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HYDROLYSIS OF CHOLINE ESTERS IN INVERTEBRATE NERVE FIBERS

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SUMMARY

The hydrolysis of acetylcholine, *dl*-acetyl-β-methylcholine, butyrylcholine and triacetin by various homogenized invertebrate nerve tissues has been compared. The invertebrate species used were: lobster, spider crab and squid. The enzyme of the different invertebrate nerves shows the characteristics of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) as indicated by the substrate concentration–activity relationship. The enzyme is inhibited by physostigmine. Hydrolysis of acetylcholine and acetyl-β-methylcholine by axoplasm of the giant axon of squid is only 20% and 10% of that of the envelope.

INTRODUCTION

Studies of the activity–substrate concentration relationships have served to distinguish the different types of ACh-hydrolyzing enzymes. The ACh-esterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) is inhibited by excess of substrate and shows a well defined optimum substrate concentration[1,2]. Another criterion is the relative substrate specificity[3]. ACh-esterase hydrolyzes MeCh. Although this substrate is hydrolyzed at a lower rate than ACh, it is considered to have a high degree of specificity for ACh-esterase[4,5]. BuCh is split at a low rate, the V_{max} is about 150 times lower than that for ACh. In contrast, other choline ester-splitting enzymes, such as *e.g.* serum esterase, are not inhibited by high concentrations of ACh and show less activity at low concentrations. Serum esterase splits BuCh but not MeCh[6]. In the present study both substrate concentration–activity relationships and substrate specificity have been tested to characterize the choline ester-splitting enzymes in the nerves of lobster, and spider crab, and in the giant axon of the squid to determine to what extent the properties of choline ester-splitting enzymes of three so closely related unmyelinated invertebrate nerve fibers can be compared.

METHODS

The walking leg nerve of lobster (*Homarus americanus*) and spider crab (*Libinia emarginata*) and the giant axon of squid* (*Loligo pealii*) were used. The nerves were

Abbreviations: ACh, acetylcholine; MeCh, acetyl-β-methylcholine; BuCh, butyrylcholine; Tr, triacetin.

* The giant axons used were not well cleaned and contained many small fibers of the Stellar nerve.

homogenized since the rates of hydrolysis are lower in intact than in homogenized nerves, presumably due to permeability barriers[7]. Substrates tested were: ACh, MeCh, BuCh, and Tr. The substrates were dissolved in Woods Hole sea water and adjusted with Tris buffer to a pH of 7.6–7.8. Cholinesterase activity was determined according to the method of HESTRIN[8].

The substrates were used in five different concentrations, in order to establish substrate-concentration relationships. The nerves were rapidly dried on blotting paper, weighed and then homogenized in the incubation fluid. The final mixture was then placed in a shaker and samples were taken every hour on the hour. The experiments lasted 2–10 h, depending on the rate of hydrolysis. Room temperature was 20°. Controls for spontaneous hydrolysis were run concurrently.

RESULTS

The hydrolytic activity of the various nerve fibers was tested with each substrate. Fig. 1 shows the rates of hydrolysis by spider-crab fibers. A rather sharp maximum is obtained with 5 mM ACh. The rates for the other substrates are low and show a slow linear increase in rate with concentration.

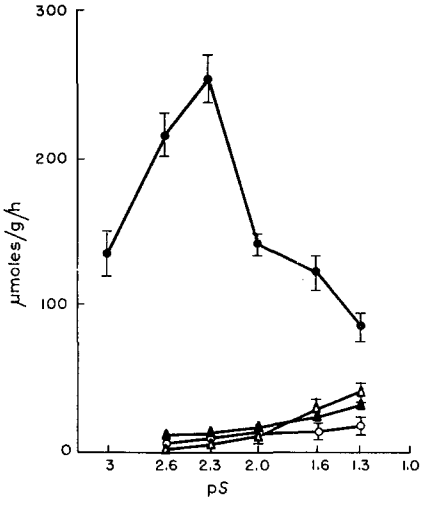

Fig. 1. Hydrolysis of ACh, MeCh, BuCh, and Tr, by homogenized walking leg nerve fibers of spider crab. p*S* negative log of molar substrate concentration. The vertical bars indicate standard deviation of the mean. ●—●, ACh; ▲—▲, MeCh; ○—○, BuCh; △—△, Tr. *T* 18°, pH 7.8.

The esterases in lobster nerve (Fig. 2) show a similar relationship. The concentrations per gram tissue are, however, markedly higher than those in the spider crab.

Physostigmine, a competitive inhibitor of ACh-esterase, inhibits the hydrolysis of 10 mM MeCh nearly completely in a range from 500 to 5 μM. At still higher substrate concentration (50 mM) the inhibition is less complete, about 92–94%. Here the high substrate concentration may be in competition with the inhibitor for the active sites and overcomes the inhibitor effect.

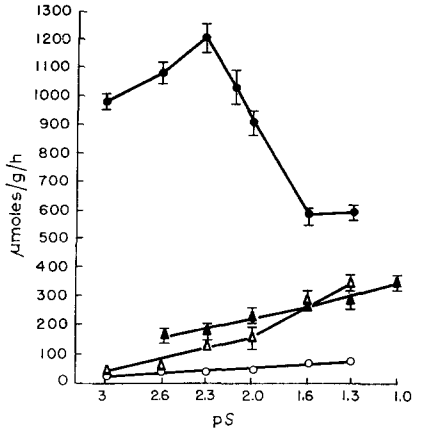

Fig. 2. Hydrolysis of ACh, MeCh, BuCh, and Tr, by homogenized walking leg nerve fibers of the lobster. Symbols as in Fig. 1; T 18°, pH 7.8.

Fig. 3 shows that ACh again is hydrolysed with the highest rate but in contrast to the preparations described before the optimum rate is reached at a concentration of 10 mM and then declines only slowly. The concentrations per g tissue are the lowest of the three nerve preparations tested. The rate of hydrolysis for Tr increases more rapidly in the higher concentration (around 25 mM).

Using the squid giant axon, the axoplasm has been separated from the surrounding

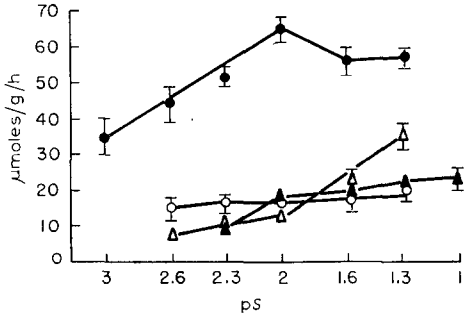

Fig. 3. Hydrolysis of ACh, MeCh, BuCh, and Tr, by homogenized giant axon of the squid. Symbols as in Fig. 1. T 18°, pH 7.8.

envelope and tested then separately for ACh-esterase activity. Table I shows that for two different concentrations of ACh (5 and 10 mM) the activity found in the axoplasm is about 20% of that in the envelope. For the two concentrations of MeCh tested (25 and 10 mM) the axoplasm activity is about 10% of that of the envelope.

A few tentative K_m values were determined for the esterases of the three different nerve fibers using the four different substrates mentioned above. Obviously, using homogenized preparations the K_m values obtained can only be considered as ap-

TABLE I

HYDROLYSIS OF ACh AND MeCh BY GIANT AXON OF THE SQUID *(L. pealii)*

Substrate	*Concentration (M)*	*μM of ester hydrolyzed per g per h*		
		Envelope	*Axoplasm*	*Whole Axon*
ACh	0.01	90.9 ± 1.5	17.7 ± 3.3	65.0 ± 3.5
ACh	0.005	97.8 ± 13.6	18.6 ± 4.3	51.1 ± 3.1
MeCh	0.025	25.95 ± 0.7	2.71 ± 0.0	19.34 ± 3.1
MeCh	0.010	18.8 ± 1.5	2.39 ± 1.0	15.15 ± 3.8

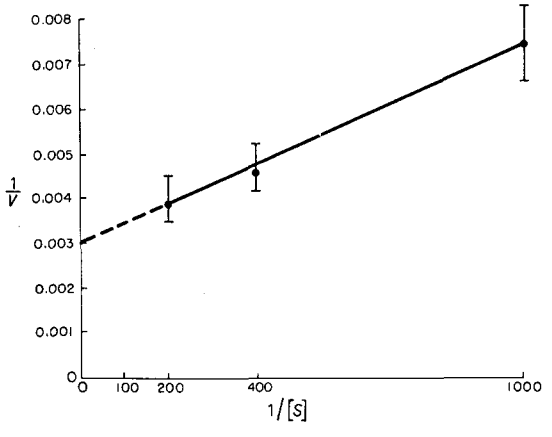

Fig. 4. Reciprocal plots of velocity of ACh hydrolysis by homogenized spider crab walking leg nerve as a function of substrate concentration.

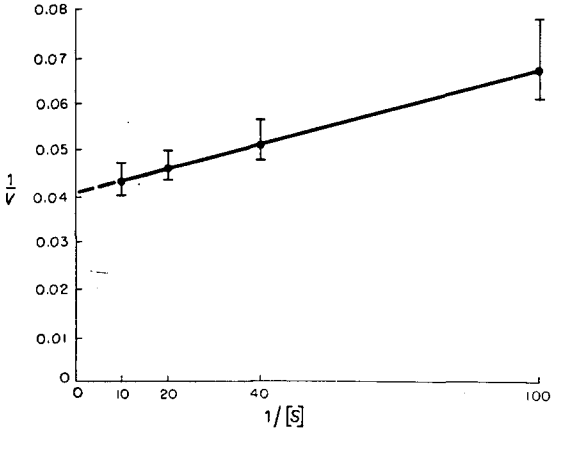

Fig. 5. Reciprocal plots of velocity of MeCh hydrolysis by homogenized giant axon of the squid as a function of substrate concentration.

proximations since presence of a different kind of esterase cannot be excluded and even with good and thorough homogenization only part of the enzyme may be solubilized and freely accessible to the substrate. The values are, therefore, referred to as to apparent K_m values. Although some of the determinations were not quite satisfactory because the range of variations of individual determinations was rather large, probably due to variations in particulate matter present, the results give relative values which appear to be a better measure of affinities of the different enzyme preparations than substrate–activity relationships. The K_m values for the enzyme preparations of the three types of fibers used were determined for all four substrates. Two sets of experiments are given as illustrations. Fig. 4 presents the data for the spider-crab fibers with ACh as the substrate, Fig. 5 those for the squid axons with MeCh as the substrate.

DISCUSSION

When the substrate concentration–activity relationship of ACh hydrolysis by homogenized suspensions of three invertebrate unmyelinated nerve fibers are compared, those of the walking leg of the spider crab and the lobster appear to be quite similar. A well defined optimum of substrate concentration and a strong inhibition of the enzyme activity by excess substrate are observed. With the squid giant axon the inhibition by excess of substrate is not nearly as marked as with the two other preparations: at 50 mM the activity is only 10% lower than that at the optimum of 10 mM, whereas in the two other cases the optimum rate is found at 5 mM and the difference is about 55%. The differences of specificity towards the other substrates tested, MeCh, BuCh and Tr are less marked; however, all these activities are rather low.

As might have been expected, the lowest K_m values obtained with all three preparations were those with ACh as a substrate, the highest those with Tr. However, even for a given substrate there are several-fold differences of the K_m values when the different preparations are compared. Additional investigations with solubilized enzyme preparations are, however, required for more accurate data.

The data presented show that there are deviations from the essential features described for ACh-esterase by AUGUSTINSSON AND NACHMANSOHN[3], even if preparations of nerve fibers are used which are so closely related as the three types studied in the present investigation. Since the whole fibers were used, it is possible that the presence of other ester-splitting enzymes may be responsible for some of the differences observed. It is, however, entirely possible that the proteins of ACh-esterase may have some differences in properties which account for the modifications of the patterns even if the active sites and the mechanism of hydrolysis as described by NACHMANSOHN AND WILSON[9] are the same as it appears almost certain. The question of differences of the proteins could only be decided by an isolation of the enzymes. The data show that some caution is necessary in classifying the type of enzyme present in the tissue and that the criteria used should not be too rigidly applied.

Another interesting result of the studies reported is the presence of ACh-esterase in the axoplasm. The concentration is small but significant. Since the hydrolysis of MeCh is more specific than that of ACh, it appears likely that the concentration is about 10% of that found in the envelope. The low absolute activity may account for the irregular and apparent negligible values when tested with the Cartesian diver technique by BOELL AND NACHMANSOHN[10] in which the activity is measured

for a very short time only. It would be interesting to study whether the enzyme is associated with some organized particles of the axoplasm or present there in free form.

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