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Inhibition of Fly Head Acetylcholinesterase by Bis-[(*m*-hydroxyphenyl)-trimethylammonium Iodide] Esters of Polymethylenedicarbamic Acids

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A series of bis-[(*m*-hydroxyphenyl)trimethylammonium iodide] esters of polymethylenedicarbamic acids and a number of (*m*-hydroxyphenyl)trimethylammonium iodide esters of straight-chain *N*-alkylcarbamic acids have been examined as inhibitors of acetylcholinesterase from fly head. Evidence is presented suggesting that inhibition of acetylcholinesterase by the bis-carbamates is due to carbamoylation of the enzyme, as is generally thought to be the case with esters of *N*-alkylcarbamic acids. Inhibition is irreversible. The (*m*-hydroxyphenyl)-trimethylammonium iodide ester of *N*-hexylcarbamic acid also inhibits fly head acetylcholinesterase irreversibly. There is therefore no need to implicate a second functional group in bis-carbamate esters to explain the irreversible inhibition of the enzyme. An unusual feature of the inhibition is that inhibition lines do not pass through 100% enzyme activity at $t = 0$, except for rather low concentrations of inhibitor (<10 μM for the octamethylene compound). Also, inhibition lines tend towards a maximum slope as inhibitor concentration is increased. The first observation indicates complex-formation, even in the presence of high concentrations of substrate, and by using measurements of inhibition at relatively high inhibitor concentrations, affinity constants K'_a have been calculated. K'_a varies from 0.1 μM for the dodecamethylene compound to 10μM for the tetramethylene compound, in the presence of 3.75 mM-acetylthiocholine, indicating high affinity for the enzyme. The second observation shows that, owing to this high affinity, the enzyme becomes saturated with inhibitor under the experimental conditions employed, and from the limiting slope values of the carbamoylation rate constant (k_2) have been calculated. k_2 varies from 0.15 min^{-1} for the tetramethylene compound to 1 min^{-1} for the decamethylene compound. Variations of potency in this series are therefore mainly due to changes in affinity (100-fold) rather than in carbamoylation rate (sevenfold). The observation that large molecules may acylate the enzyme raises certain problems, which are discussed.

The mechanism by which carbamates inhibit acetylcholinesterase has been the subject of much research over the past decade. The results appear to indicate that inhibition proceeds via a rapidly formed reversible complex that is converted, at a rate that is slow compared with the first stage, into a carbamoylated enzyme with concomitant release of the hydroxylic moiety of the carbamate ester (Wilson, Hatch & Ginsburg, 1960; Wilson, Harrison & Ginsburg, 1961; O'Brien, 1965, 1968; O'Brien, Hilton & Gilmour, 1966; Main & Hastings, 1966; Hellenbrand, 1967). This carbamoylated enzyme has a half-life that may be measured in minutes, which leads to fairly rapid recovery of carbamate-inhibited enzymes (Wilson *et al.* 1960, 1961).

Bis-carbamate esters of the series (I) and (II) are also known to be effective inhibitors of acetylcholinesterase and are unusual in that incubation of inhibitor and enzyme leads to progressive increase of inhibition over many hours, an observation suggesting a very slow rate of recovery of inhibited enzyme (Stumpf, 1959; Kraup, Schwarzacher & Stumpf, 1955; Klupp, Kraup, Schwarzacher & Stumpf, 1955; Herzfeld & Stumpf, 1956). Little detailed work has appeared, and the mechanism by which these esters act is not known, although Wilson *et al.* (1961) have shown that the very slow rate of recovery of enzyme inhibited by compounds of series (II) is accelerated by hydroxylamine. This

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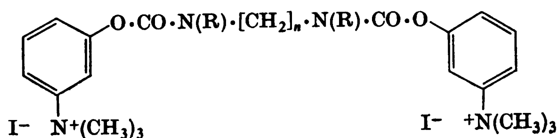
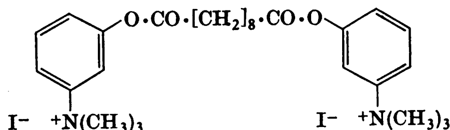
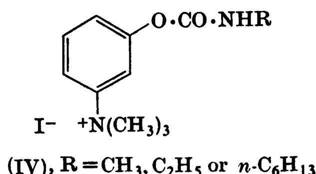


(I), R = H; (II), R = CH_3



(III)



(IV), R = CH_3, C_2H_5 or n-C_6H_{13}

finding suggests that inhibition involves formation of a stable carbamate ester. Were it not for this observation, the suggestion that bis-carbamates (formulae I and II) could act in the same way as, say, *N*-methylcarbamates might be greeted with scepticism, since these are large molecules and the substrate specificity of acetylcholinesterase (for a review see Cohen & Oosterbaan, 1963) suggests that molecules above a certain size would be unlikely to acylate the enzyme. We decided to look for more evidence about the mechanism of action of bis-carbamates, both because this mechanism is of interest in itself, as it bears upon the steric requirements for interaction at the active site of acetylcholinesterase, and because inhibition may involve functional groups outside the active site. In the latter case one might hope to discover informative differences in the behaviour of enzymes from various sources towards bis-carbamate esters.

The most notable property of this group of bis-carbamates is that they appear to produce virtually irreversible inhibition of acetylcholinesterase. There is some temptation to ascribe this property to the presence of a second functional group on the inhibiting molecule, but this need not be the explanation since these compounds may be considered simply as long-chain *N*-alkylcarbamates, and it could be that long-chain *N*-alkylcarbamate derivatives of acetylcholinesterase are much more stable than their short-chain homologues. Since no evidence has appeared in the literature about inhibition of acetylcholinesterase by long-chain *N*-alkylcarbamates, we included a number of *N*-alkylcarbamate esters of group (IV) (R = CH_3, C_2H_5 and n-C_6H_{13}) in the study reported here.

As a beginning it was clearly necessary to confine the examination to acetylcholinesterase from one source. We chose fly head acetylcholinesterase and began by studying the kinetics of inhibition of this enzyme by compounds of groups (I) and (IV). This paper describes the results of the kinetic study, together with the results of a brief examination of a dicarboxylic acid ester analogue (III) as a substrate for acetylcholinesterase from fly head and from the electric organ of the electric eel.

To avoid the correct but cumbersome terminology, the compounds of series (I) are referred to by the length of the alkyl chain, i.e. C_4 compound etc., and collectively as bis-carbamates.

MATERIALS

Acetylthiocholine iodide was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. *m*-Dimethylaminophenol (Aldrich Chemical Co.) was purified by recrystallization of the soluble portion of the commercial product from light petroleum (b.p. 60–80°C) before use.

Bis-isocyanatoalkanes were made from the appropriate dicarboxylic acid chlorides by the wet method (Allen & Bell, 1955). Physical properties corresponded well with those given in the literature (Siefken, 1949).

Bis-(*m*-dimethylaminophenyl) esters of polymethylenedicarbamic acids were prepared by reaction of bis-isocyanatoalkanes with *m*-dimethylaminophenol in dichloromethane in the presence of a few drops of triethylamine at room temperature for several hours. Crude materials were purified by column chromatography on silica gel in dichloromethane containing an increasing proportion of ether, and by recrystallization. All compounds gave single spots on silica-gel t.l.c. plates (Merck GF_{254}) in dichloromethane–ether (10:1, v/v) and had $R_F \simeq 0.5$. All compounds had similar i.r. spectra, showing peaks at $3350\,cm^{-1}$ (NH) and $1700\,cm^{-1}$ (C=O). Melting points (uncorrected) and analytical results are given below in the form: length of alkyl chain (n in structure I), melting point, literature melting point where known (Schmied, Bilek & Seifried, 1956) (Found: C, H, N; Calc. C, H, N%): C_4 compound, 137–138°C, 93–95°C (Found: C, 63.7; H, 7.4; N, 13.5. Calc. for $C_{22}H_{30}N_4O_4$: C, 63.75; H, 7.3; N, 13.5%); C_5 compound, 115–116°C (Found: C, 64.3; H, 7.5; N, 13.2. $C_{23}H_{32}N_4O_4$ requires C, 64.5; H, 7.5; N, 13.1%); C_6 compound, 142–143°C, 141–144°C (Found: C, 65.7; H, 7.9; N, 12.8. Calc. for $C_{24}H_{34}N_4O_4$: C, 65.1; H, 7.7; N, 12.7%); C_8 compound, 105–106°C (Found: C, 66.45; H, 8.1; N, 12.05. Calc. for $C_{26}H_{38}N_4O_4$: C, 66.5; H, 7.9; N, 11.9%); C_{10} compound, 99.5–100°C (Found: C, 67.4; H, 8.5; N, 11.4. Calc. for $C_{28}H_{42}N_4O_4$: C, 67.4; H, 8.5; N, 11.2%); C_{12} compound, 95°C (102°C after melting and resolidifying) (Found: C, 68.4; H, 9.0; N, 10.7. Calc. for $C_{30}H_{46}N_4O_4$: C, 68.4; H, 8.8; N, 10.6%).

The tertiary amino compounds were dissolved in acetone and treated with an excess of methyl iodide. After about 16h at room temperature the bis-methiodide had crystallized out in almost quantitative yield in each case, and was filtered off, washed with acetone, and dried

in vacuo. Length of alkyl chain, measured and literature melting points, and analytical results are given below (melting points vary with the rate of heating): C_4 compound, 198–199°C (decomp.), 150–152°C (Found: I, 36.7. Calc. for $C_{24}H_{36}I_2N_4O_4$: I, 36.35%); C_5 compound, 153–155°C (Found: I, 36.0. $C_{25}H_{38}I_2N_4O_4$ requires I, 35.6%); C_6 compound, 120°C (decomp.), 130–135°C (Found: I, 33.6. Calc. for $C_{26}H_{40}I_2N_4O_4$: I, 35.0%); C_8 compound, 115–117°C (decomp.) (Found: I, 33.1. Calc. for $C_{28}H_{44}I_2N_4O_4$: I, 33.6%); C_{10} compound, 114–116°C (decomp.), 115°C (Found: I, 31.4. Calc. for $C_{30}H_{48}I_2N_4O_4$: I, 32.4%); C_{12} compound, 113–115°C (Found: I, 30.2. $C_{32}H_{52}I_2N_4O_4$ requires I, 31.3%).

(*m*-Hydroxyphenyl)trimethylammonium iodide esters of *N*-alkylcarbamic acids were prepared by the method described above. *N*-alkyl group, melting point and analytical results are given below: CH_3, 166–167°C (Found: C, 39.4; H, 5.1; N, 8.5; I, 37.9. Calc. for $C_{11}H_{17}IN_2O_2$: C, 39.3; H, 5.1; N, 8.3; I, 37.8%); C_2H_5, 163–166°C (Found: C, 41.2; H, 5.6; N, 8.3; I, 35.7. $C_{12}H_{19}IN_2O_2$ requires: C, 41.2; H, 5.5; N, 8.0; I, 38.2%): *n*-C_6H_{13}, 119–121°C (Found: C, 47.1; H, 6.7; N, 6.9; I, 31.0. $C_{16}H_{27}IN_2O_2$ requires: C, 47.3; H, 6.7; N, 6.9; I, 31.2%).

Bis-(*m*-dimethylaminophenyl) sebacate (III) was made in the usual manner from the acid chloride, purified by chromatography on silica gel with dichloromethane and treated with an excess of methyl iodide in acetone, as described above. The *bis-methiodide* (Found: C, 46.85; H, 5.85; N, 4.0. $C_{28}H_{42}I_2N_2O_4$ requires C, 47.4; H, 5.8; N, 3.9%) had m.p. 147–148°C.

Enzyme. The enzyme (EC 3.1.1.7, acetylcholine acetyl-hydrolase), obtained from the head of *Musca domestica* L. (housefly) and purified approx. 2500-fold (C. W. Kearns & W. R. Campbell, unpublished work), met with all of the criteria previously established for 'true' acetylcholinesterase (Nachmansohn, 1959). The specific activity varied from 10 to 100mmol/h per mg of protein. The enzyme was made up in 0.01M-sodium phosphate buffer, pH 7.4.

METHODS

Inhibition of acetylcholinesterase was examined by the method of Ellman, Courtney, Andres & Featherstone (1961) by using a Hitachi Perkin–Elmer Coleman 124 double-beam spectrophotometer, thermostatically maintained at 26°C.

Substrate (3.75mM) was made up freshly for each concentration of each inhibitor, in order to avoid errors introduced by progressive spontaneous solvolysis of the substrate. The solvent was 0.1M-sodium phosphate buffer, pH 7.0. The substrate solution contained 1mg of chromogenic agent/ml (3mM).

In a typical experiment 0.09ml of enzyme solution (in 0.01M-phosphate buffer, pH 7.4) and 0.01ml of a suitable inhibitor solution in 0.1M-NaCl were incubated at 25°C in a tapered centrifuge tube. Mixing was achieved by rapid vibration with a micro shaker. Samples (10 or 20μl) were removed at intervals and introduced into a spectrophotometer cell containing 3ml of substrate and chromogenic agent solution (300- or 150-fold dilution) and the cell was inverted completely several times to ensure mixing. The time that elapsed between introduction of the sample (zero time by definition) and measurement of the rate was, on average, 8s. Rate lines were each measured for 10–20s and involved a change in E_{412} of up to 0.6 unit. Separate experiments established that rate lines were linear for at least 1 min. The final substrate concentration was at least 3.65mM.

For zero-time inhibition studies a similar procedure was followed. A sample of inhibitor was introduced into the cell and followed immediately by enzyme, the cell inverted several times and the rate measured. Again rate lines were linear for at least 1 min, even in the presence of a substantial amount of enzyme–inhibitor complex.

The technique described permitted rate measurements to be made for four portions from each enzyme–inhibitor sample. For quantitative calculations each run was repeated to give an inhibition line consisting of seven or eight points, to which a line was fitted by the method of least squares. In such cases standard deviations are given in the text. The standard deviations represent, as appropriate, the probable error in the slope, or the intercept at $t = 0$, of a line fitted to the determined points. They do not represent experimental error, which must be greater.

In order to examine the spontaneous recovery of enzyme inhibited with various simple *N*-alkylcarbamates (group IV) and the C_{10} bis-carbamate (group I, $n = 10$), the following technique was employed. A sample of enzyme was incubated with inhibitor ($\leqslant 10\mu$M) until 90–100% inhibition was obtained. The solution was placed on a column (10cm × 1.2cm) of Bio-Gel P-2 (exclusion limit 2600), of which the void volume had previously been measured with uninhibited enzyme, and the appropriate enzyme-containing fraction collected. This fraction was kept at 25°C and samples were removed at various times for assay of enzymic activity by the Ellman *et al.* (1961) procedure. Each of the above experiments was repeated.

Spontaneous solvolysis of bis-carbamates was measured by observing the increase in E_{273} due to release of phenol, with the appropriate solvent as reference, after calibration of the instrument with pure phenol.

RESULTS

The bis-carbamates that form the subject of most of this study are known to be unstable in aqueous solution (Kraup *et al.* 1955; Klupp *et al.* 1955; Herzfeld & Stumpf, 1956; Stumpf, 1959). Therefore, since the concentration must be known with reasonable accuracy for inhibition experiments, it was necessary to examine the spontaneous hydrolysis of these compounds under various appropriate conditions. The appropriate conditions were those obtaining in the stock solution (pH 6.8, water or 0.1M-sodium chloride), which might normally be used for several hours; in the incubation vessel (0.01M-phosphate buffer, pH 7.4), in which the compound might remain for some minutes, and in the spectrophotometer cell (0.1M-phosphate buffer, pH 7.0) for up to 30s. Decomposition during this last stage of the experiment is sufficiently small to be neglected entirely.

The results of the investigation relevant to the inhibition study are that below concentrations of about 0.5mM (except for the C_{12} compound) decomposition takes place to the extent of 3% (i.e. the release of 0.03mol of phenol/mol of bis-carbamate) in 1h at 25°C in 0.1M-sodium chloride, pH 6.8, and about 5% in 2min in 0.01M-phosphate buffer, pH 7.4. At higher concentrations the rate of decomposition increases in a marked fashion as concentration increases, and this is noticeable with the C_{12} compound, even at a concentration of 0.5mM. It is quite possible that this behaviour is due to aggregation, which might be expected to promote reactions, between solvolysed and intact molecules, which compete with and are faster than simple solvolysis. The fact that initial rates (up to 5min) are not greatly affected by concentration supports this explanation, as does the observation that strong ($\geqslant$1mM) solutions of these compounds deposit insoluble polymers of high melting point on standing, which possess peaks characteristic of urea as well as of carbamate linkages in the i.r. spectrum. At a given pH, increase in ionic strength slightly decreases solvolysis rates. Stock solutions were therefore made up in 0.1M-sodium chloride and used for up to 1h, if below 0.5mM, and for much shorter times if above that concentration. The slight decrease of concentration with time was corrected for where necessary. The decomposition in buffer was more important, but in practice progressive inhibition lines were indistinguishable from straight lines if the period of measurement was kept below 2min. Later deviations were satisfactorily accounted for by decomposition, and there was no reason to suppose that non-linearity, observed after 3–4min, was due to any other factor. Inhibition lines up to 2min, although apparently linear, must in fact have been shallow curves, but the error produced by the assumption of linearity should be small compared with other experimental errors inherent in the experimental procedure.

Spontaneous regeneration experiments with (*m*-hydroxyphenyl)trimethylammonium iodide esters of *N*-alkylcarbamic acids, with Bio-Gel columns to remove excess of inhibitor, demonstrated that enzyme inhibited with the *N*-methyl- and *N*-ethyl-carbamate esters recovered to a perceptible extent over a few hours (Fig. 1). The enzyme inhibited with *N*-hexyl ester and the C_{10} bis-carbamate did not recover to any detectable extent over 20h when treated in the same manner. In another experiment, a sample of enzyme was inhibited completely by incubation with the C_8 bis-carbamate at 1μM. Dialysis against 0.01M-phosphate buffer, pH 7.4, for 16h at 4°C produced no recovery, although in this time solvolysis alone would have decreased the inhibitor concentration to 1pM. A control experiment with uninhibited

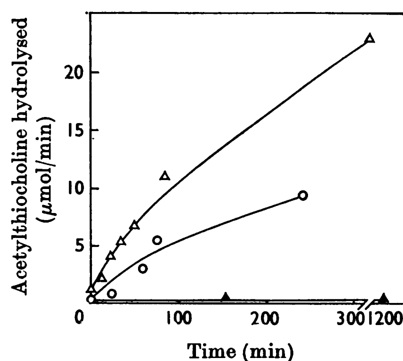


Fig. 1. Spontaneous recovery of fly head acetylcholinesterase inhibited by the (*m*-hydroxyphenyl)trimethylammonium iodide esters of *N*-methylcarbamic acid (△), *N*-ethylcarbamic acid (○) and *N*-hexylcarbamic acid (▲). Solid triangles (▲) also represent the results obtained with the bis-[(*m*-hydroxyphenyl)trimethylammonium iodide] ester of decamethylenedicarbamic acid.

enzyme showed a decrease in activity of 25% after the same treatment for the same time. It is therefore apparent that recovery of the enzyme inhibited with bis-carbamates is extremely slow and, for the purpose of a study involving a time-scale of minutes, may be regarded as zero.

An examination of the kinetics of inhibition by bis-carbamates over a wide range of concentrations reveals a number of features not usually seen in carbamate inhibition of acetylcholinesterase. First, owing to the very high affinities of bis-carbamates for the enzyme it is possible to achieve complete complexing and reach a stage where an increase in inhibitor concentration brings about no further increase in the rate of progressive inhibition of the enzyme. Secondly, measurement of two parameters reflecting the affinities of bis-carbamates for the enzyme under different conditions suggests that, in this case, more than one type of complex may be formed between inhibitor and enzyme. The reasons underlying these statements are considered in the Discussion section, but they are mentioned here since they dictate the manner in which the results are presented.

Inhibition lines for low concentrations of inhibitor all pass through the origin, indicating that complexing of enzyme and inhibitor is abolished in the spectrophotometer cell because of dilution, or the presence of excess of substrate, or both (Fig. 2). At higher concentrations (Fig. 3) lines intercept the axis at points below 100% activity, i.e. the enzyme is inhibited at zero time. This must be taken to demonstrate the presence of complexed enzyme in the spectrophotometer cell, and the relationship

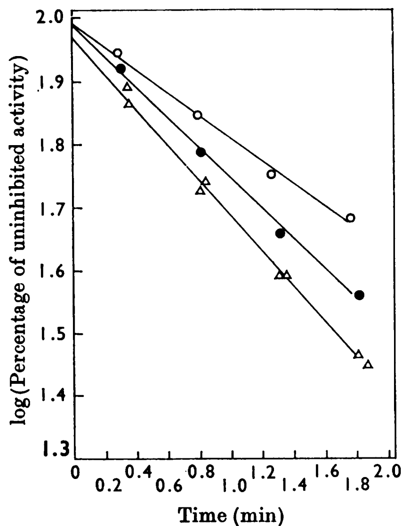


Fig. 2. Inhibition of fly head acetylcholinesterase at 25°C by the bis-[(*m*-hydroxyphenyl)trimethylammonium iodide] ester of decamethylenedicarbamic acid at concentrations of 0.232μM (○), 0.464μM (●) and 0.9μM (△).

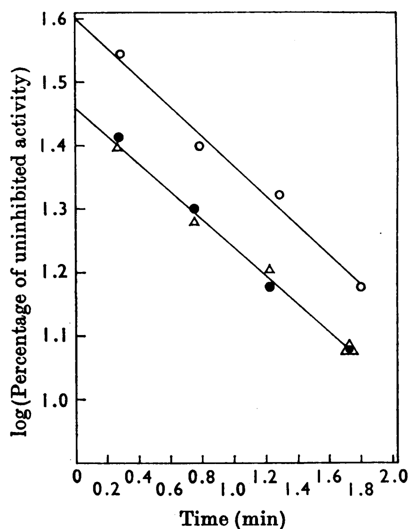
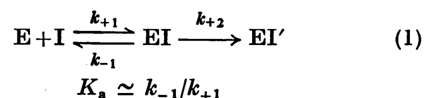


Fig. 3. Inhibition of fly head acetylcholinesterase at 25°C with the bis-[(*m*-hydroxyphenyl)trimethylammonium iodide] ester of octamethylenedicarbamic acid at concentrations of 0.233mM (○) and 0.466mM (●, △).

between the concentrations of free enzyme, complexed enzyme and inhibition should reflect the conditions in the spectrophotometer cell, rather than in the incubation vessel. The intercept should be log (percentage of free enzyme), and (100 − percentage of free enzyme) will be the percentage of reversibly complexed enzyme. Thus, by using the expression $K'_a = ([E]/[EI])[I']$ (where [I] is the inhibitor concentration in the spectrophotometer cell) one may calculate an apparent affinity constant K'_a which reflects affinity of enzyme for inhibitor at pH 7.0, in the presence of 0.1M-phosphate buffer, 3.75mM- acetylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic acid). If this is done for various concentrations of one inhibitor, the calculated values for K'_a are similar (±10% of the mean value). If the position of intersection represents a true equilibrium, then it should be possible to approach from either side and obtain the same result. In order to determine whether this was the case, the zero-time inhibition was measured for the C_{10} compound. An appropriate quantity of inhibitor was added to the substrate–chromogenic agent solution in the cell and this was followed by enzyme solution. Four repeats of this procedure gave reasonable reproducible values for the zero-time rate and the mean value was found to lie on the progressive inhibition line at $t = 0$ (the value of t is in fact 10s), indicating that equilibration does take place in the cell and that the values calculated for K'_a do reflect conditions in the cell.

This experiment was not carried out with the enzyme sample used to obtain the values shown in Table 1, and consequently zero-time points do not appear in the figures, since values of K'_a vary from one enzyme sample to another. Whether the values obtained for K'_a may be converted into a true K_a value will depend upon whether the inhibition is competitive or not.

At sufficiently high concentrations of inhibitor (I) the progressive inhibition lines become parallel (Fig. 3) indicating saturation of the enzyme (E). This phenomenon is predicted by the kinetic scheme (1), normally assumed to represent carbamate inhibition, which involves rapid reversible formation of a complex EI, followed by a slower step to give the carbamoylated enzyme EI′, but is not usually seen because of relatively low solubilities and affinities and relatively high carbamoylation rates.



$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EI' \quad (1)$$

$$K_a \simeq k_{-1}/k_{+1}$$

However, it is most convenient to be able to obtain and measure a limiting rate since it indicates that a concentration of inhibitor has been reached such that complexing of enzyme in the incubation vessel is virtually complete. Consequently, the only factor controlling the progressive increase of inhibition must be the rate of the slow irreversible step (i.e. k_{+2} in the scheme above) and the slope of

Table 1. *Rate constants* (k_{+2}), *affinity constants* (K_a) *and relative affinity constants* (K'_a) *for inhibition of fly head acetylcholinesterase with a series of bis*-[(m-*hydroxyphenyl*)*trimethylammonium iodide*] *esters of polymethylenedicarbamic acids* (I)

Results are given ±S.D.

No. of carbon atoms in alkyl chain	k_{+2} (min^{-1})	K'_a (M)	K_a (M)
4	0.15±0.02	1.25 (±0.05)×10^{-5}	—
5	0.28±0.02	6.9 (±0.3)×10^{-6}	—
6	0.27±0.03	4.55 (±0.25)×10^{-6}	—
8	0.51±0.02	1.25 (±0.05)×10^{-6}	1.05×10^{-6}
10	0.96±0.02	3.9 (±0.2)×10^{-7}	2.6 ×10^{-7}
12	0.46±0.04	1.2 (±0.1)×10^{-7}	—

a series of parallel lines should be a direct, accurate, and easily made measurement of the value of this rate constant.

Since one may see the whole range of behaviour predicted by eqn. (1) there are two ways in which one may obtain reaction constants from the observed results. As discussed above, one may calculate nominal affinity constants (K'_a) and true rate constants (k_{+2}) from inhibition lines obtained at high concentrations of inhibitor. Alternatively, one may employ eqn. (2), relating progressive inhibition to time and inhibitor concentration, as derived by Main (1964) for the kinetic scheme given in eqn. (1) assuming that $[I] \gg [E]$ and $k_{-1} \gg k_{+2}$.

$$\frac{1}{[I]} = \left(\frac{1}{2.3} \cdot \frac{dt}{d\log V} \cdot \frac{k_{+2}}{K_a}\right) - \frac{1}{K_a} \quad (2)$$

where [I] is inhibitor concentration; $dt/d\log V$ is the reciprocal slope of progressive inhibition lines and $K_a = k_{-1}/k_{+1}$ if the assumptions made above are correct. See the Appendix for a discussion of the meaning of K_a.

By using a series of lines obtained with relatively low concentrations of inhibitor one may plot $(1/2.3)\cdot(dt/d\log V)$ against $1/[I]$ to obtain intercepts on the abscissa and ordinate of $-1/K_a$ and $1/k_{+2}$ respectively. The limiting slope at high concentrations will appear on such plots as a point on the $dt/d\log V$ axis (ordinate at $1/[I] = 0$) and removes the necessity of determining this intercept by extrapolation. Values of k_{+2} obtained by the two methods will therefore by definition be the same. The values of K_a obtained will reflect conditions in the incubation vessel (i.e. 0.01 M-phosphate buffer, pH 7.4, no substrate) and would be expected to differ from the corresponding values of K'_a. Fig. 4 shows that plots of this kind appear to give straight lines for which K_a values may be estimated (Table 1).

Calculation of K'_a and k_{+2} values for the whole of this series from parallel inhibition lines obtained at high concentrations gave the values shown in Table 1. The values were obtained by carrying out each experiment twice with the highest convenient concentration of each inhibitor and fitting a line to the resulting eight points. Rather surprisingly the values of K_a and K'_a obtained for the C_8 and C_{10} bis-carbamates are very similar, but too much emphasis should not be placed on this for the moment, since there are several possible reasons for this coincidence, which are discussed below.

If inhibition of acetylcholinesterase by this series of compounds is due, in part, to carbamoylation, it might be expected that the analogous dicarboxylic acid esters would be more or less effective substrates for the enzyme. This is indeed the case, as Fig. 5 shows, for the C_8 bis-ester (III). A direct simultaneous comparison of the rate of phenol production in 0.01 M-phosphate buffer, pH 7.4, at 26°C in the presence and absence of enzyme shows that for the fly head enzyme the initial rate of phenol production for the catalysed reaction (after subtracting the spontaneous rate) is about 0.35 mmol of phenol/h per mg of protein at 0.5 mM. The rate of release of thiocholine from acetylthiocholine at the optimum substrate concentration (3.75 mM) for the same enzyme sample was 3.4 mmol/h per mg of protein. Thus the hydrolysis rate for the dicarboxylic acid ester by fly head acetylcholinesterase is about one-tenth that of acetylthiocholine. We have as yet no information about the optimum concentration for the ester (III), but it must in any case be regarded as a fairly good substrate. Fig. 5 also shows a similar experiment carried out with a rather more concentrated sample of acetylcholinesterase from the electric eel, which demonstrates the same behaviour even more markedly. In both cases the overall rate falls sharply after 1 mol of phenol/mol of ester has been produced, but too much cannot be read into this, since the phenol produced is itself a good reversible inhibitor and may, when it reaches a sufficiently high concentration, decrease the hydrolysis rate of the ester (III).

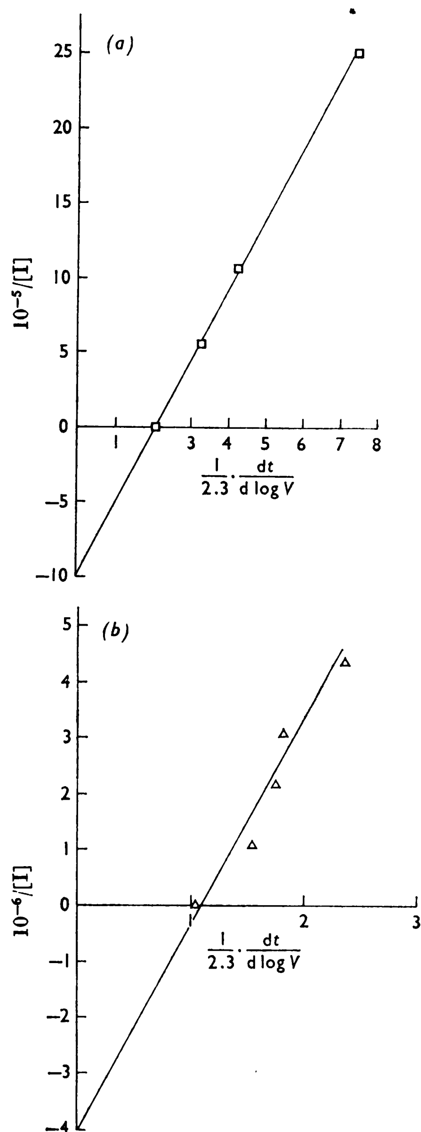


Fig. 4. Double-reciprocal plots for bis-[(*m*-hydroxyphenyl)trimethylammonium iodide] esters of (*a*) octamethylenedicarbamic acid and (*b*) decamethylenedicarbamic acid.

DISCUSSION

The inhibition of acetylcholinesterase by carbamate esters is considered to involve formation of a reversible complex EI, followed by conversion of EI into the carbamoylated enzyme EI′.

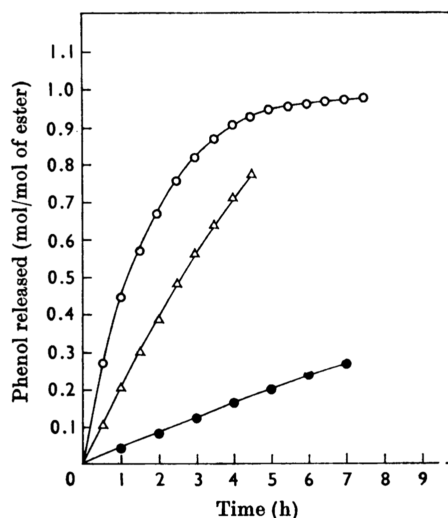
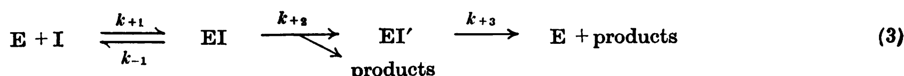


Fig. 5. Hydrolysis of the bis-[(*m*-hydroxyphenyl)trimethylammonium iodide] ester of sebacic acid at 26°C and 0.5mM concentration in 0.01M-phosphate buffer, pH7.4, in the presence of fly head acetylcholinesterase (△); electric eel acetylcholinesterase (○), and in the absence of enzyme (●).

where E is enzyme, I is a carbamate inhibitor and $k_{-1}/k_{+1} = K_a$, provided $k_{-1} \gg k_{+2}$ (see the Appendix). Finally, regeneration of the enzyme may take place by reaction of EI′ with water. In the case of the bis-carbamates (I) and the *N*-hexylcarbamic acid ester (IV) (R = *n*-hexyl) we have shown that k_{+3} is either zero or is very small.

Since the dicarboxylic acid ester (III) is a good substrate for the enzyme, it seems reasonable to suppose that the bis-carbamates (I) may carbamoylate the enzyme, and to ascribe the slow progressive inhibition, described by k_{+2}, to carbamoylation. It is possible that other first-order processes contribute to k_{+2}, but as we have no evidence of this, there is no reason to consider them at this moment. The values obtained for k_{+2} (Table 1) ranging from 0.1 to 1.0 min^{-1} are similar to those reported for a number of phenyl and heterocyclic esters of *N*-methyl- and *NN*-dimethyl-carbamic acid on fly head acetylcholinesterase (Hellenbrand, 1967) and bovine erythrocyte acetylcholinesterase (O'Brien *et al.* 1966).

The results of progressive inhibition studies with bis-carbamates at high concentrations demonstrate a saturation phenomenon (Fig. 3), which may only be explained by supposing that the carbamoylation



$$\mathrm{E + I} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{EI} \xrightarrow{k_{+2}} \mathrm{EI'} \xrightarrow{k_{+3}} \mathrm{E + products} \quad (3)$$
$$\searrow \text{products}$$

step is preceded by complexing. In order that the rate of progressive inhibition may reach the observed maximum, it must be assumed that a stage is reached in the incubation vessel at which all available enzyme is complexed with inhibitor.

These observations make it reasonably certain then that inhibition of acetylcholinesterase by bis-carbamates follows a pathway similar to that normally assumed for inhibition by simple *N*-alkyl-carbamates, i.e. complexing is followed by carbamoylation. The third step, regeneration, takes place very slowly or not at all, owing to the little-understood stability of the carbamoylated enzyme.

Measurement of a parameter reflecting the affinity of enzyme for inhibitor was carried out in two ways (see the Results section) with rather surprising results. An apparent affinity constant K'_a, reflecting conditions in the spectrophotometer cell, in the presence of substrate, was measured for the whole series of bis-carbamates (Table 1) for the purposes of comparison. Not unexpectedly, the results showed a progressive and large increase in affinity (decreasing values of K'_a) with increasing length of the polymethylene chain. It was expected that the values of K'_a would be much larger than than the true K_a values, since the presence of substrate should depress complex-formation, but in the two cases examined (C_8 and C_{10} bis-carbamates) evaluation of K_a by the usual double-reciprocal plot (Fig. 4) gave values of K_a very similar to those obtained for K'_a for the same compounds. There are several possible explanations for this unlooked-for coincidence. In addition to the presence or absence of substrate, the conditions in the spectrophotometer cell differ from those in the incubation vessel with respect to pH and ionic strength. It is conceivable, but not probable, that these differences could result in an increase in affinity that would compensate for depression of complex-formation by substrate. Alternatively, if the assumption made in the double-reciprocal-plot method, that $k_{-1} \gg k_{+2}$, is not correct, then an erroneous value will be obtained for K_a. Lastly, and perhaps most probably, since the compounds under discussion are large amphiphilic molecules, it may be that several types of complex are formed between enzyme and inhibitor that lead to reversible inhibition, but not all of these are capable of conversion into carbamoylated enzyme. Such a situation would not be revealed if the kinetic study were to be carried out with only the double-reciprocal plot, but the values obtained for k_{+2} and K_a would be fictitious and of uncertain meaning. The same is true of values of K'_a and k_{+2} obtained by using inhibitor concentrations high enough to saturate the enzyme, since the proportion of complex capable of conversion into carbamoylated enzyme would be unknown. For the reasons given above, the exact meanings of the constants k_{+2}, K_a and K'_a, in the context of this study, are unknown. The values obtained must therefore be regarded only as a means of comparison within the series of bis-carbamates.

Apart from the uncertainty about the nature of complexing between acetylcholinesterase and bis-carbamate esters of group (I), there is another property that distinguishes these compounds from *N*-methyl- and *NN*-dimethyl-carbamic acid esters. Whereas the latter groups produce an inhibited enzyme that is spontaneously regenerated, bis-carbamates inhibit the fly head enzyme irreversibly. The temptation to ascribe this irreversibility to the presence of a second functional group may be put aside since the (*m*-hydroxyphenyl)trimethyl-ammonium iodide ester of *N*-hexylcarbamic acid also produces irreversible inhibition of the enzyme. It must therefore be a property of the long-chain carbamic acid ester and further examination of the problem might be most conveniently carried out with a fairly short series of simple *N*-alkyl-carbamates.

The probability that esters of long-chain di- and mono-carbamic acids can transfer to the enzyme a carbamoyl group, which is not easily removed, raises some interesting points.

First, the substrate specificity of acetylcholinesterase from various sources leads one to suppose that the esteratic site of the protein will only accept acyl groups of a fairly small limiting size. This is apparently not true and it may therefore be that the effect on hydrolysis rate of increasing the size of the acyl group in a series of choline esters is due to the effect of the acyl group on the preferred conformation of the choline ester, and not directly to any size limit for the accommodation of an acyl group at the esteratic site.

The second point concerns the irreversible nature of inhibition of acetylcholinesterase by esters of long-chain carbamic acids. The detailed mechanism of carbamoylation and decarbamoylation of the enzyme is not known but the work which has been carried out on the solvolysis of *N*-alkyl- and *NN*-dialkyl-carbamates at least indicates which mechanisms may have to be considered. Solvolysis of aromatic esters of *N*-alkylcarbamates under basic conditions is known to involve rapid abstraction by OH^- of the proton attached to the nitrogen atom, followed by a rate-limiting cleavage of the carbonyl carbon–oxygen bond. On the other hand, aliphatic esters of *N*-alkylcarbamic acids and all esters of *NN*-dialkylcarbamic acids are hydrolysed via attack of OH^- on the carbonyl carbon atom to give the intermediate characteristic of carboxylic acid ester hydrolysis (Dittert & Higuchi, 1963; Christenson, 1964; Bender & Howe, 1965; O'Brien *et al.* 1966). One therefore has a choice of two gross mechanisms for carbamoylation of an enzyme, i.e.

by proton abstraction, bond cleavage and nucleophilic attack by the appropriate group on the isocyanate produced, or by direct nucleophilic attack on the carbonyl carbon atom. For decarbamoylation one has no choice since a carbamoylated enzyme will very probably be an aliphatic *N*-alkylcarbamate and must decompose by nucleophilic attack of water on the carbonyl carbon atom. Whatever the mechanisms, one would expect very little difference between carbamoylation rates (k_{+2}) and between decarbamoylation rates (k_{+3}) for a series of carbamates differing only in the length of the *N*-alkyl chain if these rates were controlled only by electronic factors, since the difference in inductive effects between *n*-alkyl groups is small. If, on the other hand, steric factors affect k_{+2} and k_{+3} as well as affinity (K_a), changes in *N*-substitution would have a marked effect. Unfortunately, there seems to be no evidence in the literature about this point. The evidence about bis-carbamates (Table 1) suggests that steric factors do control carbamoylation rates in this series, since k_{+2} increases, up to a point, as alkyl-chain length increases, a change in the opposite direction to that expected if only electronic factors needed to be taken into account. Decarbamoylation rates clearly are greatly affected by the nature of the *N*-alkyl substituent in view of the irreversible inhibition observed, but the reason for this is not clear. Possibly the approach of water molecules to the carbonyl group of the carbamoylated enzyme is hindered by the hydrophobic aliphatic chain.

CONCLUSIONS

Bis-carbamate esters of group (I) appear to inhibit fly head acetylcholinesterase in a manner similar to that generally accepted for inhibition by simple *N*-alkylcarbamates. Complexing is followed by transfer of a carbamoyl group to give an enzyme derivative, which is sufficiently stable for the inhibition to be irreversible. Irreversible inhibition is also produced by a simple ester of *N*-hexylcarbamic acid (IV, $R = n$-hexyl) and it is not therefore necessary to implicate the second functional group of bis-carbamates in order to explain irreversibility of inhibition by these compounds.

The bis-[(*m*-hydroxyphenyl)trimethylammonium iodide] ester of sebacic acid (III) is a reasonable substrate for the fly head enzyme, and this observation suggests that there may be no severe restriction on the size of the acyl group that may be transferred to the enzyme.

REFERENCES

Allen, C. F. H. & Bell, A. (1955). In *Organic Syntheses, Collective Vol.* 3, p. 846. Ed. by Horning, E. C. New York: John Wiley and Sons Inc.

Bender, M. L. & Howe, R. B. (1964). *J. org. Chem.* **30**, 3975.

Christenson, I. (1964). *Acta chem. scand.* **18**, 904.

Cohen, J. A. & Oosterbaan, R. A. (1963). In *Handbuch der experimentellen Pharmakologie*, vol. 15, p. 308. Ed. by Koelle, G. B. Berlin, Göttingen and Heidelberg: Springer-Verlag.

Dittert, L. W. & Higuchi, T. (1963). *J. pharm. Sci.* **52**, 852.

Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. M. (1961). *Biochem. Pharmac.* **7**, 88.

Hellenbrand, K. (1967). *J. agric. Fd Chem.* **15**, 825.

Herzfeld, E. & Stumpf, C. (1956). *Archs int. Pharmacodyn. Thér.* **107**, 33.

Klupp, H., Kraup, O., Schwarzacher, H. G. & Stumpf, C. (1955). *Archs int. Pharmacodyn. Thér.* **101**, 205.

Kraup, O., Schwarzacher, H. G. & Stumpf, C. (1955). *Arch. exp. Path. Pharmak.* **225**, 117.

Main, A. R. (1964). *Science, N.Y.*, **144**, 992.

Main, A. R. & Hastings, F. L. (1966). *Science, N.Y.*, **154**, 400.

Nachmansohn, D. (1959). *Chemical and Molecular Basis of Nerve Activity*, pp. 20–26. New York: Academic Press Inc.

O'Brien, R. D. (1965). *Expl Med. Surg.*, Suppl. Issue Dec. 1965, p. 117.

O'Brien, R. D. (1968). *Molec. Pharmac.* **4**, 121.

O'Brien, R. D., Hilton, B. D. & Gilmour, L. P. (1966). *Molec. Pharmac.* **2**, 593.

Schmied, O., Bilek, L. & Seifried, W. (1956). *Austrian Patent* 185371 (to Österreichische Stickstoffwerke).

Siefken, W. (1949). *Justus Liebigs Annln Chem.* **562**, 75.

Stumpf, C. (1959). *Proc. west. pharmac. Soc.* **2**, 92.

Wilson, I. B., Harrison, M. A. & Ginsburg, S. (1961). *J. biol. Chem.* **236**, 1498.

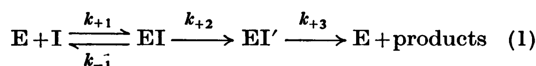
Wilson, I. B., Hatch, M. A. & Ginsburg, S. (1960). *J. biol. Chem.* **235**, 2312.

APPENDIX

Meaning of K_a

This question has been thoroughly examined by Main (1969), who concluded that unless $K_a = k_{-1}/k_{+1}$ eqn. (7) (below) was not valid. In particular, the commonly used version in which K_a is assumed to be $(k_{-1}+k_{+2})/k_{+1}$ is not valid. Main's (1969) examination was comprehensive but it is possible to arrive at the same conclusion in a simpler manner.

The generally accepted view of carbamate inhibition of acetylcholinesterase is expressed by eqn. (1):



$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EI' \xrightarrow{k_{+3}} E + \text{products} \quad (1)$$

where E is enzyme, I a carbamate ester, EI a reversible enzyme–inhibitor complex and EI′ the carbamoylated enzyme. If k_{+3} is assumed to be small, or if it is known to be zero (as for bis-car-

bamate inhibitors), then the following differential and conservation equations describe the process:

$$d[EI]/dt = k_{+1}[E][I] - k_{-1}[EI] - k_{+2}[EI] \quad (2)$$

$$d[EI']/dt = k_{+2}[EI] \quad (3)$$

$$E_0 = [E] + [EI] + [EI'] \quad (4)$$

where E_0 is initial enzyme concentration.

If no assumptions are made about the relative magnitudes of k_{+1}, k_{-1} and k_{+2}, then the above equations have no explicit solution (see, e.g., Mahler & Cordes, 1966). If, however, under experimental conditions such as those described above, it is observed that $\log[100 \times (E_0 - [EI])/E_0]$ [i.e. log (% activity)] varies in a linear fashion with time, i.e. the reaction is first-order, then certain helpful assumptions may be made. Since [EI′] increases with time in a first-order manner, then [EI] must decrease in a first-order manner (eqn. 3). This implies (eqn. 1) that

$$[EI] = a[E] \quad (5)$$

where a is some proportionality constant. If it is assumed that $k_{-1} \gg k_{+2}$ then equilibrium is approximately maintained between [E], [I] and [EI] and one may write

$$[EI] = [E][I]/K_a \quad (6)$$

where $K_a = k_{-1}/k_{+1}$. Since it is arranged that [I] is so much larger than [E] that it is virtually constant, $[EI] = a[E]$, where $a = [I]/K_a$. By making these assumptions Main (1964) derived eqn. (7):

$$\frac{1}{[I]} = \left(\frac{1}{2.303} \cdot \frac{\Delta t}{\Delta \log V} \cdot \frac{k_{+2}}{K_a}\right) - \frac{1}{K_a} \quad (7)$$

where $\Delta \log V$ is the change in the velocity of hydrolysis of substrate after incubation of the enzyme with a concentration [I] of inhibitor for a time Δt.

It should be noted that the assumption of approximate equilibrium between E, I and EI does not involve the assumption that $d[EI]/dt = 0$. The assumption is only that the rate $k_{+2}[EI]$ is much smaller than the rate $k_{-1}[EI]$. Thus, if first-order kinetics are observed, eqn. (7) may be valid over a wide range of concentrations of EI.

In order to obtain the expression $K_a = (k_{-1} + k_{+2})/k_{+1}$ in eqn. (7), it is necessary to make the assumption that $d[EI]/dt = 0$ and hence, from eqn. (3):

$$[EI] = [E][I]k_{+1}/(k_{-1} + k_{+2}) \quad (8)$$

Now suppose that $k_{-1} \simeq k_{+2}$, but that first-order kinetics are observed, then the concentration of EI must vary with time in a first-order manner, but at any given time must be less than that predicted by the equilibrium (eqn. 6). Therefore, $K_a > k_{-1}/k_{+1}$ and

$$K_a = (k_{-1} + x)/k_{+1} \quad (9)$$

If during a typical experiment the enzyme activity decreases from 100% to say 50%, as it may well do, then the values of [E] and [EI] must decrease to one-half of the initial value in each case, regardless of the value of x. If one puts $x = k_2$ then the result is absurd, since it has to be assumed that $d[EI]/dt = 0$ in order to derive the expression (8).

The only case in which expression (8) could be held to be correct is that in which the observed kinetics are zero-order.

REFERENCES

Mahler, H. E. & Cordes, E. H. (1966). *Biological Chemistry*, chapter 6, p. 224. New York, Evanston and London: Harper and Row–John Weatherhill Inc.

Main, A. R. (1964). *Science, N.Y.*, **144**, 992.

Main, A. R. (1969). *Can. med. Ass. J.* **100**, 161.