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The Hydrolysis of Non-Choline Esters by Acetylcholine-Esterase from Human Erythrocytes

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It is now well established that the esterase which has been regarded as specific for choline esters also splits non-choline esters. This new approach to the specificity problem of the acetylcholine-splitting enzymes was initiated by the observation of Bodansky [1], who found that triacetin is hydrolysed by the acetylcholine-esterases of brain and erythrocytes. The esterase of certain snake venoms, the properties of which are similar to those of the acetylcholine-esterase of mammalian nervous tissue and erythrocytes, was shown by Bovet-Nitti [2] to hydrolyse a number of aliphatic esters, in particular acetyl esters. Augustinsson [3] has demonstrated that the acetyl-ester linkage in acetylsalicylcholine and acetylsalicylic acid itself are split by the brain and erythrocyte esterases. The splitting of acetylsalicylic acid has recently been confirmed by Zeller *et al.*[4] with erythrocytes and snake venom. A most careful study of the substrate specificity of these enzymes has been made by Adams and Whittaker [5,6]. The carbon analogue of acetylcholine, 3,3-dimethyl butyl acetate, is the most rapidly split next to acetylcholine itself. It was stated by these authors that in general the more closely the alcohol group simulates the choline configuration, the more rapidly is the ester hydrolysed. However, Zeller *et al.*[4] have shown that the acetylcholine-esterases of human erythrocytes and certain snake venoms catalyse the hydrolysis of the acetates of very different hydroxyl derivatives. Therefore, Zeller has considered his "e-Type" cholinesterase (equivalent to acetylcholine-esterase used in this paper) to be an "acetylesterase".

A study of the enzymic hydrolysis of triacetin by Blaschko and Holton [7] and Augustinsson [8] confirmed the assumption that the acetylcholine-esterases present in brain (caudate nucleus), electric organ, erythrocytes, and snake venom are responsible for the hydrolysis of this ester. The observation of

Bodansky [1] that human brain splits triacetin even more rapidly than acetylcholine was explained by Augustinsson [8]. He showed that there is a great difference between the activity-substrate concentration relationships for these two substrates.

The main purpose of the present investigation is to give further information about the hydrolysis by the acetylcholine-esterase of non-choline esters, exemplified by acetylsalicylcholine, acetylsalicylic acid, and triacetin.

METHODS

The Warburg manometric method was used for the determination of the esterase activity [3]. The enzyme activity is expressed in terms of the amount of CO_2 in μl ($= b_{30}$), evolved during 30 minutes minus the corresponding value for non-enzymic hydrolysis. Measurements were made at 25° C.

The enzyme preparation was obtained by washing human erythrocytes (the blood was taken up in heparin) four times with 0.9 per cent sodium chloride solution and then haemolysing them with distilled water to form a solution of the original blood volume. This haemolysate was subsequently diluted with distilled water to give a suitable activity for each substrate used.

The following substrates were employed:

Substrate	Abbreviation	Mol. wt.	
Acetylcholine chloride	ACh	181.66	Hoffmann-La Roche & Co.
Acetylsalicylcholine chloride	ASaCh	301.77	LKB, Stockholm (prepared according to Euler *et al.* [9])
Acetylsalicylic acid	ASa	180.15	Bofors AB
Triacetin	TA	218.20	Fisher Scientific Co.

The acetylsalicylic acid was neutralised with 0.1 *M* NaOH and then used immediately after neutralisation.

HYDROLYSIS OF ACETYLSALICYLCHOLINE (ASaCh)

Total hydrolysis at various substrate concentrations

The author [3] has recently found that ASaCh gives a familiar dissociation curve when the enzyme activity is plotted against the logarithm of molar substrate concentration. This is in sharp contrast to the bell-shaped curve obtained with acetylcholine. It has been suggested that at high ASaCh concentrations only the acetyl-salicylic linkage is split, the breakdown of the choline-ester linkage occurring no more than it does in the case of high con-

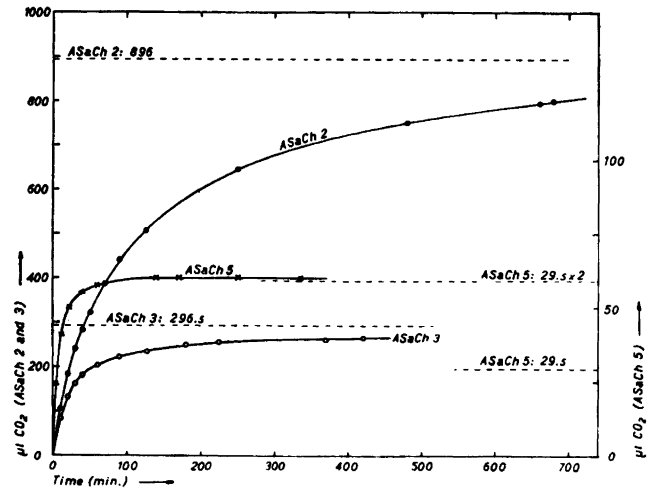

Fig. 1. Total (enzymic + spontaneous) hydrolysis of acetylsalicylcholine of various concentrations. Total volume of the reaction mixture 2.00 ml. Key to the various substrate concentrations in Table 1, which also gives the values found experimentally for spontaneous hydrolysis (30 minutes) are found.

centrations of acetylcholine. At low substrate concentrations, on the other hand, both ester linkages of ASaCh are supposed to split at the same time. Support of this hypothesis has now been obtained by the following experiments.

The hydrolysis of ASaCh was allowed to proceed for a time period long enough to give complete destruction of the substrate. Fig. 1 shows the results obtained with various substrate concentrations. It is seen that at high substrate concentrations (1.99×10^{-2} *M* and 6.62×10^{-3} *M*; ASaCh 2 and 3 respectively) only one of the ester linkages is split enzymically. At low substrate concentration (6.62×10^{-4} *M*; ASaCh 5), on the other hand, both ester linkages are broken, because the hydrolysis proceeds till the amount of CO_2 liberated corresponds to the calculated value for the breakdown of two ester linkages. Both linkages also are split when the ASaCh concentration is 1.99×10^{-3} *M* (ASaCh 4; not shown in the figure). After 1345 minutes, 155 μl CO_2 was evolved; the calculated value for complete hydrolysis of one of the linkages is 89.5 μl.

From these experiments we conclude that when the ASaCh concentration is below 1.99×10^{-3} *M* both ester linkages of this substrate are split. When the concentration is above that value only one of the linkages is broken. This substrate concentration is approximately the same as has been shown to be the optimum for the enzymic hydrolysis of acetylcholine by this enzyme

(2—3 $\times$ 10^{-3} M). We know that high concentrations of acetylcholine inhibit the enzymic hydrolysis. It is now suggested that a similar situation is valid for the hydrolysis of ASaCh, *i. e.*, at high ASaCh concentration the hydrolysis is retarded when only the hydrolysis of the choline-ester linkage is concerned. Therefore, at very high ASaCh concentrations only the acetyl-salicylic linkage is broken. Further support for this hypothesis has been obtained in experiments when choline was used as an enzyme inhibitor. Thus, it has been demonstrated [3] that at high substrate concentrations the inhibition by choline is much less than expected in a competitive inhibition. At low and medium ASaCh concentrations the inhibition is considerable or of the same order of magnitude as has been observed for the hydrolysis of acetylcholine.

The hypothesis put forward by Murray and Haldane [10] to explain the bell-shaped curve obtained by plotting the enzyme activity against the logarithm of molar acetylcholine concentration is based on the assumption of *two* active centres (in the following termed centre I and II) of the enzyme molecule. This proposal is well supported by the results described above and by those which will be discussed in the following.

Competition Experiments

A preliminary report on competition experiments carried out with mixtures of substrates and with esterases from various sources has recently been published by the present author [3]. A theory applicable to such experiments has been worked out by Whittaker and Adams [11]. The experimental results of the present investigations are summarised in Table 1.

Let us first consider the hydrolysis of mixtures of acetylcholine and ASaCh. In the presence of high concentrations of both substrates the rate of hydrolysis is between the rate of hydrolysis of each substrate measured separately, *i. e.*, lower than the ASaCh hydrolysis and higher than the acetylcholine hydrolysis. Hence, there is a competition between these two substrates. When the initial substrate concentrations are lower, the rate of hydrolysis of the substrate mixture becomes higher than each of the separate rates. At these concentrations also there is a competition between the two substrates, *i. e.*, acetylcholine and ASaCh compete for the same active groups of the enzyme molecule. The mechanisms of the two reactions, however, are not the same at high and low substrate concentrations. This is further evidence in support of the hypothesis put forward above for the enzymic hydrolysis of the two ester linkages of ASaCh.

Table I also shows that acetylsalicylic acid (ASa) is split by erythrocyte haemolysate, as demonstrated previously [3,4]. In the experiments with the

Table 1. Total (enzymic + spontaneous) hydrolysis of acetylsalicylcholine and acetyl- in molarity according

Time (min.)	μl CO_2											
	1				2				3			
	ACh	ASaCh	ASa	ACh + ASaCh	ACh	ASaCh	ACh + ASaCh	ASa + ASaCh	ACh	ASaCh	ASa	ACh ASa
6	6.5	24.5	5.5	23	12.5	24.5	19	10.5	19	26	2	2
12	16	45.5	11	41	26.5	44	33	28	39	38.5	4.5	4
18	23	63.5	17	55	39	60.5	42.5	42	60	54	7	6
24	31.5	79	21.5	67	52	75	53.5	53	80	66.5	8.5	8
30	38	91	26	77.5	65	88	62.5	63.5	98.5	77.5	10	9
36	45.5	105	30.5	88	77	101	72.5	74	114	88	12	11
42	—	117	—	100	—	111.5	84	85	—	99	—	12
88		200		166		185.5	154	152		148		21
100		—		181		199.5	173	169		158		24
130		—		—		234.5	213	203		181		29
163		310		268		269	257	237		199		34
181		330		278		286	282	253		210		37
237		—		—		332	353	—		—		44
Autohydrolysis μl CO_2 per 30 min.	9.5	13.5	16	23	6	8	14	20.5	4.5	4.5	9	
Calculated values for complete hydrolysis	4 928	2 966		7 894	1 479	896	2 375		493	296.5		79

Soln. no.	1	2	3	4	5
ACh chloride	1.10×10^{-1}	3.30×10^{-2}	1.10×10^{-2}	3.30×10^{-3}	1.10×10^{-3}
ASaCh chloride	6.62×10^{-2}	1.99×10^{-2}	6.62×10^{-3}	1.99×10^{-3}	6.62×10^{-4}
ASa (as Na salt)	1.11×10^{-1}	3.33×10^{-2}	1.11×10^{-2}	3.33×10^{-3}	1.11×10^{-3}

These values refer also to those of the substrate mixtures. Enzyme concentration: 0.013 ml haemolysate per ml reaction mixture (total volume 2.00 ml). The calculated values (for ASaCh) at the bottom of the table refer to the hydrolysis of one ester linkage.

mixtures of acetylcholine and ASa it is seen that even here competition occurs. This also happens when ASa and ASaCh are present at the same time.

The affinity constants for each substrate, using the formula introduced by Whittaker and Adams, have not been determined, mainly because it has been difficult to evaluate

alicylic acid and mixtures of these compounds with acetylcholine. Substrate concentrations the following:

μl CO_2

		4						5					
+ a	ASa + ASaCh	ACh	ASaCh	ASa	ACh + ASaCh	ACh+ ASa	ASa + ASaCh	ACh	ASaCh	ASa	ACh + ASaCh	ACh+ ASa	ASa + ASaCh
)	10	23.5	23	2	25	15	11	23	15	1	30.5	23.5	10
)	26	47.5	37.5	2	46	38.5	24	43	24	1	53	46.5	21
'	37	71	48.5	4	66.5	59.5	32	52	32	3	68.5	58	27
'	47	90	56.5	4.5	84	79	38	54.5	36	4	77.5	63.5	32
.5	58.5	108	63	5	101.5	95	43	56	38	5	85	65.5	36
:	64	122.5	68	6.5	115	110	49	56	42.5	6	89	67.5	38
	72	—	72.5	—	131	—	53	—	45	—	93	—	40
	123		93		183		71		57		108		49
	132		96		189		74		60		109		50
	150		102		201		80		64		111		—
	170		103		211		82		65		113		
	184		104		215		—		65		114		
	—		105		223				—		—		
.5	13.5	4	3.5	4	7.5	8	7.5	3	2.5	4	5.5	7	6.5
		148	89.5		237.5			49.5	29.5		79		

the initial rate of hydrolysis for acetylsalicylcholine and acetylsalicylic acid (the velocity falls off rapidly with time).

HYDROLYSIS OF TRIACETIN (TA)

Acetylcholine-esterases from various sources have been shown recently to split triacetin. This was demonstrated in competition experiments by Blaschko and Holton [7] using dog brain and cobra venom. With the purified electric tissue esterase (*Electrophorus*) the present author has given a similar evidence [8].

In order to get further information about the mechanism of action of the acetylcholine-esterase of human erythrocytes, competition experiments have

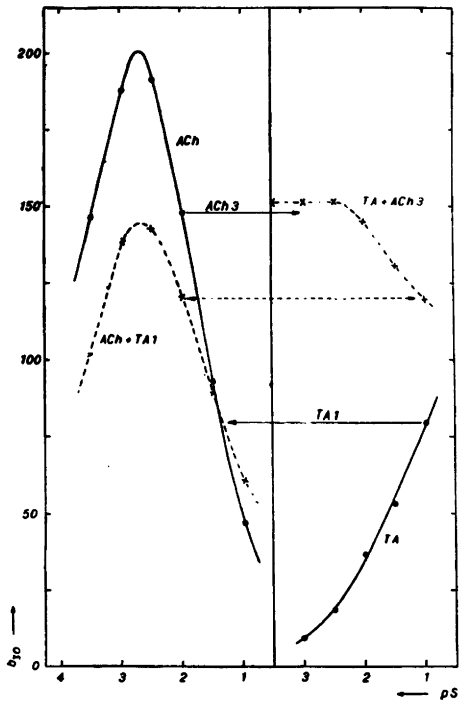

Fig. 2. Activity-pS curves for the enzymic hydrolysis of acetylcholine (ACh) and triacetin (TA) and mixtures of these esters by the human erythrocyte acetylcholine-esterase.

been carried out at various substrate concentrations. The results are shown in Fig. 2. Triacetin competes with acetylcholine both at low and high acetylcholine concentrations. The experimental results also demonstrate that triacetin of increasing concentrations decreases the enzymic hydrolysis of 1.10×10^{-2} *M* acetylcholine.

As pointed out above and according to the tentative model for acetylcholine esterase introduced by Zeller and Bissegger [10] there are two active centres in the enzyme molecule. One of them (centre I) is a negatively charged group which combines with the positive nitrogen atom of the choline ester, probably by an electrostatic attraction. This active enzyme centre is characteristic of the acetylcholine-esterase; cf. the discussion of Adams and Whittaker [11]. The second active centre (centre II) is supposed to attract the acyl group of the ester. Characteristic of centre II is its ability to split acetates at a higher rate than other esters. This is the reason why triacetin is split.

Further studies by the present author [12] have demonstrated that choline inhibits the enzymic hydrolysis of triacetin by this enzyme. This inhibition is not competitive. The inhibition is independent of the triacetin concentration. With increasing choline concentration the degree of inhibition increases up to

0.25 *M* choline; a still higher choline concentration does not further change the hydrolysis rate of triacetin.

As long as we consider only the centre II it is quite justifiable to speak about an acetylase or acetylesterase rather than a choline-esterase, as proposed by Bovet-Nitti [2] and Zeller *et al.* respectively [4]. This centre controls the specificity of the enzyme for the acyl portion of the substrate. We are indebted to the work of Adams and Whittaker for the observation that only a relatively small variation is possible on that part of the ester. The observation, especially that made by Zeller *et al.*, that the alcoholic part can be varied considerably without any appreciable loss of enzyme activity is not too surprising.

We must remember, however, that it is the centre I that is really characteristic of this esterase. No esters other than those of choline and probably those of the phosphorus and arsenic analogues of choline have yet been discovered which give the characteristic activity-substrate concentration relationship of acetylcholine-esterase. Centre I — in combination with centre II — is responsible for this. The distance between these two centres is probably about the same as that between the nitrogen atom (of choline) and the carbonyl group (of a choline ester), *i. e.*, 5.8 Å. As far as we know there exists in the living body only one compound which consists of a positively charged atom, a chain of two carbon atoms, and an alcoholic group esterified with acetic acid. That compound is acetylcholine. Most probably, it is the physiological substrate of the enzyme which therefore is called acetylcholine-esterase [13].

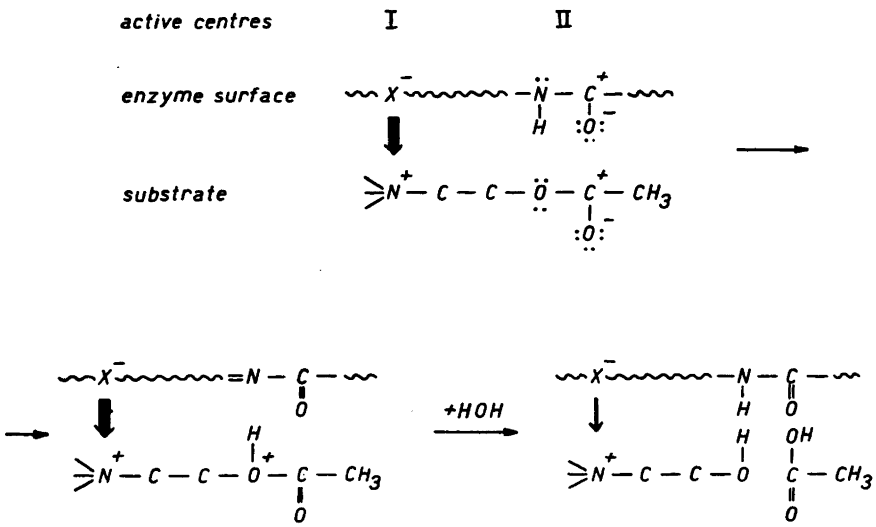

Fig. 3. Tentative model for the mechanism of enzyme action.

SUMMARY

The enzymic hydrolysis of acetylsalicylcholine, acetylsalicylic acid, and triacetin by acetylcholine-esterase has been studied using human erythrocytes as an enzyme source.

Acetylsalicylcholine at high concentration does not inhibit the enzyme. Both ester linkages of this substrate are split at low concentrations, while at high concentration the choline ester linkage only is split. In competition experiments it has been demonstrated that both acetylsalicylcholine and acetylsalicylic acid compete with acetylcholine for the active groups of the enzyme molecule. This occurs both at high and low substrate concentration.

Triacetin and acetylcholine compete for one of the two active groups (centre II) of the enzyme.

Proof has been given for the existence of two active centres of the acetylcholine-esterase. Centre I carries the active group which combines with the positively charged nitrogen atom of choline, and centre II the group which combines with the acyl (acetyl) group of the ester.

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