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INHIBITION OF CHOLINESTERASE BY CARBAMATES. A NEW KINETIC APPROACH

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1. A mathematical model, describing the inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by carbamates in the presence of a substrate, is presented.

2. This model was used for determining the affinity, carbamylation, and decarbamylation constants of *N*-hydroxypropoxur with spider mite cholinesterase, and the affinity and carbamylation constants of eserine with bovine erythrocyte cholinesterase.

3. The merits of this method are compared with those of the preincubation method used by I. B. WILSON, M. A. HATCH AND S. GINSBURG (*J. Biol. Chem.*, 235 (1960) 2312 for the determination of decarbamylation constants and by A. R. MAIN AND F. L. HASTINGS (*Science*, 154 (1966) 400) for the determination of affinity and carbamylation constants.

INTRODUCTION

WILSON *et al.*[1] have furnished strong evidence in support of the assumption by MYERS AND KEMP[2] that the inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by certain esters of carbamic acid follows the reaction sequence

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EC \xrightarrow{k_3} E + \text{products} \quad \text{(leaving group)} \qquad (1)$$

where E, I, EI, and EC stand for free enzyme, inhibitor, reversible enzyme–inhibitor complex, and carbamylated enzyme, respectively. To prove this mechanism WILSON *et al.*[1] developed a differential equation for the kinetics of the inhibition based on the reaction sequence(1).

This equation can be integrated to the form

$$\ln\left(1 - \frac{EC}{EC_{ss}}\right) = -\left[\frac{k_2}{\frac{K_I}{I} + 1} + k_3\right] t \qquad (2)$$

Abbreviation: DTNB, 5,5'-dithio-bis(nitrobenzoate).

where EC and EC_{ss} denote the concentrations* of carbamylated enzyme at time t and in the steady state, respectively, and K_I is the dissociation constant of the reversible enzyme–inhibitor complex. In the steady state the rate of carbamylation equals that of decarbamylation.

Since the residual acetylcholinesterase activity is measured after extensive dilution of the enzyme–inhibitor mixture with substrate solution of relatively high concentration, the reversible complex EI can then be neglected and, in Eqn. 2 EC and EC_{ss} are equal to the percentages of inhibition of the enzyme at time t and in the steady state.

A measurable residual enzyme activity in the steady state is, of course, essential for the experimental determination of the kinetic constants in Eqn. 2. In practice this condition can be encountered only when the inhibitor concentration is much lower than the value of K_I; but when $I \ll K_I$, k_2 cannot be determined from Eqn. 2.

Accordingly, MAIN AND HASTINGS[3] developed an experimental method whereby much larger inhibitor concentrations were used, the enzyme being fully inhibited within less than 1 min. Now, when I is of the same order of magnitude as K_I, k_3 in Eqn. 2 becomes small as compared to the other term in the coefficient of t, and the same is, of course, true in Wilson's original differential equation from which Eqn. 2 has been derived. The reciprocal of this differential equation then takes the form

$$\frac{dt}{d\ln(E_0 - EC)} = \frac{1}{k_2} + \frac{K_I}{k_2 \cdot I} \tag{3}$$

MAIN AND HASTINGS[3] used this equation in the incubation of the enzyme with inhibitor for varying intervals within 0.5 min and measuring the residual enzyme activity $v_i \sim (E_0 - EC)$ following extensive dilution with substrate solution, thus allowing EI to be neglected. By doing this for a range of inhibitor concentrations and plotting $\Delta t/\Delta \log v$ against the reciprocal inhibitor concentrations they obtained a straight line from which K_I and k_2 could be determined.

This method has a few drawbacks. (1) It is not very easy to time exactly the intervals between the introduction of inhibitor and the dilution with substrate solution, if such intervals are of the order of a few seconds. Such very small incubation intervals are, in fact, required with inhibitors of any consequence. And even if exact timing were possible, then mixing could still pose a problem that could be resolved only by the use of a stopped-flow device. (2) The extensive dilution required for adequate depression of the reversible enzyme–inhibitor complex makes it necessary to work with an amount of enzyme about two orders of magnitude larger than is actually used for the determination of residual enzyme activities. Thus the method is wasteful and is hardly applicable when only small samples of enzyme are available, as is often the case with the small animals like insects and acarids.

We have therefore developed a rate equation for the inhibition of acetylcholinesterase in the presence of substrate. This method permits of the determination of affinity, carbamylation, and decarbamylation constants from a series of progression curves obtained on incubating the enzyme simultaneously with substrate and inhibitor, and varying the concentration of either agent.

* Brackets indicating concentrations have been omitted in this and the following equations.

THEORY

For the reaction of acetylcholinesterase with a substrate the reaction sequence is

$$E + S \leftrightharpoons ES \rightarrow E\text{Ac} \rightarrow E + \text{Ac}$$

where S, ES and EAc stand for substrate, reversible enzyme–substrate complex, and acylated enzyme, respectively. If the enzyme activity is determined by the Ellman method the development of the absorbance (A) at 412 nm is proportional to the reaction velocity

$$\frac{dA}{dt} = v = k \cdot ES \tag{4}$$

Thus a differential equation has to be developed for $ES = f(t)$ during the progress of the inhibition.

The following equations apply to the mixture of enzyme, substrate, and inhibitor

$$E_0 = E + ES + EI + EC \tag{5}$$

hence

$$\frac{dEC}{dt} = -\left(\frac{dE}{dt} + \frac{dEI}{dt} + \frac{dES}{dt}\right) \tag{6}$$

Furthermore

$$K_I = \frac{E \cdot I}{EI} \tag{7}$$

$$K_s = \frac{E \cdot S}{ES} \tag{8}$$

and

$$\frac{dEC}{dt} = k_2 \cdot EI - k_3 \cdot EC \tag{9}$$

the change in concentration of the carbamylated enzyme being determined by its formation from EI and by its spontaneous hydrolysis. Finally I and S are assumed to remain constant during the reaction.

Now from Eqn. 9

$$EC = \frac{k_2}{k_3} \cdot EI - \frac{1}{k_3} \cdot \frac{dEC}{dt} \tag{10}$$

and from Eqn. 8

$$E = \frac{K_s}{S} \cdot ES$$

Combination of Eqns. 7 and 8 leads to

$$EI = \frac{I}{K_I} \cdot \frac{K_s}{S} \cdot ES$$

Thus, Eqn. 5 can be written as

$$E_0 = \left[\frac{K_s}{S} + \frac{I \cdot K_s}{K_I \cdot S} + 1\right] ES + EC \tag{5a}$$

Let the term inside the brackets be represented by p. Then, with Eqn. 10

$$E_0 = p \cdot ES + \frac{k_2}{k_3} \cdot \frac{I \cdot K_s}{S \cdot K_I} \cdot ES - \frac{1}{k_3} \cdot \frac{dEC}{dt}$$

in this equation let us replace

$$\frac{k_2}{k_3} \cdot \frac{I \cdot K_s}{K_I \cdot S}$$

by q.

With Eqn. 6 it then follows that

$$E_0 = (p + q)\, ES + \frac{p}{k_3} \cdot \frac{dES}{dt}$$

On integration with respect to t, this becomes:

$$ES = C \cdot \exp\left(-\frac{p + q}{p} k_3 \cdot t\right) + \frac{E_0}{p + q} \tag{11}$$

At $t = 0$, $EC = 0$; hence from Eqn. 5: $E_0 = p \cdot ES_0$; and

$$\frac{E_0}{p} = C + \frac{E_0}{p + q}$$

therefore

$$C = E_0 \frac{q}{p(p + q)}$$

Since $k \cdot E_0 = v_{max}$, it follows that

$$v_i = k \cdot ES = \frac{v_{max}}{p + q} \cdot \frac{q}{p} \exp\left(-\frac{p + q}{p} k_3 t\right) + \frac{v_{max}}{p + q} \tag{12}$$

If the progress of the enzyme reaction is recorded as a change of absorbance $v = dA/dt$, and Eqn. 12 is integrated with respect to t, we obtain

$$A = \int v_i dt = \frac{v_{max}}{p + q} \cdot \frac{q}{p} \int \exp\left(-\frac{p + q}{p} k_3 t\right) dt + \frac{v_{max}}{p + q} \int dt$$

$$= -\frac{v_{max}}{(p + q)^2 k_3} \exp\left(-\frac{p + q}{p} k_3 t\right) + \frac{v_{max}}{p + q} \cdot t + C \tag{13}$$

Since the absorbance is zero at $t = 0$,

$$C = \frac{v_{max}}{(p + q)^2 k_3}$$

This is an exponential function with a straight line superimposed (Fig. 1).
At $t \to \infty$, the curve straightens out.

In order to determine the kinetic constants of the inhibition it is convenient to revert to Eqn. 12, where

$$\frac{v_{max}}{p + q} = (v_i)_{t = \infty}$$

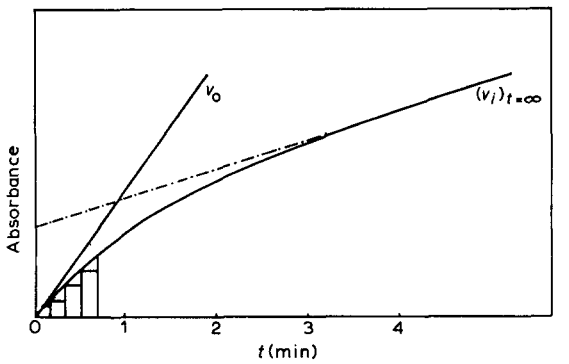


Fig. 1. Example of a progression curve for the hydrolysis of acetylthiocholine by cholinesterase in the presence of a carbamate inhibitor. Spider mite acetylcholinesterase with 1 mM acetylthiocholine and $2.86 \cdot 10^{-4}$ M *N*-hydroxypropoxur.

is the direction coefficient of the straight line. Thus Eqn. 12 can be written as follows:

$$\left(\frac{dA}{dt}\right)_t - \left(\frac{dA}{dt}\right)_{t=\infty} = \frac{v_{max}}{p+q} \cdot \frac{q}{p} \exp\left(-\frac{p+q}{p} k_3 t\right) \tag{12a}$$

or in logarithmic form:

$$\ln\left[\left(\frac{dA}{dt}\right)_t - \left(\frac{dA}{dt}\right)_{t=\infty}\right] = \ln \frac{v_{max}}{p+q} \cdot \frac{q}{p} - \frac{p+q}{p} k_3 t \tag{12b}$$

With the aid of this equation, affinity, carbamylation, and decarbamylation constants can, in principle, be determined from the progression curves of substrate hydrolysis by acetylcholinesterase in the presence of a simultaneously added inhibitor. The procedure is as follows: (1) From the progression curves obtained with a range of inhibitor concentrations, the increments per unit of time are determined for a series of time intervals, and, after subtraction of the increment of the steady-state reaction, plotted semilogarithmically against t. According to Eqn. 12b a linear plot results with

$$\frac{p+q}{p} k_3$$

as direction coefficient. (2) The velocity of the uninhibited enzyme reaction v_0 is divided by the steady-state velocity of the inhibited reaction $(v_i)_{t=\infty}$.
But

$$v_0 = \frac{v_{max}}{1 + \frac{K_s}{S}} \text{ and } v_i = \frac{v_{max}}{p+q}; \text{ therefore } \frac{v_0}{(v_i)_{t=\infty}} = \frac{p+q}{1 + \frac{K_s}{S}}$$

For each inhibitor concentration this quotient is divided by

$$\frac{p+q}{p} k_3$$

The resulting quotients, *i.e.*

$$\frac{p}{k_3\left(1+\frac{K_s}{S}\right)} = \frac{1 + \frac{K_s}{S} + \frac{I}{K_I} \cdot \frac{K_s}{S}}{k_3\left(1 + \frac{K_s}{S}\right)} = \frac{1}{k_3} + \frac{I}{K_I k_3\left(1 + \frac{S}{K_s}\right)} \tag{14}$$

are plotted against the inhibitor concentration, resulting in a straight line with $1/k_3$ as the intercept on the ordinate. The slope of this line is often too small to permit of evaluation of K_I with reasonable accuracy. (3) The value of k_3 is subtracted from $(p+q)k_s/p$, and the reciprocals of the resulting values, multiplied by the pertinent inhibitor concentrations are plotted against the inhibitor concentration.
Since

$$\frac{I\,p}{q\cdot k_3} = \frac{1 + \frac{K_s}{S} + \frac{I}{K_I}\cdot\frac{K_s}{S}}{\frac{k_2}{K_I}\,\frac{K_s}{S}} = \frac{I}{k_2} + \frac{K_I}{k_2}\left(1 + \frac{S}{K_s}\right) \qquad (15)$$

this plot is a straight line, with $1/k_2$ as the direction coefficient.

Note: When $k_2/k_3 \gg 1$, k_3 cannot be determined by this procedure, since $v_0/(v_i)_{t=\infty}$ then becomes very large and inaccurate.

The method outlined by WILSON *et al.*[1]—*i.e.* preincubation of the enzyme with low concentrations of inhibitor and determination of the progress of the inhibition by dilution with substrate solution at a series of time intervals—is then more accurate. In such cases the above procedure is reduced to the items (1) and (3).

EXPERIMENTAL

The following examples illustrate the application of the procedure outlined in the preceding section: (a) the inhibition of acetylcholinesterase from spider mites by 2-isopropoxyphenyl-*N*-hydroxy-*N*-methylcarbamate (*N*-hydroxypropoxur); (b) the inhibition of acetylcholinesterase from bovine erythrocytes by eserine.

Materials

Cholinesterase from spider mites (*Tetranychus cinnabarinus* Bois D.) was obtained by extraction of a freeze-dried homogenate from 5 g of spider mites with acetone at −16°, and discarding of the solvent fraction. The residue, after evacuation, was treated as described by SMISSAERT[4] to yield a preparation with a specific activity of 0.096 μmole acetylthiocholine per min per mg of protein. The Michaelis constant with this substrate was 0.12 ± 0.01 mM* at 25°.

Acetylcholinesterase from bovine erythrocytes was preparation No. 10220 from Serva, with a specific activity of 2 enzyme units/mg. The K_m value with acetylthiocholine was 0.34 ± 0.02 mM. Eserine was purchased from Brocades, and *N*-hydroxypropoxur was prepared by Mr. W. H. Dekker of the Institute of Organic Chemistry TNO, Utrecht. Acetylthiocholine was a preparation from Koch-Light, and 5,5′-dithio-bis(nitrobenzoate) (DTNB) was supplied by Aldrich.

Incubation method

Acetylcholinesterase activity was determined by the method according to ELLMAN[5], with acetylthiocholine used as the substrate and DTNB as the chromogenic reagent. Absorbance was measured at 412 nm in a 1.5-ml cuvette (1 cm light path) with a thermostatted Zeiss PMQ II spectrophotometer, and recorded *via* a Unicam

* In this paper all margins of error have been caculated at 95% confidence levels.

TABLE I

PROGRESSION AND STEADY-STATE INHIBITION PARAMETERS FROM THE INCUBATION OF ACETYLCHOLINESTERASE FROM SPIDER MITES AND *N*-HYDROXYPROPOXUR

See text.

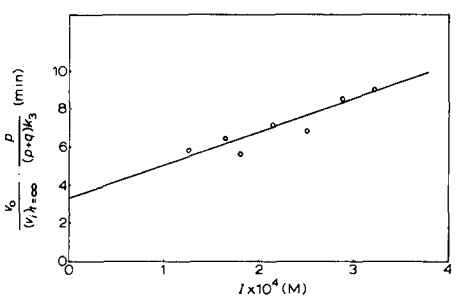
$I \times 10^4$ (M)	$\frac{p+q}{p} \cdot k_3$ (min^{-1})	$\frac{v_0}{(v_i)_{t=\infty}}$	$\frac{v_0}{(v_i)_{t=\infty}} \cdot \frac{p}{(p+q)k_3}$ (min)
3.21	0.83	8.20	9.05
2.86	0.78	6.76	8.70
2.50	0.90	6.18	6.85
2.14	0.76	5.44	7.19
1.79	0.75	4.60	5.64
1.61	0.69	4.46	6.47
1.25	0.63	3.66	5.78

SP 45 log converter on a Philips PM 8100 flat bed recorder. A digital voltmeter with printer was used occasionally to record absorbances at discrete time intervals.

The incubation mixtures consisted of 1.4 ml enzyme solution in 0.08 M pyrophosphate buffer (pH 7.5), with 3% NaCl added. To start the reaction 0.1 ml of a solution containing both acetylthiocholine (to a final concentration of 1 mM unless specified otherwise), and the inhibitor was added to the enzyme solution and mixed quickly. All the incubation mixtures contained 1% ethanol. Recording of the absorbance was started immediately after mixing, and it was continued until it followed a linear course.

RESULTS

In Table I and Figs. 2 and 3 are presented the results of an incubation of spider mite acetylcholinesterase with acetylthiocholine and *N*-hydroxypropoxur, at 25°.



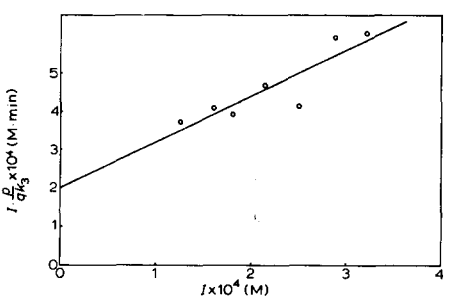


Fig. 2. Plot of the quantity

$$\frac{1}{k_3} + \frac{I}{K_I k_3}\left[1 + \frac{S}{K_s}\right]$$

against the inhibitor concentration. Spider mite cholinesterase with *N*-hydroxypropoxur as inhibitor and acetylthiocholine as the substrate.

Fig. 3. Plot of

$$\frac{I}{k_2} + \frac{K_I}{k_2}\left[1 + \frac{S}{K_s}\right]$$

against the inhibitor concentration. Same incubation experiment as in Fig. 2.

The tangents of the semilogarithmic plots and the quotients of uninhibited and steady-state-inhibited enzyme activity are presented in Table I. The quotients resulting from these sets of data have been plotted against the inhibitor concentration in Fig. 2. By means of regression analysis the value of the intercept is calculated as 3.3 ± 1.3 (min); hence $k_3 = 0.3$ (0.2–0.5) min^{-1}.

The slope is $(1.7 \pm 0.7) \cdot 10^{-4}$ $min \cdot M^{-1}$. Fig. 3 is a plot of $I \cdot \frac{p}{qk_3}$ against I for the same experiment. The ordinate values of this plot have been obtained by subtracting the value of k_3, which has been taken as 0.30 min^{-1}, from the data in the second column of Table I, and multiplying the reciprocals of the resulting values by the pertinent inhibitor concentrations. The intercept of the resulting straight line (correlation coefficient 0.90) is $(2.0 \pm 1.5) \cdot 10^{-4}$ M·min; the regression coefficient

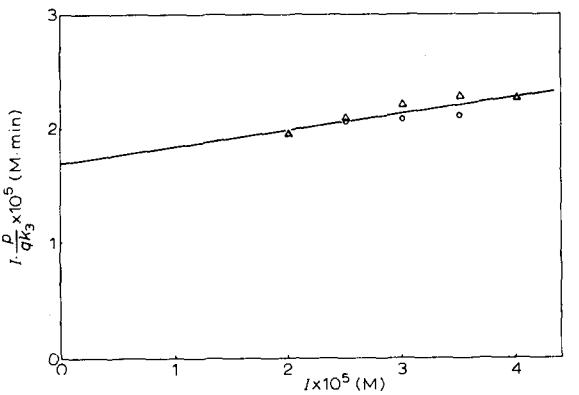


Fig. 4. Inhibition of bovine erythrocyte acetylcholinesterase by eserine. Plot of

$$\frac{I}{k_2} + \frac{K_I}{k_2}\left[1 + \frac{S}{K_s}\right]$$

against the inhibitor concentration.

$1/k_2$ is 1.2 ± 0.6 min; hence $k_2 = 0.8$ (0.6–1.7) min^{-1}, and with $K_m = 0.12$, $K_I = (1.8 \pm 1.4) \cdot 10^{-5}$ M.

The second example is the inhibition of bovine erythrocyte acetylcholinesterase by eserine. Here the value of k_3 is known, *i.e.* 0.018 min^{-1} at 25° (ref. 6).

Fig. 4 is a plot of $I(p/qk_3)$ against the inhibitor concentration from progression curves of this reaction. The regression coefficient is calculated as 0.15 ± 0.05; hence $k_2 = 6.9$ (5.0 — 10.9) min^{-1}. The intercept is $(1.70 \pm 0.16) \cdot 10^{-5}$ M, and with $K_m = 0.34$ this yields $K_I = (2.87 \pm 0.27) \cdot 10^{-5}$ M.

In a similar experiment (Fig. 5) the inhibitor concentration was kept constant and the substrate concentration was varied up to 1 mM, where substrate inhibition of the enzyme becomes manifest. The regression line of this plot cuts the ordinate at 0.30 ± 0.11 min; the regression coefficient is $(0.43 \pm 0.14) \cdot 10^3$ $min \cdot M^{-1}$. Since now, according to Eqn. 14, the intercept is $\frac{1}{k_2}\left[1 + \frac{K_I}{I}\right]$ and the slope is $\frac{K_I}{Ik_2K_s}$, it follows that $k_2 = 6.5$ (4–23) min^{-1}, and $K_I = (2.9 \pm 0.9) \cdot 10^{-5}$ M. This agrees well with the result of the incubation with variable inhibitor concentration*.

* The K_I values found by us differ an order of magnitude with that found by MAIN AND HASTINGS[3]. We have checked that this difference is entirely due to the use of different buffer systems.

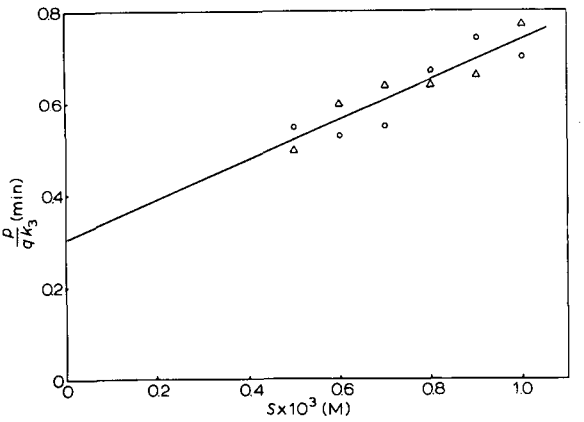


Fig. 5. Inhibition of bovine erythrocyte acetylcholinesterase by $3.06 \cdot 10^{-5}$ M eserine. Plot of

$$\left[\frac{1}{k_2} + \frac{K_I}{Ik_2}\right] + S\frac{K_I}{Ik_2K_s}$$

against the concentration of acetylthiocholine.

DISCUSSION

From the results presented in this communication it is apparent that the procedure described here permits determination of the affinity and carbamylation constants, and in certain cases also the decarbamylation constants, of cholinesterase inhibition by carbamates. No other assumptions are required than that the concentration of substrate and inhibitor remain constant during the incubation. This is in contrast to the preincubation method of MAIN AND HASTINGS[3], where it has to be assumed that on dilution with substrate solution the concentration of the reversible enzyme–inhibitor complex is negligible; moreover, the effect of decarbamylation is assumed to be negligibly small.

It is of practical interest that our method requires only a relatively small number of incubations as compared to the preincubation method. Moreover, each incubation requires only a fraction of the amount of enzyme that is needed in the preincubation method, to allow the dilution necessary to validate the assumption that the reversible complex is absent.

Since the results depicted in Figs. 4 and 5 have both been obtained by duplicate experiments with different enzyme concentrations, the reproducibility of the method is also fairly good.

From the example of *N*-hydroxypropoxur (Figs. 2 and 3) it is clear that, in particular, the determination of k_3 is somewhat precarious; the determination of K_I and k_2 also suffers from the fact that in this case the steady state is reached very quickly. It is, however, apparent that with such high rates of decarbamylation the preincubation method also yields less accurate results. Again, with carbamylation constants of over 30 min^{-1}, no appreciable accuracy can be expected from either approach, the margins of error in $1/k_2$ exceeding the absolute values.

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