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# THE INHIBITION OF FROG TISSUE CHOLINESTERASES AND THE INFLUENCE OF ESERINE AND PROSTIGMINE ON THE ACTION OF ACETYLCHOLINE ON THE FROG HEART*

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**Abstract**—The activity and type of cholinesterases in various tissues of the frog (*Rana esculenta*) and possible seasonal differences in the activity were investigated. The activity is highest in the sympathetic chain, the brain and spinal ganglia, somewhat lower in Bidder's ganglia of the heart, and low in cardiac muscle, skeletal muscle, blood and liver. Cholinesterases in frog tissues are acetylcholinesterases; their sensitivity to eserine is lower than that of mammalian cholinesterases, which might be responsible for the failure of eserine to increase the negative inotropic effect of acetylcholine in the frog heart.

Frog tissues are conventional test systems for a number of pharmacological experiments providing fundamental information on cholinergic mechanisms. However, the data in the literature on the activity and type of frog tissue cholinesterases [acetylcholinesterase, acetylcholine hydrolase (EC 3.1.1.7, AChE); butyrylcholinesterase, acylcholine acyl-hydrolase (EC 3.1.1.8, BuChE)] [1–3] is scarce and often contradictory. Likewise there is no uniform view in the literature concerning the influence of eserine on the activity of cholinesterases and on the effect of acetylcholine (ACh) in frog or other amphibian tissues. Mendel and Hawkins [4] and later others [5, 2, 6] observed a relatively low sensitivity of cholinesterases in the amphibian tissues to eserine. In pharmacological experiments reported in the literature either an enhancement [7] or no enhancement of the ACh effect was observed [8–11]. Considering the fact that frog tissues and eserine have been the tools most frequently used by pharmacologists during the last half of the century, such inconsistency of information is difficult to comprehend.

For this reason we investigated the activity and the type of cholinesterases in various frog tissues as well as the sensitivity of cholinesterases to eserine. In parallel experiments on the frog heart the influence of eserine and prostigmine on the effect of acetylcholine was studied. Further, the activity of cholinesterases in the frog tissues was investigated during various seasons, for it has long been known that in winter the sensitivity of the frog heart to ACh is considerably higher than in summer.

## MATERIALS AND METHODS

The experiments were carried out on frogs (*Rana esculenta*) all the year round. The frogs were decapitated and the tissues removed under a dissecting microscope. Small tissue samples (spinal ganglia, sympathetic chain, atrium, ventricle and Bidder's ganglia of the heart) were immediately frozen and dried. In the case of the brain, liver, whole heart and skeletal muscle, the entire organ was homogenized, frozen and dried. Immediately before measuring cholinesterase activity the dried samples were weighed and homogenized in small glass homogenizers containing 30–200 $\mu$l of frog Ringer [11] (108·7 mM NaCl, 4·3 mM $NaHCO_3$, 3·8 mM KCl, 1·6 mM $CaCl_2$, 0·22 mM $NaHPO_4$, 5·0 mM glucose). For each measurement 10 $\mu$l of the homogenate containing 20–300 $\mu$g of dry tissue were used.

Citrate was added to the blood samples obtained by tapping the heart and the erythrocytes separated from the plasma. The erythrocytes were washed three times with Ringer and brought to the original volume of blood with distilled water. The results were extrapolated to the amount of plasma and the erythrocytes contained in 1 $\mu$l of whole blood. The samples were used either immediately afterwards or were kept at $-20°$ for periods up to several months.

The amount of each tissue per assay was adjusted to such a manner that no more than 30% of the substrate was hydrolized. In measuring the hydrolysis of butyrylcholine (BuCh) 10–100 times larger tissue samples were used than in measuring the hydrolysis of ACh. Each sample was run in three parallel assays. The activity of cholinesterase was determined by the radiometric method [12]. The two substrates acetyl-1-[$^{14}$C]choline iodide, and butyryl-1-[$^{14}$C]choline iodide (sp. act. 4·55 mCi/m-mole and 3·59 mCi/m-mole respectively) were obtained from New England Nuclear Corp. and were diluted with the corresponding cold cholines esters (3 mM). The inhibitors used were eserpine sulphate, 0·1 mM and 10 $\mu$M, prostigmine sulphate, 10 $\mu$M; 1,5-bis-4-allyl dimethylammonium phenyl pentan-3-one dibromide (BW284C51) (10 $\mu$M), and tetra-isopropylpyrophosphoramide (izo-

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Table 1. Activity of cholinesterases in frog tissues

| Tissue | ACh | BuCh |
|---|---|---|
| Brain | 53·00 ± 9·4 (25) | — |
| Spinal ganglia | 90·70 ± 19·8 (21) | — |
| Sympathetic chain | 82·40 ± 18·9 (27) | — |
| Heart: | | |
| Whole heart | 1·80 ± 0·8 (22) | — |
| Atrium-muscle | 3·30 ± 0·6 (20) | |
| Bidder's ganglia | 25·50 ± 11·1 (17) | - |
| Ventricle | 1·60 ± 0·3 (15) | — |
| *M. sartorius* | 2·20 ± 0·7 (9) | - |
| Liver | 3·90 (1) | 0·80 (1) |
| | 3·10 (1) | 0·60 (1) |
| Blood: | | |
| Plasma | 0·10 ± 0·0 (6) | 0·02 ± 0·01 (4) |
| Erythrocytes | 0·20 ± 0·1 (7) | — (5) |

Rate of hydrolysis of acetylcholine (ACh) and butyrylcholine (BuCh), $\mu$mole × $10^4/\mu$g dry wt per hr. Results are expressed as mean ± S.D. Figures in parentheses indicate the number of measurements.

OMPA, 10 $\mu$M. The preincubation time with inhibitors was 60 min.

Parallel experiments were made on the isolated, spontaneously beating frog heart. Frog Ringer was used at 16°. The negative inotropic effect of ACh was measured by recording the contraction amplitudes by means of an isotonic lever on the smoked paper of a kymograph. The effect of ACh was expressed as the per cent by which the amplitude was lowered. At the beginning of each experiment the excitability of the preparation was tested with ACh in a suitable concentration until the effect was stabilized. During the experiment the response of the preparation to ACh showed no essential changes. AChE (1 mM and 0·1 $\mu$M) was tested three or four times before and after adding the inhibitor. Thereafter the Ringer solution was replaced by the inhibitor, prepared in Ringer solution. The response to ACh was tested three or four times, 20 min after adding the inhibitor. The concentrations of both eserine and prostigmine were 1 $\mu$M and 10 $\mu$M.

## RESULTS

The rate of ACh and BuCh hydrolysis is shown in Table 1. The activity of cholinesterases is highest in the nervous system. The activity is somewhat lower in the nerve structures of the heart, that is, in Bidder's ganglia. During the isolation of these ganglia not all non-nervous tissue can be removed so that actual activity in Bidder's ganglia might be higher. The cholinesterase activity in the muscle fibers of the heart atrium and ventricle and in the skeletal muscle are of the same order of magnitude and considerably lower than that in the nervous system (Table 1). The low activity in the homogenate of the whole heart is due to the fact that in the heart the amount of nervous tissue (Bidder's ganglia) is very small compared with the amount of muscle (1:8000). The activity in liver and blood is similar to that in muscle.

The rate of BuCh hydrolysis in all the tissues was negligible (less than 2% of the rate of ACh hydrolysis) except in the liver and plasma, where the percentage was 19 and 14, respectively.

Table 2 shows that frog tissue AChE's are considerably less sensitive to eserine than the AChE's of the corresponding mammalian tissues [4]. Prostigmine and BW284C51 are at least ten times more potent inhibitors of frog tissue AChE than eserine. The fact that these two substances in low concentrations inhibit ACh hydrolysis indicates that frog tissue cholinesterase is AChE. The BuCh hydrolysis measured

Table 2. The influence of inhibitors on the activity of cholinesterases in frog tissues, expressed as per cent of inhibition

| Tissue | Eserine | | Prostigmine | BW284C51 |
|---|---|---|---|---|
| | 0·1 mM | 10 $\mu$M | 10 $\mu$M | 10 $\mu$M |
| Brain | 88 (1) | 33 (1) | 88 (1) | 99 (1) |
| | 86 (1) | 41 (1) | 86 (1) | 99 (1) |
| Heart: | | | | |
| Whole heart | 91 ± 3 (17) | — | — | 93 ± 5 (17) |
| Atrium-muscle | 86 ± 11 (4) | 42 ± 10 (1) | 100 | 92 ± 10 (4) |
| | | | 86 | |
| Bidder's ganglia | 97 ± 2 (5) | 68 ± 19 (5) | - | 99 ± 1 (5) |
| Ventricle | 86 ± 11 (5) | 53 ± 21 (4) | 81 ± 6 (3) | 91 ± 9 (5) |
| Liver | — | 26 (1) | 94 (1) | 99 (1) |
| | — | 23 (1) | 94 (1) | 97 (1) |
| Blood: | | | | |
| Plasma | 76 (1) | 14 ± 8 (4) | 88 ± 2 (3) | 98 ± 2 (4) |
| Erythrocytes | 74 ± 0 (3) | 13 ± 8 (5) | 83 ± 8 (6) | 99 ± 1 (5) |

Results are expressed as mean ± S.D. Figures in parentheses indicate the number of measurements.

Table 3. The influence of eserine (a) and prostigmine (b) on the negative inotropic effect of acetylcholine in isolated spontaneously beating frog heart

(a)

| Experiment No. | ACh (0·1 μM) | ACh (0·1 μM) Eserine (1 μM) | ACh (0·1 μM) Eserine (10 μM) |
|---|---|---|---|
| 1 | 91 ± 2 (4) | 95 ± 0 (4) | — |
| 2 | 69 ± 6 (4) | 49 ± 5 (4) | — |
| 3 | 39 ± 6 (4) | 32 ± 5 (4) | |
| 4 | 89 ± 3 (3) | | 87 ± 4 (4) |
| 5 | 88 ± 5 (3) | | 86 ± 2 (4) |
| 6 | 83 ± 7 (4) | | 86 ± 7 (4) |

(b)

| | | | Prostigmine (1 μM) | | Prostigmine (10 μM) | |
|---|---|---|---|---|---|---|
| Experiment No. | ACh (1 μM) | ACh (0·1 μM) | ACh (1 μM) | ACh (0·1 μM) | ACh (1 μM) | ACh (0·1 μM) |
| 1 | — | 50 ± 2 (4) | — | 71 ± 3 (4) | — | 64 ± 2 (4) |
| 2 | 37 ± 4 (4) | — | 75 ± 1 (3) | — | 74 ± 2 (4) | — |
| 3 | — | 44 ± 6 (4) | — | 79 ± 1 (3) | — | 79 ± 0 (4) |

Results are expressed as per cent (mean ± S.D.) by which the amplitude is lowered. Figures in parentheses indicate the number of test doses of ACh on the same preparation.

in the liver and plasma is possibly due to the activity of AChE and not to the activity of BuChE:izo-OMPA up to the concentration of 10 μM does not affect BuCh hydrolysis.

The rate of ACh hydrolysis by muscle enzyme was tested in concentrations ranging from 10 to 0·1 mM and the optimal concentration was found to be between 10 and 1 mM. This result is in good agreement with the inhibition by the excess of substrate described earlier for frog brain ChE [4]. The ability to hydrolize acetyl-β-methylcholine but not BuCh, inhibition of the hydrolysis of ACh by BW284C51, by prostigmine and by the excess of the substrate suggest strongly that the ACh-splitting enzyme in frog excitable tissues should be classified as AChE regardless of its relative insensitivity to eserine.

Parallel experiments on frog heart when the influence of eserine and prostigmine on the negative inotropic effect of ACh (Table 3 a and b) was investigated, show that 1 μM and 10 μM eserine does not enhance the effect of ACh at a concentration of 0·1 μM; prostigmine at a concentration of 1 μM and 10 μM increases the effect of ACh (0·1 and 1 μM). 10 μM prostigmine did not increase further the effect of ACh.

The results of the experiments in which the activity of cholinesterases in frog tissues during different times of the year investigated showed no essential seasonal differences. The activity of cholinesterases varied from animal to animal irrespective of the season so that in spite of a large number of experiments possible minor seasonal differences were not observed.

## DISCUSSION

In the light of our experiments, it can be concluded that the frog tissues investigated contain no BuChE. Similar results were obtained for various amphibian tissues: frog and toad *medulla spinalis* [1], frog brain [13], the sympathetic ganglia of the frog [2] and, with the cytochemical method, also in Bidder's ganglia of the frog heart [6]. However, it was reported that in the bladder of the toad about 10% of the hydrolysis of ACh is due to the activity of BuChE [5] and that the sympathetic ganglia of the frog contain predominantly BuChE [3]. Our experiments show that in frog plasma and liver ACh hydrolysis is completely inhibited by BW284C51 (10 μM), which indicates that even there the type of cholinesterases resembles AChE as defined for mammalian tissue. This observation supports the theory [14, 1] that nonspecific cholinesterase begins to appear in phylogenetically higher vertebrates in both the nervous system and non-nervous tissues.

The weak inhibition of frog AChE by eserine may be responsible for the findings that in some amphibian tissues eserine does not enhance the response to ACh. Thus it was reported that eserine influences neither the ACh effect nor the response of the electrically stimulated pelvic nerve of the toad bladder [10], and that it did not sensitize the toad large intestine for the ACh effect. Lissak [8] found no significant sensitization of the frog heart to ACh by eserine. Furthermore it was reported that eserine does not enhance the inotropic effect of ACh in the frog heart [11]. This is in keeping with our findings that eserine in concentrations of 1 and 10 μM, in which it does not inhibit the activity of AChE, does not enhance the negative inotropic effect of ACh.

Some authors obtained a weak sensitization to ACh by using relatively high concentrations of eserine, e.g. on the *m. rectus abdominis* of the toad (eserine 15 μM), but they obtained a sensitization by using prostigmine in considerably lower concentrations (0·3 μM) [15]. A profound difference between the influence of eserine and prostigmine on the effect of ACh was observed also in experiments with the toad

bladder [5]. Our pharmacological experiments on the frog heart likewise show that 1 $\mu$M prostigmine considerably enhances the effect of ACh.

The differences between the influence of eserine and prostigmine on the effect of ACh observed in pharmacological preparations of amphibian tissue are in good agreement with biochemical evidence which showed that at the concentrations employed frog AChE is completely inhibited by prostigmine but only slightly, if at all, by eserine.

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## REFERENCES

1. L. W. Chacko and J. A. Cerf, *J. Anat.* **94,** 74 (1960).
2. M. Brzin, V. M. Tennyson and P. E. Duffy, *J. Cell Biol.* **31,** 215 (1966).
3. E. Giacobini, *Ann. N.Y. Acad. Sci.* **144,** 646 (1967).
4. R. D. Hawkins and B. Mendel, *J. cell. comp. Physiol.* **27,** 69 (1946).
5. C. Bell and G. Burnstock, *Biochem. Pharmac.* **14,** 79 (1965).
6. Ž. Majcen and M. Brzin, *Histochemie* **25,** 217 (1971).
7. P. Zapata and C. Eyzaguirre, *Can. J. Physiol. Pharmac.* **45,** 1021 (1967).
8. K. Lissak, *Am. J. Physiol.* **127,** 263 (1939).
9. G. Burnstock, J. O'Shea and M. Wood, *J. exp. Biol.* **40,** 403 (1963).
10. H. Boyd, G. Burnstock and D. Rogers, *Br. J. Pharmac.* **23,** 151 (1964).
11. I. A. Boyd and C. L. Pathak, *Q. Jl exp. Physiol.* **50,** 330 (1965).
12. M. W. McCaman, L. R. Tomey and R. E. McCaman, *Life Sci.* **7,** 233 (1968).
13. S. C. Shen, P. Greenfield and J. E. Boell, *J. Comp. Neurol.* **102,** 717 (1955).
14. T. O. Sippel, *J. exp. Zool.* **128,** 165 (1955).
15. E. Xavier, P. M. Freire and J. R. Valle, *Arch. int. Pharmacodyn.* **147,** 388 (1964).