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THE ESTERASES OF GUINEA PIG LIVER HYDROLYSING LOCAL ANAESTHETIC ESTERS

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Abstract—The local anaesthetic esters Novesine, Nesacaine and Ophthaine are rapidly hydrolysed by guinea pig liver homogenates at rates greater than the rate for benzoylcholine. Procaine is hydrolysed at a much lower rate. The enzyme hydrolysing benzoylcholine in guinea pig liver is known to have a low affinity for its 'substrate'[10] and experiments with the competitive inhibitors Cinchocaine and Chlorpromazine suggest that its affinity for the local anaesthetics studied is considerably greater. Inhibition experiments using the organophosphates Dimethyl Coroxon and Haloxon show that the enzyme hydrolysing BzCh is mainly responsible for local anaesthetic ester hydrolysis but indicate the presence of two other local anaesthetic esterases.

INTRODUCTION

THERE has been much work on the hydrolysis of local anaesthetic esters by human plasma and serum since the original investigation of procainesterase by Kisch, Koster and Strauss.[1] Kalow[2, 3] has presented evidence that procaine hydrolysis in human plasma is due to cholinesterase and has stressed the structural similarity of procaine and benzoylcholine. There is a considerable variation in the rate of hydrolysis of different local anaesthetic esters by human serum.[4, 5] Becker[5] has suggested the existence of several cholinesterases in human plasma having variable activity in regard to the hydrolysis of ester type local anaesthetics.

The hydrolysis of procaine by the plasma of a variety of laboratory and domestic animals has been investigated by Aven, Light and Foldes.[6] These workers found that, in contrast to human plasma, the hydrolysis of procaine by the plasma of other mammals appears to be very slow, even though acetylcholine and benzoylcholine may be hydrolysed at comparable or greater rates as in the guinea pig and horse.[7]

Little evidence is available about the relative hydrolysis rates of different ester type local anaesthetics by animal tissues, but recently it has been shown that in many species liver esterases appear to be more active in the metabolism of local anaesthetics than the plasma enzymes.[8]

Guinea pig liver homogenates rapidly hydrolyse benzoylcholine but show little activity towards acetylcholine.[7, 9–11] It was, therefore, thought to be of interest to determine whether the "benzoylcholinesterase" of guinea-pig liver was the enzyme responsible for the rapid hydrolysis of local anaesthetic esters previously observed.[8]

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EXPERIMENTAL

Materials

Adult guinea pigs of either sex were used. Diet 41, greenstuff and tap water were allowed *ad libitum*. All animals were killed by a blow on the head and bled out after decapitation.

Livers were removed from bled animals and homogenized with distilled water in an all glass apparatus, 1 ml of homogenate corresponding to 100 mg of fresh tissue.

Unless otherwise stated liver homogenates were tested for esterase activity within 2 hr after slaughter.

Estimation of esterase activity

The measurement of esterase activity in liver homogenates was performed at 37° in the presence of 0·067 M phosphate buffer at pH 7·2, by the modified method of Hestrin[12] previously described.[8] Results are expressed as μmole of ester hydrolysed/g fresh tissue/hr.

Inhibition experiments

In all experiments the time of inhibition was 15 min and the temperature 37°. The stated concentration of inhibitor was the concentration during incubation before the addition of substrate. The effect of different inhibitor concentrations was observed at least eight times on one homogenate and the results confirmed by duplicate experiments on at least three other homogenates. The results are expressed as residual activity, percent of original, this being the ratio of esterase activity in the presence of inhibitor to esterase activity of a control containing the uninhibited enzyme preparation, expressed as a percentage.

Substrates

1. Acetylcholine perchlorate (ACh) (British Drug Houses Ltd.)
2. *n*-Butyrylcholine chloride (BuCh) (British Drug Houses Ltd.)
3. Benzoylcholine chloride (BzCh) (British Drug Houses Ltd.)
4. 2-Diethylaminoethyl-4-aminobenzoate (Procaine) (British Drug Houses Ltd.)
5. 2-Diethylaminoethyl-2-chloro-4-aminobenzoate (Nesacaine). (A gift from Strasenburgh Laboratories, Rochester, N.Y.)
6. 2-Diethylaminoethyl-3-amino-4-propoxybenzoate (Ophthaine). (A gift from E. R. Squibb & Sons, Liverpool and London)
7. 2-Diethylaminoethyl-3-butoxy-4-aminobenzoate (Novesine). (A gift from A. Wander Ltd., King's Langley, Herts.)

Substrates 4–7 were used as the hydrochlorides.

Inhibitors

1. Diethyl 4-nitrophenyl phosphate (Paraoxon) (Koch-Light Laboratories Ltd.)
2. Diethyl 3-chloro-4-methylcoumarin-7-yl phosphate (Coroxon). (A gift from Cooper Technical Bureau, Berkhamsted, Herts., U.K.)
3. Di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate (Haloxon). (A gift from Cooper Technical Bureau, Berkhamsted, Herts., U.K.)
4. Dimethyl 3-chloro-4-methylcoumarin-7-yl phosphate (Dimethyl Coroxon). (A gift from Cooper Technical Bureau, Berkhamsted, Herts., U.K.)

5. Physostigmine salicylate (British Drug Houses Ltd.)
6. 2-Butoxy-*N*-(2-diethylaminoethyl) cinchoninamide hydrochloride (Cinchocaine) (Ward Blenkinsop & Co. Ltd.)
7. Chlorpromazine hydrochloride (Largactil) (May & Baker Ltd.)

All inhibitors were dissolved in distilled water where this was possible. Inhibitors 2, 3 and 4 were dissolved in a minimal quantity of acetone before dilution with distilled water.

RESULTS AND DISCUSSION

(a) *Hydrolysis of esters by guinea-pig liver homogenates*

The local anaesthetic esters Novesine, Nesacaine and Ophthaine were rapidly hydrolysed by guinea-pig liver at rates considerably in excess of the rate for Procaine

TABLE 1. HYDROLYSIS OF ESTERS BY GUINEA-PIG LIVER HOMOGENATES

	Hydrolytic activity (μ mole/g/hr)		
Ester	*Mean ± S.E.	(n)	Range
Nesacaine	949 ± 45·8	(14)	710–1156
Ophthaine	456 ± 41·0	(11)	272–716
Novesine	789 ± 75·7	(13)	456–1303
Procaine	93 ± 4·8	(38)	45–151
BzCh	423 ± 44·4	(17)	115–698
BuCh	231 ± 28·1	(13)	80–396
ACh	12 ± 2·3	(12)	0–24

* Mean hydrolytic activity ± S.E.M. of liver homogenates obtained from (n) animals.

(Table 1). Although the animals were bled thoroughly before removal of the liver the possibility exists that part of the observed activity towards acetylcholine, benzoylcholine and butyrylcholine was due to residual blood. Because of the relatively very low hydrolytic activity of guinea-pig plasma towards the local anaesthetic esters[8] this possibility does not arise for Procaine, Ophthaine, Nesacaine and Novesine.

Acetylcholine was hydrolysed at a very low rate. An homogenate from one animal which hydrolysed all the other esters at rates approximating to the mean values showed no detectable activity towards acetylcholine. No correlation was found for different homogenates between the hydrolysis rate for Procaine and the rate for acetylcholine. A correlation existed between the hydrolysis rate for Procaine and the rates for the other local anaesthetic esters: Novesine ($r = 0{\cdot}9703$, $P < 0{\cdot}001$), Nesacaine ($r = 0{\cdot}9572$, $P < 0{\cdot}001$), and Ophthaine ($r = 0{\cdot}9517$, $P < 0{\cdot}001$). There was also correlation between the procaine hydrolysis rates and the rates for benzoylcholine ($r = 0{\cdot}6986$, $P < 0{\cdot}01$) and butyrylcholine ($r = 0{\cdot}7330$, $P < 0{\cdot}01$). The existence of correlation is evidence of a constant relationship between the hydrolysis rates of the various substrates in liver homogenates from different animals. It seems unlikely that the enzyme hydrolysing acetylcholine is responsible for any local anaesthetic hydrolysis in guinea-pig liver.

Statistical examination of the results provided no evidence for a sex difference in the hydrolysis of the esters.

(b) *The effect of storage of liver homogenates at 3°–5° on the hydrolysis of the esters*

It was consistently observed that storage of the homogenates at 3°–5° resulted in a loss of activity towards benzoylcholine and butyrylcholine, whereas the hydrolysis of Novesine, Nesacaine, Ophthaine and Procaine was little affected (Table 2). This

TABLE 2. THE EFFECT OF STORAGE OF LIVER HOMOGENATES AT 3°–5° ON THE HYDROLYSIS OF ESTERS

Ester	Period of storage (hr)			
	0	24	120	144
	Residual activity (%)			
Procaine	100	99	97	96
BzCh	100	66	39	27
BuCh	100	70	33	25
Novesine	100	102	96	96
Ophthaine	100	100	98	97
Nesacaine	100	100	99	97

observation alone does not necessarily indicate the existence of two different protein species involved in the hydrolysis of the substrates. It may indicate that different groups on the enzyme molecule are involved in the formation of the enzyme substrate complex in either case.

(c) *The effect of physostigmine on the hydrolysis of esters by guinea pig liver homogenates*

The pattern of inhibition was similar for all of the esters studied, benzoylcholine hydrolysis being rather less sensitive and butyrylcholine hydrolysis more sensitive than local anaesthetic ester hydrolysis to the action of the inhibitor (Table 3).

TABLE 3. THE EFFECT OF PHYSOSTIGMINE SALICYLATE ON THE HYDROLYSIS OF ESTERS BY GUINEA-PIG LIVER HOMOGENATES

Ester	Physostigmine concentration (μM)*						
	2	4	6	8	10	40	100
	Residual activity (%)						
Novesine	100	76	65	62	52	20	0
Ophthaine	100	75	64	60	51	24	0
Nesacaine	100	76	63	60	53	24	0
Procaine	100	78	67	64	55	23	0
BzCh	100	86	76	67	61	20	0
BuCh	75	60	—	49	—	—	—

* Liver homogenates were incubated at 37° for 15 min with the stated concentration of physostigmine before the addition of the substrate.

(d) *The effect of Chlorpromazine on the hydrolysis of the esters*

Chlorpromazine is known to inhibit human plasma cholinesterase.[13] Hydrolysis of all the esters was inhibited, to different extents, by Chlorpromazine (Table 4). Within the group of local anaesthetic esters procaine hydrolysis was somewhat less sensitive

to Chlorpromazine inhibition than was the hydrolysis of Ophthaine, Nesacaine and Novesine. Butyrylcholine hydrolysis and, to a lesser extent, benzoylcholine hydrolysis was significantly inhibited at Chlorpromazine concentrations which had no effect on local anaesthetic ester hydrolysis.

TABLE 4. THE EFFECT OF CHLORPROMAZINE ON THE HYDROLYSIS OF ESTERS BY GUINEA-PIG LIVER HOMOGENATES

Ester	Chlorpromazine concentration (μM)*							
	1	10	40	80	100	200	300	400
	Residual activity (%)							
Novesine	—	—	100	—	67	53	43	37
Ophthaine	—	—	100	—	72	55	45	38
Nesacaine	—	—	100	—	76	54	45	36
Procaine	—	—	100	—	86	72	60	50
BzCh	—	92	67	46	15	—	—	—
BuCh	96	72	55	38	10	—	—	—

* Liver homogenates were incubated at 37° for 15 min with the stated concentration of chlorpromazine before the addition of the substrate.

(e) *The effect of Cinchocaine on the hydrolysis of the esters*

Benzoylcholine hydrolysis was completely inhibited by 10 μM Cinchocaine whereas the concentration required for complete inhibition of local anaesthetic ester hydrolysis was in excess of 800 μM (Table 5). Butyrylcholine hydrolysis was somewhat more sensitive to the inhibitor than the hydrolysis of the local anaesthetics.

TABLE 5. THE EFFECT OF CINCHOCAINE ON THE HYDROLYSIS OF ESTERS BY GUINEA-PIG LIVER HOMOGENATES

Ester	Cinchocaine concentration (μM)*						
	0·1	1	2	10	100	400	800
	Residual activity (%)						
Novesine	—	—	—	96	78	52	30
Ophthaine	—	—	—	95	70	56	40
Nesacaine	—	—	—	97	75	58	32
Procaine	—	—	—	96	67	45	30
BzCh	84	40	27	0	—	—	—
BuCh	—	—	92	66	26	5	—

* Liver homogenates were incubated at 37° for 15 min with the stated concentration of Cinchocaine before the addition of the substrate.

Cinchocaine inhibits the enzymic hydrolysis of procaine and other local anaesthetic esters by human serum cholinesterase and this inhibition is thought to be competitive.[14] If Cinchocaine acts mainly by competitive inhibition, then the difference in the inhibition patterns of benzoylcholine and local anaesthetic hydrolysis seen in the present study (Table 5) may be due to differing relative affinities of the substrates and Cinchocaine for a single enzyme, and not to the presence of more than one enzyme.

(f) *Inhibition of esterase activity by organophosphates*

The inhibitory activity of organophosphates on mammalian enzymes possessing carboxylic esterase activity has been fully described and reviewed.[15, 16]

The final inhibitor concentration required to reduce the enzymic hydrolysis of the esters by 50 per cent (I_{50}) shows relatively little variation between the different substrates for a given inhibitor (Table 6). Esterase activity was most strongly inhibited

TABLE 6. INHIBITION OF THE ENZYMIC HYDROLYSIS OF CHOLINE AND LOCAL ANAESTHETIC ESTERS BY ORGANOPHOSPHORUS COMPOUNDS

Ester	Inhibitors			
	Haloxon	Dimethyl coroxon	Coroxon	Paraoxon
	I_{50}. (μM)*			
BzCh	8·7	1·5	0·12	0·35
BuCh	6·9	1·0	0·12	0·35
Novesine	10·0	4·2	0·09	0·18
Ophthaine	4·6	1·4	0·06	0·26
Procaine	4·1	4·8	0·07	0·14
Nesacaine	7·4	8·9	0·06	0·26

* Liver homogenates were incubated at 37° for 15 min with a range of concentrations of the inhibitors before the addition of substrate. The concentration of inhibitor required to produce 50 per cent inhibition of enzymic hydrolysis (I_{50}) was determined graphically.

by Coroxon and least strongly by Haloxon: the concentration of Haloxon required to produce 50 per cent inhibition of ester hydrolysis being 58–120 times the requisite concentration of Coroxon.

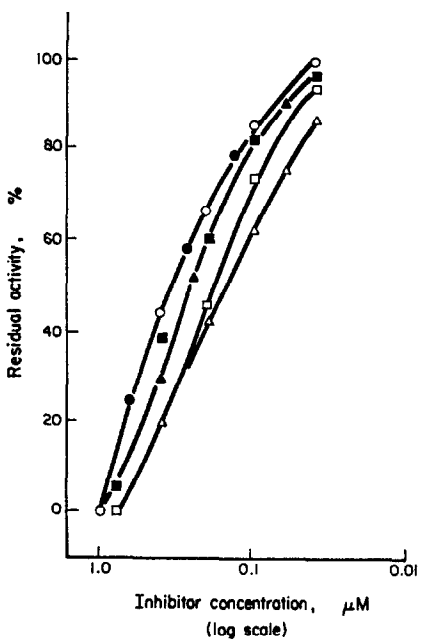

FIG. 1. Inhibition of ester hydrolysis by Paraoxon. ○ = BzCh; ● = BuCh; □ = Novesine; ■ = Ophthaine; △ = Procaine; ▲ = Nesacaine.

In experiments with the diethyl phosphates Paraoxon and Coroxon the variation of residual activity with inhibitor concentration followed a similar course for benzoylcholine, butyrylcholine and the local anaesthetic esters (Figs 1 and 2).

The curves for benzoylcholine and butyrylcholine were identical in each case. The hydrolysis of the choline esters was less strongly inhibited than the hydrolysis of the local anaesthetics at a given concentration of either Coroxon or Paraoxon.

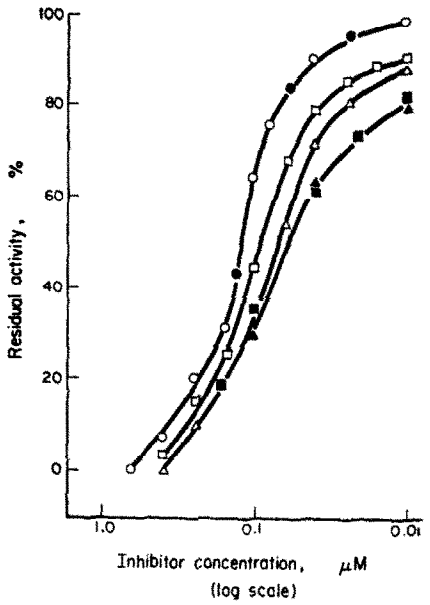

FIG. 2. Inhibition of ester hydrolysis by Coroxon. ○ = BzCh; ● = BuCh; ⊡ = Novesine; ■ = Ophthaine; △ = Procaine; ▲ = Nesacaine.

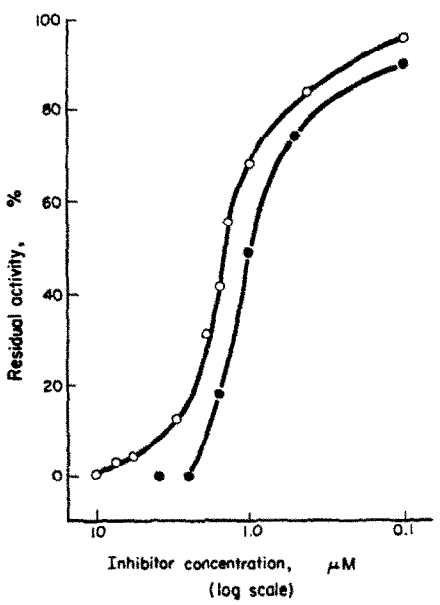

FIG. 3. Inhibition of choline ester hydrolysis by Dimethyl Coroxon. ○ = BzCh; ● = BuCh.

Dimethyl Coroxon inhibited the hydrolysis of the choline and local anaesthetic esters in a similar way up to an inhibitor concentration of 1 μM (Figs 3–5). Increasing

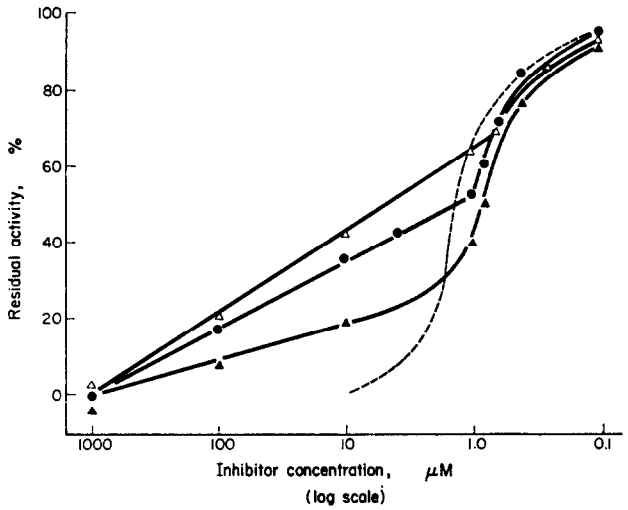

FIG. 4. Inhibition of local anaesthetic ester hydrolysis by Dimethyl Coroxon. ● = Ophthaine; △ = Procaine; ▲ = Nesacaine; — — — = BzCh (cf. Fig. 3).

the inhibitor concentration, in the range 1–10 μM, caused a steep fall in the residual enzymic activity to zero for benzoylcholine and butyrylcholine (Fig. 3). At a final concentration of 10 μM Dimethyl Coroxon the hydrolysis of the local anaesthetic esters, expressed as a percentage of the rate in the absence of the inhibitor, was Nesacaine 20%, Opthaine 35%, Procaine 43% and Novesine 47% (Figs 4 and 5). Enzymic hydrolysis of the substrates Ophthaine, Nesacaine and Procaine was reduced to zero at a final concentration of 1 mM Dimethyl Coroxon. At this concentration the residual activity towards Novesine was 28 per cent (Fig. 5).

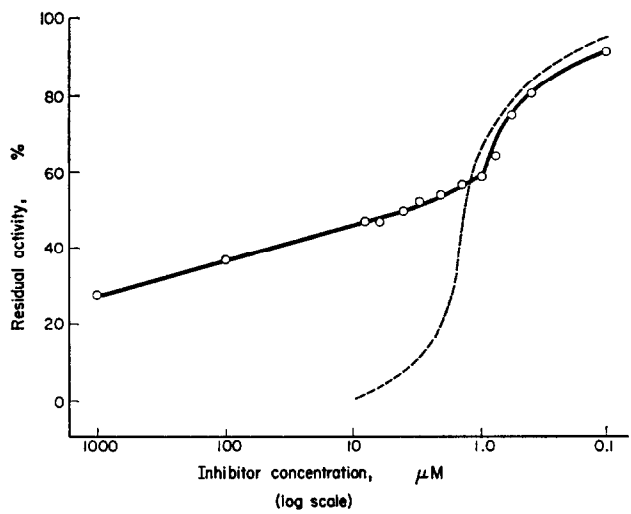

FIG. 5. Inhibition of Novesine hydrolysis by Dimethyl Coroxon. ○ = Novesine; — — — = BzCh (cf. Fig. 3).

Similar results were obtained with the Di-(2-chloroethyl) phosphate Haloxon (Figs 6 and 7). The hydrolysis of benzoylcholine was reduced to zero by 48 μM Haloxon (Fig. 6), at which concentration the residual hydrolysis of the local anaesthetic esters was Procaine 10%, Ophthaine 11%, Nesacaine 23% and Novesine 27% (Fig. 7).

There is evidence that organophosphate inhibition of enzyme activity is due to the phosphorylation of enzymes.[15, 16] The inhibition resulting from organophosphate

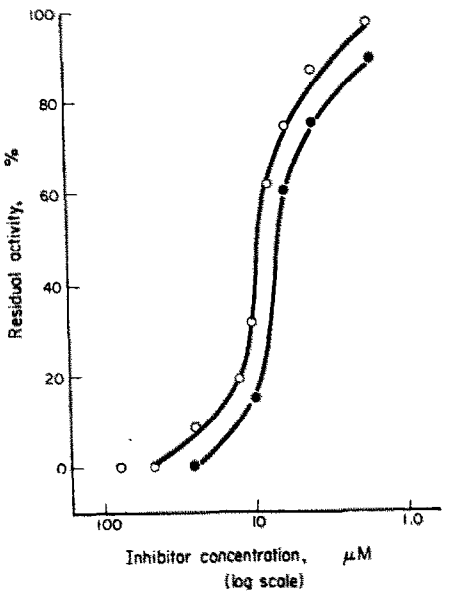

FIG. 6. Inhibition of choline ester hydrolysis by Haloxon. ○ = BzCh; ● = BuCh.

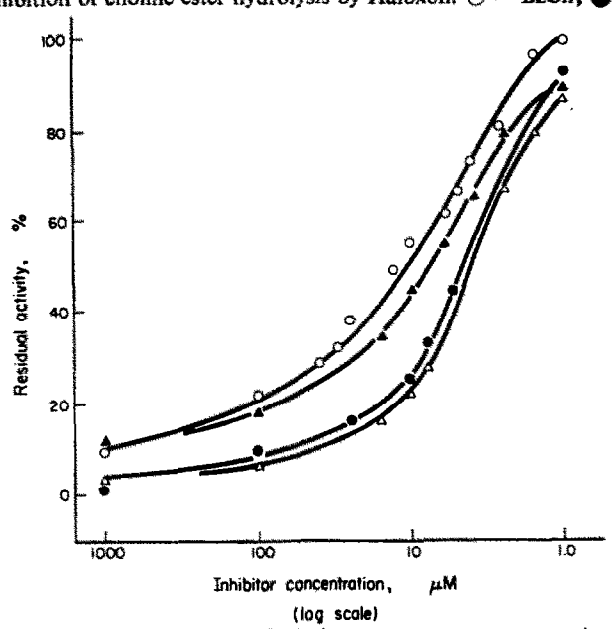

FIG. 7. Inhibition of local anaesthetic ester hydrolysis by Haloxon. ○ = Novesine; ● = Ophthaine; △ = Procaine; ▲ = Nesacaine.

action is generally non-reversing or slowly reversing depending on the type of phosphorylated enzyme formed.[17–19] If the inhibition is due to phosphorylation of the enzyme then the extent of inhibition, of a given enzyme, produced by pre-incubation under standard conditions will be the same for any substrate. The present results show that at certain concentrations of Dimethyl Coroxon and Haloxon the degree of inhibition was not the same for all of the substrates (Figs 3–7) and indicate the presence, in guinea-pig liver, of at least three enzymes capable of hydrolysing the local anaesthetic esters studied. One of these is the enzyme, also hydrolysing benzoylcholine and, presumably, butyrylcholine which is completely inhibited by 10 μM Dimethyl Coroxon. This point is well illustrated by the superimposition of the curve for benzoylcholine (Fig. 3) on the curves for Novesine (Fig. 5) and the other local anaesthetic esters (Fig. 4). The second esterase, which does not hydrolyse benzoylcholine and butyrylcholine, is completely inhibited by 1 mM Dimethyl Coroxon (Fig. 4). Although the hydrolysis of Ophthaine, Nesacaine and Procaine was completely inhibited by 1 mM Dimethyl Coroxon, the residual enzymic hydrolysis of Novesine at this inhibitor concentration was 28 per cent (Fig. 5) suggesting the presence of a third esterase with specificity requirements fulfilled by this substrate. The results obtained with Haloxon, although a less potent inhibitor than Dimethyl Coroxon, provide further evidence for the existence of at least two enzymes active in the hydrolysis of the local anaesthetic esters studied (Figs 6 and 7).

The finding that a large proportion of the hydrolytic activity of homogenates towards benzoylcholine is lost during cold storage, the hydrolysis of the local anaesthetics being little affected (Table 2), conflicts with the idea of an enzyme hydrolysing both benzoylcholine and the local anaesthetics unless it is assumed that different sites on the enzyme molecule are involved in the formation of the enzyme substrate complexes. That the relative affinity of this enzyme for benzoylcholine is very much less than for the local anaesthetic esters is suggested by the experiments with the competitive inhibitors Cinchocaine and Chlorpromazine.

In view of the results presented in this paper and in agreement with the findings of Augustinsson[7] it is considered that benzoylcholinesterase is a poor name for any of the esterases of guinea-pig liver involved in the hydrolysis of benzoylcholine.

REFERENCES

1. B. KISCH, H. KOSTER and E. STRAUSS, *Expl Med. Surg.* **1**, 51 (1943).
2. W. KALOW, *J. Pharmac. exp. Ther.* **104**, 122 (1952).
3. W. KALOW, *Pharmacogenetics*. Saunders, London, (1962).
4. F. F. FOLDES, D. L. DAVIS and O. J. PLEKSS, *Anesthesiology* **17**, 187 (1956).
5. C. E. BECKER, *J. dent. Res.* **40**, 190 (1961).
6. M. H. AVEN, A. LIGHT and F. F. FOLDES, *Fedn Proc.* **12**, 299 (1953).
7. K.-B. AUGUSTINSSON, *Acta physiol. scand.* **15**, *suppl.* 52 (1948).
8. R. M. LEE and B. H. LIVETT, *Biochem. Pharmac.* **16**, 1757 (1967).
9. C. H. SAWYER, *Science* **101**, 385 (1945).
10. S. ELLIS, *J. Pharmac. exp. Ther.* **91**, 370 (1947).
11. H. BLASCHKO, T. C. CHOU and I. WADJA, *Br. J. Pharmac.* **2**, 108 (1947).
12. S. HESTRIN, *J. biol. Chem.* **180**, 249 (1949).
13. W. KALOW and R. O. DAVIES, *Biochem Pharmac.* **1**, 183 (1959).
14. W. KALOW and M. O. MAYKUT, *J. Pharmac. exp. Ther.* **116**, 418 (1956).
15. D. F. HEATH, *Organophosphorus Poisons*. Pergamon Press, Oxford (1961).
16. R. D. O'BRIEN, *Toxic Phosphorus Esters*. Academic Press, New York (1960).
17. A. N. DAVIDSON, *Biochem. J.* **60**, 339 (1955).
18. M. VANDEKAR and D. F. HEATH, *Biochem. J.* **67**, 202 (1957).
19. R. M. LEE, *Biochem. Pharmac.* **13**, 1241 (1964).