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# COMPETITIVE IRREVERSIBLE INHIBITION OF ENZYMES IN THE PRESENCE OF A SUBSTRATE: SCOPE AND LIMITATIONS

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Rapid irreversible inhibition of enzymes constitutes a difficult problem and demands sophisticated techniques to meet contemporary expectations of accuracy and precision. Modern computerized, analytical techniques now allow inhibition to be measured in the presence of a chromogenic substrate, the decomposition product of which can be followed by a conventional method and in a continuous mode. This article has been written to fulfill a need for guidelines to aid the designer of experiments for the irreversible inhibition of enzymes. Thus the scope and limitations of the continuous competitive method for the irreversible inhibition of enzymes is examined here. Examples of acetylcholinesterase inhibition by two diagonally different phosphonate inhibitors are used for illustrating accuracy and precision of the competitive irreversible inhibition technique at different levels of enzyme saturation with inhibitor and substrate.

KEY WORDS: Competitive irreversible inhibition, substrate, acetylcholinesterase, phosphonates, sarin, soman.

## INTRODUCTION

Methods for the study of irreversible inhibition of enzymes have been developed fairly recently and their use is still rare. Slow irreversible inhibition of enzymes can be monitored directly under first or zero order condition[1] and is straightforward, the only exception being the circumstance in which non-enzymic decomposition of the inhibitor also occurs simultaneously.[2]

The rapid irreversible inhibition of enzymes, however, constitutes a difficult problem and demands sophisticated techniques to meet contemporary expections of accuracy and precision. Currently a conventional method introduced by Main[3], for the study of rapid irreversible inhibition of enzymes, is gaining popularity among enzyme kineticists. The essence of the method is to allow inhibition to take place in the presence of a substrate, whose decomposition product can be followed now continuously by modern, computerized, analytical techniques.[1,4–6] Nevertheless, the original derivation[3] of the relationship between an observed rate constant and concentrations and kinetic microconstants has remained in use. Originally, data were collected, and frequently still are, after the mixing of enzyme with a deadend inhibitor, by discontinuous sampling of enzyme activity as a function of time. Only the bimolecular rate constant for the encounter of enzyme and inhibitor was calculated initially.[3a] An extension of the derivation[3b,c] to include the case of enzyme saturation, that is the application of Michaelis-Menten kinetics, was further developed by Hart and

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O'Brian,[7] de Jong *et al.*,[8] and others.[9-11] However, these authors used the pre-equilibrium assumption for the examples reported in their papers. Another and worrisome practice is the use of the so called "zero time method" as introduced[7] to the inhibition of acetylcholinesterase (AChE) by paraoxon (4-nitrophenyl diethylphosphate) in the presence of 4-nitrophenyl acetate. The unwarranted premise of this approach is that both the dissociation constant $K_d$ and the unimolecular rate constant $k_i$ can be determined from monitoring one single kinetic run if the enzymic turnover of the substrate is also known in the absence of the inhibitor. In fact, only the ratio $k_i/K_i$, the bimolecular rate constant, can be obtained at inhibitor concentrations less than $K_i$. Furthermore, $k_i/K_i$ can be obtained with greater accuracy if the same equation is used in a different form.* A better use of the method is presented by Brufani *et al.*,[11] who studied a system under enzyme saturation for the evaluation of the two parameters, $k_i$ and $K_i$.

The most general description of irreversible enzyme inhibition, based on the steady-state assumption, has been given by Tian and Tsou.[6] These authors provide the most useful relationship between observed rate constants and their constituents, bimolecular and unimolecular rate constants. The derivation is based on the steady state assumption, but it is presented in a complete form only in the Chinese literature. It is pointed out in the paper that irreversible enzyme inhibition can also be competitive, uncompetitive, or noncompetitive with substrate reactions, similar to the case with reversible inhibition. All types of inhibition are discussed in some detail in the paper, with the emphasis again on cases when the enzyme is not saturated with the inhibitor. These authors also provide some guide for the experimenter; they relate the magnitude of product release to initial concentrations and steady-state parameters. A thorough and critical analysis of the competitive irreversible inhibition of enzymes is still needed though for the practical kineticist, because the algebraic complexity and scope of application of the irreversible competitive inhibition method are non-trivial. What the experimental kineticist will need and not find in the literature, in any of the papers currently available on this topic, is information on either one or more of the following: (1) common biochemical symbolism for kinetic terms, (2) derivation in an accessible publication or language, (3) guidance for the selection of substrate to compete with an inhibitor, (4) guidance for the choice of concentration of enzyme, substrate, or inhibitor and, (5) a clue to the attainment of optimal precision. Occasionally, examples of unacceptably large changes (30%) in the steady-state concentration of substrate are given in the literature (resulting in poor accuracy), because of a lack of awareness of the conditions controlling precision and accuracy in the competitive method.

In this report I would like to remedy the deficiencies in the available literature by pointing out the scope and limitations of the competitive method for the irreversible inhibition of enzymes. The guidelines described below should aid the designer of experiments for the irreversible inhibition of enzymes.

*The experiment, in the example, was conducted at a concentration well below $K_m$ of the substrate and below $K_d$ of the inhibitor, when, as we shall show, neither of the parameters targeted at in the paper could be determined accurately. Although selfconsistent data is given in the paper, only the value of $k_i/K_i$ has any agreement with values previously reported in the literature. Data collected under conditions of the experiments reported in the paper, can only support evaluation of the bimolecular rate constant, consequently, the best use of the relationship between observed rates and micro-rate constants would be in the form which yield directly the best defined parameter. But even under optimal conditions, one kinetic determination lends little confidence to any claim.

## THEORETICAL ASPECTS

Irreversible enzyme inhibition by a potent inhibitor I in the presence of a substrate S is outlined in Scheme I:

$$\begin{array}{l} E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_i} P_1 + EI^* \\ \quad + S \\ k_3 \Big\| \Big\| k_4 \\ ES \xrightarrow{k_c} \quad P_2 + E \end{array}$$

**Scheme 1**

We may denote the initial enzyme concentration $E_0$, the inhibited enzyme $EI^*$ and write the mass balance, summarizing the distribution of uninhibited enzyme $E$, ($E = E_0 - EI^*$), among the free $E_f$ and modified $ES$ and $EI$ forms as in eq. (1).

$$[E] = [E_f] + [ES] + [EI] \tag{1}$$

The two differential equations of interest are (2) and (3)

$$d[P_2]/dt = k_c[ES] \tag{2}$$

$$d[EI^*]/dt = k_i[EI] \tag{3}$$

As customary in derivations for enzyme kinetics, $E_f$ and $EI$ are readily derived in terms of $ES$ from steady-state approximations;

$$[E_f] = [ES]\, K_m/[S] \text{ and } [EI] = [ES]\, K_m/[S]\{[I]/K_i\}$$

$$\text{where } K_m = \{k_4 + k_c\}/k_3 \text{ and } K_i = \{k_2 + k_i\}/k_1$$

and upon substitution into eq. (1), eq. (4) is obtained:

$$E = [ES]\{K_m/[S] + K_m/[S]\{[I]/K_i\} + 1\} \tag{4}$$

or

$$[ES] = E[S]\, K_i/\{[I]K_m + K_mK_i + [S]K_i\} \tag{5}$$

Substitution of eq. (5) into eq. (2) gives eq. (6), the rate of product release as a function of the, also time-dependent, concentration of uninhibited enzyme ($E_t$).

$$d[P_2]/dt = [E_t]\, k_c[S]\, K_i/\{[I]\, K_m + K_mK_i + [S]K_i\} \tag{6}$$

The distribution of the different forms of enzyme as a function of time, on the other hand, is governed by eqs. (1), (2), and (3). Therefore, eq. (3) is also expressed in terms of steady-state parameters of enzyme kinetics as follows. Since $[E_f] = [EI]K_i/[I]$, $[ES] = [EI][S]/K_m\{K_i/[I]\}$ and $[E] = [E_0] - [EI^*]$ are also true, thus;

$$[EI] = [E_0 - EI^*][I]K_m/\{[I]K_m + K_mK_i + [S]\, K_i\} \tag{7}$$

and

$$d[EI^*]/dt = [E_0 - EI^*]k_i[I]K_m/\{[I]K_m + K_mK_i + [S]K_i\} \tag{8}$$

The similarity between eqs. (6) and (8) is readily recognizable. Provided that $[S] = [S_0]$ and $[I] = [I_0]$, where $[S_0]$ and $[I_0]$ are the initial concentrations, through the

reaction:

$$k_{obs} = k_i[I_0]K_m/\{[I_0]K_m + K_mK_i + [S_0]K_i\} \quad (9)$$

After separation of terms in eq. (8) and integration between limit 0 and $t$ and 0 and $EI_t^*$ (eq. (10)), eq. (11) is obtained:

$$\int d[EI^*]/[E_0 - EI_t^*] = k_{obs}\int dt \quad (10)$$

$$\ln([E_0]/[E_0 - EI_t^*]) = k_{obs}t \text{ (or } \ln([E_t]/[E_0]) = -k_{obs}t) \quad (11)$$

Now, the time-dependent product formation can also be related to the initial concentration of enzyme $E_0$ by substitution of eq. (11), in the exponential form $[E_t] = [E_0] \exp(-k_{obs}t)$, into eq. (6), which gives eq. (12).

$$d[P_2]/dt = [E_0] \exp(-k_{obs}t)\, k_c[S_0]k_i/\{[I_0]K_m + K_mK_i + [S_0]K_i\} \quad (12)$$

i.e., $d[P_2]/dt = \text{const}, \exp(-k_{obs}t)$.

Separation of terms and integration of eq. (12) between the limits 0 and $t$ and 0 and $P_2$ yields eq. (13).

$$[P_{2t}] = [E_0]k_c[S_0]K_i/\{k_i[I_0]K_m\}\{1 - \exp(-k_{obs}t)\} \quad (13)$$

i.e., $[P_{2t}] = \{\text{const}/k_{obs}\}\{1 - \exp(-k_{obs}t)\}$ and at infinite time,

$$[P_{2\infty}] = \text{const}/k_{obs} = [E_0]k_c[S_0]K_i/\{k_i[I_0]K_m\} \quad (14)$$

Since the denominator for the constant in eq. (12) and that of $k_{obs}$ in eq. (9) are identical, they cancel out in eqs. (13) and (14). In these equations, the concentration of product released in time is related to experimentally definable kinetic parameters and concentrations factored by the fraction of reaction progress $\{1 - \exp(-k_{obs}t)\}$ in eq. (13).

## PRACTICAL CONSIDERATIONS

### *Evaluation of Microscopic Rateconstants*

A convenient expression for the determination of the second-order ($k_i/K_i$) and first-order ($k_i$) rate constant is obtained by inversion of eq. (9) and separation of terms as in eq. (15).

$$1/k_{obs} = 1/k_i + \{K_i/(k_i[I_0])\}\{1 + [S_0]/K_m\} \quad (15)$$

In the broadest application, particularly when inhibition is studied at a level of about half saturation of the enzyme with the inhibitor, a plot of $k_{obs}^{-1}$ versus $[I_0]^{-1}$ gives $k_i^{-1}$ as intercept and $(1 + [S_0]/K_m)K_i/k_i$ as slope. A knowledge of $[S_0]/K_m$ or a replot of a series of slopes against corresponding values of $[S_0]$ can lead to a determination of $K_i$. Alternatively, if $K_i/[I_0] > 1$, a plot of $k_{obs}^{-1}$ versus $[S_0]$ approximates $K_i/k_i$ as intercept and $K_i/k_i[I_0]K_m$ as slope. In the reverse case, if $K_i/[I_0] < 1$, a plot of $k_{obs}^{-1}$ versus $[S_0]$ approximates $k_i^{-1}$ as intercept and $K_i/(k_i[I_0]K_m)$ as slope.

### *Scope and Limitations for the Measurement of Microscopic Rateconstants*

It is convenient to discuss limitations on the parameters in terms of reaction constants,

$\tau_0$ ($k_{obs}^{-1}$), and intrinsic reaction constants, $\tau_i$ ($k_i^{-1}$), in units of time;

$$\tau_0 = \tau_i\{1 + K_i/[I_0] + K_i/[I_0]([S_0]/K_m)\} \quad (16)$$

for which lower limits are set by conventional kinetic techniques. For example, $\tau_0 = 29$ s, half lives $> 20$ s, is an achievable limit for conventional measurements. Only if both $[S_0]/K_m$ and $K_i/[I_0]$ are much smaller than 1 will $\tau_0 = \tau_i$ be approximated in eq. (16). Table I shows calculated minimal values for $\tau_i$ (and their inverse, maximal values of $k_i$) for $\tau_0 = 29$ s and feasible levels of saturation of an enzyme with substrate and inhibitor. Ideally, $[S_0]/K_m > 1$ is required for the sake of a good approximation of the first order monitoring of remaining enzyme activity by a substrate, in all cases. Thus, unless $K_i/[I_0]$ gets very small, the term in parenthesis is $> 1$ and therefore minimal values of $\tau_i$ can be much smaller than 29 s. This implies that inhibitors with high reactivity toward the enzyme can be measured only at enzyme saturations high with respect to substrate, independently from the reactivity of the substrate with the enzyme, and low with respect to inhibitor. It also follows from Table I, that conventional kinetics can be applied at high saturation by the inhibitor only if the inhibitor has a unimolecular rate constant less than $\sim 0.35\ s^{-1}$. The unimolecular rate constants and binding constants can be determined with greater accuracy for these (slower) inhibitors. For most inhibitors, the middle part of Table I is useful. Inhibitors of a relatively broad range of reactivity can be studied under saturation, if the appropriate substrate is available with properties to give the required level of saturation. For over half of the cases in Table I, the $K_i/[I_0] \times [S_0]/K_m$ term dominates eq. (16) and therefore $\tau_i \times K_i/[I_0] \times [S_0]/K_m$ approaches $\tau_0$ i.e. it is 9–29 s.

*Accuracy and Precision*

If there is an analytically useful property, A, of the product, which is linearly related to concentration through a proportionally constant, $\alpha$, i.e. $A = P_2\alpha$, then the magnitude of $A$, the signal of $P_2$ at time $t$, is related to the proportionality constant $\alpha$ times the right hand side of eq. (13). Consequently, the total signal $P_\infty\alpha$ at infinite time is predictable from the constants that constitute eq. (17) ($\alpha$ times the right hand side of eq. (14));

$$A_\infty = \alpha[E_0]k_c[S_0]K_i/([I_0]k_iK_m) \quad (17)$$

Whereas the affinity of the substrate for the enzyme seems to be the only decisive factor in rate considerations for competitive irreversible inhibition of enzymes (eq. (16)), the reactivity of the substrate with the enzyme ($k_c$) is also of consequence to the magnitude of the signal. From eq. (17), it is clearly discernible that the choice of a

TABLE I

Minimal Values of $\tau_i$, s (Maximal Values of $k_i$, $s^{-1}$) for Different Levels of Enzyme Saturation with Substrate and with Inhibitor, when $\tau_0 > 20$ s.

| $[I]/K_i$ / $[S]/K_m$ | 0.5 | 1.0 | 10.0 | 100.0 |
|---|---|---|---|---|
| 0.01 | 0.19 (5.2) | 0.14 (7.0) | 0.03 (38.0) | 0.003 (348 ) |
| 0.10 | 1.81 (0.55) | 1.38 (0.72) | 0.26 (3.83) | 0.029 (34.9) |
| 1.00 | 11.60 (0.091) | 9.67 (0.10) | 2.42 (0.41) | 0.28 (3.52) |
| 10.00 | 25.2 (0.04) | 24.17 (0.04) | 13.81 (0.07) | 2.84 (0.35) |

substrate to compete with an inhibitor should be based on its propensity for competition to an optimal extent and on its premise for a large proportionality constant for a measurable physical property. Whereas the former is related to both attainable accuracy and precision the latter mostly bears on the question of precision (*vide infra*). Thus the conditions required by the approximations as well as demands on accuracy and precision all set the restriction on the range of choices. These are (*vide supra*): (1) the steady-state approximation, $[S_0]/K_m > 1$ and, if a full kinetic characterization is to be obtained for the inhibitor, also $[I_0]/K_i > 1$ and (2) constancy of substrate and inhibitor concentration during the entire reaction requires that $[S_0]$ and $[I_0] \gg E_0$ and, (3) $P_{2\infty} \ll [S_0]$ and thus $A_\infty \ll \alpha[S_0]$.

For an analysis of the interdependence of accuracy, precision and the parameters and variables in eq. (17), we also need to note that the magnitude of the signal is directly proportional to the initial concentrations of both the enzyme and the substrate. We can then designate a fraction **a** of the substrate to react for a given and desired signal, $A_\infty = \alpha\mathbf{a}[S_0]$. We also need to set the enzyme concentration to be $[E_0] = \mathbf{b}[S_0]$, a fraction of substrate concentration. Both, parameters **a** and **b** control accuracy and precision. If for a lowest limit of precision we allow for the total signal $A_\infty$ to be $> 0.1$, then for $\mathbf{a} = 0.1$, $\alpha[S_0]$ has to be at least 1.0 and for $\mathbf{a} = 0.01$, $\alpha[S_0]$ has to be at least 10. Thus for the latter case, when a 1% of substrate consumption would permit 99% accuracy, either a large proportionality constant or much substrate is required. The latter demand can be limited further by solubility and rate considerations (eq. (16)) in an absolute sense. In relation to the inhibitor concentration, relative affinities of the substrate and of the inhibitor for the enzyme can also restrict $[S_0]$. For example, a substrate concentration of 10 mM, which is probably extreme, should be combined with $\alpha > 10^3$ *signal* $M^{-1}$ to get 99% accuracy. Thus for a signal of 0.1, **a** will have to remain in the range of 0.01–0.1 for a majority of cases. This sets the limit on accuracy to 99–90% if reasonable precision is also to be obtained.

Substitution for $A_\infty = \alpha\mathbf{a}[S_0]$ and for $[E_0] = \mathbf{b}[S_0]$ into eq. (17) gives eq. (18).

$$\mathbf{a}/\mathbf{b} = k_c \times \tau_i \times [S_0]/K_m \times K_i/[I_0] \tag{18}$$

Again, for a majority of cases $\tau_i \times [S_0]/K_m \times K_i/[I_0] = 9$–$29$ if optimal conditions for the kinetic measurements are also to be maintained (*vide supra*) and therefore eq. (18) will be restricted to $(0.01\text{–}0.1) = \mathbf{b}k_c(9\text{–}29)$ for practical purposes. Thus the balance between **b** and $k_c$ leaves a fairly large range of possibilities even when restricted to the common circumstances discussed above: the product $\mathbf{b}k_c$ can take values $2 \times 10^{-4}$–$2 \times 10^{-2}$ for the restricted cases. If a poor substrate is to be used, ($k_c > 0.002$), **b** can not be very small (much enzyme is needed), but it has to be smaller than 0.1 in order to obtain a minimum of 90% accuracy. In this latter case, then, $\mathbf{a} < 0.1$ offers no improvement in accuracy. Inversely, small values of **b** are needed to accommodate substrates of high reactivity, i.e., large values of $k_c$. Nonetheless, $\mathbf{b} < \mathbf{a}$ does not grant the system any improvement in accuracy.

In the most ideal case, of course, both $\alpha$ is very large $> 10^4$ *signal* $M^{-1}$ and the substrate is soluble up to 10 mM concentration. Then either the signal can be enhanced to improve precision, or a smaller fraction of substrate can be permitted to react for $\mathbf{a} < 0.01$ to increase accuracy above 99%, provided that $k_c$ is large enough, and therefore **b** can also be $< 0.01$.

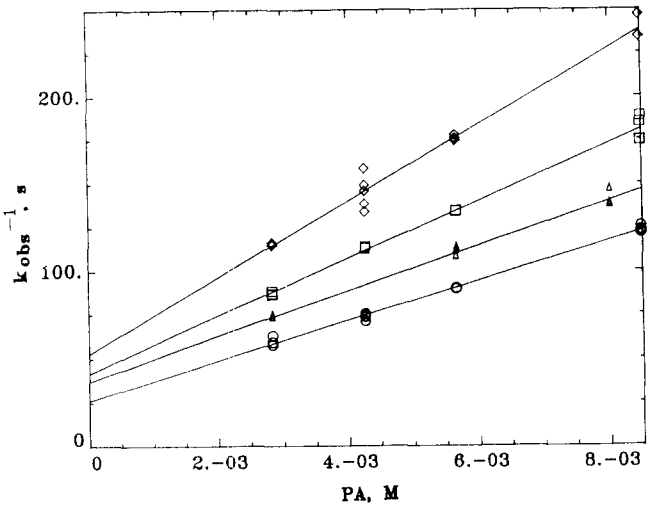


FIGURE 1 The dependence of the inverse observed rate constants on phenyl acetate concentration for the inhibition of the electric eel AChE with sarin: ◇ $4.89 \times 10^{-8}$ M; □ $6.22 \times 10^{-8}$ M; △ $7.56 \times 10^{-8}$ M; ○ $8.89 \times 10^{-8}$ M.

## EXAMPLES

For a typical example, the inhibition of AChE by 2-propyl methylphosphonofluoridate (sarin) is presented here. From a cursory investigation the approximate value of $\tau_i$ or $k_i$ can be obtained for a rational design; they are 0.5–5 s and 0.2–2 $s^{-1}$ respectively in our case and are in agreement with values reported earlier.[7] For convenient rates one should choose saturation levels for the inhibitor and substrate from the right of the diagonal in Table I. A further restriction of choices for spectroscopic measurements of this inhibition reaction is the fact that chromogenic substrates of AChE are insoluble in buffers at $\sim [S_0]/K_m > 4$, which sets the limit of choices for $[I_0]/K_i$ and $[S_0]/K_m$ to the values at the upper middle section of Table I. The $K_m$ value of phenyl acetate, a good chromogenic substrate of AChE with a solubility of $< 10$ mM at pH 7.6 in 0.05 M phosphate buffer with 5% methanol as cosolvent, is $2.28 \pm 0.10$ mM.[4b,c] According to the predictions of Table I, at substrate concentrations $1K_m - 5K_m$, the concentrations of the inhibitor should be kept at $0.1K_i - 1K_i$ for spectroscopic measurements. Sarin concentrations were, thus, set in the range between $5 \times 10^{-8}$ and $1 \times 10^{-7}$ M and phenyl acetate concentrations were at 2–8.5 mM. Figure 1 shows the plot of the inverse observed rate constants versus phenyl acetate concentrations according to eq. (16) for four concentrations of sarin. The calculation of $k_i/K_i$ was from 1/(slope $\times$ $K_m[I_0]$) and $k_i$ from 1/{intercept-(slope $\times$ $K_m$)} of the least squares fit of the data. The parameters obtained with their standard deviations are listed in Table II. As it could be expected from the discussions above and the cursory values from the kinetic parameters, $k_i/K_i$ is the best defined parameter under the circumstances. The reproducibility of $k_i$ is poor and the error associated with $k_i$ is too large to permit a good assessment of this parameter. Consequently, $K_i$ can not be obtained with any reproducibility either. Since $K_i/[I_0] > 4$–12, the inter-

TABLE II

Linear Least-Squares Parameter[a] for the Inhibition of AChE with Sarin[b] and First and Second Order Rate Constants Calculated as Described in the Text.

| $10^8$ Sarin, M | $10^{-4}$ S1 (SD) | Int (SD) | $k_i$, $s^{-1}$ (SD) | $k_i/K_i$, $M^{-1}s^{-1}$ (SD) | $10^7$ $K_i$, M |
|---|---|---|---|---|---|
| 4.89 | 2.18 | 52.8 | 0.33 | 41150 | 8.02 |
| | (0.09) | (5.43) | (0.66) | (17710) | |
| 6.22 | 1.64 | 41.9 | 0.22 | 43030 | 5.11 |
| | (0.06) | (3.41) | (0.18) | (14360) | |
| 7.56 | 1.29 | 37.4 | 0.12 | 45140 | 2.65 |
| | (0.04) | (2.7) | (0.03) | (14000) | |
| 8.89 | 1.14 | 26.4 | 2.00 | 43440 | 4.60 |
| | (0.03) | (1.6) | (6.64) | (9880) | |
| | | Avg. | 0.66 | 43190 | 5.1 |
| | | | (0.9) | (1638) | (2.2) |

[a]S1 = slope; Int = intercept of plots according to eq. (16) (Figure 1).
SD = standard deviation; propagated for $k_i$ and $k_i/K_i$ according to the calculation described in the text.
[b]pH 7.70, 0.05 M phosphate buffer, 25°C.

cept approaches the value of $K_i/(k_i[I_0])$. Since the slope equals $K_i/(k_i[I_0]K_m)$, the value of $K_m$ can be calculated from intercept/slope. The $K_m$ values obtained from the data in Table II are 2.32–2.52 mM in good agreement with that measured independently with phenyl acetate (*vide supra*).

From another careful determination of the inhibition rate constants at 8.12 mM phenyl acetate concentration and higher concentrations of sarin, the inverse observed rate constants were plotted against the inverse of the sarin concentration as illustrated in Figure 2.[4C] The intercept of the plot then gives the inverse of $k_i$ and the slope equals $K_i/k_i(1 + [S_0]/K_m)$. From this measurement, the following values were calculated: $k_i = 0.4 \pm 0.26\,s^{-1}$, $k_i/K_i = (4.65 \pm 0.16) \times 10^5 M^{-1}s^{-1}$, and $K_i = 8.7 \times 10^{-7}$ M (Table III). These are in good agreement with literature reports and with the (best) data in the first line of Table II. The difficulty involved in evaluating $k_i$ of this magnitude (close to 1 or larger) from the intercept of plots of the kind shown in Figure 2 is that the reciprocal is also quite small and frequently negative since the precision is still inadequate (65% standard error for this case and often higher). Data analyzed in the same manner as in Figure 2 for two other inhibitors of AChE, 3,3-dimethyl-2-butyl methylphosphonofluoridate (soman) and 4-nitrophenyl 2-propyl methylphosphonate (IMN) are also listed in Table III.[4c] For these cases too, the precision in the value of $k_i$ is lacking regardless of how the data is worked up. Thus, it is not the precision in the calculation of a certain kinetic parameter that one can alter by rearranging the equation to another form or by choosing to vary one variable versus the other under a set of conditions (enzyme saturation), but it is rather the number of parameters for which physically meaningful values can be obtained even with poor precision. In the first example (Figure 1), although with poor precision, one obtains physically meaningful values for $k_i$.

The estimate of accuracy in these measurements with $A_\infty$ always $= 0.100$ and $\alpha = 1.34 \times 10^3$ OD $M^{-1}$ is as follows: for phenyl acetate 2.2 mM $\mathbf{a} = 0.1/(1.34 \times 2.2) = 0.034$, 3.4% change in the constancy of the concentration of phenyl acetate. At phenyl acetate concentrations of 8.4 mM, if $A_\infty$ is kept at 0.1 OD, the accuracy could be enhanced to > 99%. For easier data manipulation and for the sake of greater precision, the signal was allowed to go higher to ~0.400 and thus the same

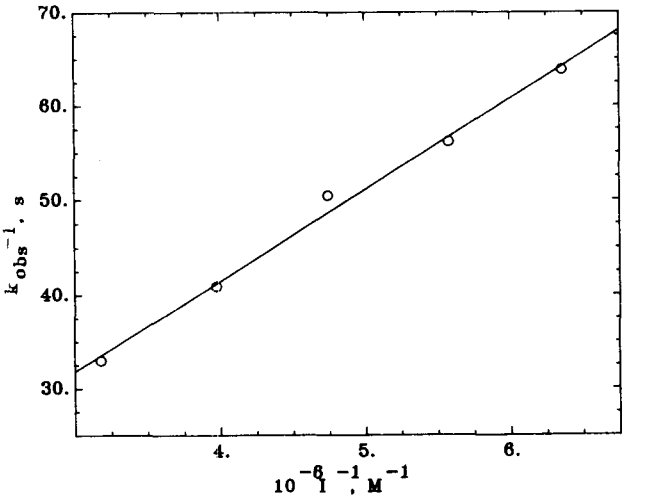


FIGURE 2 The dependence of the average of the inverse observed rate constants on the inverse of the concentration of sarin for the inhibition of electric eel AChE at 8.2 mM phenyl acetate.

accuracy was obtained throughout this set of measurements. The enzyme concentration required for these experiments is very small because the value of $k_c$ is large, $1.7 \times 10^4 s^{-1}$. For example, in the first case, **a** = 0.034, $\tau_i \sim 2.5$ s (Table III), $[S_0]/K_m \sim 1$, and when $K_i/[I_0] = 10$, then **b** = $0.034/(1.7 \times 10^4 \times 2.5 \times 1 \times 10) = 8 \times 10^{-8}$ fraction of the substrate concentration corresponding to $1.76 \times 10^{-10}$ M active-sites. The accuracy obtained for the limiting cases in the studies for which data is given in Tables II–IV, corresponding values of **b**, and concentration of AChE active-sites are tabulated in Table V.

Other substrates that have better chromophores as a leaving group, such as 4-nitrophenyl or naphthyl, could not be used in concentrations to give saturation levels of AChE, comparable to what could be obtained with phenyl acetate, due to their lower solubility in the medium. Other substrates are also less efficient and have lower values for $k_c$ so that higher AChE concentrations would be needed for the experiments. Lastly, the necessary correction for the background hydrolysis of 4-nitrophenyl acetate would further decrease precision.

A slower inhibitor of AChE, 4-nitrophenyl methyl propylphosphonate (MPN), permitted the study of inhibition with concentrations close to $K_i$, while the saturation level of AChE with phenyl acetate was kept close to $K_m$ and below. The data for

TABLE III
First and Second Order Rate Constants and Binding Constants for the Inhibition of AChE by Organophosphorus Compounds at 25° C and pH = 7.70.

| Inhibitor | $k_i$, $s^{-1}$ | $k_i/K_i$ $M^{-1}s^{-1}$ | $K_i$, $\mu M$ |
|---|---|---|---|
| Sarin[a,b] | 0.4 ± 0.26 | (4.65 ± 0.16) × $10^5$ | 0.87 |
| Soman[a,b] | 0.6 ± 0.4 | (1.56 ± 0.03) × $10^6$ | 0.39 |
| IMN[a,c] | 0.26 ± 0.17 | 8131 ± 385 | 32 |

[a]According to eq. (16); PA 8.12 mM; inhibitor 0.1–0.4 $K_i$. [b]Ref. 4c. [c]Ref. 4b.

TABLE IV
Linear Least-Squares Parameters[a] for the Inhibition of AChE with MPN[b] and First and Second Order Constants Calculated as Described in the Text.

| $10^5$ MPN, M | $10^{-3}$ Sl | Int | $k_i$, $s^{-1}$ | $k_i/K_i$, $M^{-1}\,s^{-1}$ | $10^5$ $K_i$, M |
|---|---|---|---|---|---|
| 2.04 | 34.0 | 97.8 | 0.05 | 633 | 7.1 |
| 3.87 | 16.6 | 58.2 | 0.05 | 683 | 7.3 |
| 7.76 | 10.5 | 33.5 | 0.10 | 542 | 18 |
| 8.03 | 7.5 | 27.3 | 0.10 | 734 | 13.6 |
| 11.1 | 6.6 | 24.4 | 0.10 | 600 | 17 |
| 11.5 | 7.0 | 23.4 | 0.12 | 552 | 22 |
| 12.0 | 5.6 | 24.5 | 0.09 | 650 | 13 |
| | | Avg. | 0.09 ± 0.03 | 628 ± 69 | 12.6 ± 7 |

[a]Sl = slope, Int = intercept of plots according to eq. (16) (Figure 2). Standard errors for the data at $2.04 \times 10^{-5}$ M MPN were < 2% for Int and < 4% for Sl, but for the other data sets of fewer points they were < 10% for Int and < 28% for Sl.
[b]25°C, pH 7.70, 0.05 M phosphate buffer, 25°C.

measurements in water at seven different MPN concentrations were plotted according to eq. (16) and are illustrated in Figure 3. The linear least squares parameters and kinetic constants, calculated from the data, as described above, are given in Table IV. Since $K_m = 25K_i$, the slopes of plots of inverse rate constants versus $[S_0]$ become very small, insensitive at MPN levels of $10^{-4}$ M. The two lowest concentrations and the highest of MPN were studied in more detail to give higher precision; errors were 1.3% in the intercept and 3.9% in the slope at an MPN concentration of $2.04 \times 10^{-5}$ M. The value of $k_i/K_i$ was determined with a satisfactory reproducibility and even the value of $k_i$ was reproduced reasonably well for the two cases where more information exists. The value of $K_i$ is around $10^{-4}$ M.

At the concentration of MPN = $1.2 \times 10^{-4}$ M, approximately at $K_i$, with phenyl acetate $2.5 \times 10^{-3}$ M, $\tau_0 \sim 60$ s and at a phenyl acetate concentration of $5 \times 10^{-4}$ M, $\tau_0 \sim 44$ s; these are close to the limit of conventional techniques.

The accuracy obtainable for the concentration extremes of phenyl acetate used in these experiments, if $A_\infty = 0.1$ and since $\alpha = 1.34 \times 10^3$ OD $M^{-1}$, are: 97% since $\mathbf{a} = 0.1/(1.34 \times 2.5) = 0.030$, 3.0% violation of the assumption of the constancy of substrate, and 85% when $\mathbf{a} = 0.1/(1.34 \times 0.5) = 0.15$, 15% violation of the assumption of the constancy of substrate. Since $k_c$ is large, $1.7 \times 10^4 s^{-1}$, the enzyme concentration needed is only a very small fraction of substrate, **b** in Table V, or that

TABLE V
Accuracy of the Steady-State Condition and AChE Concentration Calculated[a] from the Data in Tables II-IV for the Inhibition of AChE.

| Inhibitor | $\tau_i$ (s) | $[S_0]/K_m$ | $[I_0]/K_i$ | Accuracy % | $10^8$**b** | $10^{11}$ $E_0$ (M) |
|---|---|---|---|---|---|---|
| Sarin | 2.5 | 1.0 | 0.1 | 96.6 | 80 | 18 |
| Sarin | 2.5 | 3.7 | 0.1 | 99.1 | 6 | 5 |
| Sarin | 2.5 | 3.7 | 0.4 | 99.1 | 23 | 19 |
| NPM | 10 | 0.22 | 1.0 | 85.0 | 4000 | 200 |
| NPM | 10 | 0.22 | 6.0 | 85.0 | 24000 | 1200 |
| NPM | 10 | 1.1 | 1.0 | 97.0 | 160 | 40 |
| NPM | 10 | 1.1 | 6.0 | 97.0 | 960 | 240 |

[a]Eq. (19). Also see text.

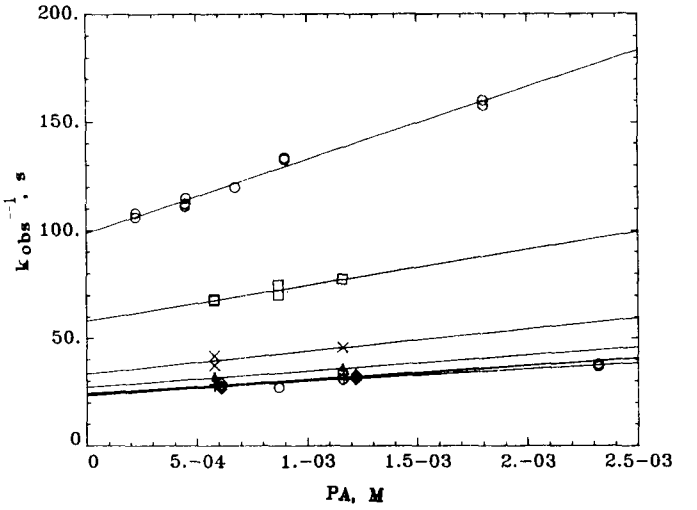


FIGURE 3 The dependence of the inverse observed rate constants on phenyl acetate concentration for the inhibition of the electric eel AChE with MPN: ○ $2.04 \times 10^{-5}$ M; □ $3.87 \times 10^{-5}$ M; × $7.76 \times 10^{-5}$ M; △ $8.03 \times 10^{-5}$ M; ◇ $11.08 \times 10^{-5}$ M; + $11.45 \times 10^{-5}$ M; ○ $12.00 \times 10^{-5}$ M.

of inhibitor (also note from Table IV that $\tau_i = 10$). In all cases, more enzyme would induce an enhanced signal, but also a larger value of **a** and, consequently, a greater violation of the constancy of substrate. Conversely, less enzyme generates a smaller signal, less precision and higher accuracy.

## CONCLUSIONS

A simple analysis of the first order rate expression describing competitive irreversible inhibition of an enzyme in the presence of a substrate is provided in this paper. In the context of a set limit for precision and accuracy a range of desirable substrate parameters and concentrations has been selected for inhibitors with a range of reactivity and affinity for enzymes. The examples for the inhibition of AChE by phosphonate esters of significantly different reactivities demonstrate the best use of a set of data under a given condition. An overview of the scope and limitations of the method is thereby provided.

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