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A METHOD FOR THE COLORIMETRIC ESTIMATION OF LOCAL ANAESTHETICS CONTAINING AN ESTER LINK, AND ITS USE IN THE DETERMINATION OF ESTERASE ACTIVITY

R. M. LEE* and B. H. LIVETT

School of Pharmacy, College of Technology, Portsmouth, Hants.

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Abstract—The colorimetric method of assay for carboxylic acid esters, originally published by Hestrin[1], has been modified, and made applicable to the assay of local anaesthetic esters such as procaine.

The determination of local anaesthetics by this method is a less rapid procedure than when used for choline and aliphatic esters, but is, nevertheless, a convenient and accurate means of estimating anaesthetic ester concentrations.

The modified Hestrin method has been used in the present study to investigate the enzymic hydrolysis of local anaesthetic esters by liver and plasma samples from various species. Of the two, the liver was found to be the much more potent source of esterases which would hydrolyse local anaesthetics; the liver of the guinea-pig was particularly active in this respect, with hydrolysis rates for certain of the anaesthetic esters in excess of 1000 μmole/g/hr.

HESTRIN[1] reported a method of assay for carboxylic acid esters, including choline esters, based on the formation of an hydroxamate under strongly alkaline conditions, followed by reaction in acid solution with ferric chloride to give a coloured derivative.

This method has found application in the determination of cholinesterase activity,[2,3,4] but has not been used for the estimation of enzyme-catalysed degradation of those local anaesthetics which have an ester linkage.

The only anaesthetic studied by Hestrin[1] was larocaine (3-diethylamino-2,2-dimethylpropyl-4-aminobenzoate) which gave a poor yield of the ferric hydroxamate derivative under the conditions employed.

In an effort to obtain a simple, rapid method of assaying procaine and related anaesthetics, the technique of Hestrin was re-examined. The modification described in this paper permits an accurate assessment of the rate of enzymic hydrolysis of many ester-linked local anaesthetics, and has been used to compare the hydrolytic activities of liver homogenates and plasma from a variety of species.

Previous estimations of esterase activity[5–10] against local anaesthetics have indicated that, in plasma, their hydrolysis is slow, compared with the rate of degradation of choline esters. The present work was undertaken to demonstrate that, in many species, the liver is considerably more active in the metabolism of local anaesthetic esters than plasma.

* Present address Smith, Kline and French Laboratories Ltd., Welwyn Garden City, Herts.

EXPERIMENTAL

Materials

All animals were killed by a blow on the head and bled out after decapitation. Plasma was obtained by centrifuging blood rendered incoagulable with heparin (0·1 mg/ml) at 4000*g* for 15 min.

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

Plasma samples and liver homogenates were tested for esterase activity within 2 hr after slaughter.

Subtrates

(1) Acetylcholine perchlorate (ACh) (British Drug Houses, Ltd.); (2) *n*-Butyrylcholine iodide (BuCh) (British Drug Houses, Ltd.); (3) Benzoylcholine chloride (BzCh) (British Drug Houses, Ltd.); (4) Atropine sulphate (British Drug Houses, Ltd.); (5) Methyl *n*-butyrate (British Drug Houses, Ltd.); (6) Methyl benzoate (British Drug Houses, Ltd.); (7) Methyl 4-aminobenzoate (Koch-Light Laboratories, Ltd.); (8) Methyl 4-hydroxybenzoate (British Drug Houses, Ltd.); (9) Methyl 3-amino-4-hydroxybenzoate (Orthocaine) (Hopkin and Williams, Ltd.); (10) Ethyl 4-aminobenzoate (Benzocaine) (British Drug Houses, Ltd.); (11) 2-Dimethylaminoethyl-4-*n*-butylaminobenzoate (Amethocaine) (British Drug Houses, Ltd.); (12) 2-Diethylaminoethyl-4-aminobenzoate (Procaine) (British Drug Houses, Ltd.); (13) 2-Diethylaminoethyl-2-chloro-4-aminobenzoate (Nesacaine) (A gift from Strasenburgh Laboratories, Rochester, N.Y.); (14) 2-Diethylaminoethyl-3-amino-4-propoxybenzoate (Ophthaine) (A gift from E. R. Squibb & Sons, Liverpool and London); (15) 2-Diethylaminoethyl-2-propoxy-4-aminobenzoate (Ravocaine) (A gift from Pfizer Ltd., Sandwich, Kent); (16) 2-Diethylaminoethyl-3-butoxy-4-aminobenzoate (Novesine) (A gift from A. Wander, Ltd., King's Langley, Herts.); (17) 2-Di-*n*-butylaminopropyl-4-aminobenzoate (Butacaine) (Samoore, Ltd., London, N.15); (18) Cocaine hydrochloride (British Drug Houses, Ltd.).

Substrates 9–17 were taken into solution as the hydrochloride, being supplied as such or having an equimolar amount of HCl added.

Assay of esters

The basic principle of the assay method was unchanged from that of Hestrin[1]; all reagents used were as described by this author.

The major modification was in the time allowed for reaction of the ester with the hydroxylamine reagent; the temperature at which this reaction took place was also varied.

A typical investigation of the rate and extent of hydroxamate formation was as follows:

10 ml of ester solution (4×10^{-3} or 8×10^{-3}M), 10 ml of 0·067M phosphate buffer at pH 7·2 and 40 ml of alkaline hydroxylamine reagent were incubated at the required temperature, with constant shaking of the flask. At intervals, 6 ml aliquots were withdrawn from the reaction mixture, and acidified with 2 ml of 4N HCl.

The coloured ferric-hydroxamate derivative was then formed by the addition of ferric chloride reagent, and the extinction of the solution measured at 540mμ, using a Unicam SP 600 spectrophotometer.

When three consecutive extinction values were equal, the reaction was considered to have reached equilibrium.

The assay was carried out under the conditions which would apply after enzyme activity determinations, hence the inclusion of phosphate buffer in the reaction mixture.

Estimation of esterase activity

The measurement of esterase activity in plasma and liver samples was performed at 37° in the presence of 0·067M phosphate buffer at pH 7·2 in all experiments. The results reported are the mean values of at least three experiments on tissues from at least three animals of each species.

Enzyme preparation, phosphate buffer and ester (final concentration : 4×10^{-3}M) in a total volume of 2 ml were incubated for a suitable time before the addition of 4 ml of hydroxylamine reagent. The mixture was then further incubated under optimal conditions for the maximum reaction between ester and hydroxylamine as determined in the previous section, before the addition of HCl and ferric chloride.

Controls for non-enzymic hydrolysis of the esters were run concurrently, and the difference in extinction values between control and enzyme tubes used to calculate esterase activity in terms of μmole of ester hydrolysed/g. of tissue/hr or for plasma asμ mole/ml/hr. This was done by reference to standard calibration curves relating extinction to μmole of ester per ml of final solution. The validity of these curves was checked at three points every day.

RESULTS AND DISCUSSION

(a) *Assay of local anaesthetic esters*

The results given in Table 1 show the time taken for maximum hydroxamate formation by local anaesthetic and other esters at different concentrations and temperatures. The time course of hydroxamate formation by Ophthaine and Benzocaine are given in Fig. 1. Increasing the ester concentration by a factor of two did

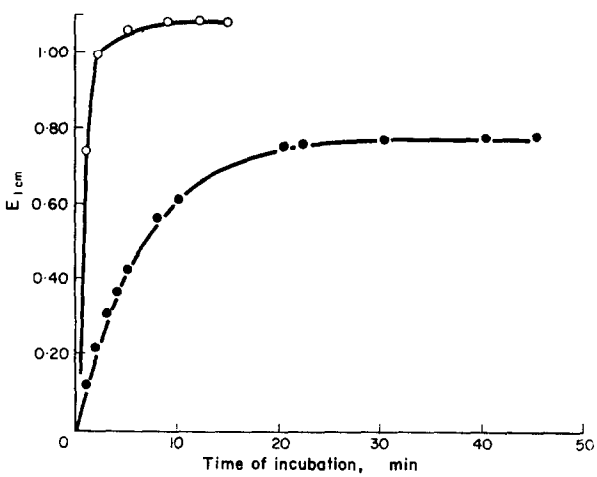


FIG. 1. The rate and extent of hydroxamate formation by Ophthaine and Benzocaine.
○ = 0·008 M Ophthaine at 20°; ● = 0·008 M Benzocaine at 20°.

not increase the time for maximum reaction with hydroxylamine to any great extent except in the case of Novesine, solutions of which are initially turbid due to liberation of the free base by the alkalinity of the hydroxylamine reagent. At the higher concentration of Novesine the turbidity took a considerable time to clear.

In an attempt to speed up the reaction times for the slower-acting anaesthetics, a temperature of 40° was employed. Although the attainment of equilibrium was advanced at the higher temperature, the maximum colour which developed was less than at 20° (Table 1, Figs. 2 and 3) suggesting that hydrolytic breakdown of the ester had also been increased by the rise in temperature.

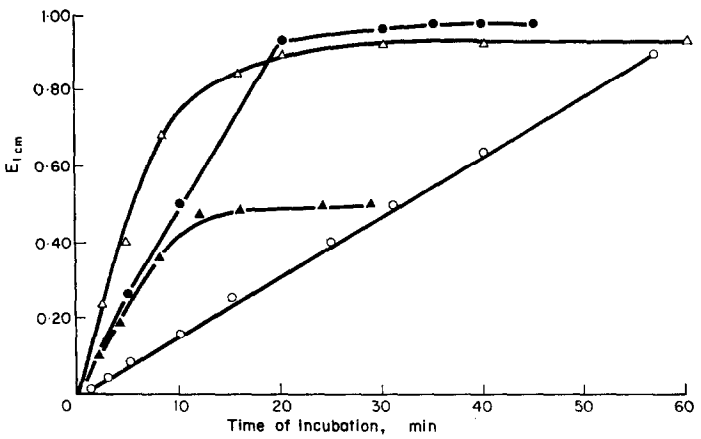


Fig. 2. The effect of temperature and ester concentration on the rate and extent of hydroxamate formation by Novesine.
○ = 0·008 M Novesine at −5°; ▲ = 0·004 M Novesine at 20°; ● = 0·008 M Novesine at 20°; △ = 0·008 M Novesine at 40°.

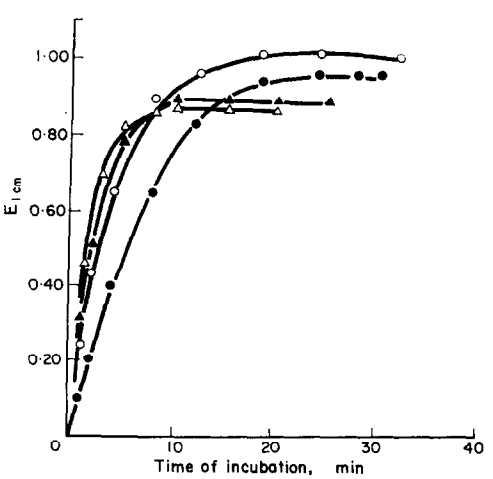
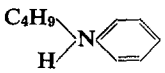
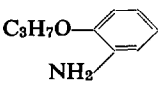
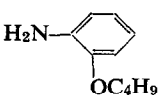
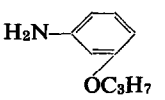
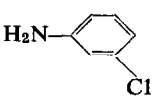
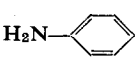
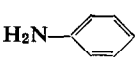
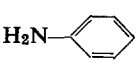
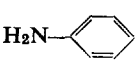
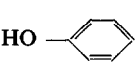
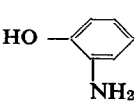


Fig. 3. The effect of temperature on the rate and extent of hydroxamate formation by Amethocaine and Ravocaine.
● = 0·008 M Amethocaine at 20°; △ = 0·008 M Amethocaine at 40°; ○ = 0·008 M Ravocaine at 20°; ▲ = 0·008 M Ravocaine at 40°.

TABLE 1. THE RELATIONSHIP BETWEEN STRUCTURE AND TIME OF MAXIMUM HYDROXAMIC ACID FORMATION FOR LOCAL ANAESTHETIC, AND OTHER ESTERS

Ester	Structure R.COO.R′ R	R′	Time for maximum hydroxamate formation (min) 20° 0·004M	20° 0·008M	40° 0·008M
Amethocaine HCl		$(CH_2).N.(CH_3)_2$	20	24	8
Amylocaine HCl		C_2H_5 / CH_3 $C.CH_2.N.(CH_3)_2$			>180
Ophthaine HCl		$(CH_2)_2.N.(C_2H_5)_2$	5	8	
Novesine HCl		$(CH_2)_2.N.(C_2H_5)_2$	15	35	25
Ravocaine HCl		$(CH_2)_2.N.(C_2H_5)_2$	15	18	10
Nesacaine HCl		$(CH_2)_2.N.(C_2H_5)_2$	10	10	
Procaine HCl		$(CH_2)_2.N.(C_2H_5)_2$	10	12	
Butacaine HCl		$(CH_2)_3.N.(C_4H_9)_2$			160
Benzocaine HCl		C_2H_5	30	30	
Methyl amino benzoate		CH_3	7	7	
Methyl benzoate		CH_3	<1	<1	
Methyl hydroxy benzoate		CH_3	30	35	
Orthocaine HCl		CH_3	45	35	
Atropine sulphate			25*	25†	
Cocaine HCl			10	12	

The concentrations of atropine used were 0·0126M* and 0·0063M†

This result raised the question of how important hydrolysis of the esters was during the long periods necessery for hydroxamation. Relative to acetylcholine, which is fully hydroxamated within one minute, colour production by the local anaesthetic esters was high (Table 2). In all cases the calibration of ester concentration with extinction was linear up to a final concentration of 1 μmole of ester per ml. It is concluded that at 20° hydrolysis is relatively insignificant and proportional to ester concentration, and therefore, need not be considered in calculating enzyme activities.

TABLE 2. THE EXTENT OF FERRIC HYDROXAMIC ACID COMPLEX FORMATION BY ESTERS

Ester	Extinction per O-acyl equivalent relative to ACh=100*
ACh ClO_4	100
BuCh Cl	111
BzCh Cl	129
Amethocaine HCl	145
Ophthaine HCl	162
Novesine HCl	146
Ravocaine HCl	151
Nesacaine HCl	112
Procaine HCl	137
Benzocaine HCl	115
Methyl 4-amino benzoate	131
Methyl benzoate	137
Methtl 4-hydroxy benzoate	136
Orthocaine HCl	153
Atropine SO_4	75
Cocaine HCl	90
Triacetin	119

*Data derived from calibration curves relating E_{1cm} to μmole of ester per ml of final solution over the range 0·1–1·0 μmole. 1 μmole of ACh per ml of final solution gave E_{1cm} 0·838.

In general, the results of Table 1 indicate that the esters with the more complex R or R′ groups react more slowly with alkaline hydroxylamine. Benzocaine, Orthocaine and methyl-4-hydroxybenzoate did not conform to this pattern, however, and their times of reaction contrast strongly with that of the rapidly reacting methyl benzoate.

The maximum extinction value for methyl-4-hydroxybenzoate was 136 per cent of that for acetylcholine; this figure is greatly in excess of that found by Hestrin[1], who gives a value of 28 per cent of that for acetylcholine. However, Hestrin allowed a reaction time of only 4 min, at which time we found that methyl-4-hydroxybenzoate gave an extinction value which was 35 per cent of that for acetylcholine.

Two of the local anaesthetic esters studied, Butacaine and Amylocaine, reacted so slowly with alkaline hydroxylamine as to render the investigation of their enzymic hydrolysis too lengthy a procedure, but for the others this method of assay is accurate, relatively rapid and does not suffer from the disadvantages described by Kalow[5] for his method utilising the ultra-violet absorption spectra of the esters.

The reaction rate of Novesine with alkaline hydroxylamine was also determined at −5° (Fig. 2) and little or no colour formation with ferric chloride was obtained

in the first minute. The choline esters, on the other hand, still produced maximum colour at −5° within one minute, so that it would be possible to assay for choline esters in the presence of the local anaesthetic esters which react slowly with hydroxylamine under strongly alkaline conditions.

(b) *Enzymic hydrolysis of local anaesthetic esters*

The rates of hydrolysis of esters with local anaesthetic properties by liver and plasma samples from a number of animal species are shown in Tables 3–5. The rates of hydrolysis for choline and other esters are included for comparison.

The livers of the non-mammalian species (Table 3) were generally less active in the hydrolysis of local anaesthetic esters than were the mammalian livers (Table 4). No overall pattern was evident, but for most species Nesacaine and Ophthaine were the best esterase substrates.

No correlation between hydrolysis of anaesthetic esters and hydrolysis of choline

TABLE 3. THE HYDROLYSIS OF CHOLINE, LOCAL ANAESTHETIC AND OTHER ESTERS BY LIVERS OF NON-MAMMALIAN SPECIES

Ester	Frog	Dogfish	Hen	Pigeon	Budgerigar
ACh	126	15	97	226	116
BuCh	0	10	110	276	182
BzCh	0	0	37	44	38
Atropine	0	0	0	0	0
Methyl butyrate	–	–	–	986	–
Methyl 4–amino benzoate	0	0	33	18	0
Amethocaine	8	0	37	0	20
Benzocaine	11	0	81	6	14
Nesacaine	21	0	142	46	0
Novesine	14	0	12	21	3
Ophthaine	15	0	115	52	6
Procaine	14	0	16	16	17
Ravocaine	13	0	27	4	0

Results given are hydrolytic activities expressed as μ mole ester degraded/g of liver/hr.

TABLE 4. THE HYDROLYSIS OF CHOLINE, LOCAL ANAESTHETIC AND OTHER ESTERS BY LIVERS OF MAMMALIAN SPECIES

Ester	Mouse	Rat	Guinea pig	Hamster	Rabbit 1	Rabbit 2
ACh	520	72	12	24	106	179
BuCh	580	81	231	47	164	201
BzCh	163	7	423	3	30	171
Atropine	0	0	16	0	0	63
Methyl butyrate	963	762	1542	1031	–	–
Methyl 4–amino benzoate	51	36	116	91	184	194
Amethocaine	80	18	136	60	60	60
Benzocaine	60	38	48	110	191	101
Nesacaine	122	69	1137	199	165	145
Novesine	27	21	1174	10	74	134
Ophthaine	170	134	456	171	112	120
Procaine	27	7	93	29	17	36
Ravocaine	51	0	135	32	102	124

Results given are hydrolytic activities expressed as μmole ester degraded/g of liver/hr.

esters was observed, and in most cases identity of the esterase responsible for the hydrolysis of the local anaesthetic esters is still a matter for conjecture.

The outstanding hydrolysis rates of Nesacaine, Ophthaine and Novesine by guinea-pig liver suggested that 'benzoylcholinesterase' might be involved, since this enzyme is known to be present in guinea-pig liver [11–14], and the present study confirms a high activity against benzoylcholine. Evidence to demonstrate that this hypothesis is only partly correct will be presented in a subsequent paper. Atropinesterase activity in rabbit plasma is genetically determined and is completely absent from some rabbit plasmas[15]. Liver taken from rabbits with no atropinesterase activity in the plasma, was inactive in the hydrolysis of atropine, but possessed esterases capable of breaking down the local anaesthetic esters particularly benzocaine. The liver of rabbits, whose plasma contained an atropinesterase, had higher rates of hydrolysis for acetyl, butyryl and benzoyl choline as well as for atropine, but did not have a significantly increased ability to hydrolyse the local anaesthetics with the exception of Novesine and Procaine, and had considerably less activity against benzocaine than those rabbits without an atropinesterase.

Preliminary studies with rabbit plasma (Table 5) have indicated that the hydrolysis of procaine and other local anaesthetic esters by plasma containing an atropinesterase was higher than by plasma without atropinesterase, as was found by Daniell *et al.*[10] There is evidence to show that the elevated hydrolysis of atropine and

TABLE 5. THE HYDROLYSIS OF SOME LOCAL ANAESTHETIC ESTERS BY PLASMAS SAMPLES FROM VARIOUS SPECIES

Ester	Rat	Guinea pig	Hamster	Pigeon	Rabbit 1	Rabbit 2
Nesacaine	<1	<1	<1	13	3	19
Novesine	4	<1	10	8	<1	4
Ophthaine	4	<1	13	8	2	4
Procaine	<1	<1	4	6	<1	3
Ravocaine	–	<1	2	–	<1	3
Benzocaine	<1	<1	<1	13	2	<1
Atropine	–	–	–	–	<1	9

Results given are hydrolytic activities expressed as μmole ester degraded/ml of plasma/hr.

TABLE 6. HYDROLYSIS RATES OF ESTERS BY PLASMA SAMPLES FROM ATROPINESTERASE 'HIGH' AND 'LOW' RABBITS

Rabbit No.	Atropine	BzCh	Procaine
1	8·2	20	3·0
2	12·7	25	3·0
3	11·9	29	3·1
4	0·8	2	0·8
5	0·4	3	0·3
6	1·0	2	0·4
7	0·9	2·5	0·4
8	10·3	22	2·9
9	1·6	2	0·5

Results given are hydrolytic activities expressed as μmole ester degraded/ml of plasma/hr.

benzoyl choline in certain rabbit plasma samples is linked genetically but that the esterases mediating these hydrolyses are not identical[12,15,16]. In this paper Table 6 shows that whenever the activity against benzoylcholine and atropine is high, then the plasma possesses an enhanced ability to hydrolyse procaine. Thus, the rate of degradation of local anaesthetic esters by rabbit plasma should also be subject to genetic control.

The hydrolysis of local anaesthetic esters by plasma of four other species are all given in Table 5. It is notable that guinea-pig plasma had little or no activity in this respect, in contrast to the liver of this species. In view of this it is possible that elevated rate of local anaesthetic ester hydrolysis by guinea-pig plasma may be indicative of liver damage in this animal.

REFERENCES

1. S. HESTRIN, *J. biol. Chem.* **180,** 249 (1949).
2. J. H. FLEISHER, E. J. POPE and S. F. SPEAR, *A.M.A. Archs ind. Hlth* **11,** 332 (1955).
3. J. C. SABINE, *Blood* **10,** 1132 (1955).
4. R. M. LEE, *Biochem. Pharmac.* **13,** 1551 (1964).
5. W. KALOW, *J. Pharmac. exp. Ther.* **104,** 122 (1952).
6. M. H. AVEN, A. LIGHT and F. F. FOLDES, *Fedn Proc.* **12,** 299 (1953).
7. W. KALOW and M. O. MAYKUT, *J. Pharmac. exp. Ther.* **116,** 418 (1956).
8. C. E. BECKER, *J. dent. Res.* **40,** 190 (1961).
9. F. MARGOLIS and P. FEIGELSON, *J. biol. Chem.* **238,** 2620 (1963).
10. H. B. DANIELL, A. E. WADE and F. FORD MILLIKAN, *J. pharm. Sci.* **53,** 1341 (1964).
11. C. H. SAWYER, *Science* **101,** 385 (1945).
12. S. ELLIS, *J. Pharmac. exp. Ther.* **91,** 370 (1947).
13. H. BLASCHKO, T. C. CHOU and I. WAJDA, *Br. J. Pharmac.* **2,** 108 (1947).
14. K. B. AUGUSTINSSON, *Acta physiol. scand.* **15,** *suppl.* 52 (1948).
15. P. B. SAWIN and D. GLICK, *Proc. natn Acad. Sci. U.S.A.* **29,** 55 (1943).
16. C. I. WRIGHT, *J. Pharmac. exp. Ther.* **75,** 328 (1942).