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THE AFFINITY OF ATROPINE-LIKE ESTERS FOR ESTERASES

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In a recent paper from this department observations on a number of esters with atropine-like actions were reported (Ing, Dawes, and Wajda, 1945). The substances studied included the choline esters of benzilic, tropic, and atrolactinic acid, as well as esters of benzilic acid in which choline was replaced by other basic alcohols. In the experiments to be described we have investigated the behaviour of these substances towards esterases, and in particular their affinity for the cholinesterases, by testing whether or not they inhibited the hydrolysis of esters known to be substrates of these enzymes. In addition, we have examined two other esters of interest to the pharmacologist: (a) cocaine, and (b) the diethylaminoethyl ester of *cyclo*hexyl-phenylacetic acid (trasentin 6H).

Mammalian tissues contain a number of different enzymes which will hydrolyse acetylcholine and other choline esters (Alles and Hawes, 1940; Richter and Croft, 1942; Mendel and Rudney, 1943; Zeller and Bissegger, 1943; Nachmansohn and Rothenberg, 1945). Mendel and Rudney have shown that two different types of cholinesterase can be characterized by using acetylcholine and benzoylcholine as substrates; they distinguish the "true" cholinesterase from the "pseudo"-cholinesterase. The former will hydrolyse acetylcholine only, the latter both acetylcholine and benzoylcholine. We have found Mendel's nomenclature useful in describing our results and it has therefore been employed in this paper. In addition to these two types of enzymes, preparations have been described which will hydrolyse benzoylcholine, but not acetylcholine. The guinea-pig liver (Sawyer, 1945) and the ox kidney (Gunter, 1946) each contain an enzyme of this kind.

Rabbit serum contains an enzyme, tropinesterase, which hydrolyses atropine, and it was naturally of interest to study the behaviour of the synthetic atropine substitutes towards this enzyme. Tropinesterase does not occur in all rabbits; its presence is genetically determined (Sawin and Glick, 1943).

A few observations were made on an enzyme which hydrolyses tropacocaine in horse serum. The occurrence of this enzyme, first reported by Glick and Glaubach (1941), was confirmed.

The tissues and substrates used are listed in Table I and the substances tested in Table II.

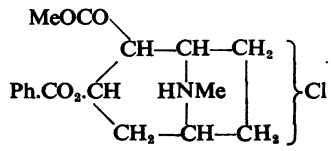
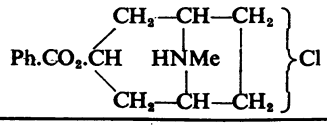
TABLE I

LIST OF TISSUES AND SUBSTRATES USED

Species	Tissue	Amount of tissue used	Substrate	Concentration
Dog ..	Caudate nucleus	5–10 mg.	Acetylcholine bromide	$6 \times 10^{-3}M$
Horse ..	Serum	0.2 ml.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Guinea-pig	Liver	12.5–25 mg.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Ox	Kidney	1.0–1.3 g.	Benzoylcholine chloride	$6 \times 10^{-3}M$
Rabbit* ..	Serum*	0.15 ml.	Atropine sulphate	1 g./100 ml.
Horse ..	Serum	0.3 ml.	Tropacocaine hydrochloride	$10^{-2}M$

* tropinesterase-positive.

TABLE II

	Substance	Structure
Quaternary bases	Lachesine (E3) or benzilyloxyethyldimethylethylammonium chloride	$Ph_2.C(OH).CO_2.CH_2.CH_2.NMe_2Et\}Cl$
	Benzilylcholine chloride (C1)	$Ph_2.C(OH).CO_2.CH_2.CH_2.NMe_3\}Cl$
	Tropylcholine chloride	$Ph.CH(CH_2OH).CO_2.CH_2.CH_2.NMe_3\}Cl$
	Atrolactylcholine chloride	$Ph.CMe(OH).CO_2.CH_2.CH_2.NMe_3\}Cl$
Tertiary bases	Dimethylaminoethyl benzilate hydrochloride (C4)	$Ph_2.C(OH).CO_2.CH_2.CH_2.NMe_2H\}Cl$
	Diethylaminoethyl benzilate hydrochloride (E1)	$Ph_2.C(OH).CO_2.CH_2.CH_2.NEt_2H\}Cl$
	Trasentin 6H or diethylaminoethyl phenyl*cyclo*hexylacetate hydrochloride	$Ph(C_6H_{11})C(OH).CO_2.CH_2.CH_2.NEt_2H\}Cl$
	Cocaine hydrochloride	
	Tropacocaine hydrochloride	

Serial numbers in parentheses are taken from Ing *et al.* (1945).

MATERIALS AND METHODS

Enzyme extracts were prepared by grinding the appropriate organs with sand; Krebs's Ringer-bicarbonate was used as the medium. No sand was added in preparing extracts of caudate nucleus, a suspension of the thoroughly ground tissue being used. No attempt was made to purify the enzyme preparations used, and it should be borne in mind that impurities may have influenced the affinities of the substances tested in this and the following paper.

Non-enzymic hydrolysis during the experiments occurred to a very small extent with the quaternary ammonium bases, but benzilic esters with a tertiary basic group showed a larger spontaneous hydrolysis which gave rise to a small blank. Trasentin 6H gave a slight precipitate with the Ringer solution, and its actual concentration is therefore somewhat uncertain.

The hydrolysis was followed manometrically; the experiments were carried out at a temperature of 38° with a gas mixture containing 95 per cent N_2 and 5 per cent CO_2.

RESULTS

(1) *Hydrolysis of acetylcholine by dogs' caudate nucleus* (Table III).—Preparations of the caudate nucleus contain a powerful cholinesterase (Nachmansohn, 1937). This enzyme is a true cholinesterase (Mendel and Rudney, 1943).

Not one of the esters examined was hydrolysed by this preparation. We also tested their affinity for the enzyme by measuring their effect on the rate of hydrolysis of acetylcholine. They had very little action. The strongest effect was found with cocaine, which, in a concentration equimolecular to that of acetylcholine, caused an inhibition of 50 per cent. With trasentin 6H the inhibition was less, and none of the benzilic esters had any inhibitory activity in the concentration used.

TABLE III

EXTRACT OF THE CAUDATE NUCLEUS OF THE DOG

Hydrolysis of acetylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	0
Benzilylcholine (C1)	$6 \times 10^{-3}M$	0
Tropylcholine	$6 \times 10^{-3}M$	0
C4	$6 \times 10^{-3}M$	0
E1	$6 \times 10^{-3}M$	0*
Trasentin 6H	$6 \times 10^{-3}M$	15
Cocaine	$6 \times 10^{-3}M$	50

* This experiment was carried out with an extract of rabbit's basal ganglia.

(2) *Hydrolysis of benzoylcholine by horse serum* (Table IV).—Horse serum contains an active pseudo-cholinesterase, and in order to exclude the action of true cholinesterase we used benzoylcholine as substrate. The same sample of serum was used in all these experiments.

Horse serum hydrolysed atrolactylcholine; these observations are described separately in section (6). None of the other esters was hydrolysed, but they all had an affinity for the horse serum esterase: all the substances tested inhibited the enzyme, and with cocaine and trasentin the percentage inhibitions were higher than for true cholinesterase.

TABLE IV

HORSE SERUM

Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	71
Benzilylcholine (C1)	$6 \times 10^{-3}M$	33
Tropylcholine	$6 \times 10^{-3}M$	43
Trasentin 6H	$6 \times 10^{-3}M$	81
Cocaine	$6 \times 10^{-3}M$	85

(3) *Hydrolysis of benzoylcholine in guinea-pig liver* (Table V).—This preparation contains no pseudo-cholinesterase (Blaschko, Chou, and Wajda, 1947); the hydrolysis of benzoylcholine is solely due to the benzoylcholinesterase described by Sawyer (1945). Whether or not benzoylcholine is the normal substrate of the enzyme in the living animal is unknown.

All the esters examined acted as inhibitors of the enzyme (see Table V) and the percentage inhibitions were higher than for either true or pseudo-cholinesterase. Not one of the esters was hydrolysed by the enzyme, with the possible

TABLE V

GUINEA-PIG'S LIVER EXTRACT

Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	94
" "	$6 \times 10^{-4}M$	91
" "	$6 \times 10^{-5}M$	66
C4	$6 \times 10^{-3}M$	100
E1	$6 \times 10^{-3}M$	100
Trasentin 6H	$1.2 \times 10^{-3}M$	97
" "	$1.2 \times 10^{-4}M$	88
" "	$1.2 \times 10^{-5}M$	80
" "	$1.2 \times 10^{-6}M$	45
Cocaine	$6 \times 10^{-3}M$	100
"	$6 \times 10^{-4}M$	92
"	$6 \times 10^{-5}M$	44

exception of trasentin 6H, where with the lowest concentration used ($1.2 \times 10^{-6}M$) the percentage inhibitions during the first 15 min. period were consistently higher than in the second.

(4) *Hydrolysis of benzoylcholine in ox kidney* (Table VI).—The benzoylcholinesterase of ox kidney described by Gunter (1946) has not yet been fully analysed. The enzymic activity of the tissue extracts is very much less than that of the extracts of guinea-pig liver, and it was therefore necessary to use much larger amounts of tissue (see Table I).

Our results with this preparation showed a striking difference from those with the guinea-pig liver extract: the esters examined had little or no effect on the rate of hydrolysis of benzoylcholine.

TABLE VI

OX KIDNEY EXTRACT

Hydrolysis of benzoylcholine ($6 \times 10^{-3}M$)

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$6 \times 10^{-3}M$	12
Benzilylcholine (C1)	$6 \times 10^{-3}M$	19
C4	$6 \times 10^{-3}M$	0 (approx.)
E1	$6 \times 10^{-3}M$	29 (approx.)
Trasentin 6H	$10^{-3}M$	0
Cocaine	$10^{-3}M$	14

(5) *Hydrolysis of atropine in rabbit serum* (Table VII).—All experiments were carried out with the sera of two animals which were found to contain tropinesterase; they were among a group of about a dozen animals tested for the presence of the enzyme. Not one of the substances included in Table VII showed any enzymic hydrolysis. Cocaine was not included, as it was found to be hydrolysed enzymically; we confirmed Glick and Glaubach's (1941) observation that this hydrolysis also occurs in the serum of animals without tropinesterase.

The benzilic esters had no inhibitory action on tropinesterase (see Table VII); of all the substances examined only trasentin 6H had a slight inhibitory effect.

TABLE VII

RABBIT'S SERUM

Hydrolysis of atropine sulphate (1 g./100 ml.) by tropinesterase

Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$10^{-2}M$	0
Benzilylcholine (C1)	$10^{-2}M$	0
Atrolactylcholine	$6 \times 10^{-3}M$	0
Trasentin 6H	$10^{-3}M$	60

(6) *Enzymic hydrolysis of atrolactylcholine in horse serum.*—None of the synthetic esters examined showed any detectable enzymic hydrolysis with the exception of the choline ester of atrolactinic acid in horse serum. Only small amounts of the substance were at our disposal, but the following facts were established: the ester showed an appreciable hydrolysis in Ringer solution, but the rate of liberation of carbon dioxide was consistently higher in the presence of horse serum. That this hydrolysis in excess of the blanks was due to an enzyme is supported by the following observations:

(i) the hydrolysis increased with increasing amounts of serum; in one experiment the additional amount of CO_2 liberated in 15 min. by 0.2 ml. of horse serum was 9.5 μl., in another experiment with 0.4 ml. of serum it was 19 μl. (the figures for spontaneous hydrolysis in the blanks were 7.5 and 9.5 μl. respectively);

(ii) the additional hydrolysis was abolished by boiling the serum; and

(iii) it was reduced in the presence of $1.8 \times 10^{-5}M$ eserine.

In addition the following facts were established: there was no enzymic hydrolysis of atrolactylcholine in rabbit serum (0.15 ml. per flask) and in dog's caudate nucleus extract (equivalent of 40 mg. fresh weight of tissue per flask).

These results strongly suggest that atrolactylcholine is a substrate of pseudo-cholinesterase. Mendel *et al.* (1943) have shown that the serum of the horse has about 5 times as much pseudo-cholinesterase activity as that of the rabbit. Moreover, the eserine inhibition of the hydrolysis of both benzoyl- and atrolactyl-cholines were of the same order: in the presence of $1.8 \times 10^{-5}M$ eserine the hydrolysis of $6 \times 10^{-3}M$ benzoylcholine was reduced, the CO_2 output falling from 102 to 38.5 μl. in 15 min., and for atrolactylcholine, used in the same concentration, the corresponding figures were 15 and 9 μl. CO_2 respectively. It was also shown that atrolactylcholine had an affinity to pseudo-cholinesterase, as the hydrolysis of benzoylcholine was reduced in the presence of the atrolactinic ester. The amounts of carbon dioxide liberated by 0.2 ml. of horse serum in 15 min. were:

with $6 \times 10^{-3}M$ benzoylcholine—117 μl.
with $6 \times 10^{-3}M$ atrolactylcholine—9.5 μl.
with both esters—58.5 μl.

(7) *Hydrolysis of tropacocaine in horse serum* (Table VIII).—None of the substances examined had any affinity for the enzyme in the concentrations used

TABLE VIII

HORSE SERUM

Hydrolysis of tropacocaine ($10^{-3}M$)

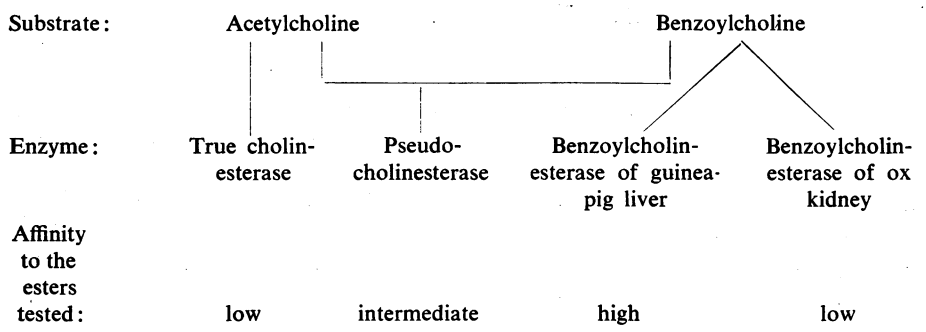
Substance added	Concentration	Percentage inhibition
Lachesine (E3)	$10^{-3}M$	0
Benzilylcholine (C1)	$10^{-3}M$	0
Cocaine	$10^{-3}M$	0
Atropine	$10^{-3}M$	0

in our experiments. The enzyme responsible for the hydrolysis of tropacocaine must therefore be distinct from pseudo-cholinesterase, as this enzyme is inhibited by some of the substances listed in Table VIII, e.g., cocaine, lachesine, and benzilylcholine.

Discussion

Our observations provide an example of the usefulness of inhibitors for distinguishing related enzymes. All the enzymes studied were esterases and yet great differences in their affinities to the esters tested were found. The degree of inhibition is determined not by the substrate used, which may or may not be common to two enzymes, but by the specific affinities of the enzyme itself. With acetylcholine as substrate this was first clearly demonstrated in experiments by Zeller (1942), who found that the human serum cholinesterase was much more sensitive to *iso*propyl-antipyrin than the cholinesterase of the central nervous system.

Our results can be summarized in the following scheme:



All the substances tested had little affinity to true cholinesterase, more to pseudo-cholinesterase, and the highest affinity to the benzoylcholinesterase of guinea-pig liver; the benzoylcholinesterase of ox kidney was little or not at all inhibited. The activity of the ox kidney was very low and the amount of tissue used had therefore to be very much greater than with any of the other preparations (Table I). The possibility that the small inhibitor action in this case is due to a reaction of the esters with some other constituents in the ox kidney cannot therefore be excluded, but it seems more likely that the benzoylcholinesterase of ox kidney differs from the corresponding enzyme in the liver of the guinea-pig.

We have not found any enzymic hydrolysis of lachesine or any of the other esters of benzilic acid tested as atropine substitutes by Ing *et al.* (1945). It is interesting that these substances which must have a great affinity for the tissue receptors on which atropine acts have little or no affinity for the enzyme tropinesterase.

Our experiments on atrolactylcholine increase the number of choline esters known to be hydrolysed in animal tissue ; it seems interesting that the chemically closely related esters of tropic and benzilic acids were not hydrolysed.

Cocaine, an ester of benzoic acid, has a much higher affinity for pseudo-cholinesterase, which hydrolyses the benzoic ester of choline, than for the true esterase which does not (see also Nachmansohn and Schneemann, 1945). Cocaine also inhibits the benzoylcholinesterase of guinea-pig's liver, but neither the benzoylcholinesterase of ox kidney nor the tropacocainesterase of horse serum was inhibited by cocaine ; this shows that affinity for cocaine is not a general property of esterases which hydrolyse esters of benzoic acid.

SUMMARY

1. The action of cocaine, benzilylcholine, lachesine, and a number of related atropine-like esters on cholinesterases and on tropinesterase has been studied.

2. These substances were found to have little affinity to the " true " cholinesterase of brain tissue, more affinity to the " pseudo "-cholinesterase of horse serum, and a high affinity to the benzoylcholinesterase of guinea-pig's liver. They had little or no effect on the hydrolysis of benzoylcholine by ox kidney extracts.

3. They had little or no affinity to the tropinesterase of rabbit's serum.

4. Evidence is given which suggests that atrolactylcholine is hydrolysed by pseudo-cholinesterase of horse serum.

5. The tropacocainesterase of horse serum was not inhibited by these substances, which shows that it is not identical with pseudo-cholinesterase.

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