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CHOLINESTERASE ACTIVITY IN THE CENTRAL NERVOUS SYSTEM OF *ANODONTA CYGNEA L.*

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The studies on the central and peripheral nervous system in molluscs refer primarily to the gastropods and cephalopods. Due to experimental and methodical difficulties much less is known of the construction and acitivity of the nervous system of Pelecypods (Lamellibranchiates). Thus, we have no satisfactory knowledge and exact data on the chemical specificities of active substances involved in the activity of cells and synapses of the central nervous system, though the presence of 5-HT and of catecholamines in the ganglia and the activating effect of these agents when administered from outside has been demonstrated by several authors (DAHL et al. 1962, SALÁNKI 1963, SWEENEY 1963, PUPPI 1964, ZS.-NAGY et al. 1965).

Studies performed by HORRIDGE (1961) on *Mya*, and PUPPI (1963) on *Anodonta* with the objective of establishing the possibility of the presence of a cholinergic mechanism in the central nervous system ended with positive results. The same indications were obtained by the electron microscopic studies of ZS.-NAGY (1964) demonstrating in the synapses of central nervous system the presence of structures corresponding to the ACh vesicles of vertebrates.

Nevertheless, it was not possible to histochemically demonstrate the presence of cholinesterase in the ganglia of *Anodonta* either by the thiocholine or by the indoxyle acetate methods (ZS.-NAGY and SALÁNKI 1965).

It is generally accepted that the presence of ACh-decomposing enzyme in the postsynaptic field is incident to the cholinergic mechanism, thus, in order to obtain more knowledge on the central neuronal regulation it is essential to decide whether acetylcholinesterase is present in the ganglia (which represents the central nervous system in *Anodonta)* or not. In certain cases this might offer some basis in the indetification of the above mentioned structures which are of 200—300 Å diameter and ultrastructurally resemble ACh-vesicles.

Material and methods

The whole central nervous system (cerebral, visceral, and pedal ganglia) of *Anodonta cygnea L.* was examined. The samples were homogenized for 10—15 minutes with distilled water in glass-potter placed into icy water. Incubation conditions: 1 ml homogenized sample consisting of the whole central

nervous system of two animals (about 40 mg) and the required quantity of substrate was added into 5 ml incubation solution, and the mixture obtained was adjusted to the wanted pH (5.0—8.5) value by the addition of trismaleate buffer. Incubation temperature was 37 $\pm$ 0.1 °C and the duration of incubation 3 hours. The self-hydrolysis of enzyme-free samples was determined. In case inhibitors were used they were added both to the incubation solution and the control tube. This was necessitated by the colour change of eserine during its decomposition.

Measurement: The decomposition of substrate was determined by the method of HESTRIN (1949). Calibration curve was taken with $AChClO_4$. The coloured product was measured photometrically with BECKMAN G 2400 spectrophotometer at 530 mμ wavelength.

In the experiments the following substrates and inhibitors were used:

acetylcholine Cl (ACh; Merck)
acetylthiocholine J (AThCh; Fluka)
butyrylthiocholine J (BuThCh; Fluka)
acetyl- β-methylcholine (MeCh; Light)
benzoylcholine (BeCh; Light)
eserine salicylate (BDH)
diizopropyl-fluoro-phosphate (DFP)

To avoid the differences between individuals a homogenizate was prepared of the central nervous system of 50—100 animals and this was used in the measurements. Nitrogen determinations were performed according to KJELDAHL'S method. NH_3 was distilled into saturated boric acid solution and was titrated with 0.01 n HCl solution.

Results

1. Rate of the enzyme activity with various substrates and the effect of inhibitors.

In every case incubation was performed at pH 8 and lasted for 3 hours. The concentration of substrate was 4 mM. Considering the relatively long time needed for the excision and collection of ganglia (2—3 hour's) from the animals and becasue of changes in wet-weight content occurring also in iced physiological solution, the decomposition rates obtained are expressed in terms of N content. From this value the rate of ChE-activity might be expressed also approximatively on wet-weight basis by computation (N content of ganglia = 8—10 mg N/g wet-weight).

Rate of hydrolysis

Substrate	μg substrate/mg N/hour	mg substrate/g wet-weight/hour
ACh	226	1.8—2.3
MeCh	184	1.5—1.8
AThCh	214	1.7—2.1
BuThCh	217	1.7—2.2
BeCh	no hydrolysis	—

The effect of inhibitors was measured in the presence of 4 mM ACh at 37°C and at pH 8.

Cholinesterase activity was completely inhibited by 10^{-5} M eserine and 10^{-6} M DFP. An about 40—60% inhibition was produced by 10^{-6} M eserine and 10^{-7} M DFP and no inhibition was observed in case of 10^{-7} M eserine and 10^{-8} M DFP.

2. Substrate-inhibition

Because the activity of acetyl cholinesterase is specifically inhibited by high substrate concentrations, which phenomenon is not characteristic of non-specific ChE (AUGUSTINSSON 1949) the activity of ChE was measured at different ACh concentrations. Incubation series was made at 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 10 mM AChCl concentrations. The results were related to the rate of decomposition at 4 mM concentration. *Fig. 1* illustrates decomposition rates

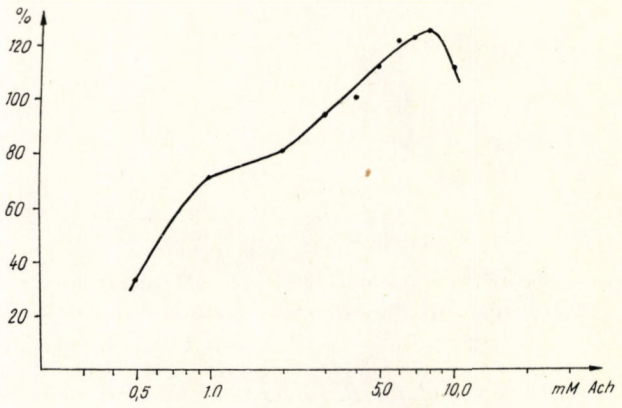

Fig. 1. The ACh splitting of homogenized ganglion depending on the substrate concentration (pH 8, 37° C)

1. ábra Ganglionhomogenizátum ACh bontása a szubsztrátkoncentráció függvényében (pH 8, 37° C)

in the percentage of values obtained at 4 mM. It appears that decomposition has a maximum which is followed by a decrease. Maximum was, however, obtained at about 8 mM substrate concentration. The shape of the curve is not identical with the bell-shaped curve generally accepted for specific ChE in literature (AUGUSTINSSON 1963), moreover, by increasing concentration further the descending slope of the curve becomes undefinite because of great deviation of data. For that very reason we shall abstain here from presenting these data. Besides the maximum at about 8 mM concentration, an inflexion point is observable on the ascending slope near to 1 mM.

3. pH dependency of ChE activity

The experiments were performed at different pH-values (5—8.5 pH), at 4 mM AChCl concentration and at 37°C, and decompostion rates were related to values obtained at pH 8. The results are presented in *Fig. 2.* A maximum activity is observable at pH 7 and only a decrease of pH below 5.5 or a rise above 8.5 produces considerable loss of activity.

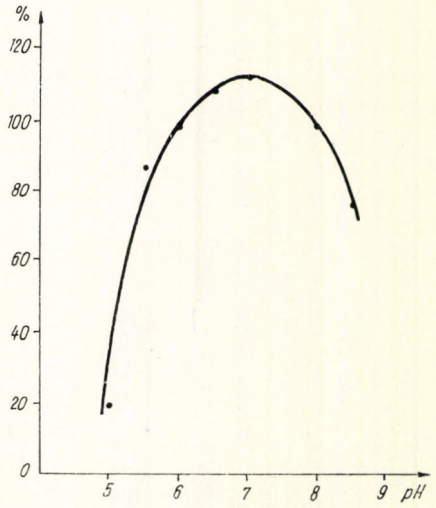

Fig. 2. pH dependency of ACh decomposition (4 mM substrate concentration, 37° C)
2. ábra ACh bontás pH függése (szubsztrátkoncentráció 4 mM, 37 °C)

4. The effect of formalin fixation on ChE activity

Previous histochemical studies on ChE in the ganglia of *Anodonta cygnea* (Zs.-Nagy and Salánki 1965) resulted in negative reactions. In these methods formalin fixation and also acide pH range were used. To elucidate the source of these negative results experiments were performed at different pH values also on homogenizates of ganglia pretreated by formalin.

Excised ganglia were treated with 4% neutral formalin solution for 1 hour. They were washed subsequently in running tap-water for 2 hours and were rinsed with distilled water. After homogenization the samples were incubated in the presence of 4 mM AChCl at 8.7 and 6 pH values. It was found that incubation at pH 6—8 after formalin fixation showed decreased activity with about 50%.

Discussion

As it is evidenced by the experiments cholinesterase exists in the central nervous system of *Anodonta cygnea L.* On basis of data obtained this is most probably a specific cholinesterase, though the properties of the demonstrated

enzyme do not comply with every criteria of it. The following phenomena are indicative of the presence of ChE: a considerable acetyl-β-methylcholine (MeCh) decomposition, the absence of benzoylcholine decomposition and the fact that highest values were observed in case of acetylcholine decomposition. Examining the rate of decomposition in the function of the substrate a maximum was observable at 8 mM and not at 4 mM regarded characteristic of AChE. Definite inhibition was not observed above 10 mM concentration either. Here decomposition rate was equal to that observed at 5 mM concentration. Occasionally a 70—100 per cent inhibition or no inhibition at all was observed at concentrations higher than 10 mM. The strong deviation of data obtained at high substrate concentration is obviously of methodical origin.

The dependency of decomposition on substrate concentration is not linear even at low concentrations and an inflexion is observable at 1 mM. It is suggested that perhaps two or more ChE-es of different nature exist in the homogenizate and they may be responsible for the deviation of the substrate curve from the conventional one. It was evidenced by previous studies (Zs.-Nagy and Salánki 1965) that non-specific esterase content in gastropods fluctuates considerably according to the functional condition of the animal. A similar phenomenon exists presumably in case of *Anodonta* and the alterations in the amount of this enzyme, induced by changes in the functional condition of the animal, may explain the undefinite descending slope of the substrate curve at concentrations above 10 mM.

Rate of enzyme activity was relatively low, 226 μg ACh/mgN/hour at pH 8 and at 4 mM substrate which expressed on wet-weight basis corresponds to about 2 mg ACh /g/hour. Considering that substrate concentration has an optimum at 8 mM where the observed activity is about 25% higher as compared to values obtained at 4 mM, further that an about 10% activity increase was obtained by incubation at pH optimum (pH 7) in comparison to pH 8, it is inferred that under optimum conditions we might reckon with an about 30% higher enzyme activity (i. e. about 2.6 mg ACh/g wet-weight/hour).

It might be of interest to compare the ACh activities of different invertebrates. The rate of the actvity of ChE expressed in mg ACh/g wet-weight/hour unit was in case of *Loligo* brain 2—4 mg, in the ganglion of *Limulus* heart 0.14 mg and in case of the mantle nerve of *Loligo* 0.006 mg (Prosser and Brown 1961).

In accordance with literary data (Taxi 1952) a decrease of activity by 50% was observed after formalin treatment and a considerable decrease of similar dimension was demonstrable below pH 6 and above pH 8.

The fact that ChE was not demonstrable histochemically in the ganglia of *Anodonta cygnea* can be attributed probably to the relatively low rate of activity, to the inhibitory effect of formaldehyde treatment and to the influence of acidic pH. It is assumable, however, that with a more suitable technique (without formaldehyde treatment or electron microscopic examination respectively) the chemically demonstrable amount would suffice for the demonstration of the localization of this enzyme.

We have no knowledge of the localization of ChE-activity demonstrated in these experiments, nonetheless the results obtained and the literary data (Horridge 1961, Puppi 1963, Zs.-Nagy 1964) suggest that it has a synaptic localization. The actual activity values suggest, however, that in the neural processes of the ganglia of *Anodonta cygnea* primarily not a cholinergic mecha-

nism, but chemical compounds different from ACh are involved. This assumption is supported by investigations demonstrating considerable amounts of dopamine (DAHL et al. 1962), catecholamine (PUPPI 1964) and 5-HT (DAHL et al. 1962; ZS.-NAGY et al. 1965) further the effect of 5-HT and of catecholamines on the central nervous system of *Anodonta* (SALÁNKI 1963). It is not to be ignored, however, that ACh may possibly have a more important role. Its decomposition may take place not in loco, but outside of the nervous system in the lymph, and the cessation of its effect in the synapses is due possibly to the adaptation of the postsynaptic structure and not to the decomposition by ChE (SAKHAROV and NISTRATOVA 1963).

The possibility of the presence of cholinerg mechanism in the central nervous system of Pelecypods is still an unsolved problem and further extensive studies are needed for the satisfactory solution of this problem.

Summary

The cholinesterase activity of the central nervous system of *Anodonta cygnea* (Pelecypoda) was investigated by biochemical methods. The cholinesterase activity observed is characterized by the followings:

1. ACh decomposition does not show linear increase parallel with the rise of substrate-concentration.
2. ACh-decomposition is most definite.
3. Acetyl-β-methylcholine (MeCh) decomposition is considerable.
4. There is no benzoylcholine decomposition.
5. 50% inhibition by 10^{-6} M eserine and by 10^{-7}M DFP.
6. There is an optimum at pH 7, and a considerable decrease in activity below pH 6 and above pH 8.
7. An about 50% decrease in activity after one hour treatment in 4% formaldehyde.

The above results indicate that a specific cholinesterase exists in the homogenized samples of the cerebral, visceral and pedal ganglia. At pH 8, at 37°C temperature and in case of 4 mM substrate concentration the rate of activity is 226 μg ACh /mg N/ 1 hour, which is equivalent to about 1.8—2.2 mg ACh/1 hour/1 g wet-weight.

The results suggest that cholinergic synapses may also be present in the ganglia of *Anodonta*. The low rate of decomposition and other factors indicate that they constitute only the smaller part of synapses in this animal and probably the central mechanisms are realized by chemical transmitters different from ACh.

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CHOLINESZTERÁZE-AKTIVITÁS *ANODONTA CYGNEA L.* KÖZPONTI IDEG RENDSZERÉBEN

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Összefoglalás

Anodonta cygnea (Pelecypoda) központi idegrendszerének cholineszteráze aktivitását vizsgáltuk biokémiai módszerrel.

A talált cholineszteráze aktivitás jellemzői:

1. A szubsztrát-koncentráció emelésével az ACh bontás nem mutat lineráis emelkedést
2. Legkifejezettebben az ACh-t bontja
3. Acetyl-β-methilcholint (MeCh) jól bontja
4. Benzoilcholint nem bontja
5. Eserin 10^{-6}, DFP 10^{-7} M koncentrációban 50%-os gátlást okoz
6. Optimuma pH 7-nél van, s az aktivitás pH 6 alatt, valamint 8 felett jelentősen csökken
7. 4%-os formaldehidben történt egy órás kezelés az aktivitást kb. 50%-kal csökkenti.

Fentiek alapján valószínű, hogy a cerebrális, viscerális és a pedális ganglionok homogenizátuma specifikus cholineszterázét tartalmaz. Az aktivitás mértéke pH 8-nál, 37° C-on, 4mM szubsztrát-koncentráció esetén 226 μg ACh/mg N/óra, ami 1 g nedves súlyra számítva kb. 1,8—2,2 mg ACh/órának felel meg.

Fentiek alapján feltehető, hogy *Anodonta* ganglionjaiban cholinerg szinapszisok is vannak. A bontás alacsony foka — más tényezőkkel együtt — arra utal, hogy ezek a szinapszisoknak csak kisebb részét képezik, s a központi ingerületáttevődés zömében az ACh-tól eltérő kémiai transzmitter-anyag részvételével valósul meg.

ХОЛИНЭСТЕРАЗНАЯ АКТИВНОСТЬ ЦЕНТРАЛЬНОЙ НЕРВНОЙ СИСТЕМЫ *ANODONTA CYGNEA L.*

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Исследовали холинэстеразную активность центральной нервной системы *Anodonta cygnea L. (Pelecypoda)* биохимическим методом. Характеристики обнаруженной холинэстеразной активности:

1. Отсутствует линейная зависимость между концентрацией субстрата и расщеплением ацетилхолина.
2. Из всех использованных субстратов наиболее выраженное расщепление проявлял ацетилхолин.
3. Ацетил-β-метилхолин расщепляется хорошо.
4. Бензоилхолин не расщепляется.
5. Эзерин 10^{-6} М и ДФП 10^{-7} М вызывают 50%-ное торможение активности фермента.
6. Оптимум активности при pH 7; ниже pH 6 и выше pH 8 активность значительно снижается.
7. Обработка 4% формальдегидом в течение часа снижает активность примерно наполовину.

На основе вышеизложенного представляется вероятным, что гомогенаты педального, висцерального и церебрального ганглиев содержат специфическую холинэстеразу. При pH 8, 37° С и концентрации субстрата 4 мМ активность соответствует 226 μg АХ /mg N/ час, что в пересчете на 1 г сырого веса равно примерно 1,8—2,2 мг АХ/час.

Полученные данные позволяют предполагать, что в ганглиях беззубки имеются и холинэргические синапсы. Низкий уровень расщепления ацетилхолина и другие факты указывают на то, что холинэргические синапсы составляют лишь небольшую часть синапсов и что в других случаях центральная передача возбуждения осуществляется каким-то другим медиатором, отличающимся от ацетилхолина.