STUDIES ON CHOLINESTERASE IN *CARCINUS MAENAS*

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The problem whether cholinergic nerves are found among the Crustacea must be considered to be as yet unsolved. One of the necessary conditions to the existence of such nerves is the presence of a cholinesterase. In this investigation some properties of the cholinesterase (ChE) in *Carcinus maenas* are examined in the light of the recent findings regarding the cholinesterases of mammals.

Between 1940 and 1943 it was ascertained that in the human body there are two kinds of cholinesterase, the e-type or true ChE in erythrocytes and brain and the s-type or pseudo ChE in blood serum (ALLES AND HAWES, RICHTER AND CROFT, MENDEL AND RUDNEY, ZELLER AND BISSEGGER)[1]. The s-type also hydrolysed non-choline esters, while the e-type proved to be specific to a number of choline esters. Acetyl-β-methylcholine (mecholyl) was hydrolysed by the e-type but not by the s-type, whereas benzoylcholine was hydrolysed by the s-type only (MENDEL AND RUDNEY)[1]. The e-type (true ChE) displayed its maximum of activity at low substrate concentrations, an excess of acetylcholine or mecholyl inhibited the hydrolysis. On the other hand the unspecific pseudo ChE showed its maximum of activity at high substrate concentrations.

Before the existence of two kinds of ChE had been demonstrated, it had already been established that the tissues of Crustacea contained an enzyme capable of hydrolysing acetylcholine. The haemolymph was found to contain no or hardly any enzyme (SIMONART, BACQ, KOCHTOJANZ, BACQ AND OURY)[2]. The tissues on the contrary, contained cholinesterase; that is to say the enzyme was usually found in a considerable amount in the nervous system, to a lesser degree also in muscle (BACQ AND NACHMANSOHN, MARNAY, SMITH AND GLICK, JULLIEN AND VINCENT)[2]. When after 1943 the specifity of the enzymes capable of hydrolysing acetylcholine was also examined in the case of the invertebrates NACHMANSOHN AND ROTHENBERG[3] found a specific enzyme in the lobster which hydrolysed mecholyl but did not attack benzoylcholine; no more details are mentioned. AUGUSTINSSON[4] examined a number of marine invertebrates; for these animals he found a hydrolysis of mecholyl, benzoylcholine and non-choline esters in such varying proportions that he was forced to assume the existence of a number of enzymes, all of different specificity. Especially in muscles of *Carcinus maenas* he found a hydrolysis of both mecholyl and benzoylcholine. It seemed important to the present authors to study some properties of the *Carcinus*-cholinesterase more in detail.

METHODS

The crabs were placed at our disposal by the Zoological Station at Den Helder and were kept in a sea water aquarium of the Amsterdam Zoological Gardens. The animals were dissected and the

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required tissues were removed; after being weighed these tissues were ground with quartz sand in a mortar, taken up in a quantity of Ringer-solution (containing 6 g NaCl, 150 mg $CaCl_2$, 100 mg KCl and 150 mg $NaHCO_3$ in one litre of water) and centrifuged. The ChE activity in the decanted liquid was in most cases determined the same day, sometimes after the liquid had been stored in the refrigerator for 24 hours. The amounts of tissue used were usually about 1 g (*e.g.*, leg muscle, heart, digestive gland); only with the central nervous system the amounts used varied from 100 to 300 mg, for which quantities a number of animals were required.

The ChE activity was measured by the Warburg manometric method, based on the manometric estimation of the volume of CO_2 evolved from a system containing bicarbonate by the acid formed by hydrolysis of the ester. In the main compartments of the flasks 1.40 ml of fluid was brought; this 1.40 ml contained 0.2 or 0.4 ml of enzyme solution, the rest was a $NaHCO_3$ solution. In the side bulbs of the flasks 0.2 ml of the substrate solution was introduced. The final concentration of the $NaHCO_3$, after mixing the two compartments, was 0.025 M. The hydrolysis was carried out in a gas mixture of 95% N_2 and 5% CO_2 (% by volume) at a temperature of 27° C. The manometers were read at five-minute intervals, usually for an hour, the first reading taking place 4 minutes after mixing. In some flasks the enzyme solution was always replaced by Ringer-solution in order to determine the spontaneous hydrolysis of the substrate solutions.

The substrates used were: acetylcholine chloride (HOFFMANN-LA ROCHE), benzoylcholine chloride (HOFFMANN-LA ROCHE), acetyl-β-methylcholine chloride (MERCK), methylbutyrate (AMSTERDAMSCHE CHININE FABRIEK).

RESULTS AND DISCUSSION

A. CHOLINESTERASE CONTENT OF THE VARIOUS TISSUES

The enzyme activity of the various tissues was expressed as $b_{30'}$, representing the amount of CO_2 in μl evolved during 30 minutes by the quantity of enzyme present in 100 mg tissue (when the course of the evolved CO_2-time curve is linear). The substrate used was acetylcholine in a final concentration of 0.006 M (*i.e.*, the optimal concentration; see under B). The results are shown in Table I.

TABLE I

Tissue	$b_{30'}$
Haemolymph	0
Liver	< 10
Heart	50
Leg muscle	90
Cerebral ganglion	250
Abdominal ganglion	1400
(Brain of *Rana*	290)

The table shows that with the exception of muscles and nervous system the tissues of Carcinus only contained very low concentrations of an enzyme hydrolysing acetylcholine. A large amount of enzyme is found in the ganglia; their activity is 15 times as large as that of the leg muscles, the ChE content of which tissue approaches most nearly that of the nervous system. For comparison the $b_{30'}$ of brain of *Rana esculenta* was determined and found to be 290, which is of the same order of magnitude as that of the Carcinus cerebral ganglion. It is furthermore interesting that the activity of the abdominal ganglion is more than 5 times as large as that of the cerebral ganglion. The cause of this considerable difference in activity between both ganglia is not yet fully clear to us.

The further experiments were usually carried out with the two kinds of ganglion (cerebral + abdominal ganglion) put together; the activity usually amounted to 1300 (values of 1100 to 1350 were found).

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B. CHOLINESTERASE ACTIVITY OF NERVOUS SYSTEM AND LEG MUSCLE WITH RESPECT TO VARIOUS SUBSTRATES

The enzymic hydrolysis caused by enzyme solutions obtained from nervous system and leg muscle was examined in case of acetylcholine 0.0056 M, benzoylcholine (BCh) 0.006 M, mecholyl (Mech), 0.03 M and methylbutyrate (Mb) 0.003 M (all concentrations are the final molar concentrations in the reaction flasks).

The results are shown in Table II.

TABLE II

Substrate	$b_{30'}$ muscle	$b_{30'}$ nervous system
ACh	89	780
BCh	0	0
Mech	40	440
Mb	0	0

This table, which represents the results of a number of experiments, demonstrates that mecholyl is hydrolysed well, benzoylcholine and methylbutyrate not at all on the contrary. So with respect to the substrate specificity the ChE of Carcinus behaves like the specific true ChE of mammals.

This result contradicts the statement of AUGUSTINSSON that the ChE of Carcinus would also be able to hydrolyse benzoylcholine. When our enzyme solutions acted upon benzoylcholine in concentrations ranging from 0.003 to 0.09 M, no enzymic hydrolysis was ever observed. Even with extracts prepared by grinding the whole crabs (which extracts showed a good activity towards acetylcholine) we could not demonstrate a hydrolysis of benzoylcholine.

C. CHOLINESTERASE ACTIVITY AS A FUNCTION OF THE SUBSTRATE CONCENTRATION

We studied the ChE activity of extracts of nervous system and of leg muscle towards acetylcholine in concentrations of 0.0011, 0.0028, 0.0056, 0.009 and 0.056 M. When the amount of CO_2 evolved in μl is plotted against the time, curves are obtained which run a linear course at first, then begin to bend and finally proceed parallelly to the time-axis (naturelly the reason for this is that the acetylcholine is gradually completely

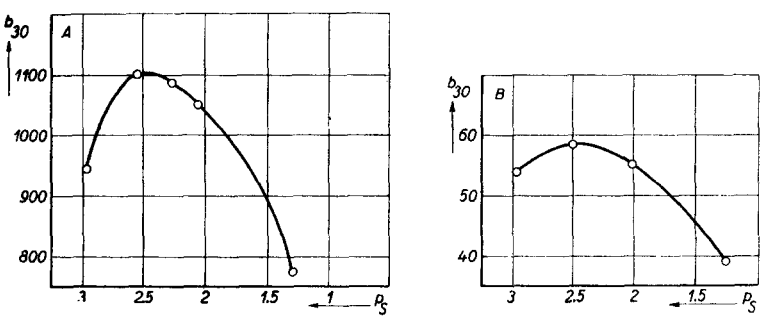

Fig. 1. Cholinesterase activity as a function of the acetylcholine concentration. A: ChE from nervous system; B: ChE from leg muscle

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hydrolysed). The curves for 0.0056 M acetylcholine had the steepest inclination in the linear part. For each curve the $b_{30'}$ has been calculated and in Fig. 1 the values for $b_{30'}$ of the enzyme solutions obtained from nervous system and leg muscle have been plotted graphically against the p_S (negative logarithm of the molar substrate concentration).

Fig. 1 shows that with both kinds of enzyme solution an excess of acetylcholine inhibits the activity. As regards a varying concentration of acetylcholine, our ChE-solutions also behave like the specific true ChE of mammals. For both kinds of enzyme solution we find an optimum of activity for $p_S = 2.5$, *i.e.*, an optimum in the same region as the optimum of the mammalian true ChE.

Next we studied the activity of extracts obtained from nervous system and leg muscle towards mecholyl of varying concentrations. With mecholyl we did not find a maximum in the activity-p_S-curve; at higher concentrations the curve tends to become parallel to the p_S-axis and remains so up to the high concentration of 0.2 M. See Fig. 2.

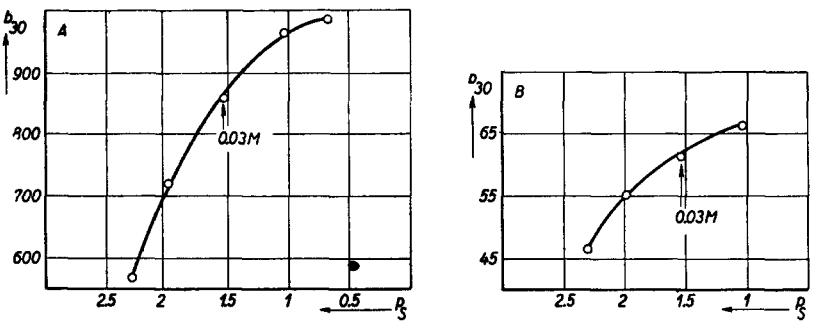

Fig. 2. Cholinesterase activity as a function of the mecholyl concentration. A: ChE from nervous system; B: ChE from leg muscle

This progress is different from that displayed by the activity-p_S-curve of mecholyl of the mammalian true ChE; this last curve shows a maximum at 0.03 M mecholyl. The shifting of this maximum with different species of animals is in itself not so surprising; in so far the shifting is of importance that the place of the maximum is no longer at 0.03 M. As it is necessary in case of comparative ChE-studies to work with the optimal substrate concentrations, a full activity-concentration curve must always be determined. When comparing the ratio of the hydrolysis of acetylcholine in a certain concentration and that of mecholyl in a certain concentration for different species of animals, a great deal of its importance may be lost if the concentrations used differ largely from the optimal ones. This fact is not clearly recognized in all publications on this subject, as is shown in one of the papers of AUGUSTINSSON[4].

D. STABILITY

The activity of a great number of our enzyme solutions had decreased to fifty per cent after storage in a refrigerator at 2–4° C for eight days.

E. INHIBITON BY PHYSOSTIGMINE

The inhibition by physostigmine sulphate (SMITH, Edinburgh) was studied with a number of enzyme solutions obtained from abdominal ganglia. The substrate used was acetylcholine in a concentration of 0.0056 M. Table III shows the results.

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The table shows a fifty per cent inhibition at a concentration of 10^{-6} M physostigmine. In our experiments the physostigmine was left in contact with the enzyme for 35 to 45 minutes before adding the substrate. In the same way we studied the inhibition by physostigmine of the true ChE of human erythrocytes (temperature also 27° C). Here we found a fifty per cent inhibition at 10^{-6} M physostigmine too. Consequently the behaviour of both kinds of enzyme is the same with respect to the inhibition by physostigmine.

TABLE III

Molar concentration of physostigmine	% inhibition
10^{-4}	100
10^{-5}	94
10^{-6}	48
10^{-7}	9
10^{-8}	0

The authors wish to express their thanks to Dr. SUNIER for his assistance in the preservation of the crabs used in this investigation.

SUMMARY

1. In *Carcinus maenas* a cholinesterase was found. Especially in the nervous system it was present in high concentrations. The activity $b_{30'}$ in the central nervous system was 1300, as against 90 in the leg muscle, the tissue following next in activity.

2. The activity of the abdominal ganglion is more than five times that of the cerebral ganglion.

3. With respect to the substrate specificity the enzyme solutions behave like the human specific true cholinesterase; mecholyl is hydrolysed well, benzoylcholine not at all.

4. Also with respect to the acetylcholine concentration the enzyme solutions behave like the specific true cholenesterase.

5. The activity-mecholyl concentration curve does not show a maximum at 0.03 M; we did not observe any inhibition by an excess of mecholyl.

6. The 50% inhibition by physostigmine of the Carcinus cholinesterase takes place at the same concentration of physostigmine as does the inhibition of the human true cholinesterase.

RÉSUMÉ

1. On a trouvé une cholinestérase chez les *Carcinus maenas*. C'est dans le système nerveux qu'elle est surtout présente à forte concentration. L'activité $b_{30'}$ dans le système nerveux central est de 1300, alors qu'elle est de 90 dans le muscle de la jambe; le tissu suivant immédiatement les tissus nerveux au point de vue activité.

2. L'activité du ganglion abdominal est supérieure à 5 fois celle du ganglion cérébral.

3. En ce qui concerne la spécificité des substrats, les solutions d'enzyme se comportent comme la vraie cholinestérase humaine; le mecholyl est nettement hydrolysé, la benzoylcholine ne l'est pas du tout.

4. En ce qui concerne aussi la concentration en acétylcholine, les solutions d'enzyme se comportent comme la vraie cholinestérase humaine.

5. La courbe d'activité, en fonction de la concentration en mecholyl, ne montre pas un maximum pour 0.03 M. On n'a pas observé d'inhibition par un excès de mecholyl.

6. L'inhibition de 50% par la physostigmine de la cholinestérase des *Carcinus* se produit pour la même concentration de physostigmine que celle qui inhibe la vraie cholinestérase humaine.

ZUSAMMENFASSUNG

1. In *Carcinus maenas* wurde eine Cholinesterase gefunden. Besonders im Nervensystem war sie in hohen Konzentration vorhanden. Die Aktivität $b_{30'}$ betrug im zentralen Nervensystem 1300, während sie im Beinmuskel, dem nächstaktiven Gewebe, 90 betrug.

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2. Die Aktivität des abdominalen Nervenknotens ist mehr als fünfmal grösser als die des Gehirnnervenknotens.

3. Was die Substratspezifität betrifft, so verhalten sich die Enzymlösungen wie die wahre menschliche Cholinesterase; Mecholyl wird deutlich hydrolysiert, Benzoylcholin gar nicht.

4. Auch in Bezug auf die Acetylcholinkonzentration betragen sich die Enzymlösungen wie die spezifische wahre Cholinesterase.

5. Die Kurve der Aktivität in Funktion der Mecholylkonzentration zeigt kein Maximum bei 0.03 M; Hemmung durch Mecholylüberschuss konnte nicht festgestellt werden.

6. Die 50%-ige Hemmung der Carcinuscholinesterase durch Physostigmin tritt bei derselben Physostigminkonzentration auf wie die Hemmung der wahren menschlichen Cholinesterase.

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