Acetylcholinesterase

TWO TYPES OF INHIBITION BY AN ORGANOPHOSPHORUS COMPOUND: ONE THE FORMATION OF PHOSPHORYLATED ENZYME AND THE OTHER ANALOGOUS TO INHIBITION BY SUBSTRATE

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1. The kinetics of the reaction of di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate (haloxon) and related compounds with acetylcholinesterase were studied and found to be unusual. 2. By a progressive reaction haloxon produces a di-(2-chloroethyl)phosphorylated enzyme. The influence of substrate on this reaction leading to a phosphorylated active centre was studied. From competition experiments between inhibitor and substrate values of K_m for acetylcholine and acetylthiocholine of 0.79 mM and 0.23 mM respectively were derived. 3. Haloxon also combines with acetylcholinesterase by a non-progressive reaction, producing a complex that is reversible by dilution and by high concentrations of acetylcholine and acetylthiocholine. From this non-progressive reaction the competition between haloxon and substrate was studied, and it was shown that haloxon combines with a site involved in inhibition by substrate. From competition experiments the following dissociation constants were derived: for combination of haloxon and this site K_i is 4.9 μ M and for the combination of substrates with this site K_{ss} values are 12 mM and 3.3 mM for acetylcholine and acetylthiocholine respectively. 4. The non-phosphorus-containing compound 3-chloro-7-hydroxy-4-methylcoumarin was shown to be a good reagent for the site involved in inhibition by substrate; its dissociation constant for the combination with this site is 30 μ M. 5. In order to interpret the experimental results, theoretical equations were derived for an enzyme with two binding sites to both of which substrate and inhibitor can combine. The equations correlate the activity of the enzyme with the concentration of substrate and inhibitor, for both progressive and non-progressive inhibition. These equations are applicable to reactions of acetylcholinesterase with organophosphorus compounds, carbamates etc. and may be applicable to other enzymes possessing two binding sites.

All cases of phosphorylation of cholinesterases by organophosphates so far described are consistent with the following equation (Main, 1964; Reiner & Aldridge, 1967):

EH and EA stand for the free and phosphorylated enzyme respectively; $(EH \cdots AB)$ is a Michaelis-type complex between enzyme and inhibitor (AB). The integrated differential equation for reaction (1) is:

$$\ln \left(\frac{[EH_0]}{[EH_0] - [EA]} \right) = \frac{k_{+2}[AB] \cdot t}{K_a + [AB]} \quad (2)$$

where $[EH_0]$ is the total enzyme concentration, t is the time of the reaction and K_a is:

$$K_a = (k_{+2} + k_{-1})/k_{+1} \quad (3)$$

When the concentration of the Michaelis complex is negligible eqns. (1) and (2) become respectively (Aldridge, 1950):

and

$$\ln \left(\frac{[EH_0]}{[EH_0] - [EA]} \right) = k_a \cdot t \cdot [AB] \quad (5)$$

When $[AB] \ll K_a$, it follows from eqns. (2) and (5) that:

$$k_a = k_{+2}/K_a \quad (6)$$

If the phosphorylated enzyme is capable of hydrolysis:

The integrated differential equation for reaction (7) is:

$$\ln \left(\frac{[\text{EA}_0]}{[\text{EA}]} \right) = k_{+3} \cdot t \quad (8)$$

where $[\text{EA}_0]$ stands for the initial concentration of the phosphorylated enzyme.

The present paper describes the kinetics of inhibition of acetylcholinesterase by some di-(2-chloroethyl) phosphates. It has been shown that inhibition of the enzyme by these compounds produces di-(2-chloroethyl)phosphorylated cholinesterase, which is unstable and spontaneously dephosphorylates (Lee & Hodsden, 1963; Lee, 1964; Reiner & Aldridge, 1967; Pickering & Malone, 1967). The kinetics of inhibition by haloxon obtained by Lee (1964) were abnormal, and he attributed the

abnormality to spontaneous reactivation of the phosphorylated enzyme. This explanation is not possible when the time-scale of inhibition and reactivation is taken into account. A re-examination of the kinetics of inhibition by organophosphorus compounds containing a 3-chloro-4-methylcoumarin moiety has shown some unusual features, which cannot entirely be explained on the basis of the general reactions outlined above.

MATERIALS AND METHODS

Enzyme preparation. The enzyme preparation was purified bovine erythrocyte cholinesterase (Winthrop Laboratories Inc., New York, N.Y., U.S.A.). Stock solutions were prepared in water and kept at 4° no longer than 1–2 days. Further dilutions were prepared in buffer immediately before use. The enzyme concentration during assay was 0.015 mg./ml. (spectrophotometric determination) or 0.15–0.40 mg./ml. (manometric determination). The enzyme concentration during inhibition varied according to the experimental procedure, which is described in the Results section.

Table 1. Chemical names and structural formulae of inhibitors

Di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate (haloxon)

Diethyl 3-chloro-4-methylcoumarin-7-yl phosphate (coroxon)

Di-(2-chloroethyl) 4-nitrophenyl phosphate (nitrophenylhalon)

Di-(2-chloroethyl) 2,4,5-trichlorophenyl phosphate (trichlorophenylhalon)

3-Chloro-7-hydroxy-4-methylcoumarin

Buffers. The phosphate-pyrophosphate buffer was prepared by titrating 20 mM- Na_2PO_4 with 20 mM- $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ to pH 7.4 or 8.0. The phosphate buffer was prepared by titrating 0.5 M- Na_2HPO_4 with 0.5 M- NaH_2PO_4 to pH 7.4. The bicarbonate buffer, pH 7.4, contained 18 mM- NaHCO_3 and 0.14 M- NaCl , and was gassed with $\text{CO}_2 + \text{N}_2$ (5:95).

Inhibitors. Haloxon, coroxon, nitrophenylhalon, trichlorophenylhalon and 3-chloro-7-hydroxy-4-methylcoumarin were obtained from Cooper Technical Bureau, Berkhamsted, Herts. (see Table 1 for structural formulae and chemical names). Stock solutions (10 mM) were prepared in ethanol. Further dilutions were prepared in buffer immediately before use.

Determination of enzyme inhibition. The spectrophotometric and manometric methods were used for measuring inhibition of cholinesterase.

In the spectrometric procedure enzyme and inhibitor were incubated in phosphate-pyrophosphate buffer. The concentration of ethanol in the enzyme-inhibitor mixture was 2.0% (v/v) or less; control samples contained no inhibitor but the same ethanol concentration. At suitable time-intervals the substrate (acetylthiocholine iodide) solution was added, and the enzyme activity was measured by the method of Ellman, Courtney, Andres & Featherstone (1961). Details of the volume of enzyme, inhibitor and substrate solutions are given with the particular experiments, which were all carried out at 25° and pH 7.4. The extinction at 412 nm. was read on a Unicam SP.600 spectrophotometer or on a modified Unicam SP.1400 spectrophotometer at 15 sec. intervals for 1 min., at least three readings being taken beginning 15 or 30 sec. after the addition of substrate. When the substrate concentration was 10 μM , 4.0 cm. cells were used and the time of assay was 0.5 min. The extinction increased linearly for 1 min. for both inhibited and uninhibited enzyme samples. Corrections for spontaneous hydrolysis were necessary only when the substrate concentration was 10 mM.

For the manometric procedure substrate (acetylcholine chloride or perchlorate) and inhibitor in 1.8 ml. of bicarbonate buffer were put into the main compartment of the Warburg flask, and the enzyme solution (0.2 ml.) was added from the side arm. The final concentration of ethanol during enzyme assay was 2.0% (v/v) or less; control samples contained no inhibitor, but the same ethanol concentration. All experiments were carried out at 25° and pH 7.4.

Measurements of spontaneous reactivation. The enzyme was incubated with a suitable concentration of inhibitor in phosphate-pyrophosphate buffer, pH 7.4 to obtain about 80–90% inhibition. The enzyme-inhibitor solution was then diluted 1:500 with phosphate-pyrophosphate buffer, pH 8.0. At suitable time-intervals 3.0 ml. samples were withdrawn and 7.0 ml. of phosphate buffer, pH 7.4, containing the substrate (acetylcholine iodide) was added. Enzyme activity was determined by the spectrophotometric method of Ellman *et al.* (1961). The procedure is described in detail elsewhere (Reiner & Aldridge, 1967). All experiments were carried out at 25°.

RESULTS

Kinetics of acetylcholinesterase inhibition by haloxon and coroxon. Enzyme and inhibitor were incubated in a total volume of 10.0 ml. of phosphate-pyrophosphate buffer for a certain time, before

addition of substrate (0.1 ml. of acetylthiocholine iodide). The final substrate concentration during enzyme assay was 1.0 mM. This procedure of measuring inhibition of cholinesterase is referred to below as the no-dilution procedure (addition of substrate to the enzyme-inhibitor mixture produces a dilution of only 1%).

At each concentration of haloxon the loss of enzyme activity followed first-order kinetics, but the first-order rates so obtained were not linearly related to the concentration of haloxon (Fig. 1). Further, the first-order lines did not pass through the origin [$\log(100\% \text{ activity}) = 2.0$]. To obtain the enzyme activity at zero-time inhibition, the enzyme solution (1.0 ml.) was added to a mixture of haloxon and substrate (9.1 ml.) and the activity assayed as described above. In individual experiments the intercept on the vertical axis obtained by extrapolation of the first-order lines was always identical with that obtained experimentally by adding enzyme to a mixture of haloxon and substrate (Fig. 1). Such kinetics are abnormal.

The usual reaction between cholinesterase and

Fig. 1. Inhibition of acetylcholinesterase by haloxon. The no-dilution technique was used (see the text for details). The results presented are for one particular experiment. The concentration of haloxon and the derived first-order constant, k , are shown against each line. The reproducibility in different experiments is given below; the values in parentheses are the numbers of experiments.

Concn. of haloxon (μM)	k (min. ⁻¹)		Intercept (% activity at $t = 0$)	
	Mean $\pm$ s.e.m.	Range	Mean $\pm$ s.e.m.	Range
1	0.18 $\pm$ 0.01 (8)	0.15–0.22	83 $\pm$ 1 (13)	78–87
5	0.52 $\pm$ 0.02 (9)	0.41–0.60	54 $\pm$ 2 (14)	43–63
20	0.84 $\pm$ 0.06 (7)	0.71–1.03	28 $\pm$ 2 (14)	18–38

Fig. 2. Inhibition of acetylcholinesterase by coroxon. The no-dilution technique was used (see the text for details). The concentration of coroxon and the derived first-order constant, k , are shown against each curve.

Fig. 3. Inhibition of acetylcholinesterase by nitrophenylhalon. The no-dilution technique was used (see the text for details). The concentration of nitrophenylhalon and the derived first-order constant, k , are shown against each curve.

organophosphorus compounds shows the characteristics defined by eqns. (4) and (5); nitrophenylhalon (Fig. 3) and trichlorophenylhalon behave in this way. The second-order rate constants for these inhibitors are 4.7×10^5 and $4.8 \times 10^5 \text{ l. mole}^{-1} \text{ min}^{-1}$ respectively. When the first-order rates are not linearly related to inhibitor concentration (cf. eqns. 2 and 3), this indicates the presence of an appreciable amount of a Michaelis-type complex between enzyme and inhibitor (eqn. 1). An example of this type of kinetics is inhibition of cholinesterase by the methyl sulphate of diethyl 3-trimethylaminophenyl phosphate (cf. Fig. 2 of Reiner & Aldridge, 1967). However, in this instance there are no intercepts, all lines passing through the origin.

The kinetics of inhibition of cholinesterase by coroxon (Fig. 2) (containing ethoxy groups) is analogous to those of haloxon (containing 2-chloroethoxy groups), whereas those for nitro-

Fig. 4. Inhibition of acetylcholinesterase by haloxon. The inhibitor concentration before the addition of acetylthiocholine (final concn. 1.0 mM) was $10 \mu\text{M}$. The enzyme activity was measured after different dilutions as indicated against each line. The times are those for the incubation of inhibitor and enzyme before dilution.

phenylhalon (Fig. 3) and trichlorophenylhalon are those of the simplified reaction described by eqns. (4) and (5). This suggests that the abnormal kinetics obtained with haloxon and coroxon are associated with the coumarin part of the molecule and not the chloroethoxy group. This possibility is made more likely by the observation that 3-chloro-7-hydroxy-4-methylcoumarin is also inhibitory, though less so than haloxon or coroxon; the inhibition is non-progressive.

Progressive inhibition of acetylcholinesterase by haloxon and coroxon. In the experiments described (Figs. 1 and 2) the no-dilution technique was employed. By incubating the enzyme and inhibitor in a small volume and diluting with substrate, various degrees of dilution were obtained [3 ml. of (enzyme + inhibitor) + 7 ml. of substrate solution = 3.3 times dilution; 0.5 ml. + 10 ml. = 21 times dilution; 0.4 ml. + 40 ml. = 101 times dilution]. These different procedures ensure that the concentration of free inhibitor during the determination of activity may be varied independently of its concentration during the preincubation with enzyme. Typical results are shown in Fig. 4. For a given concentration of inhibitor, the rate of the progressive inhibition is independent of the subsequent dilution. However, the intercept on the vertical axis [$\log(100\% \text{ activity}) - \log(\% \text{ activity at } t=0)$] is much decreased; the inhibition indicated by the intercept is therefore reversible and the extent of this inhibition is proportional to the concentration of inhibitor during the determination of activity.

Fig. 5. Inhibition of acetylcholinesterase by haloxon. Enzyme activity was measured by the no-dilution technique and the concentration of haloxon was $5\mu\text{M}$. The concentration of acetylthiocholine present during the determination of enzyme activity is shown against each line.

Fig. 6. Effect of substrate on progressive inhibition by haloxon during the determination of enzyme activity. Enzyme was added to a mixture of haloxon and substrate (final concns. $5\mu\text{M}$ and 1mM respectively). Line A is the control activity in the absence of inhibitor. The slope of the broken line B yields the initial enzyme activity in the presence of inhibitor and is the tangent to the curve C. The solid line D with no experimental points is the calculated loss of activity in the absence of substrate (cf. Fig. 1; $k = 0.57\text{ min.}^{-1}$ for $5\mu\text{M}$ -haloxon).

Increasing the concentration of substrate during assay from 1.0 to 10.0mM does not affect the rate of progressive inhibition, but decreases the intercept on the vertical axis (Fig. 5).

For all experiments with acetylthiocholine, the

determination of activity was complete within 1min. after adding the substrate. In the absence of inhibitor the activity was linear for at least 6min. (Fig. 6). In the presence of inhibitor some departure from linearity was found; this was due to progressive inhibition, which takes place in spite of the presence of substrate, but this rate of inhibition is greatly diminished compared with the rate in the absence of substrate (Fig. 6). It is clear that errors due to inhibition during the 1min. assay will be very small.

Progressive inhibition of cholinesterase by organophosphorus compounds involves phosphorylation of the enzyme. To verify that the enzyme obtained by progressive inhibition with haloxon is di-(2-chloroethyl)phosphorylated acetylcholinesterase, the rate of the spontaneous reactivation of the inhibited enzyme was measured. The enzyme was inhibited 80 – 90% by haloxon and the rate of reactivation of the inhibited enzyme followed eqn. (8) (Fig. 7). The rate constant of reactivation obtained graphically, k_{+3} , is 0.017 min.^{-1} . The rate of spontaneous reactivation of acetylcholinesterase inhibited by nitrophenylhalon was identical with that of acetylcholinesterase inhibited by haloxon (Fig. 7). This proves that the two inhibitors produced di-(2-chloroethyl)phosphorylated acetylcholinesterase, the leaving groups being the coumarin and 4-nitrophenol respectively.

Reversible inhibition of acetylcholinesterase by haloxon and coroxon. The phenomenon of a reversible inhibition in addition to progressive inhibition is very unusual for organophosphorus compounds. The only previous instance when kinetics analogous to those presented in Figs. 1 and 2 was found has subsequently been shown to be due to an impurity in the inhibitor (Aldridge, 1950; Aldridge & Davison, 1952). Therefore considerable attention was given to the purity of the haloxon and coroxon. Different preparations have always given the same results, even when the starting materials were specially purified before synthesis. Both haloxon and coroxon are solids; repeated recrystallization has no influence on their activity. The possibility of an impurity of low stability was examined by testing activity after incubation of a stock solution of haloxon in ethanol at 38° for 4 weeks; activity was unaltered.

One other theoretical possibility is that the inhibitor and substrate react to produce a reversible inhibitor. The following observations lead us to the view that this is unlikely: (1) if haloxon is added to the enzyme just after or together with the substrate, the same inhibition is obtained; (2) if the substrate and haloxon are preincubated, before the enzyme is added, inhibition is independent of the time of preincubation; (3) the phenomenon occurs with haloxon, coroxon and the coumarin; (4) inhibition decreases with increasing substrate concentration.

Fig. 7. Spontaneous reactivation of di-(2-chloroethyl)phosphorylated acetylcholinesterase. Enzyme was incubated before dilution for 4 min. with $10\mu\text{M}$ -haloxon (○), for 20–30 min. with $2.5\mu\text{M}$ -haloxon (Δ) or for 4 min. with $1.2\mu\text{M}$ -nitrophenylhalon (□). The derived first-order rate constant is shown.

Fig. 8. Representation of a theoretical case of the effect of dilution. Parallel lines *A* and *B* correspond to inhibition of acetylcholinesterase by haloxon obtained by the 100-times-dilution procedure (*A*) and no-dilution procedure (*B*). In the no-dilution procedure the sum of the phosphorylated and reversibly inhibited enzyme is measured. Below are given the percentage activities corresponding to the lines.

Re- action time	Enzyme activity (%)		Col. 1— Col. 2 = reversibly inhibited enzyme	Col. 3 as % (74.9 = 100%)	Col. 3 as % of Col. 1
	Curve A	Curve B			
	(1)	(2)	(3)	(4)	(5)
0	100.0	25.1	74.9	100.0	74.9
1	83.1	20.9	62.2	83.1	74.9
2	69.2	17.4	51.8	69.2	74.9
3	57.5	14.4	43.1	57.5	74.9
4	47.9	12.0	35.9	47.9	74.9
5	39.8	10.0	29.8	39.8	74.9

From these observations we conclude that the abnormal kinetics are due to haloxon and coroxon.

Relationship between reversible and progressive inhibition. Before evaluation of the experimental results so far described, it is important to appreciate the meaning of the type of kinetics demonstrated in Fig. 4. Fig. 8 illustrates a theoretical case where application of the no-dilution and the 100-times-dilution technique leads to parallel inhibition lines in a logarithmic plot. In other words in both anticipated cases the first-order rate constants of inhibition are the same.

In actual experiments the conditions of incubation of enzyme and haloxon in the two cases were identical except for the enzyme concentration. In the 100-times-dilution technique (cf. Fig. 4) it was 100-fold higher and was approx. 6 nM [the calculation is based on a catalytic-centre activity for bovine erythrocyte cholinesterase at 37° of 300 000 (Cohen & Warringa, 1953); acetylthiocholine is hydrolysed at a similar rate to acetylcholine (Koelle, 1950; Ellman *et al.* 1961); the temperature co-efficient for acetylcholinesterase is approx. 1.4 (Shukuya, 1953)]. The lowest concentration of inhibitor was $1\mu\text{M}$, which is about 200-fold higher than the enzyme concentration. In both instances, therefore, the inhibitor is in large excess compared with the enzyme and the bimolecular reaction will show first-order kinetics. The determination of activity by using the no-dilution and 100-times-dilution techniques, when the enzyme is incubated with the same concentration of inhibitor, may logically be used to provide information about the type of inhibited enzyme present in the solution.

The theoretical line *A* in Fig. 8 passing through the origin gives the rate of loss of enzyme activity

due to the formation of phosphorylated enzyme. In the legend to Fig. 8 the activities are given from which the logarithmic plot was derived. Column 3 is the measure of the reversible inhibition. As the reaction proceeds, the amount of reversible inhibition decreases (column 3) and the rate of its decrease is the same as the rate of phosphorylation (compare columns 1 and 4), and therefore at all times the reversibly inhibited enzyme is in equilibrium with the non-phosphorylated enzyme (column 5, cf. eqns. 1 and 11).

In our experiments the rate of progressive inhibition is dependent only on the concentration of inhibitor during the incubation period and not on concentration during assay (Fig. 4). The rate constants are, however, not linear with respect to inhibitor concentration (Figs. 1 and 2). Two different expressions can be derived to give the relation between the first-order rate constant and inhibitor concentration. Accepting the usual mechanisms of the reaction given in eqn. (1), the first-order rate constant, k , for a given inhibitor concentration is from eqn. (2):

$$k = \frac{(k_{+2}/K_a)[AB]}{1 + [AB]/K_a} \quad (9)$$

Rearrangement according to Wilkinson (1961) yields:

$$\frac{[AB]}{k} = \frac{K_a}{k_{+2}} + \frac{[AB]}{k_{+2}} \quad (10)$$

When $[AB]/k$ is plotted against $[AB]$ the slope is $1/k_{+2}$ and the intercept K_a/k_{+2} .

However, another mechanism of inhibition of acetylcholinesterase is possible and is defined by eqn. (11):

In this mechanism the Michaelis-type complex is omitted (cf. eqn. 4) and $(\text{EH} \cdots \cdots \text{AB})^R$ is another reversible complex between enzyme and inhibitor that does not lead to phosphorylated enzyme. The dissociation constant, K_i for this complex is given by:

$$K_i = [\text{EH}][\text{AB}]/[(\text{EH} \cdots \cdots \text{AB})^R] \quad (12)$$

and the first-order rate constant for progressive inhibition at a given inhibitor concentration, k , is (see Appendix 2 for derivation):

$$k = \frac{k_a[\text{AB}]}{1 + [\text{AB}]/K_i} \quad (13)$$

Eqn. (13) is analogous to eqn. (9) and rearrangement yields:

$$\frac{[\text{AB}]}{k} = \frac{1}{k_a} + \frac{[\text{AB}]}{k_a K_i} \quad (14)$$

Depending on whether the mechanism defined by eqn. (11) or eqn. (1) holds, a graph of $[\text{AB}]/k$ against $[\text{AB}]$ will therefore yield k_a and K_i (eqn. 14) or K_a and k_{+2} (eqn. 10) respectively. The alternative values obtained for haloxon and coroxon by such graphs are given in Table 2.

Influence of substrate on reversible inhibition. Since substrate influences the reversible inhibition (Fig. 5), a detailed study has been made of the effect of various concentrations of acetylthiocholine and acetylcholine on the reversible inhibition by haloxon, coroxon and the coumarin. The results have been analysed by the method of Hunter & Downs (1945) by using eqn. (15) for competitive inhibition with respect to substrate:

$$\frac{v[\text{AB}]}{v_0 - v} = K_i + \frac{K_i}{K_m}[\text{S}] \quad (15)$$

where v_0 is the enzyme activity in the absence of inhibitor and v is the activity in the presence of inhibitor. When acetylthiocholine is used as a substrate both v_0 and v are the activities over the

Table 2. Kinetic constants derived from progressive inhibition of acetylcholinesterase by haloxon and coroxon in the absence of substrate

The numbers of observations are indicated by n and the results are expressed as means $\pm$ s.e.m. $[\text{AB}]$ indicates the range of concentrations of inhibitor used. See the text for eqns. (10) and (14).

Constants calculated from	
Eqn. (10)	Eqn. (14)
Haloxon ($n = 79$; $[\text{AB}] = 0.75\text{--}20 \mu\text{M}$)	
$K_a = 4.60 \pm 0.49 \mu\text{M}$	$K_i = 4.60 \pm 0.49 \mu\text{M}$
$k_{+2} = 0.97 \pm 0.14 \text{ min.}^{-1}$	$k_a = (2.11 \pm 0.24) \times 10^5 \text{ l. mole}^{-1} \text{ min.}^{-1}$
Coroxon ($n = 21$; $[\text{AB}] = 5\text{--}100 \mu\text{M}$)	
$K_a = 12.5 \pm 2.9 \mu\text{M}$	$K_i = 12.5 \pm 2.9 \mu\text{M}$
$k_{+2} = 0.99 \pm 0.05 \text{ min.}^{-1}$	$k_a = (7.9 \pm 1.9) \times 10^4 \text{ l. mole}^{-1} \text{ min.}^{-1}$

Table 3. *Kinetic constants for the reversible inhibition of acetylcholinesterase by haloxon, coroxon and 3-chloro-7-hydroxy-4-methylcoumarin*

The results are calculated by using eqn. (15). The numbers of observations are indicated by n and the results are expressed as means $\pm$ s.e.m. [AB] and [S] indicate the range of concentration of inhibitor and substrate studied respectively.

Inhibitor and substrate	n	K_i (μM)	K_{ss} (mM)	[AB] (μM)	[S] (mM)
Haloxon and acetylcholine	62	5.12 ± 0.73	10.2 ± 3.3	1.25–40	3–100
Haloxon and acetylthiocholine	105	4.95 ± 0.37	3.6 ± 0.4	1–20	0.01–10
Coroxon and acetylcholine	35	19.1 ± 2.4	19.7 ± 3.2	5–80	10–100
Coumarin and acetylcholine	34	32 ± 16	5.6 ± 3.0	15–500	7–100
Coumarin and acetylthiocholine	28	28 ± 4	2.3 ± 0.4	10–100	0.01–10

first minute after adding enzyme to a mixture of inhibitor and substrate. For acetylcholine as substrate v_0 and v were the initial rates of carbon dioxide liberation assessed graphically. K_i is the dissociation constant for the enzyme-inhibitor complex and K_m is the Michaelis constant for substrate (Dixon & Webb, 1964). The substrate concentrations ranged from $10 \mu\text{M}$ to 10mM for acetylthiocholine and from 3mM to 100mM for acetylcholine.

When $v[\text{AB}]/(v_0 - v)$ is plotted against [S] straight lines are obtained (illustrated for coumarin in Fig. 9) and according to Hunter & Downs (1945) K_i and K_m may be evaluated. For a given inhibitor the same K_i values were obtained irrespective of whether acetylcholine or acetylthiocholine was used as substrate (Table 3). Further, the K_i values obtained for haloxon and coroxon approximate to the values for K_a or K_i derived from the rates of progressive inhibition in the absence of substrate (Table 2). The reversible inhibition may be therefore due to a complex between enzyme and inhibitor that is not a Michaelis-type complex (eqn. 11). This conclusion is substantiated by the fact that the concentrations of substrate competitive with this complex are much higher than K_m for substrate and enzyme determined by standard methods. The effective concentrations in our experiments are similar to those that cause inhibition by excess of substrate (usually described by K_{ss} ; Table 3). This leads us to the conclusion that the Hunter & Downs (1945) equation (eqn. 15) does not, in this case, yield K_m , but may give K_{ss} . As shown in Appendix 1 an equation of the same type as that of Hunter & Downs (1945) may be derived if the inhibitor competes for the site at which high concentration of substrate causes inhibition. Therefore in Fig. 9 and Table 3 the derived constants for substrates are designated K_{ss} and in Table 2 the derived constants are K_i and not K_a .

Influence of substrate on progressive inhibition. For reversible inhibition by haloxon or coroxon high concentrations of substrate are competitive and K_{ss} was evaluated (Table 3). For progressive inhibi-

Fig. 9. Relation between reversible inhibition of acetylcholinesterase by the coumarin and the concentration of acetylcholine. Enzyme was added to a mixture of coumarin and acetylcholine and the enzyme activity was determined manometrically. Readings were taken at 2 min. or 5 min. intervals depending on the concentration of acetylcholine used. The derived constants from the regression line shown are K_i $32 \pm 16 \mu\text{M}$ and K_{ss} $5.6 \pm 3.0 \text{mM}$ (cf. eqn. 15). In all 34 determinations were made and the range of concentrations of the coumarin was $15\text{--}500 \mu\text{M}$. Numbers in parentheses indicate the numbers of identical values.

tion that is due to phosphorylation of the active centre, a study of the influence of substrate should allow us to derive K_m . The first-order rate constant for progressive inhibition in the presence of substrate, k' , for a system described by eqn. (11) is given by eqn. (16) (see Appendix 3 for derivation):

$$k' = \frac{k_a[\text{AB}]}{1 + \frac{[\text{S}]}{K_m} + \frac{[\text{S}]}{K_{ss}} + \frac{[\text{S}]^2}{K_m K_{ss}} + \frac{[\text{AB}]}{K_i} + \frac{[\text{AB}][\text{S}]}{K_m K_i}} \quad (16)$$

The rates of progressive inhibition in the presence of substrate, k' , were measured for haloxon and coroxon by using the spectrophotometric method (with acetylthiocholine as substrate) and the

Table 4. *Kinetic constants derived from the influence of substrate on progressive inhibition of acetylcholinesterase by haloxon and coroxon*

k' is the measured first-order constant; k is the first-order constant with no substrate present (calculated from the constants in Table 2). K_m is calculated by using eqn. (16). The numbers of observations are given in parentheses and the results are expressed as the means $\pm$ s.e.m. $[S]$ and $[AB]$ are the concentrations of substrate and inhibitor used respectively. The constants used in the calculation of K_m are derived from the mean of the results in Tables 2 and 3 and are: K_i (haloxon) $4.9 \mu\text{M}$; K_i (coroxon) $16.6 \mu\text{M}$; K_{ss} (acetylcholine) 12 mm ; K_{ss} (acetylthiocholine) 3.3 mm ; k_a (haloxon) $2.11 \times 10^5 \text{ l. mole}^{-1} \text{ min.}^{-1}$; k_a (coroxon) $7.9 \times 10^4 \text{ l. mole}^{-1} \text{ min.}^{-1}$.

$[S]$ (mm)	$[AB]$ (μM)	k (min.^{-1})	k' (min.^{-1})	K_m (mm)
Acetylthiocholine and haloxon				
1.0	5	0.505	0.091 (4)	0.25 ± 0.01
	20	0.790	0.135 (3)	0.21 ± 0.02
			Mean	0.23 ± 0.01 (7)
Acetylcholine and haloxon				
10	5	0.505	0.021 (1)	0.60
	10	0.664	0.032 (5)	0.62 ± 0.07
	20	0.790	0.042 (1)	0.63
	40	0.870	0.078 (1)	1.05
			Mean	0.67 ± 0.07 (8)
Acetylcholine and coroxon				
10	20	0.608	0.040 (12)	0.84 ± 0.08
	40	0.752	0.056 (11)	0.82 ± 0.09
			Mean	0.83 ± 0.06 (23)

manometric method (with acetylcholine as substrate). Inhibition was followed for up to 5 min. in the spectrophotometric procedure (readings being taken every 15 or 30 sec.; cf. Fig. 6) and for up to 20 or 30 min. in the manometric procedure (readings taken every 2 or 3 min.). The results presented in Table 4 show clearly that both substrates slow down the rate of progressive inhibition by haloxon and coroxon. The calculated mean K_m for acetylcholine is 0.79 mm and for acetylthiocholine 0.23 mm .

The values of K_m thus obtained (Table 4) are more than one order lower than the values for K_{ss} given in Table 3. The great difference between the concentrations of substrate effective in competing with the progressive and reversible inhibition (K_m and K_{ss} respectively) reflect two different reactions between enzyme and inhibitor.

Reproducibility of the results. It has been our experience that the reproducibility of the results is less than we expected (see the legend to Fig. 1) and we have no explanation for the variability.

In presenting the results according to eqn. (15) small variations in enzyme activity lead to large changes in the ratio $v/(v_0 - v)$. This is part of the

explanation of the large scatter in Fig. 9 and contributes to the range of K_{ss} values for a given substrate (Table 3).

For a given inhibitor all values of K_i determined from different types of experiment are within 1 s.e.m. (Tables 2 and 3). All K_m values for acetylcholine and acetylthiocholine are about 15-fold smaller than the K_{ss} for these substrates and the statistical significance of this difference is certain.

DISCUSSION

A detailed examination of the kinetics of inhibition of acetylcholinesterase by haloxon and coroxon has been made. Since the kinetics were unusual, many experiments were carried out (see Tables 2 and 3) before we were convinced that the observed phenomenon did not originate in errors in the technique or because of impurities. These possibilities have been eliminated.

Haloxon and coroxon react with acetylcholinesterase in two ways, one to yield a phosphorylated enzyme and the other to produce an inactive but reversible complex. The reversible complex is revealed by a non-progressive inhibition and is distinct from a Michaelis-type complex between enzyme and inhibitor. The concentrations of substrate that compete effectively are those that cause substrate inhibition. For acetylcholine our mean derived K_{ss} is 12 mm and the range of published values is $11\text{--}22 \text{ mm}$ (Shukuya, 1951; Myers, 1952; Augustinsson, 1948). For acetylthiocholine our mean derived K_{ss} is 3.3 mm but the published value is 30 mm (Ellman *et al.* 1961; see below for discussion of this value). It seems probable therefore that haloxon, coroxon and the coumarin are reacting in a similar way to acetylcholine or acetylthiocholine when they cause inhibition. The derivation of the equation in Appendix 1 shows that, when inhibitor and substrate compete for a site other than the active centre, the Hunter & Downs (1945) plot will yield K_{ss} . Since inhibitor and high concentrations of substrate compete, presumably they combine with the same site. Our results indicate that the site is the substrate inhibition site. The complex produced by the reaction between cholinesterase and high concentrations of substrate is usually thought to be inactive. It was difficult for us to understand how competition for this site could lead to a decrease in inhibition by haloxon; we therefore considered that the substrate-inhibited complex (ES_1S_2 ; see Scheme 1 for symbols) decomposed to give products. However, derivation of the Hunter & Downs (1945) type of equation (see Appendix 1) shows that the same equation is obtained whether the substrate-inhibited complex decomposes or not. When ES_1S_2 is stable (i.e. $k'_0 = 0$; Scheme 1), the derived K_{ss} will be a dissociation

Fig. 10. Relation between the concentration of substrate and activity of acetylcholinesterase. v is the relative rate of hydrolysis of substrate, $[S]$ is the concentration of substrate, and k_0 and k'_0 are rate constants defined in Scheme 1. The experimental results for acetylcholine (a) are from our manometric experiments and those for acetylthiocholine (b) are taken from Ellman *et al.* (1961). The solid lines have been calculated by using the equation derived by Webb (1963) and the following constants taken from Tables 3 and 4: for acetylcholine, K_m 0.79 mm and K_{ss} 12 mm; for acetylthiocholine, K_m 0.23 mm and K_{ss} 3.3 mm. The symbols (●) indicate experimental points corrected to coincide with the calculated line at 10 mm-acetylcholine (a) and 5 mm-acetylthiocholine (b).

constant. When ES_1S_2 has activity ($k'_0 > 0$) the derived constant will be a very complex parameter, having the meaning of a Michaelis constant (K_{ms} ; see Appendix 4). It is not possible to be certain which view is correct, but there is some evidence in favour of the latter. Frequently the curve relating $\log[S]$ to activity is asymmetric and at high concentrations of substrate activity decreases less than expected. Myers (1952) showed that this form would be obtained if ES_1S_2 breaks down. By using our derived constants for acetylcholine [K_m 0.79 mm (Table 4); K_{ss} 12 mm (Table 3)] theoretical relationships between substrate concentration and

activity have been derived, as described by Webb (1963), when $k'_0/k_0 = 0, 0.1$ and 0.2 (Fig. 10a). Our experimentally determined values are consistent with ES_1S_2 decomposing at one-tenth of the rate of ES_1 . This conclusion agrees with the interpretation by Myers (1952). Our derived constant, K_{ss} , for acetylthiocholine is 3.3 mm whereas the published value is 30 mm (Ellman *et al.* 1961). However, when the results of Ellman *et al.* (1961) are plotted as described by Webb (1963) with our derived constants [K_m 0.23 mm (Table 4); K_{ss} 3.3 mm (Table 3)] results consistent with $k'_0/k_0 = 0.09 - 0.1$ are obtained (Fig. 10b). Whether asymmetry of curves relating $\log[S]$ to activity is caused by the breakdown of the ES_1S_2 complex is not proved by these results. However, whatever the cause there is no doubt for these two cases in Figs. 10(a) and 10(b) that a more accurate value of K_{ss} is derived from the tangent to the curves and not by the straight lines through the experimental points.

The rate of progressive inhibition by haloxon and coroxon is decreased by acetylcholine and acetylthiocholine; the values for K_m derived from these experiments are 0.79 mm for acetylcholine and 0.23 mm for acetylthiocholine. The following values for K_m for acetylcholine and mammalian acetylcholinesterases determined under similar ionic conditions have been published: cow erythrocyte, 0.63 mm (Augustinsson, 1948); human erythrocyte, 0.37 mm (Shukuya, 1951), 0.21 mm (Wright & Sabine, 1948), 0.12 mm (Alles & Hawes, 1940) and 0.26–0.37 mm (Myers, 1952); mouse brain, 0.22 mm (Mendel & Rudney, 1943). For acetylthiocholine the published values are: bovine erythrocyte, 0.14 mm (Ellman *et al.* 1961) and 0.50 mm (Koelle, 1950). The simplest mechanism to explain our results is that given by eqn. (11). If this is accepted then it is not necessary to consider that an appreciable concentration of a Michaelis-type complex between enzyme and haloxon or coroxon is present. It is reasonable that these molecules should have a low affinity for the active site of acetylcholinesterase, as they do not resemble acetylcholine. For very few inhibitors has kinetic evidence been produced (under the conditions of our experiments) for the presence of an appreciable amount of enzyme in the form of a Michaelis-type complex. It has been shown for phosphostigmine (Reiner & Aldridge, 1967), but this has obvious structural resemblance to acetylcholine; even in that case the K_a was only 0.2 mm.

All of our results taken together imply that cholinesterase has two binding sites, site 1 and site 2. Site 1 is the active centre. Binding of substrate to site 1 leads to substrate hydrolysis and binding of haloxon and coroxon to site 1 leads to progressive phosphorylation of the enzyme. The coumarin cannot bind to site 1. Site 2 can bind acetylcholine,

Scheme 1. Diagrammatic representation of possible reactions between acetylcholinesterase and haloxon and/or substrate. *h* is haloxon, *S* is substrate and E_{-2}^{-1} is the enzyme with two independent sites. Site 1 is the active centre and site 2 is that concerned with substrate inhibition.

acetylthiocholine, haloxon, coroxon and the coumarin. Binding to site 2 produces reversible inhibition of the enzyme, thus decreasing the activity of site 1. Scheme 1 summarizes all theoretically possible reactions arising from the above statement. The reactions studied and discussed in this paper are marked by the appropriate constants, and the internal consistency of our results indicates that these reactions are probably dominating. To what extent the other reactions occur, if at all, cannot be assessed by our present experimental techniques, but some of the variations observed in determining K_{ss} might be due to routes not taken into account in our interpretations.

In the Appendices equations are derived from equilibrium conditions. This has been done because of the complexity of the reactions and of the derivations from steady-state conditions. However, experimentally it is not possible to distinguish between K_m and K_s (see Appendix 3) and between K_{ss} and K_{ms} (see Appendix 4). In Appendix 3 we have derived an equation to describe the influence of substrate on progressive inhibition in our system (cf. eqn. 11). Previously the effect of substrate on progressive inhibition has been derived by Main & Dauterman (1963). Their derivation contains only the $[S]/K_m$ term and no correction needed to be made for the influence of K_{ss} because they used phenyl acetate, which does not cause substrate inhibition. When substrate inhibition occurs the two additional terms are required that are included in eqn. (16). Only when all the terms in eqn. (16) are included is the same K_m obtained at all inhibitor concentrations (see Table 4). This success in the application of these equations persuades us that the derivations in this paper provide a general framework for the interpretation of the kinetics of the

reaction of organophosphorus compounds, carbamates etc. with acetylcholinesterase and other esterases.

The reversible inhibition by haloxon has revealed itself as an intercept that the inhibition lines produced on the ordinate when $\log(\% \text{ activity})$ was plotted against the time of inhibition (Fig. 1). Theoretically such intercepts can be due either to a Michaelis-type complex between inhibitor and the active centre, or to a reversible complex formed between the inhibitor and on another site in the enzyme. The way to distinguish between these two possibilities is described in this paper.

The affinity of acetylcholine for the site involved in substrate inhibition is much less than that for the coumarin, haloxon or coroxon. It has been brought to our attention by D. C. Watts that the distance between the phosphorus atom and the carbonyl oxygen of haloxon and coroxon approximates to the distance between the nitrogen and carbon oxygen of acetylcholine. Thus we may consider the possibility of a two-point attachment of haloxon and coroxon as is normally accepted for acetylcholine. However, the different competitive behaviour of acetylcholine or acetylthiocholine against the phosphorylation of the enzyme on the one hand and the reversible inhibition on the other indicates that site 1 and site 2 cannot be the esteratic and anionic sites respectively. Similar considerations lead to the conclusion that binding to site 1 and site 2 is independent whether there is a one-point or two-point attachment of acetylcholine or haloxon to site 1 or site 2. This is contrary to early ideas about substrate inhibition of acetylcholinesterase that required that two molecules of acetylcholine were bound to the active centre (Zeller & Bissegger, 1943). A more recent view is

that inhibition of acetylcholinesterase by acetylcholine may be attributed to binding of the quaternary nitrogen of another substrate molecule to the anionic site of the acetylated enzyme (Krupka & Laidler, 1961). If the view is accepted that acetylcholine attaches itself to a site on acylated enzyme, then our results showing the high activity of haloxon, coroxon and the coumarin make it unlikely that the anionic site alone would be an adequate site. Such a site would require affinity for the coumarin. Thus either the binding of acetylcholine, haloxon, coroxon and the coumarin to site 2 must be independent of the binding of acetylcholine to site 1 or binding to site 2 is independent of an acetyl group on part of site 1. It is therefore possible to regard the attachment of haloxon, coroxon and the coumarin to give reversible inhibition and acetylcholine and acetylthiocholine to cause substrate inhibition as an allosteric phenomenon. This hypothesis may simplify the interpretation of inhibition by any substrate of acetylcholinesterase.

It is clear that there must be some structural similarity between the coumarin and acetylcholine, but the similarities may reside in only a small part of the molecules. The high affinity of the coumarin for site 2 probably indicates that the fit to it is good. Acetylcholine can take up many possible forms in solution (Culvenor & Ham, 1966) and when absorbed. We must therefore retain an open mind about the stereochemical specificity of the site(s) involved in substrate inhibition of acetylcholinesterase.

It should be pointed out that our conclusions and views are possible because the reaction of haloxon and acetylcholinesterase has three important consequences: (1) the active centre is phosphorylated; (2) the phosphorylated enzyme is unstable and can therefore be identified; (3) haloxon combines with a site other than the active centre that is the site involved in substrate inhibition. The study of haloxon also has led to the demonstration that 3-chloro-7-hydroxy-4-methylcoumarin is a good reagent for this site.

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APPENDIX

Theoretical derivations

All the derivations depend on the following assumptions and definitions.

(1) These Appendices concern the derivation of enzyme activity as a function of substrate and/or inhibitor concentrations when the enzyme possesses two binding sites.

(2) Site 1 is the active centre and site 2 is that concerned with inhibition by substrate. Substrate bound to site 1 breaks down to products and inhibitor bound to site 1 leads to acylated enzyme.

(3) Binding to sites 1 and 2 is independent. Therefore binding of substrate or inhibitor to site 2

is the same whether site 1 is occupied or not. Similarly for combination of substrate or inhibitor on site 1 when site 2 is occupied or not.

(4) The following symbols are used throughout: E, free enzyme with two binding sites; S, substrate; I, inhibitor; ES_1 , EI_1 , complex between S or I and site 1; ES_2 , EI_2 , complex between S or I and site 2; ES_1S_2 , complex between S and sites 1 and 2.

All other symbols are defined in this way with the site where S and I are attached given as numbered subscripts.

Appendix 1. Application of the method of Hunter & Downs (1945) to combination of an inhibitor to site 2 of the enzyme

The method of Hunter & Downs (1945) allows the dissociation constant between E and I to be derived from the relation between activity in the presence of inhibitor and a range of substrate concentrations.

In the above formulation the combination of inhibitor to site 1 is not considered. Combination of inhibitor with site 2 always leads to inactive enzyme.

The rate of product formation (v) is given by:

$$v = k_0[ES_1] + k'_0[ES_1S_2] \quad (1)$$

The total enzyme concentration $[E_0]$ equals:

$$[E_0] = [E] + [ES_1] + [ES_2] + [ES_1S_2] + [EI_2] + [ES_1I_2] \quad (2)$$

The dissociation constants for the reactions shown in the scheme above are given by the following equations:

$$K_s = [E][S]/[ES_1] \quad (\text{reaction 1}) \quad (3)$$

$$K_{ss} = [E][S]/[ES_2] \quad (\text{reaction 2}) \quad (4)$$

$$K_s = [ES_2][S]/[ES_1S_2] \quad (\text{reaction 3}) \quad (5)$$

$$K_{ss} = [ES_1][S]/[ES_1S_2] \quad (\text{reaction 4}) \quad (6)$$

$$K_i = [E][I]/[EI_2] \quad (\text{reaction 5}) \quad (7)$$

$$K_i = [ES_1][I]/[ES_1I_2] \quad (\text{reaction 6}) \quad (8)$$

From eqn. (2):

$$[E_0] = [ES_1] \left(1 + \frac{[E]}{[ES_1]} + \frac{[ES_2]}{[ES_1]} + \frac{[ES_1S_2]}{[ES_1]} + \frac{[EI_2]}{[ES_1]} + \frac{[ES_1I_2]}{[ES_1]} \right) \quad (9)$$

Substitution of rearranged eqns. (3)–(8) in eqn. (9) gives:

$$[E_0] = [ES_1] \left(1 + \frac{K_s}{[S]} + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}} + \frac{K_s[I]}{K_i[S]} + \frac{[I]}{K_i} \right) \quad (10)$$

and substitution for $[ES_1S_2]$ from eqn. (6) in eqn. (1) gives:

$$\begin{aligned}
 v &= k_0[ES_1] + k'_0[ES_1] \frac{[S]}{K_{ss}} \\
 &= [ES_1] \left(k_0 + k'_0 \frac{[S]}{K_{ss}} \right) \quad (11)
 \end{aligned}$$

By substitution in eqn. (11) for $[ES_1]$ derived from eqn. (10), activity in the presence of inhibitor is given by:

$$v = \frac{[E_0] \left(k_0 + k'_0 \frac{[S]}{K_{ss}} \right)}{1 + \frac{K_s}{[S]} + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}} + \frac{K_s[I]}{K_i[S]} + \frac{[I]}{K_i}} = \frac{[E_0]A}{B} \quad (12)$$

Activity in the absence of inhibitor, v_0 , is given by:

$$v_0 = \frac{[E_0] \left(k_0 + k'_0 \frac{[S]}{K_{ss}} \right)}{1 + \frac{K_s}{[S]} + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}}} = \frac{[E_0]A}{C} \quad (13)$$

From eqns. (12) and (13) inhibition is given by:

$$v_0 - v = \frac{[E_0]A}{C} - \frac{[E_0]A}{B} = \frac{[E_0]A(B - C)}{BC}$$

Therefore:

$$\begin{aligned}
 \frac{v[I]}{v_0 - v} &= \frac{\text{Activity}[I]}{\text{Inhibition}} = \\
 &= \frac{[E_0]A}{B} \cdot \frac{BC[I]}{[E_0]A(B - C)} = \frac{C[I]}{B - C} \quad (14)
 \end{aligned}$$

Converting back into the original symbols from eqns. (12) and (13):

$$\frac{v[I]}{v_0 - v} = \frac{\left(1 + \frac{K_s}{[S]} + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}} \right) [I]}{\frac{K_s[I]}{K_i[S]} + \frac{[I]}{K_i}}$$

This simplifies to give:

$$\frac{v[I]}{v_0 - v} = K_i + \frac{K_i[S]}{K_{ss}} \quad (15)$$

Eqn. (15) allows K_i and K_{ss} to be derived by plotting $v[I]/(v_0 - v)$ against $[S]$. In the original Hunter &

Downs (1945) equation, for an enzyme with one binding site only, the same form of equation is obtained but K_s and not K_{ss} is derived.

Eqn. (15) is based on the general scheme defined above. It is important to appreciate the effect, on the form of the equation, of modifying some of the assumptions in the scheme.

(a) Eqn. (15) is not affected by the magnitude of k_0 or k'_0 . The terms containing k_0 or k'_0 cancel out (cf. eqn. 14, term A).

(b) When it is obligatory that I only combines with site 2 when site 1 is occupied by S, i.e. reaction (5) is omitted, the final equation is:

$$\frac{v[I]}{v_0 - v} = K_t + K_t \left(\frac{K_s}{[S]} + \frac{[S]}{K_{ss}} \right) \quad (16)$$

A plot of $v[I]/(v_0 - v)$ against $[S]$ will not give a straight line except when $[S]$ is high, i.e. the enzyme is substantially all in the form ES_1 , and eqn. (16) simplifies to eqn. (15).

(c) When I is unable to combine with site 2 when site 1 is occupied by S, i.e. reaction (6) is omitted, the final equation is:

$$\frac{v[I]}{v_0 - v} = K_t + \frac{K_t[S]}{K_s} \left(1 + \frac{[S]}{K_{ss}} \right) \quad (17)$$

A plot of $v[I]/(v_0 - v)$ against $[S]$ will not give a straight line except when $[S]$ is small, i.e. a substantial part of the enzyme has both sites 1 and 2 unoccupied, and eqn. (17) becomes:

$$\frac{v[I]}{v_0 - v} = K_t + \frac{K_t[S]}{K_s} \quad (18)$$

This is identical with the original Hunter & Downs (1945) equation and K_t and K_s are derived.

(d) The original derivation (eqn. 15) and those from the special cases (eqns. 16 and 18) all yield K_t .

Appendix 2. Rate of acylation of site 1 when the inhibitor combines with sites 1 and 2 of the enzyme

Binding of I to site 2 is a reversible reaction defined by the dissociation constant, K_t :

$$K_t = [\text{E}][\text{I}]/[\text{EI}_2] \quad (19)$$

Binding of I to site 1 leads to the progressive formation of acylated enzyme, EA_1 . The concentration of the Michaelis-type intermediate, EI_1 ,

is considered to be very small and the rate of progressive inhibition is therefore defined as:

$$\frac{d[\text{EA}_1]}{dt} = k_a[\text{E}][\text{I}] \quad (20)$$

where k_a is the second-order rate constant of inhibition.

The total enzyme concentration $[\text{E}_0]$ is:

$$[\text{E}_0] = [\text{E}] + [\text{EA}_1] + [\text{EI}_2] \quad (21)$$

Rearrangement of eqn. (21) and inserting it into eqn. (19) gives:

$$[\text{E}] = \frac{[\text{E}_0] - [\text{EA}_1]}{1 + [\text{I}]/K_t} \quad (22)$$

Substitution of eqn. (22) into eqn. (20) gives:

$$\frac{d[\text{EA}_1]}{[\text{E}_0] - [\text{EA}_1]} = \frac{k_a[\text{I}].dt}{1 + [\text{I}]/K_t} \quad (23)$$

Integration of eqn. (23) gives:

$$\ln \left(\frac{[\text{E}_0]}{[\text{E}_0] - [\text{EA}_1]} \right) = \frac{k_a[\text{I}].t}{1 + [\text{I}]/K_t} \quad (24)$$

At a given inhibitor concentration the first-order rate constant of inhibition is:

$$k = \frac{k_a[\text{I}]}{1 + [\text{I}]/K_t} \quad (25)$$

Rearrangement of eqn. (25) according to Wilkinson (1961) gives:

$$\frac{[\text{I}]}{k} = \frac{1}{k_a} + \frac{[\text{I}]}{k_a K_t} \quad (26)$$

The same equation will be obtained when two inhibitors are used, one combining only with site 1 to give acylated enzyme and the other combining only with site 2.

Appendix 3. Effect of substrate on the rate of acylation of site 1 for enzymes that have two independent binding sites for inhibitor and substrate

The above scheme combines the reactions described separately in Appendices 1 and 2. The rate of formation of acylated enzyme EA_1 is given by:

$$\frac{d[EA_1]}{dt} = k_a[E][I] \quad (27)$$

The total enzyme concentration $[E_0]$ equals:

$$[E_0] = [E] + [EA_1] + [ES_1] + [ES_2] + [ES_1S_2] + [EI_2] + [ES_1I_2] \quad (28)$$

The dissociation constants for reactions (1)–(6) shown in the scheme above are given in Appendix 1 (eqns. 3–8). From eqn. (28):

$$[E_0] - [EA_1] = [E] \left(1 + \frac{[ES_1]}{[E]} + \frac{[ES_2]}{[E]} + \frac{[ES_1S_2]}{[E]} + \frac{[EI_2]}{[E]} + \frac{[ES_1I_2]}{[E]} \right) \quad (29)$$

Substitution of rearranged eqns. (3)–(8) (Appendix 1) into eqn. (29) yields:

$$[E_0] - [EA_1] = [E] \left(1 + \frac{[S]}{K_s} + \frac{[S]}{K_{ss}} + \frac{[S]^2}{K_s K_{ss}} + \frac{[I]}{K_i} + \frac{[S][I]}{K_s K_i} \right) \quad (30)$$

Inserting the expression for $[E]$ derived from eqn. (30) in eqn. (27):

$$\frac{d[EA_1]}{dt} = \frac{k_a[I]([E_0] - [EA_1])}{1 + \frac{[S]}{K_s} + \frac{[S]}{K_{ss}} + \frac{[S]^2}{K_s K_{ss}} + \frac{[I]}{K_i} + \frac{[S][I]}{K_s K_i}} \quad (31)$$

Integration of eqn. (31) yields:

$$\ln \left(\frac{[E_0]}{[E_0] - [EA_1]} \right) = \frac{k_a[I].t}{1 + \frac{[S]}{K_s} + \frac{[S]}{K_{ss}} + \frac{[S]^2}{K_s K_{ss}} + \frac{[I]}{K_i} + \frac{[S][I]}{K_s K_i}} \quad (32)$$

At a given inhibitor concentration the first-order rate constant for acylation in presence of a given $[S]$ will be:

$$k' = \frac{k_a[I]}{1 + \frac{[S]}{K_s} + \frac{[S]}{K_{ss}} + \frac{[S]^2}{K_s K_{ss}} + \frac{[I]}{K_i} + \frac{[S][I]}{K_s K_i}} \quad (33)$$

For simpler situations when equations have been derived from equilibrium and steady-state conditions, K_s is evaluated from the former and K_m from the latter. By analogy we assume that in eqn. (33) K_s has the meaning of K_m , and K_{ss} may have the meaning of K_{ms} (see Appendix 4).

Appendix 4. Rate of product formation when site 1 and site 2 of the enzyme are occupied by substrate

Only the rate of product formation from the ES_1S_2 complex, v_{ss} , will be considered, and this rate is:

$$v_{ss} = k'_o[ES_1S_2] \quad (34)$$

v_{ss} will be maximal when all the enzyme is in the ES_1S_2 form:

$$V_{ss} = k'_o[E_0] \quad (35)$$

The dissociation constants for reactions (1)–(4) in the above scheme are given in Appendix 1 (eqns. 3–6). The total enzyme concentration equals:

$$[E_0] = [E] + [ES_1] + [ES_2] + [ES_1S_2] \quad (36)$$

Substituting eqns. (3)–(6) from Appendix 1 into eqn. (36):

$$[E_0] = [ES_1S_2] \left(1 + \frac{K_s}{[S]} + \frac{K_{ss}}{[S]} + \frac{K_s K_{ss}}{[S]^2} \right) \quad (37)$$

Inserting eqns. (37) and (35) into eqn. (34) gives the rate of product formation due to ES_1S_2 :

$$\begin{aligned} v_{ss} &= \frac{k'_o[E_0]}{1 + \frac{K_s}{[S]} + \frac{K_{ss}}{[S]} + \frac{K_s K_{ss}}{[S]^2}} \\ &= \frac{V_{ss}}{1 + \frac{K_s}{[S]} + \frac{K_{ss}}{[S]} + \frac{K_s K_{ss}}{[S]^2}} \end{aligned} \quad (38)$$

The substrate concentration at which v_{ss} equals $V_{ss}/2$ corresponds to the Michaelis constant, K_{ms} , for eqn. (34). Inserting this condition into eqn. (38):

$$\frac{V_{ss}}{2} = \frac{V_{ss}}{1 + \frac{K_s}{[S]} + \frac{K_{ss}}{[S]} + \frac{K_s K_{ss}}{[S]^2}} \quad (39)$$

the following expression is obtained:

$$[S]^2 - (K_s + K_{ss})[S] - K_s K_{ss} = 0 \quad (40)$$

or

$$[S] = \frac{1}{2}[(K_s + K_{ss}) \pm (K_s^2 + 6 K_s K_{ss} + K_{ss}^2)^{\frac{1}{2}}] \quad (41)$$

Only the solution that makes $[S] > 0$ is meaningful and therefore:

$$K_{ms} = \frac{1}{2}[(K_s + K_{ss}) + (K_s^2 + 6 K_s K_{ss} + K_{ss}^2)^{\frac{1}{2}}] \quad (42)$$

The expression for K_{ms} given by eqn. (42) is applicable to values of K_s and K_{ss} of any magnitude relative to one another. For the case when $[S] \gg K_s$ and $K_{ss} \gg K_s$ and incorporating the definition that

$K_{ms} = [S]$ when $v_{ss} = V_{ss}/2$ eqn. (39) can be simplified, and the expression for K_{ms} becomes $[S] = K_{ss} = K_{ms}$.

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