is controlled by keeping the volume of the diver's gas charge constant, using a manometer to change the pressure during the experiment. The magnetic diver microgasometer is operated at a constant pressure so that the gas can expand or contract freely, depending on the type of reaction in the ampulla. The resulting changes in the buoyancy of the diver are compensated by changing the strength of the magnetic field acting on a small permanent magnet attached to the diver.

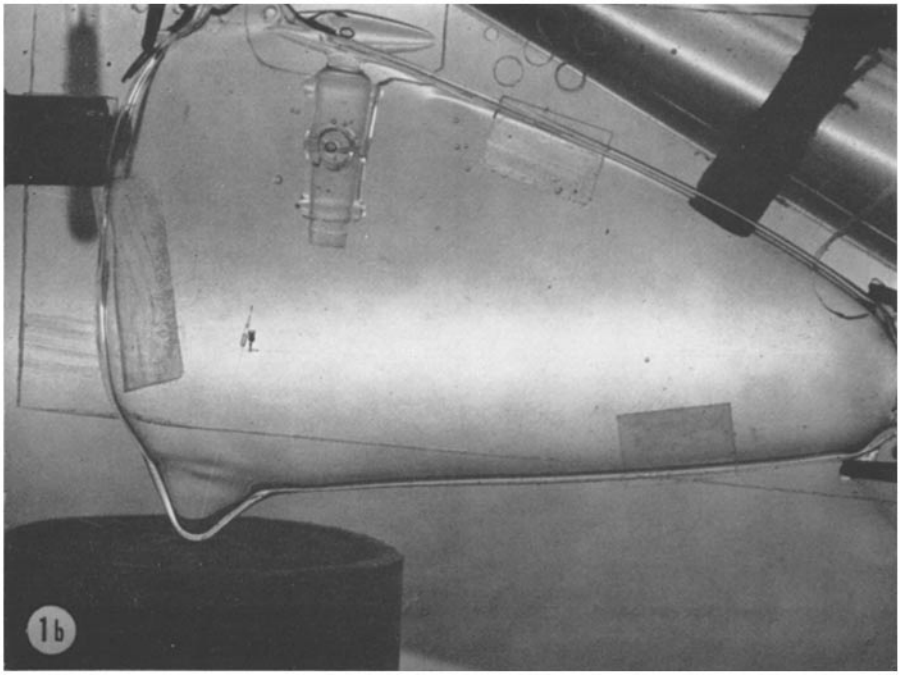
As shown in Fig. 1 *a* and *b*, the equipment in its present shape (Brzin, in preparation) consists of an ampulla-shaped gasometric chamber (*c*) and a thin glass fiber (*a*) with a loop to which a small polyethylene-coated magnet is sealed (*b*). Within the polyethylene coating is enclosed an air bubble, to carry the weight of the magnet. The opposite end of the glass fiber is sealed into a hollow glass stopper (*d*) inserted in the end of a flat, elongated flotation vessel (*g*). By changing the thickness of the glass fiber and the size of the magnetic float the sensitivity of the instrument can be regulated according to expected enzyme activity of the various samples. Below the flotation vessel a coil of copper wire (*e*) protected against water is mounted and connected to a direct current source, a resistor, and a milliamperemeter.

The charged and sealed ampulla is transferred to the flotation vessel by means of glass tubing, as shown in Fig. 2, steps I and II. When the pressure in the tubing is released by removing the fingertip from the opening of the glass tubing, the flotation fluid enters the tubing, and the ampulla, which adheres to the surface of the fluid and the wet inside wall, is dropped slowly into the loop of the glass fiber (Fig. 2, step III; Fig. 3). To prevent the ampulla from following the rising fluid surface, a plug of sintered glass is sealed into the lower part of the tubing.

After the instrument is loaded the system is saturated with the gas mixture (Fig. 1 *a*, stopcock *h*) and closed (stopcock *j*). The position of the diver is determined by means of a horizontally mounted microscope. Readings of the current, by which the diver is kept in its initial position, are performed at appropriate intervals depending on the activity of the sample. The instrument is calibrated at the end of each measurement, using a water manometer as described elsewhere (4).

Once mounted, the flotation vessel with the fiber and the magnetic float can be used indefinitely. However, the ampulla is usually employed for only one experiment and is discarded after calibration.





FIGURES 1 *a* AND *b* Magnetic diver set-up. Explanation in text.

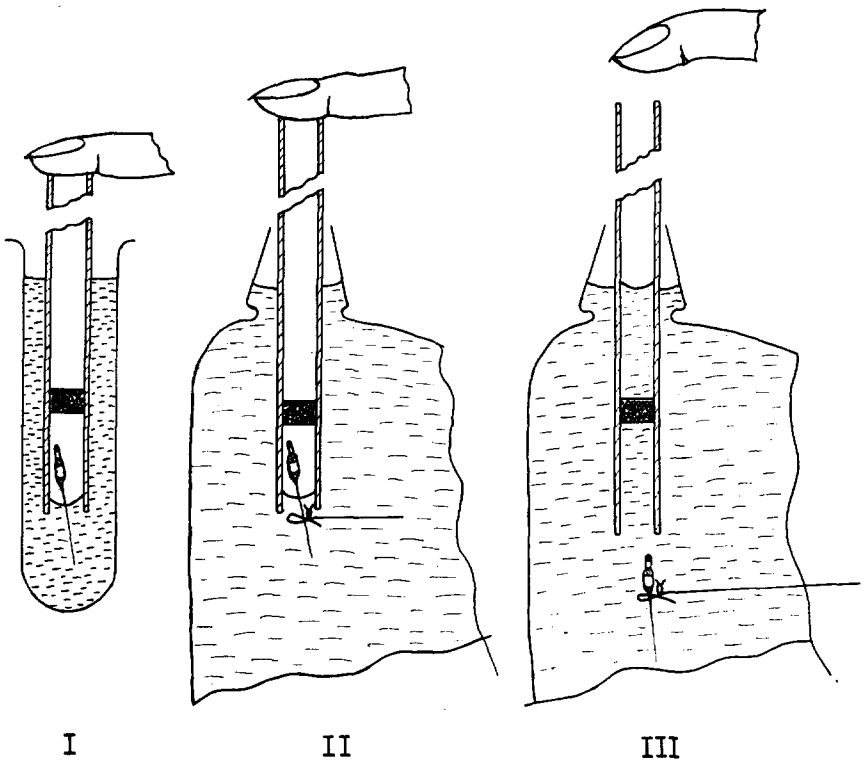


FIGURE 2 Loading of the magnetic diver with the ampulla. Explanation in text.

Biological Materials

All nerves were dissected in the usual way, and samples were taken for diver experiments. ACh bromide (5×10^{-3} M) was used as substrate in all experiments.

The squid giant axon was free of all small nerve fibers. Sections of the cleaned axon of various lengths were dissected and the axoplasm was extruded with a thin, flexible glass rod. The inner side of the axon was washed several times in sea water by alternatingly increasing and decreasing the pressure on the axon cylinder.

Ch-esterase was measured either in intact fragments of axonal cylinder (Table IV) or in homogenized samples (Table V). The homogenization was performed in two steps: First, cleaned and extruded axons were ground in a small glass homogenizer (~50 μl); then, the sample was additionally sonicated for 2 to 3 minutes in order to obtain a stable suspension of the tissue.

In order to check for the presence of contaminating small fibers adhering to the giant axon, the cleaned non-homogenized samples of giant axon were tested histochemically for Ch-esterase by Koelle's procedure (14) at the end of each experiment. Fig. 7 shows that even a few adhering small nerve fibers can be readily detected with this technique. In 12 out of the 14 experiments there were no small nerve fibers adhering, whereas in the remaining 2 experiments there were very small pieces of small fibers on the giant axon surface. The contribution to Ch-esterase activity of this contamination was very small; nevertheless the measurements from these 2 experiments were discarded and not included in the data of Table IV. We are, therefore, justified in assuming that the measured Ch-esterase activity originates from the axon cylinder itself. The axon membrane, as used in our experiments, is, however, still surrounded by other structures (Schwann cell, capillaries, connective tissue). It is not known whether these structures exhibit esterase activity and contribute to the values measured.

In those experiments in which homogenized axons were used, no histochemical check could be made after enzyme determination. The axons were, however, cleaned with particular care under a dissecting microscope before homogenization, and a number of them were not used for diver measurements but for histochemical check for small fibers. None of them was found to have been contaminated with small fibers. With some experience it is possible to dissect axons entirely free of small fibers.

Determination of the Surface Area and the Weight of the Samples

The linear dimensions of *non-homogenized* samples of the squid giant axon were measured in a blood cell counting chamber. The values were then employed for the calculation of surface area and fresh weight. The weight and the surface area of other *non-homogenized* nerve fibers in this work were calculated from the linear dimensions obtained by means of a calibrated micrometer eyepiece.

After each determination of Ch-esterase in *homogenized* samples of squid giant axon, the ampullae were cut open and the samples were blotted onto a preweighed piece of filter paper. The samples and filter paper were then dried and the dry weight was determined using a Cahn Electrobalance. It was then necessary to subtract from this weight the weight of the solutes (salts) in the substrate bicarbonate soluiton used in the ampulla. Since the concentration of these salts was known, it was only necessary to determine the volume of the ampulla. The volume was calculated from the weight of the ampulla before and after blotting of the sample.

Separate experiments were performed to obtain the relations between dry weights and linear dimensions. In three experiments, for each of two cleaned axons of the same diameter as those used for Ch-esterase determinations, the linear dimensions were measured. After drying, the ratio between dry weight and surface area was calculated and used for converting the measured dry weights of homogenized samples into the surface area and fresh weight. The direct measurement of fresh weight was inaccurate and

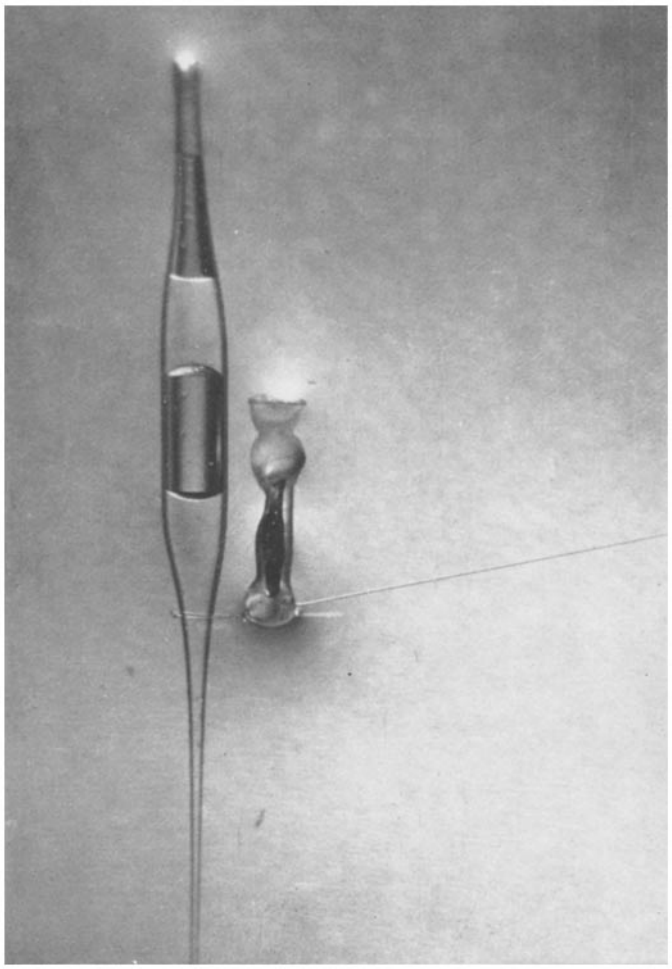


FIGURE 3 Ampulla sits in the loop on the end of the glass fiber. Polyethylene-coated magnetic float on the right.

TABLE I

Ch-Esterase in Sensory Fibers of Walking Leg of Lobster

The actual activity of enzyme observed in the diver per sample of tissue and expressed in μmoles of ACh hydrolyzed per hour is given in column 2. In column 3 the data are referred to gram nerve (fresh weight) and in column 4 to mm^2 surface area, both estimated from the linear dimensions of the sample.

Wet weight μg	μM × 10^{-3}/sample/hr.	μM/gm/hr.	μM × 10^{-3}/mm^2/hr.
1.58	1.22	772	1.3
0.95	0.61	620	0.8
1.5	1.08	720	1.2
0.96	0.758	790	1.3
0.44	0.758	805	1.6
		Mean 741	1.2
		σ = ±73.5	σ = ±0.3
		SE = ±32.6	SE = ±0.17

TABLE II

Ch-Esterase in Motor Axons of Walking Leg of Lobster

Enzyme activity given in columns 2 to 4 is expressed in the same way as in Table I.

Wet weight μg	μM × 10^{-3}/sample/hr.	μM/gm/hr.	μM × 10^{-3}/mm^2/hr.
33.0	2.02	61.2	1.14
16.4	1.53	93.2	1.93
12.5	1.55	124.5	1.78
12.4	1.45	109.2	1.88
13.2	1.30	98.4	1.90
8.1	1.16	142.8	2.36
9.2	1.26	137.3	2.05
13.9	1.76	26.5	2.39
		Mean 111.6	1.93
		σ = ±28.3	σ = ±0.41
		SE = ±10	SE = ±0.14

could not be done because of the very small dimensions of the samples, which dried very fast. After a few unsuccessful attempts this procedure was abandoned.

RESULTS

Lobster Axons

Table I summarizes the results obtained with sensory axons; Table II, those with motor axons. Although the samples have not been homogenized, the activity per gram per hour is close to that obtained previously with homogenized tissue using different techniques. The enzyme concentration of the sensory fibers is several times as high as that of the motor fibers when referred to gram nerve. In contrast, when referred to unit surface area the motor axons have slightly higher activity than the sensory axons.

Figs. 4 and 5 show the staining of the motor axons using acetylthiocholine as the substrate according to Koelle's method (14).

Squid Stellar Nerve

In experiments using the Cartesian diver technique, cholinesterase activity in the axoplasm was found to be much less than that in the cell wall. In the earlier observations in which Ch-esterase was tested, the small fibers were not removed (16). However, if it is true that the enzyme is mainly located in the cell wall, as suggested by indirect as well as by direct evidence, then the small fibers may be expected to contribute significantly to the enzyme activity, since there are very many small fibers in the stellar nerve and their total surface area is considerably greater than that of the giant axon.

As may be seen from the data of Tables III and IV, the enzyme activity per gram is about 75-fold higher in the small fibers than in intact fragments of the giant axon.

Figs. 6 and 7 show pieces of cleaned and partially cleaned giant axons, treated histochemically for Ch-esterase. Heavily stained small fibers adhering to the axonal surface are clearly visible. It appeared possible, however, on the basis of the previous experiments and some considerations to be discussed later, that even in these small fragments of the cleaned giant axon the enzyme was not saturated with substrate, or part of the enzyme could not be reached at all. Therefore, the samples were treated with an ultrasonic disintegrator. As may be seen from the data presented in Table V, the enzyme activity in the homogenized suspension was more than twice as high as that in the small fragments (Table IV), suggesting that the enzyme in the non-homogenized membrane might still be surrounded by permeability barriers. Referred to gram nerve, the activity in the small fibers (Table III) is about 40 times as high as in the homogenized suspension of the cell wall of the giant axons (Table V). However, per unit surface area the

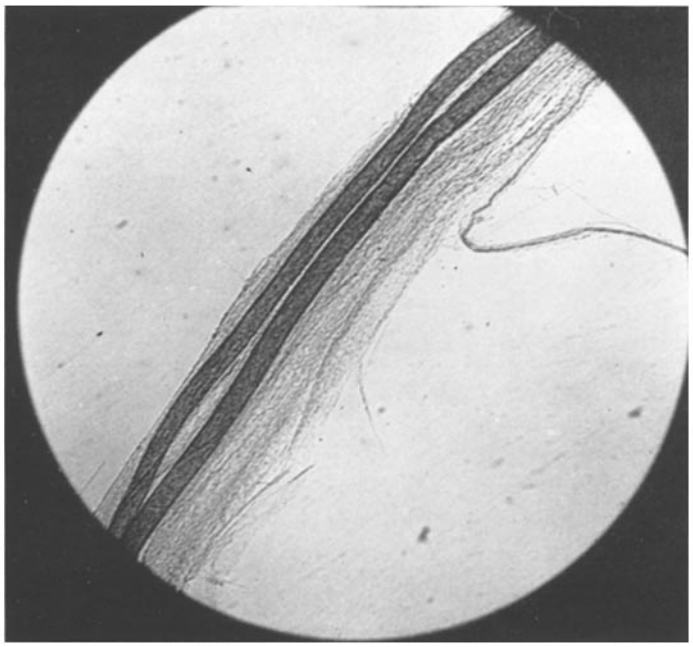


FIGURE 4 Motor axons, isolated from the nerve bundle of the walking leg of lobster. Thiocholine staining. Incubation 15 minutes. × 50.

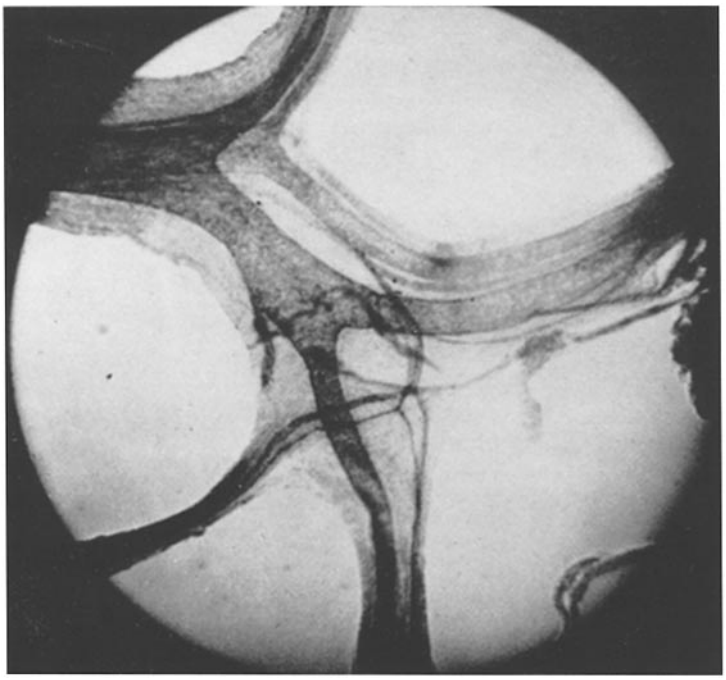


FIGURE 5 Branching of lobster motor axons before entering into the muscle. Walking leg of lobster. Thiocholine staining. Incubation 15 minutes. × 100.

TABLE III

Ch-Esterase in Small Fibers of Stellar Nerve of Squid

Enzyme activity given in colums 2 to 4 is expressed in the same way as in Table I.

Wet weight μg	μM × 10^{-4}/sample/hr.	μM/gm/hr.	μM × 10^{-4}/mm^2/hr.
12.0	7.92	66	3.4
3.0	3.57	89	4.5
8.0	7.36	92	2.8
5.0	4.15	83	4.0
10.0	6.60	66	2.3
7.0	4.97	71	2.1
		Mean 78	3.2
		σ = ±11.7	σ = ±0.96
		SE = ± 4.8	SE = ±0.41

TABLE IV

Ch-Esterase in Cleaned Fragments of Giant Axon of Squid

Enzyme activity given in columns 1 to 3 is expressed in the same way as in Table I, columns 2 to 4.

μM × 10^{-5}/sample/hr.	μM/gm/hr.	μM × 10^{-5}/mm^2/hr.
3.6	0.20	0.90
3.1	0.25	1.00
13.5	2.90	11.00
5.3	0.30	1.09
20.0	2.10	7.54
26.0	1.80	6.60
14.5	0.25	1.05
12.0	1.10	3.86
18.3	1.30	4.70
13.8	0.85	2.13
21.1	1.20	5.28
18.3	0.40	1.24
	Mean 1.05	3.85
	σ = ±0.884	σ = ±3.24
	SE = ±0.31	SE = ±0.93

small fibers have about 3 times the activity of the homogenized samples.

DISCUSSION

For reasons outlined in the Introduction it appeared desirable to obtain data referring the cholinesterase enzyme activity to unit surface area rather than to weight. Previous observations indicate that the type of esterase present in the invertebrate fibers used is essentially an ACh-esterase (see, for instance, Dettbarn, 6). However, there is no evidence excluding entirely the presence of some additional esterase of another type, although this fraction must be small. The esterase activity tested is referred to as cholinesterase, since in any event cholinesters are far more readily split at the concentration used than are other esters.

The results with lobster axons (Tables I and II) illustrate the advantage of this approach: when referred to weight there seems to be a much higher activity in sensory than in motor fibers, but when referred to surface area the enzyme activity is slightly higher in the motor axons. Also, it seems significant that on a weight basis the activity in the small fibers is about 70 times as high as in the non-homogenized giant axon, whereas when referred to surface area it is only about 10 times as high (Tables III and IV).

Even when referred to surface area it is to be expected that the enzyme concentrations will vary greatly in different types of axons. In view of the marked structural differences, such as axon diameters, ratio of the thickness of the cell wall to the total diameter, absence or presence of Ranvier nodes, etc., and in view of the great variation of physical parameters—for example, conduction velocities may vary over a range from 0.1 to 100 m/sec.—it is not surprising that the enzyme concentration varies.

The enzyme activity in the axoplasm of the squid giant axon (Table VI) is rather low as compared with that in the cell wall. In both cases there may be present some esterase other than Ch-esterase, and its amount may vary in the two types of tissue.

It must be stressed that even values obtained with highly refined methods and dissection techniques do not give us the exact concentration at the level of the excitable membrane, which is, according to the latest data of Sjöstrand (25), about 90 to 100 A thick. The samples used contained structures surrounding the membrane which are many times as thick as the membrane proper. It is probable that these structures contain Ch-esterase.

Of particular interest are the results obtained when the enzyme concentration is measured in small fragments of the cell wall before and after homogenization. In the very early observations, made in 1937, it was found that homogenization increased the enzyme activity 4 to 5 times (16).

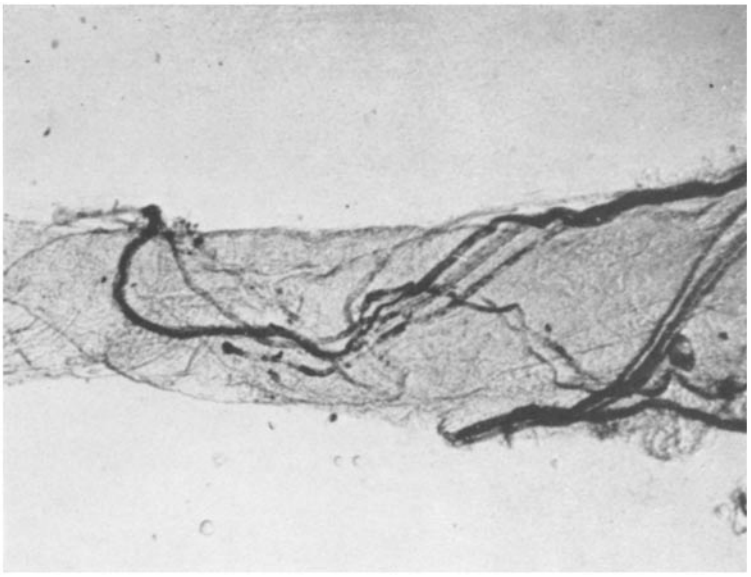


FIGURE 6 Giant axon of squid plus adhering small nerve fibers. Thiocholine staining. During 20 minutes of incubation the giant axon membrane stains only lightly, whereas the small nerve fibers show a dense deposit of precipitate. × 30.

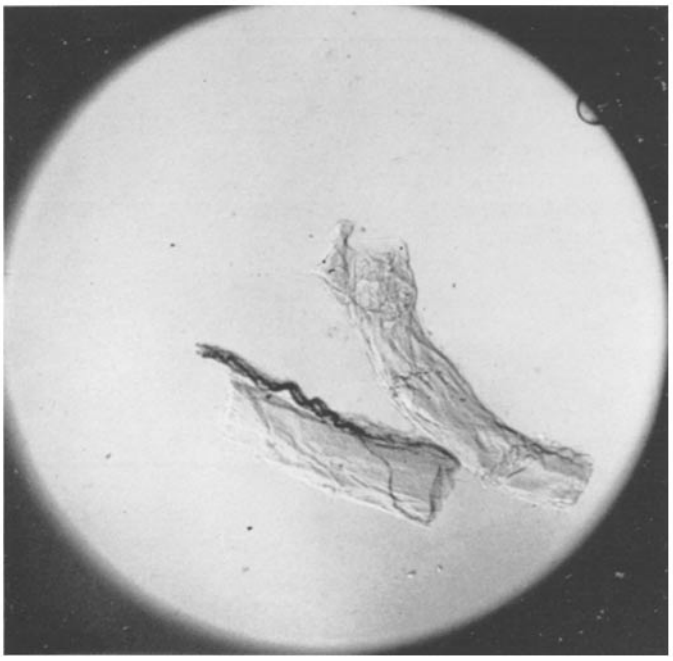


FIGURE 7 Comparison of a carefully cleaned giant axon of squid and one containing a few adhering small nerve fibers. × 12.

TABLE V

Ch-Esterase in Homogenized Giant Axon of Squid

Sample no.	Wet wt μg	Surface area mm²	Ch-esterase activity: Per sample × 10^{-5} μM/hr.	Ch-esterase activity: Per gm μM/hr.	Ch-esterase activity: Per mm² × 10^{-5} μM/hr.
1	151.8	3.3	49.1	3.2	14.2
	138.0	3.0	30.3	2.2	9.9
	161.0	3.5	37.6	2.3	10.7
2	151.8	3.3	27.1	1.8	8.2
	220.8	4.8	43.1	1.9	8.9
	225.4	4.9	46.8	2.1	9.5
3	266.8	5.8	48.3	1.8	8.4
	234.6	5.1	47.0	2.0	9.3
	248.4	5.4	43.6	1.7	8.0
4	188.6	4.1	34.0	1.8	8.3
	165.6	3.6	31.4	1.9	8.7
	174.8	3.8	44.5	2.5	11.7
	239.2	5.2	41.4	1.7	7.8
5	138.0	3.0	28.4	2.1	9.6
	124.2	2.7	24.5	2.0	9.2
	156.4	3.4	35.6	2.3	10.4
	142.6	3.1	31.2	2.2	10.1
				Mean 2.1	9.5
				$\sigma = \pm 0.36$	$\sigma = \pm 1.55$
				SE = ± 0.09	SE = ± 0.38

This is, of course, not an actual but an apparent increase, due to the fact that the substrate either is unable to reach the enzyme protected by barriers or reaches it at an inadequate rate. In the early experiments the Warburg technique was used and the tissue fragments were much bigger than in the present experiments. But even the present studies show that only 40 to 50 per cent of the enzyme activity is measured before complete homogenization. The present experiments suggest that the free access of ACh to the enzyme, even in these small fragments of the cell wall, is limited unless there is complete disintegration of structure. And if ACh penetrates slowly, it is reasonable to assume that it also leaks slowly to the outside when released within the membrane. Recently it was found, using a bioassay technique for ACh, that in the squid giant axon membrane ACh cannot diffuse out to the surrounding media, in contrast to the lobster axons, where ACh is capable of moving in both directions (9).

According to theory, ACh acts as a trigger: it initiates a series of reactions which increase the ionic permeability during electrical activity, and thus permits the movements of Na and K ions in the direction of the concentration gradient. This process differs basically from the extrusion (or uptake) of ions *against* the concentration gradients, a process requiring energy provided probably by ATP hydrolysis and not specific for conducting cells.

TABLE VI

Ch-Esterase in Axoplasm of Giant Axon of Squid

Activity is expressed in μmoles of ACh hydrolyzed per gram (fresh weight) per hour.

Wet weight mg	μM/gm/hr.
0.62	0.8
0.81	0.3
0.66	1.0
0.70	0.6
1.2	0.1
	Mean 0.56

The present data raise another interesting question. The amount of Na ions per cm^2 per impulse entering the squid giant axon during electrical activity is about 4×10^{-12} mole, as was first determined in 1950 with radioactive Na ions by Rothenberg (23, 24) and later confirmed by Keynes and Lewis (13). It would be interesting to estimate how many Na ions enter per molecule of ACh metabolized. Such an estimate requires, however, not only the knowledge of how many molecules of ACh may be hydrolyzed per cm^2 of axonal surface, but two other values which at present are not available. One is the period of time during which the ACh released by one impulse may be removed. It is, however, reasonable to assume that this period is about 1 to 2 msec. This uncertainty would not change the estimate significantly. More uncertain is the second value: the amount of enzyme present in excess. It is almost certain that the enzyme will be present in concentrations several times that required for conduction. Other factors unknown at present are the actual substrate concentration at the active site of the enzyme, and the K_m between ACh and enzyme in the organized structure. However, as recent observations of Katchalski and his associates on insoluble enzymes (10, 15) have shown, K_m's may decrease 50 to 100 times even in artificial and relatively simple systems. It appears reasonable to assume that in an organized particle of a subcellular structure the enzyme acts at a speed close to its maximum at a much lower concentration of substrate than it does in solution.

For obtaining the ratio of ACh metabolized to Na^+ entering, we shall assume that the total time available for removing the ACh released is of the same order of magnitude as the action potential, *i.e.*, 1 to 2 msec. After that period a new impulse may be conducted. Let us assume, then, that the amount of ACh released per impulse approximately equals the amount which may be hydrolyzed during 1 msec. at the maximum speed measured *in vitro*, and that there is an excess of enzyme 3 to 5 times above the minimum required for unimpaired conduction.

On the basis of these assumptions and of the Ch-esterase activity of squid giant axons measured, the following values can be calculated: 9.5×10^{-5} μmole of ACh may be hydrolyzed per cm^2 surface area per hour. This amounts to 2.5×10^{-15} mole/cm^2/msec. Assuming the amount released to be approximately that hydrolyzed in 1 msec., the ratio of Na ions entering per cm^2 per impulse (4×10^{-12} mole) to ACh released would be 1600. If we assume an excess of Ch-esterase of 3 to 5 times, the ratio would be 5000 to 8000. Thus, the action of about 3 to 5×10^9 molecules of ACh would be the trigger required per cm^2 per impulse to initiate the chain of events which permits the entrance of 2.4×10^{12} Na ions. Admittedly, there are several gaps in our knowledge, and several assumptions had to be made to obtain this figure, but even if the estimate is off by an order of magnitude, the ratio still would appear reasonable.

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