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Comparison between the Acetylcholinesterases of *Helix* Blood and Cobra Venom. II. The Hydrolysis of Certain Choline and Non-Choline Esters

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The acetylcholinesterases (aceto-cholinesterases) of various sources (*e. g.*, nervous tissues, erythrocytes and electric tissue) split acetylcholine at a much higher rate than the butyryl ester which in some cases is not split at all. The other type of acetylcholine hydrolysing enzymes is the cholinesterases (butyro-cholinesterases [1]) of certain blood plasmata which split butyrylcholine at a higher rate than acetylcholine [2–4]. Both types of esterase split non-choline esters, the enzyme affinity of which is much lower than that of the choline esters; for details, see a recent review [5]. For the hydrolysis of non-choline esters the same relationship exists between hydrolysis rate and the acyl radical of the substrate, *i. e.*, the acetates bear much the same relationship to the acetylcholinesterase as do the butyrates to the plasma cholinesterase [6,1,7].

The main esterase of *Helix pomatia* blood belongs to the group of acetylcholinesterases [3,8,9]. It has the same specificity pattern, the same activity-acetylcholine concentration relationship and is inhibited by the same enzyme inhibitors as the other enzymes of this group. The *Helix* esterase also splits non-choline esters, *e. g.*, acetic acid esters of ordinary alcohols. In certain respects, however, it differs from the other acetylcholinesterases [10]. Similar properties have been found for the cobra venom esterase. Thus this enzyme splits, in addition to acetylcholine, other esters containing acetyl groups [11–14]. Moreover, it shows the same specificity pattern and gives the same characteristic activity-substrate concentrations relationship as the other acetylcholinesterases [3]. The specific anticholinesterases also inhibit the cobra venom esterase; this has been discussed in detail in Part I of this study [10].

In the present paper the enzymic hydrolysis of certain esters, choline esters as well as non-choline esters, will be discussed. The hydrolysis of triacetin and acetylsalicylcholine is reported in some detail.

METHODS

The esterase activity was measured by the Warburg manometric method [9] and is expressed in b_{30} values. The enzyme preparations have been described in the first paper of the present study [10]. The substrates used are listed in Table 1. They were dissolved in a bicarbonate Ringer's solution. As inhibitors, choline chloride and tetraethyl pyrophosphate (pure) were used.

Results were analysed graphically as described in the previous paper [10], and according to the procedure of Lineweaver and Burk [15].

RESULTS

Specificity

Helix blood. The hydrolysis of various esters, choline as well as ordinary esters, by *Helix* blood [3,8,9] gives essentially the same substrate pattern (Table 1) as found for the acetylcholinesterases of erythrocytes and nervous and elec-

Table 1. Enzymic hydrolysis of certain esters by the blood of Helix pomatia and the venom of Naja naja. Helix blood: 25 µl; cobra venom: 0.1 mg per 2.00 ml reaction mixture.

Substrate	Molarity × 100	*Helix* blood	Cobra venom
Acetylcholine chloride	1.10	159	187.5
Propionylcholine chloride	1.02	143	151
Butyrylcholine chloride	0.95	39.5	4.5
DL-Acetyl-β-methylcholine chloride	1.02	43	78
Benzoylcholine chloride	0.82	4.5	0
N-Acetyl-*p*-aminobenzoyl choline chloride *	0.66	0	0
Salicylcholine chloride *	0.72	0	3
Acetylsalicylcholine chloride	0.66	30.5	233
Acetylsalicylic acid (Na-salt)	1.11	12	5
Acetylaneurin chloride hydrochloride	0.53	45	68
Triacetin	9.20	79.5	84.5
Tributyrin	6.62	16	2
Ethyl acetate	22.7	4	4.5
Methyl *n*-butyrate	19.6	3	0

* Synthesised according to Euler *et al.* [17]

Table 2. Competition experiments. Acetylcholine (ACh), 1.10 × 10^{-2} M; butyrylcholine (BuCh), 9.50 × 10^{-3} M; acetylaneurin (AAn), 5.28 × 10^{-3} M; triacetin (TA), 9.20 × 10^{-2} M.

Substrate(s)	*Helix* blood	Cobra venom
ACh	159	187.5
BuCh	39.5	4.5
AAn	45	68
TA	79.5	84.5
ACh + BuCh	155	76.5
ACh + AAn	168.5	135.5
ACh + TA	134.5	117
BuCh + AAn	63.5	12.5
AAn + TA	63	86.5

tric tissues. Acetyl-β-methylcholine is split at a lower rate and gives an optimum at a higher concentration (pS_{opt} 1.35) than is given by acetylcholine (pS_{opt} 2.7). Benzoylcholine is hardly split at all. Propionylcholine is hydrolysed at a somewhat lower rate than acetylcholine, and butyrylcholine at a much lower rate. Among the non-choline esters the hydrolysis rate of acetylaneurin is especially noticeable. Tributyrin, methyl butyrate, and ethyl acetate are split at very low rates or not at all. The blood splits triacetin of high concentration at a higher rate than acetylcholine of the same molar concentration, a fact explained by the different activity-substrate concentrations relationships of the two substrates.

Interestingly acetylsalicylcholine is split at a relatively high rate, whereas salicylcholine is not split at all and acetylsalicylic acid is split at a relatively low rate. An explanation for this may be that the affinity of the acetylsalicylcholine for the enzyme has been changed relative to those of the two separate esters. Probably there is a mutual action of the two ester groups in acetylsalicylcholine. It has been found previously [9] in competitions experiments, at various substrate concentrations, that acetylcholine and acetylsalicylcholine are split by the same enzyme. Moreover, it has been shown that both ester linkages of acetylsalicylcholine are split by the *Helix* blood. It has now been demonstrated that the acetate link is the most rapidly split of the two. There is a possibility that a second enzyme hydrolyses the choline ester link. Preliminary experiments suggest that the hydrolysis of tributyrin is brought about by a separate esterase present in *Helix* blood in a comparatively low concentration.

Competition experiments with certain esters, the results of which are recorded in Table 2, indicate that the hydrolysis of acetylcholine, butyrylcholine, acetylaneurin, and triacetin by *Helix* blood is probably due to one and the same enzyme. In the case of butyrylcholine and eventually also acetylaneurin the possibility may be that a butyro-cholinesterase or/and an aliesterase in addition to the acetylcholinesterase participate in the enzymic hydrolysis.

Cobra venom. Like other acetylcholinesterase, the cobra venom esterase splits in addition to acetylcholine other acetyl esters, an observation first made by Bovet-Nitti [11]. Zeller and his co-workers [14] also found that this enzyme is not a "true" or "specific" cholinesterase. It has been proposed to call this enzyme an "acetylase" [11] or "acetylesterase" [14]. The substrate pattern is, however, much the same as that of acetylcholinesterases [3]. The enzymic hydrolysis of certain esters are shown in Table 1. Moreover, it will be demonstrated in the present paper that although the snake venom esterase differs in certain respects from the other acetylcholinesterases it has many properties in common with them, *e. g.*, inhibition by compounds generally considered to be specific anticholinesterases.

The substrate specificity of the cobra venom esterase is much the same as that of the *Helix* blood esterase (Table 1). The following points are noteworthy. The characteristic relationship between the hydrolysis rates of acetyl-, propionyl-, and butyrylcholine is still more pronounced than for the *Helix* blood esterase. Thus, butyrylcholine is hardly split at all by the cobra venom, in contradistinction to the *Helix* blood. There is no indication of the existence of an aliesterase or butyro-cholinesterase in cobra venom in addition to the acetylcholinesterase. Moreover, acetyl-β-methylcholine is split but not benzoylcholine, in accordance with Mendel's method [18] for the differentiation of the two types of cholinesterases. The characteristic difference between the activity-substrate concentration relationships of acetylcholine and triacetin hydrolysis has also been demonstrated for the cobra venom [3].

This esterase splits acetylaneurin at a relatively high rate (36 % of the acetylcholine rate, as compared with 28 % for *Helix* blood). This rate of hydrolysis is the highest ever obtained for this substrate with any acetylcholinesterase preparation.

To support the idea that only a single enzyme is responsible for the hydrolysis of various esters, some competition experiments have been carried out. The results are found in Table 2.

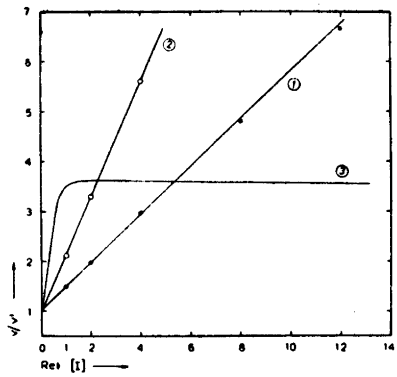

Fig. 1. Enzyme inhibition by choline chloride as function of inhibitor concentration. Substrate: 0.092 M *triacetin. v = velocity in the absence, v' = velocity in the presence of choline, expressed in μl CO_2 evolved in 30 minutes (b_{30}). Relative inhibitor concentration (Rel. [I]), 1 = 1.79 × 10^{-2} M. pI_{50} = negative logarithm of molar inhibitor concentration giving 50 % inhibition.*

Enzyme	pI_{50}
1. Helix blood	*1.45*
2. Cobra venom	*1.82*
3. Erythrocytes (human), aliesterase free	

The hydrolysis rate of acetylsalicylcholine is higher than for acetylcholine of high concentration. By contrast, the rates of hydrolysis of acetylsalicylic acid (*cf.* Zeller *et al.*[14]) and salicylcholine are only about 1/50 that of acetylcholine. As will be described later on, there is no evidence for the presence of a second esterase. It will be demonstrated that the acetate link alone of acetylsalicylcholine is split by the venom.

Hydrolysis of triacetin

Both esterases give the same relationship between triacetin concentration and hydrolysis rate. Triacetin of high concentration (> 0.1 *M*) is split at a higher rate than acetylcholine of the same high concentration. This non-choline ester is split by the acetylcholinesterase and not, even partly, by a second enzyme. Thus the enzymic hydrolysis of triacetin by this esterase is inhibited by choline (Fig. 1). The snake venom esterase seems to be inhibited more strongly than the *Helix* blood esterase. The I_{50} values obtained with 9.2 × 10^{-2} *M* triacetin are 1.52 × 10^{-2} and 3.58 × 10^{-2} *M* choline. The curves shown in Fig. 1 are typical of a competitive inhibitor [16], but the results

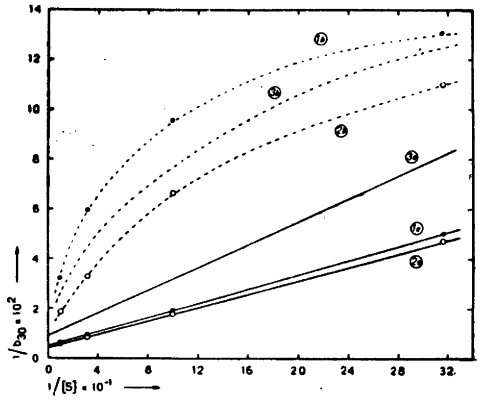

Fig. 2. Effect of choline chloride on the enzymic hydrolysis of triacetin as function of substrate concentration. Results analysed by the Lineweaver-Burk procedure. a) Controls; b) 3.58 × 10^{-2} M choline present.
1. Helix blood; 2. Cobra venom; 3. Erythrocytes (human), aliesterase free.

obtained in experiments with constant choline concentration and different substrate concentration and analysed by the Lineweaver-Burk procedure (Fig. 2) argue in favour of a more complicated mechanism. The inhibition of triacetin hydrolysis by choline may be due to steric hindrance. According to a recent theory of cholinesterase action, triacetin is attracted only to Centre II [19] (the esteratic site [20]) of the enzyme molecule, whereas choline is attracted to Centre I (the anionic site) by electrostatic attraction. The results in Fig. 2 are in accordance with this idea of the enzyme mechanism, for they show that the inhibition is not of the true competitive type.

The results obtained with an aliesterase free preparation of erythrocyte acetylcholinesterase are inserted in Figs. 1 and 2; they will be discussed in a paper to be published in due course.

Hydrolysis of acetylsalicylcholine

The enzymic hydrolysis of acetylsalicylcholine by *Helix* blood has recently been studied in detail [9]. The substrate optimum is much higher than for acetylcholine (pS_{opt} 1.35 and 2.7 respectively). Both ester linkages are split and the acetate linkage seems to be the more dapicly split. The choline ester link may be split by a butyro-cholinesterase.

As pointed out above acetylsalicylcholine is split at a high rate by the cobra venom. In Fig. 3 the total hydrolysis of this substrate of various concentrations is demonstrated. For the four lowest concentrations studied the hydrolysis stops when the calculated values for the hydrolysis of one

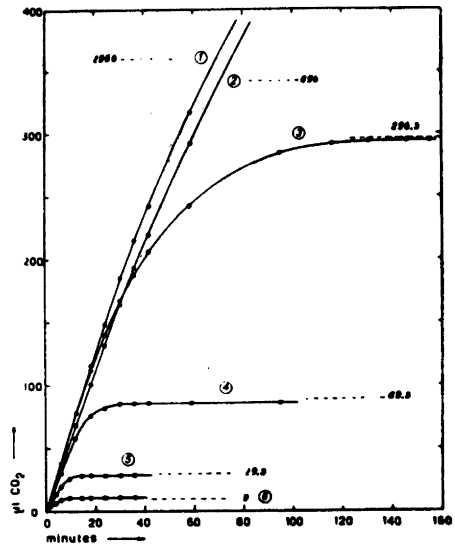

Fig. 3. Total (enzymic + spontaneous) hydrolysis of acetylsalicylcholine of various concentrations by cobra venom (0.1 mg per 2.00 ml reaction mixture). Dotted lines and numbers in µl CO_2 refer to calculated values for total hydrolysis of one ester linkage. Molar substrate concentrations:

1	*2*	*3*	*4*	*5*	*6*
6.62×10^{-2}	1.99×10^{-2}	6.62×10^{-3}	1.99×10^{-3}	6.62×10^{-4}	1.99×10^{-4}

ester linkage are reached. This link is the acetate link. Since after complete hydrolysis, the solution gives a violet colour with ferric chloride which is characteristic of a free phenolic —OH group and which is also obtained with salicylcholine, but not with acetylsalicylic acid (as sodium salt). The presence of the choline link has rendered the acetate link in acetylsalicylcholine more susceptible of enzymic hydrolysis than that in acetylsalicylic acid.

The substrate optimum for acetylsalicylcholine hydrolysis by cobra venom is somewhat higher (pS_{opt} 2.0) than for the corresponding acetylcholine hydrolysis (pS_{opt} 2.5). The hydrolysis rate is definitely depressed by high concentrations of acetylsalicylcholine (Fig. 4). Choline inhibits the hydrolysis and the results are similar to those obtained with acetylcholine. Hence, choline causes a shift of optimum acetylsalicylcholine concentration to higher concentration. At substrate concentrations lower than 10^{-2} *M*, choline inhibits the enzymic hydrolysis of acetylsalicylcholine in a true competitive way. The result suggest that both the choline part and the acetate link of this ester is attracted to the enzyme, in the same way as for acetylcholine.

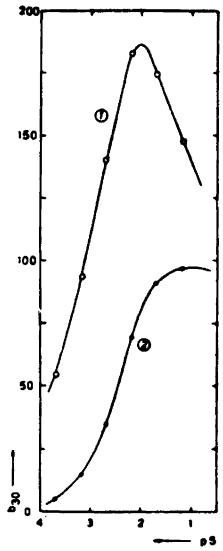

Fig. 4. Activity-pS curves for the enzymic hydrolysis of acetylsalicylcholine by cobra venom in the absence (1) and presence (2) of choline chloride (1.43 × 10^{-2} M).

Experiments with tetraethyl pyrophosphate (TEPP)

TEPP inhibits the enzymic hydrolysis of triacetin by human erythrocytes [19]. Similar results have been obtained with the esterases of *Helix* blood and cobra venom respectively. The concentration of TEPP which gives 50 % inhibition of the triacetin hydrolysis (cobra venom) is 1.73 × 10^{-9} *M*; the corresponding value for the acetylcholine hydrolysis is 4.35 × 10^{-9} *M*. It has been demonstrated in experiments similar to those described previously with acetylcholine [10] that triacetin protects the enzyme against TEPP (Table 4). Choline also protects, using either acetylcholine [10] or triacetin as substrates. The results obtained with triacetin and *Helix* blood are tabulated in Table 3. The enzyme was incubated with choline prior to the addition of TEPP and

Table 3. Protection of the Helix blood acetylcholinesterase by choline against the inactivation by TEPP tested with triacetin as substrate (0.092 M). Choline added prior to TEPP.

Choline *M*	TEPP *M*	b_{30}
—	—	178.5
—	7.15 × 10^{-9}	15.5
7.16 × 10^{-2}	—	79
7.16 × 10^{-2}	7.15 × 10^{-9}	59.5

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

Substrate	Acetyl-salicyl-choline	Acetyl-aneurin	Tri-acetin	Tri-propionin
Molarity × 100	0.66	0.53	9.20	7.69
Control (no TEPP)	156.5	53	80	13
Enzyme incubated with TEPP 70 minutes before mixed with acetylcholine	3	2	12.5	3
Substrate and TEPP simultaneously mixed with the enzyme	153	45.5	66.5	11

the hydrolysis rate compared with controls. It should be pointed out that the protection is not as good as that obtained in experiments with erythrocyte haemolysate [19].

In addition to triacetin, other esters protect acetylcholinesterases from inactivation by TEPP. Table 4 illustrates some results with acetylsalicylcholine and acetylaneurin and cobra venom. Tripropionin also seems to protect against TEPP, although the hydrolysis values are comparatively low.

A series of other compounds have been shown to protect the cobra venom esterase against TEPP action (to be published). Amongst others butyrylcholine is a protector. In addition to being an esterase inhibitor this choline ester is a TEPP protector. The b_{30} values obtained in one experiment with acetylcholine are as follows: control (acetylcholine alone), 188; incubation with TEPP, 3.5; incubation with butyrylcholine, 153; incubation with butyrylcholine prior to TEPP, 97.5. This confirms a very recent observation made by Cohen *et al.* [21] with brain acetylcholinesterase and diisopropyl fluorophosphate (the publication appeared during the preparation of this paper).

DISCUSSION

The fact that enzymes are proteins makes it inadvisable to expect complete identity between similar enzymes from different sources. The terms "species specificity" and "organ specificity" are as applicable to enzymes as to proteins in general. Proteins are classed together in groups with certain characteristics. This is also appropriate to many enzymes. The characteristics of the acetylcholine splitting enzymes so far tested have made it possible to

classify them into two types. Both types can easily be separated from the aliesterase, lipases, phosphatases, and other enzymes capable of hydrolysing ester linkages of various kinds, but future work will certainly demonstrate that within each group there exist enzymes which in certain respects differ from each other. This is already established for the phosphatases. It is a question only how far the experimenter can go in the refinement of his technique in order to trace these differences.

Certainly there exist many acetylcholinesterases [22] (aceto-cholinesterases) and the second class of cholinesterases (butyro-cholinesterases) also contains esterases which differ from each other in one or another respect [23]. The general properties of the esterases of *Helix* blood and cobra venom are those of acetylcholinesterase. In certain respects, however, they differ from the enzymes of nerve tissues, electric tissue, and erythrocytes. For instance, differences have been observed in the mechanism of action of certain esterase inhibitors.

It is still far too early to suggest that the physiological function of all these esterases is the same. For instance, what is the function of the esterase in cobra venom? For the time being there is no answer. Is there any connection between the toxic effect of cobra venom and its high acetylcholine splitting activity? Can this activity explain the different pharmacological effects of the highly esterase active venoms from Colubridae species and the non-active venoms from Viperidae species? Is the cobra venom a natural solution of the esterase coming from the glands in which it is produced? Can the solution of these problems help to solve the general problem of the function of the choline ester splitting enzymes. In itself this latter problem is part of the whole problem of the function of acetylcholine.

SUMMARY

The enzymic hydrolysis of certain choline esters and non-choline esters has been studied using the blood of *Helix* blood and cobra venom.

The substrate patterns for both esterases are similar to those of acetylcholinesterases from other sources. Acetylaneurin is split in both cases at a comparatively high rate, in contradistinction to other esterases of the same type.

The cobra venom contains only an acetylcholinesterase and is free from aliesterase activity. The presence of a butyro-cholinesterase or/and an aliesterase in addition to the acetylcholinesterase in the *Helix* blood is suggested.

Triacetin is hydrolysed by both the acetylcholinesterases. This hydrolysis is inhibited by choline, the mechanism of action of which is supposed to be a steric hindrance at the active Group II of the enzyme molecule.

Acetylsalicylcholine is hydrolysed at a relatively high rate, although both salicylcholine and acetysalicylic acid are split at a very low rate or not at all. With the cobra venom esterase the acetate link alone is split. This hydrolysis is inhibited by choline.

Tetraethyl pyrophosphate (TEPP) inhibits irreversibly the enzymic hydrolysis of non-choline esters. These esters protect the enzyme from inactivation by TEPP.

The plurality of the esterases splitting acetylcholine has been discussed.

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