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Comparison between the Acetylcholinesterases of *Helix* Blood and Cobra Venom. I. The Hydrolysis of Acetylcholine and Its Inhibition by Various Compounds

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Much work has been carried out during recent years with mammalian esterases which split acetylcholine and other choline esters. The specificities of these enzymes are now fairly well known. One type is the acetylcholinesterase or aceto-cholinesterase [1] ("specific", "true" cholinesterase), present mainly in nervous tissues and in erythrocytes. The other type (cholinesterase, butyro-cholinesterase, "pseudo" or "non-specific" cholinesterase) occurs in the blood plasma of certain animals. These two types are best differentiated by using acetylcholine and butyrylcholine as substrates and by testing the activity-substrate concentration relationship; for details, see a recent review [2].

For the erythrocyte and nerve acetylcholinesterases no characteristics so far tested have been found to separate them from each other. In certain animals, however, there occur esterases which have most of the characteristics of an acetylcholinesterase but which differ from the latter in certain respects. The blood of *Helix pomatia*, for instance, has a high acetylcholine splitting power [3–6]. The esterase responsible for this shows the same specificity pattern and a similar activity-substrate concentration relationship as the erythrocyte and nerve esterase [7], but there are certain differences (discussed below) which separate it from other known acetylcholinesterases. A similar situation holds for the esterase of cobra venom. This has a high acetylcholine splitting activity [8]. The venoms from various species do not always show this property [9]. Non-choline esters are hydrolysed by the cobra venom [10–16], the esterase of which shows the same specificity pattern as other acetylcholinesterases and gives the activity-substrate concentration relationship typical of these [7].

The main purpose of the present study is to compare some properties of the esterases of *Helix pomatia* blood and cobra venom (*Naja naja*). The first part deals with the enzymic hydrolysis of acetylcholine and the inhibition of this reaction by certain "anticholinesterases". In a second paper [19], published as a second part of this study, the hydrolysis of various choline and non-choline esters will be discussed.

METHODS

Experimental

The esterase activity was measured with the Warburg manometric technique previously described in detail [6, 17]. The enzyme activity is expressed in b_{30} values, *i. e.*, the amount of CO_2 in μl evolved during 30 minutes minus the corresponding value for non-enzymic hydrolysis. Measurements were made at 25° C. Substrates, enzymes, and inhibitors were dissolved in bicarbonate-Ringer's solution.

The edible snails, *Helix pomatia*, were collected in the early autumn. The blood was drawn from the circulus venosus and kept in the refrigerator. No change of activity was observed in 3 months.

A dried and crystallised preparation of cobra venom (*Naja naja*) was used. The venom was dissolved in bicarbonate-Ringer's solution. The solutions were stable when kept in the refrigerator for about 3 months. Fresh solutions, however, behaved differently from old ones. The solutions were made at least 4 hours before use, unless otherwise stated. The dried venoms from the Viperidae, *Crotalus horridus* and *Ancistrodon contortrix*, were used in a few preliminary experiments.

Acetylcholine chloride was used as substrate in all experiments. The inhibitors used were: choline chloride, methylene blue, physostigmine sulphate, prostigmine bromide, and tetraethyl pyrophosphate (TEPP) (pure), all dissolved in bicarbonate before use.

Graphical presentation of results

The results obtained in experiments with various inhibitor concentrations (I) and constant substrate concentration (S) have been analysed by the procedure worked out in a recent paper [6]. In the case of competitive inhibition and constant concentration of enzyme and substrate, a plot of the inhibition, expressed as v/v', against the concentration of inhibitor gives a straight line:

$$\frac{v}{v'} = 1 + [\mathrm{I}] \frac{K_\mathrm{S}}{K_\mathrm{I}\ ([\mathrm{S}] + K_\mathrm{S})}$$

The initial reaction velocities v and v' represent the enzyme activities in the absence of the inhibitor and in its presence respectively; both v and v' are expressed in b_{30} values. K_S and K_I are the dissociation constants of the enzyme-substrate and enzyme-inhibitor complexes respectively. This method has been applied recently in evaluating the action of choline [6] and other inhibitors [17, 18] on acetylcholine splitting enzymes.

The experimental results obtained by varying the substrate concentration keeping the inhibitor concentration constant have been recorded in activity-pS diagrams (pS is the negative logarithm of substrate concentration.)

RESULTS

Esterase activity

Helix blood. The blood of *Helix pomatia* is several times more active towards acetylcholine than the blood of vertebrates. Within certain limits there is a direct proportionality between blood concentration and rate of acetylcholine hydrolysis. This substate gives a bell-shaped activity-pS curve, characteristic of the acetylcholinesterases (Fig. 2). This relationship has been reported in detail in previous papers by the present author [5–7].

Snake venom. Zeller [9] showed that the venoms of the species of the Colubridae possess marked cholinesterase activity while the venoms of the species of the Viperidae show no such activity. The latter observation by Zeller was confirmed with the two viper venoms. These do not split acetylcholine even when the venom concentration is 100 times that used in the experiments with the cobra venom. It was also proved that the non-active venoms do not contain esterase inhibitors. The results are shown in Table 1.

Table 1. Comparison between the enzyme activities of three different snake venoms and mixtures of these.

Snake venom 0.1 mg of each per 2.00 ml reaction mixture	b_{30}
Naja naja	159.5
Crotalus horridus	0
Ancistrodon contortrix	0
N. naja + C. horridus	156.5
N. naja + A. contortrix	143.5

The observation has been made that the solutions of the cobra venom prepared immediately before use do not give the same activity as old solutions. A typical example is illustrated in Fig. 1. The difference between the hydrolysis curves for acetylcholine of a fresh solution and two old solutions (one week and four hours respectively) is distinct. With the fresh solution the hydrolysis rate increases during the reaction. No explanation for this induction period has been found; the enzyme may be absorbed on inert proteins and slowly go into solution.

The esterase activity of the cobra venom is comparatively very high. The b_{30} value obtained at optimum acetylcholine concentration and calculated

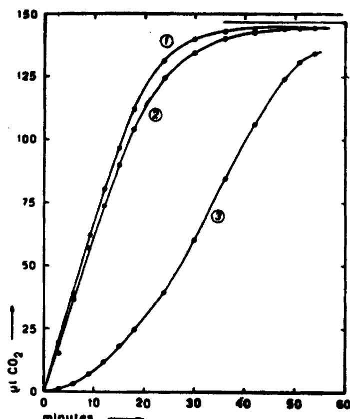

Fig. 1. Total hydrolysis of acetylcholine (3.30×10^{-3} M) by cobra venom dissolved in bicarbonate Ringer's solution. Venom concentration: 0.1 mg per 2.00 ml reaction mixture.

1. *One week old solution;*
2. *Four hours old solution;*
3. *Solution prepared immediately before filling of flask.*

for one mg dried venom is 1600—1800. This value is about the same as was found by Zeller [9]. When comparing this with the esterase activity of other material we have to remember that the natural venom contains about 70 % water, giving an activity for the native venom of 480—540. The approximate values for certain other highly active tissues under similar conditions are: horse plasma, 4; *Helix* blood, 6; the dart sac of *Helix*, 10; nucleus caudatus of human brain, 20; the electric organ of *Electrophorus*, 100. Snake venom is therefore the most active tissue known, particularly as venoms from other Colubridae species are still more active than that of the cobra [9]. The activities of the various solutions of cobra venom made at different times are not always the same, which might be explained by variations in the enzyme content of different crystals of the venom.

Direct proportionality between rate of acetylcholine hydrolysis and concentration of snake venom is obtained (Table 2) provided the enzyme concentration is not too high.

Most probably the cobra venom does not contain an ali-esterase. This will be discussed in a second paper [19].

Precipitation of the esterases by ammonium sulphate. Some preliminary experiments have been performed in precipitating the esterases with ammonium sulphate. Table 3 shows the results. In both cases the enzymes may be separated from non-active material by fractionation. The *Helix* blood enzyme

Table 2. Rate of enzymic hydrolysis of acetylcholine (1.10 × 10^{-2} M) as function of snake venom concentration.

mg cobra venom per 2.00 ml reaction mixture	b_{30}	b_{30}/0.1 mg
0.08	121.5	152
0.10	155	155
0.12	195	162.5
0.16	249	155.5
0.20	311.5	156
0.28	429	153.5
0.40	597.5	149.5

is precipitated maximally at about 60 % saturation, the snake venom esterase at a somewhat higher sulphate concentration (70 %). In both cases the enzymes can be absorbed completely onto infusorial earth from a solution of the sulphate precipitate. Bicarbonate solution brings about partial elution, but complete elution has so far proved impossible.

Esterase inhibition at various substrate concentrations

The inhibition of the enzymic hydrolysis of acetylcholine by choline chloride, methylene blue, physostigmine sulphate, prostigmine bromid, and TEPP is demonstrated in Fig. 2.

Table 3. Precipitation by ammonium sulphate. Helix blood diluted with distilled water of the same volume; the activity tested after dilution 1 to 10. Snake venom, 25 mg per 50 ml bicarbonate solution.

g ammonium sulphate per 10 ml enzyme solution	b_{30}			
	Helix blood		Cobra venom	
	Centrifugate	Precipitate	Centrifugate	Precipitate
—	193	—	294	—
2.00	190	3	291	no prec.
2.50	159	31	—	—
3.00	92	148	229	56.5
4.00	77	156	50	251
5.00	—	—	75	123

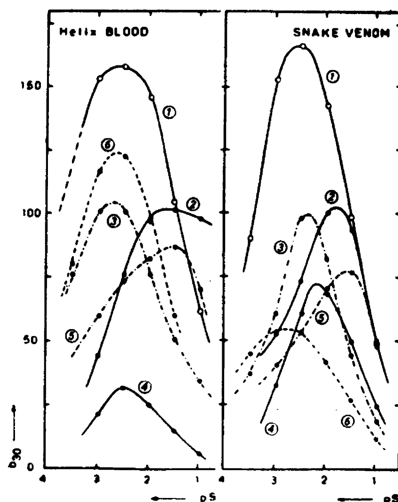

Fig. 2. Activity-pS curves for the enzymic hydrolysis of acetylcholine by Helix blood and snake venom respectively in the presence of certain inhibitors. Concentration of blood and venom per 2.00 ml reaction mixture: 25 μl blood and 0.1 mg venom respectively. The inhibitor concentration is the same in both series unless otherwise stated.

1. *Control (no inhibitor present);*
2. *Choline: 7.16 × 10^{-2} M;*
3. *Physostigmine: 3.08 × 10^{-9} M;*
4. *Prostigmine: 6.60 × 10^{-9} M, blood; 1.58 × 10^{-7} M, venom;*
5. *Methylene blue: 8.57 × 10^{-5} M, blood; 2.14 × 10^{-5} M, venom;*
6. *TEPP: 1.45 × 10^{-9} M, blood; 8.70 × 10^{-9} M, venom.*

Choline. In a recent paper [6] it has been shown that choline causes a shift to higher values of the optimum acetylcholine concentration for the esterases of erythrocytes and nerve tissues. This change of optimum substrate concentration is also observed for the snake venom esterase (Fig. 2, to the right). This shift of optimum is such that no inhibition is observed by 10^{-2} *M* choline when the acetylcholine concentration is higher that 3 x 10^{-2} *M*; at 3 x 10^{-3} *M* acetylcholine, however, the activity is inhibited 56 % by the same choline concentration.

A similar shift of optimum substrate concentration is observed with the *Helix* blood esterase. The action of choline, however, is such that at high acetylcholine concentration choline has a weak activating effect on the enzyme activity. An explanation for this may be that the blood contains a trace of a second cholinesterase (a butyro-cholinesterase) which is not inhibited by excess of acetylcholine.

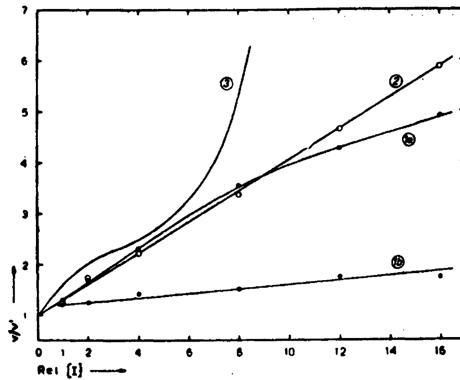

Fig. 3. Enzyme inhibition by choline chloride as function of inhibitor concentration. v = velocity in the absence, v' = in the presence of choline, expressed in μl CO_2 evolved in 30 minutes (b_{30}). Relative inhibitor concentration (Rel. [I]), 1 = 1.79 × 10^{-2} M choline. pI_{50} = negative logarithm of molar inhibitor concentration giving 50 % inhibition.

Enzyme	*Acetylcholine*	pI_{50}
1a. Helix blood	*3.30 × 10^{-3} M*	*1.27*
1b. » »	*3.30 × 10^{-2} M*	*(0.48)*
2. Cobra venom	*3.30 × 10^{-3} M*	*1.24*
3. Erythrocytes (human)	*1.10 × 10^{-3} M*	*1.47*

Methylene blue. This dye causes a shift of substrate optimum for both esterases (Fig. 2), as previously found for the erythrocyte esterase [17].

Physostigmine and prostigmine. Neither of these inhibitors shift the optimum acetylcholine concentration, except that at relatively high concentrations a slight change of substrate optimum is observed with physostigmine. This is in sharp contrast to the findings with the acetylcholinesterases of nerve tissues, erythrocytes, and electric tissue [6, 18].

TEPP. As expected, the inhibition by this irreversible inhibitor (inactivator) is independent of substrate concentration.

Esterase inhibition as a function of inhibitor concentration

The results of these experiments have been analysed by the graphical method described above. They are recorded in Figs. 3, 4, 6, and 7.

Choline. As described above, choline inhibits the *Helix* blood esterase in a way which differs from that for other acetylcholinesterases. At relatively low acetylcholine concentration (3 × 10^{-3} *M*) and a choline concentration

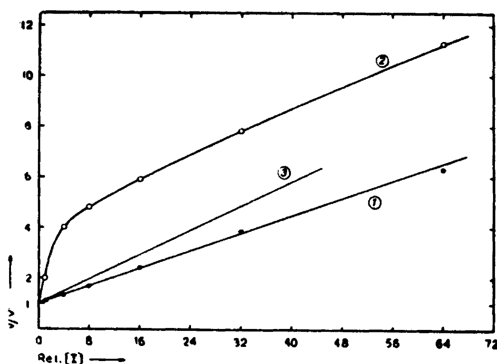

Fig. 4. Enzyme inhibition by methylene blue as function of inhibitor concentration. Acetylcholine concentration: 3.30×10^{-3} M. Rel. [I], 1 = 5.35×10^{-6} M. Description as in Fig.3.

Enzyme	pI_{50}
1. Helix blood	*4.19*
2. Cobra venom	*5.27*
3. Erythrocytes (human)	*4.36*

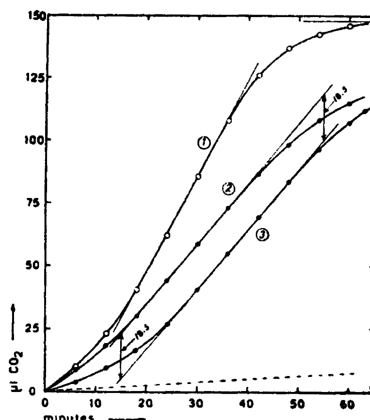

Fig. 5. The course of acetylcholine (3.30×10^{-3} M) hydrolysis by the action of cobra venom in the presence of prostigmine bromide (7.92×10^{-8} M). The parallelism between those parts of the inhibitory curves which represent equilibrium between enzyme, substrate, and inhibitor is illustrated. The dotted line refers to non-enzymic hydrolysis. Fresh solution of cobra venom.

1. *Control; b_{30} 110;*
2. *Prostigmine and acetylcholine simultaneously mixed with the enzyme; b_{30} 67.5;*
3. *Enzyme incubated 70 minutes with prostigmine before the addition of acetylcholine; b_{30} 67.5.*

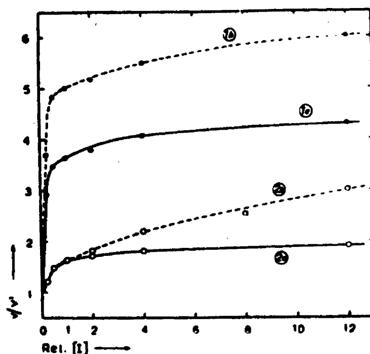

Fig. 6. Enzyme inhibition by physostigmine sulphate and prostigmine bromide as function of inhibitor concentration. Acetylcholine concentration: 3.30×10^{-3} M. Description as in Fig. 3.

Enzyme	*Inhibitor*	*Rel.* [*I*]
1a. Helix blood	*Physostigmine*	7.70×10^{-10} *M*
1b. » »	*Prostigmine*	1.65×10^{-9} *M*
2a. Cobra venom	*Physostigmine*	7.70×10^{-10} *M*
2b. » »	*Prostigmine*	1.98×10^{-8} *M*

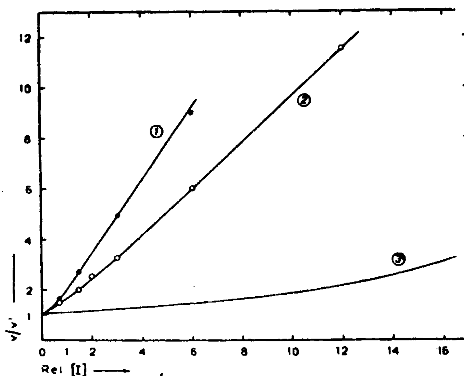

Fig. 7. Enzyme inhibition by TEPP as function of inhibitor concentration. Acetylcholine concentration: 3.30×10^{-3} M. Rel. [I], $1 = 2.9 \times 10^{-9}$ M. Description as in Fig. 3. Enzyme solutions incubated 60 minutes with TEPP before mixed with the substrate.

Enzyme	pI_{50}
1. Helix blood	*8.54*
2. Cobra venom	*8.36*
3. Erythrocytes (human)	*7.49*

not higher than 0.1 M a straight line is obtained in the graphical method, shown in Fig. 3. With increasing choline concentration the degree of inhibition does not increase in a way expected for a true competitive inhibition. When the substrate concentration is relatively high ($> 3 \times 10^{-2}$ M) the inhibition is no more of the true competitive type. This result might be due to the presence of a second enzyme, the nature of which is unknown. It may be a butyro-cholinesterase or/and an aliesterase present in low concentration. There is also a possibility that the active groups [20] of the enzyme molecule are different from those of other acetylcholinesterases.

Snake venom esterase is inhibited by choline in a competitive way. Using the same substrate concentration 50 % inhibition is attained at approximately the same choline concentration with both enzymes. In Fig. 3 the results obtained with human erythrocytes are inserted for comparison. The S-shaped curve in this case has been discussed in a previous paper [6].

Methylene blue inhibits the *Helix* blood enzyme in a truely competitive way (Fig. 4). The inhibition of the snake venom esterase seems to be more complicated. With increasing methylene blue concentration the degree of inhibition increases more rapidly at concentrations below 2×10^{-5} M than above. The results obtained with human erythrocytes have been inserted in Fig. 4 for comparison.

Physostigmine and prostigmine. It is important to investigate the time-course of inhibition not only for irreversible enzyme inactivators, but also for certain reversible inhibitors, *e. g.*, physostigmine and prostigmine. When an esterase is incubated with these inhibitors, equilibrium is not attained until about 25 minutes have elapsed; the exact time depends upon the inhibitor concentration used. If the inhibitor and substrate are simultaneously mixed with the enzyme, the period of time required for reaching the equilibrium is about the same as in the "incubation experiments". The initial reaction velocities, however, differ in the two types of experiments; after equilibrium the hydrolysis rates are the same for a given inhibitor concentration. This is illustrated with cobra venom in Fig. 5 for acetylcholine and prostigmine. It confirms the previous results obtained with the electric tissue esterase [18], human erythrocytes [21], and brain homogenates [22]. In Fig. 5 the reaction velocities at equilibrium are exactly the same. The importance of this observation is obvious. If one takes, for instance, the amount of CO_2 evolved in the first 20 minutes as the unit for enzyme activity a distorted picture is obtained. In all cases of this type of enzyme inhibition the complete hydrolysis curve must be analysed.

At constant substrate concentration the degree of esterase inhibition by physostigmine and prostigmine increases relatively rapidly up to a certain point and then continues to increase more slowly (Fig. 6). The relationship

between degree of inhibition and inhibitor concentration is different from that obtained with purified esterase preparations of electric tissue [18].

TEPP. As expected and in accordance with previous results obtained with other acetylcholinesterases, TEPP, an irreversible esterase inactivator, behaves differently from the reversible inhibitors described above. The curves in Fig. 7 show the effect on incubation with TEPP for one hour. The inhibition increases more rapidly with increasing inhibitor concentration than would be expected for a true competitive inhibitor. Both esterases studied seem to be more sensitive to the action of TEPP than are the esterases of electric tissue and erythrocytes.

When the esterases are incubated for about an hour with 10^{-8} *M* TEPP they are almost completely inactivated. When the esterases are mixed simultaneously with acetylcholine and TEPP, *i. e.*, no incubation, a much higher inhibitor concentration is necessary for enzyme destruction. Such results suggest that acetylcholine protects the enzyme against TEPP. Table 4 exam-

Table 4. Protection of the cobra venom acetylcholinesterase by acetylcholine (1.10×10^{-2} M) against the inactivation by TEPP (7.15×10^{-9} M).

	b_{30}
Control (no TEPP)	164.5
Enzyme incubated with TEPP 70 minutes before mixed with acetylcholine	9
Acetylcholine and TEPP simultaneously mixed with the enzyme	163

plifies the results obtained with cobra venom. The protective action of acetylcholine has recently been demonstrated with the electric tissue esterase [18] and the esterases of erythrocytes and brain [21, 22]. The various precautions which must be taken in evaluating the inhibition by irreversible "anticholinesterases", have also been discussed in a recent review [23].

Other esters, choline as well as non-choline, also show this protective action against TEPP inactivation. This will be demonstrated in the second paper [19]. In addition, choline seems to have the same effect although the protection is not as complete as with the other compounds tested. Table 5 shows the results obtained with *Helix* blood. When choline is added to the enzyme prior to TEPP, the hydrolysis rate is between those obtained when the enzyme is incubated with TEPP and choline respectively.

Table 5. Protection of the Helix blood acetylcholinesterase by choline against the inactivation by TEPP. Choline added prior to TEPP.

Choline M	TEPP M	b_{30}
—	—	166
—	7.15×10^{-9}	9
7.16×10^{-2}	—	87
7.16×10^{-2}	7.15×10^{-9}	57.5

A discussion of the results will be reported in connection with those described in the second paper [19].

SUMMARY

The properties of the esterases of *Helix pomatia* blood and cobra venom have been studied with acetylcholine as substrate. The results have been compared with those obtained with other esterases of the same type. Both enzymes are of the acetylcholinesterase type.

Some general characteristics of the acetylcholine hydrolysis by the action of cobra venom are reported. The venoms from two Viperidae species are inactive and do not contain esterase inhibitors. Fresh solutions of cobra venom do not show the same activity as old ones. Direct proportionality between acetylcholine hydrolysis and concentration of cobra venom is obtained.

Both the *Helix* blood and cobra venom esterases are precipitated by ammonium sulphate and can be thereby separated from non-active material.

The inhibition of the acetylcholine hydrolysis has been studied in detail with choline, methylene blue, physostigmine, prostigmine, and TEPP as inhibitors.

Choline and methylene blue cause a shift of the optimum acetylcholine concentration. For choline and *Helix* blood this shift is such that a small activation is observed at high acetylcholine concentration, probably due to the presence of a second esterase. Physostigmine and prostigmine do not give this shift in contradistinction to the results obtained with other acetylcholinesterases. The inactivation by TEPP is independent of substrate concentration.

The esterase inhibition as function of inhibitor concentration has been studied. In each case the results have been analysed graphically. In some cases the results differ from those obtained with other acetylcholinesterases. Generally, however, the degree of inhibition is of the same order of magnitude as for other acetylcholinesterases. The inhibition of both esterases by physo-

stigmine and prostigmine is a slow process. In the presence of these inhibitors the enzymic hydrolysis of acetylcholine attains an equilibrium which is the same whether or not the enzyme has been incubated with the inhibitor prior to the addition of acetylcholine. Acetylcholine and to a certain degree choline protects both enzymes against TEPP action. The latter compound is an irreversible enzyme inactivator.

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