Kinetic Evidence of Multiple Reversible Cholinesterases Based on Inhibition by Organophosphates*

(A. R. MAIN)

From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27607

SUMMARY

Inhibition of serum cholinesterase (ChE) and erythrocyte acetylcholinesterase by diisopropylphosphorofluoridate and amiton did not follow first order kinetics. Curving of the rate plots was concave upward and varied greatly. An equation was derived on the assumption that curving reflected inhibition of a multiple enzyme system. The curves obtained with partially purified horse ChE were then resolved into four linear components at 5° and into three linear components with an indication of a fourth at 25°. Similar results were obtained with human IV-6-3 ChE and bovine erythrocyte acetylcholinesterase. Each resolved component represented the first order rate plot for the inhibition of one form of ChE, indicating that four forms were present in each preparation. The phosphorylation (kₚ) and binding constants (Kᵣ) of the various forms of horse and human ChE inhibited by amiton at 5° and of horse ChE inhibited by diisopropylphosphorofluoridate at 25° were obtained and were found to vary significantly. For example, the kₚ values of horse ChE inhibited by amiton at 5° varied from 48.8 ± 2.2 min⁻¹ to 0.0028 ± 0.0003 min⁻¹ while Kᵣ varied from 7.6 ± 1.2 × 10⁻⁶ M to 3.2 ± 1.5 × 10⁻⁷ M. Binding increased as kₚ decreased with one exception. Since cholinesterases are probably agglomerates of subunits, this indicated that the kinetic properties of the various forms may differ profoundly with the state of agglomeration. However, additional results also indicated that not every inhibitor nor inhibition at every concentration is capable of bringing out these differences.

The intercepts at t = 0 of the linear first order plots were independent of the inhibitor used and of its concentration and provided a criterion by which shifts in the relative concentrations of the various forms could be followed. Changes in either temperature or the ChE concentration changed the relative concentrations of the multiple forms, suggesting that they were interconvertible. With the horse ChE preparations the rates of reversibility increased by about 60-fold when the temperature was raised from 5° to 25°.

In studies concerned with cholinesterase inhibition by organophosphate, carbamate, and organosulfonate compounds, first order kinetics with respect to the concentration of remaining free enzyme (e) has usually been approximated (1-8). Experimentally it is the velocity (v) of a substrate reaction catalyzed by (e) after inhibition for time (t) that is measured since v = e. First order kinetics were assumed when the plot of the log v against t points approximated a straight line.

For various reasons the straight line relationship may not be observed. Perhaps the most common case occurs when the inhibitor concentration (i) is of the same order as the initial enzyme concentration (e) so that kᵣe, the regeneration of the carbamylated enzyme becomes significant as the reaction approaches a steady state (9, 10).

The present paper is concerned with the relatively uncommon case in which several enzymes, each capable of hydrolyzing the substrate, are present and the observed nonlinear log v against t relationships are attributed to this condition. Regeneration would not have been significant with the inhibitors and under the conditions used (11).

There is a growing body of evidence that human and horse ChE₁ (cholinesterase, acetylcholine acetyl-hydrolase, EC 3.1.1.8) occur in multiple molecular forms (7, 12-15). Similarly, both erythrocyte and oel AcChE (acetylcholinesterase, acetylcholine acetyl-hydrolase EC 3.1.1.7) appear to exist as aggregates having a subunit structure (16, 17), and there is evidence that they too occur in differing states of aggregation (18). However, the present evidence suggests that such multiple forms do not differ greatly in their kinetic behaviors, either toward substrates or inhibitors. In addition, one form seems to predomi-

---

* Contribution from the Biochemistry Department, School of Agriculture and Life Sciences and School of Physical Sciences and Applied Mathematics. Paper 2725 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina. This work was supported in part by Research Grants AM-12355 from the National Institute of Arthritis and Metabolic Diseases and ES-00044 from the National Institute of Environmental Sciences. A preliminary report of this work has been given (Fed. Proc., 27, 590, (1968)).

---

1 The abbreviations used are: ChE, cholinesterase; AcChE, erythrocyte acetylcholinesterase; DFP, diisopropylphosphorofluoridate.
mate in most preparations while the others exist as relatively minor components. Svensmark (19) states the position as follows, "...the description of the molecular properties of serum cholinesterase may concern a mixture of proteins with nearly identical properties. However, one type predominates quantitatively, and the presence of small amounts of deviant types cannot significantly affect the results."

It was therefore surprising to observe that the log e against t plots were not linear, but began to curve significantly at about 50% inhibition when a preparation of horse serum ChE purified by the Strelitz method (20) was inhibited by the organophosphate analogue of acetylcholine, amiton,\(^2\) at 5\(^\circ\)C. This observation was made while attempting to determine the phosphorylation and binding constants for this reaction (5). Consequently, relatively high inhibitor concentrations and short inhibition times were used. Since amiton is quite stable (6) and it was known to be about 1000 times the initial enzyme concentration, the possibility that more than one enzyme was present in the mixture was considered.

That the presence of more than one enzyme could result in curving appears to be widely recognized. Krupka (4), for example, used this criteria to show that only one enzyme in a preparation of erythrocyte AcChE-hydrolyzed methyl acetate. A kinetic treatment for this case has not been given and an equation was therefore derived. With the criteria provided by this model, the effects of inhibitor concentration, temperature, different inhibitors, enzyme concentration, and the complete time course of inhibition were examined. Some earlier studies were made with Fraction IV-6-3 ChE from human serum (21) and with bovine erythrocyte AcChE and the results are also included when relevant. The present work is limited to inhibitors, although the methods used would permit studies with various substrates at different concentrations to be made.

**Experimental Procedure**

**Strelitz Horse Serum ChE**—A preparation purified by the Strelitz method (20) from horse serum was purchased from Nutritional Biochemicals. The powder did not contain added salts, buffers, or stabilizing substance such as gelatin. Solutions of 1.5 mg per ml concentration were prepared by weighing 0.15 g of powder and dissolving in about 40 ml of water at 25\(^\circ\)C. To this were added 3 ml of 200 mM sodium phosphate buffer, pH 7.0, and about 7.5 ml of 50 mM NaOH to raise the pH to 7.0. The adjusted solution was diluted to 100 ml and 1 drop of toluene was added. The solution was then maintained at 5\(^\circ\)C \(\pm 0.1\)° for at least 2 days before use and continuously thereafter when the temperature of the inhibition reaction. At 5\(^\circ\)C, for example, everything with which the enzyme solution came in contact, such as pipettes and the reaction vessels, had to be previously cooled to 5\(^\circ\)C since even momentary heating led to a significant loss of reproducibility.

**Human Fraction IV-6-3 ChE**—Type II ChE was purchased from Sigma. This was prepared from serum Fraction IV by the method of Surgenor and Ellis (21) and was Fraction IV-6-3. According to the supplier, it was about 700-fold purified with respect to plasma proteins and it did not contain added salts, buffers, or stabilizing substances. The active site concentration of the preparation used was 2.35 \(\times\) 10\(^{-7}\) moles per g of protein as determined by titration with DFP (22). La Motta et al. (23, 24) have resolved Fraction IV-6-3, also purchased from Sigma, into at least five active bands by starch gel electrophoresis. Work with Fraction IV-6-3 was terminated when the supplier was unable to fill new orders and alternative sources could not be found.

**Bovine Erythrocyte AcChE**—A preparation purified approximately 1000-fold was purchased from Sigma. One vial contained 50 mg of erythrocytic protein, 100 mg of gelatin, 30 mg of NaCl, and 250 mg of sodium phosphate salts. The contents of one vial were dissolved in about 25 ml of water, the pH was adjusted to 7.0 with 0.1 N HCl, and 1.0 ml of 200 mM sodium phosphate buffer was added. After dilution to 50 ml, 1 drop of toluene was added, and the solution was stored at 5\(^\circ\)C \(\pm 0.1\)°.

**Horse Serum**—Pooled horse serum was purchased from the Baltimore Biological Laboratory, Baltimore, Maryland. It did not contain preservatives. The pH of the undiluted serum was adjusted to 7.0 with 0.1 N HCl, and after standing overnight it was checked and readjusted. It was then maintained at 5\(^\circ\)C for 14 days before use.

**Inhibitors**—Amiton was prepared as described previously (25). The melting point was 95–99\(^\circ\)C and the preparation showed one spot by chromatography (26). Stock solutions, 0.100 M, were prepared by dissolving 0.8085 g in 25 ml of absolute ethanol. Aqueous inhibitor solutions were made by dilutions from this stock.

**Substrates**—Butyrylcholine iodide and acetylcholine chloride were purchased from Sigma. Butyrylcholine was chosen for horse and human serum ChE activity determinations. High concentrations (30 mM) and large volumes (50 ml) were used to limit inhibition in the presence of substrate.

**Determination of Rates of Inhibition**—In principle, esterase and inhibitor were allowed to react for a measured time after which inhibition was stopped by addition of substrate and the residual activity was measured.

**Rates of inhibition** were measured with a special inhibition reaction vessel as described previously (5, 28), but the variable nature of the enzyme system studied led to a few modifications. The temperature-dependent rates at which shifts occurred in the relative concentration of the multiple enzyme forms made it necessary to maintain the enzyme solution continuously at the temperature of the inhibition reaction. It is, for example, everything with which the enzyme solution came in contact, such as pipettes and the reaction vessels, had to be previously cooled to 5\(^\circ\)C since even momentary heating led to a significant loss of reproducibility.

The design of the inhibition reaction vessel was modified so that both side arms could be filled from the top with long nosed pipettes. This eliminated the side arm entry port. The vapor...
block for use with high concentrations of DFP was also eliminated by mixing enzyme and inhibitor within 45 sec of pipetting the DFP solution into its side arm. The capacity of the substrate chamber was doubled and 20 ± 2 ml of the 50 ml of substrate were placed in it.

Inhibition reactions were usually followed to 99%, or more, of completion. Since residual velocities were the measure of inhibition, it was necessary to measure velocities which ranged from 100% to less than 1% of initial with reasonable precision. To do this with Radiometer pH stats, initial velocities within the maximum attainable were used. The maximum was determined by the titrant concentration (20 nm NaOH), the delivery syringe (0.5 ml), and the rate of delivery (0.5 ml in 3 min). Sufficient esterase (0.2 to 0.5 ml) was used to give initial rates of from 2.6 to 2.8 moles of substrate hydrolyzed per min at pH 7.0.

Inhibition in the presence of substrate caused significant but modest curving of the progress curves at the higher inhibitor concentrations used. Velocities were then calculated by taking tangents to the curve at various times as described previously (29).

Both the inhibition and the substrate reactions were carried out at pH 7.0. The pH was not varied so as to avoid possible complications associated with the reversible nature of the multiple enzyme system studied. At pH 7.0, 96% of the amitron was in the charged form which inhibits most powerfully. A number of early runs made at pH 7.6 gave the same characteristic curving obtained at pH 7.0.

RESULTS

Equation for Simultaneous Inhibition of Several Enzymes by Irreversible Inhibitors

When several different enzymes are inhibited in the presence of excess inhibitor so that the inhibitor concentration remains constant, the time course for the inhibition of one particular enzyme will be independent of the others. If the inhibition of each enzyme then follows first order kinetics, the first order rate constant ($\rho$) of the individual reactions would be defined experimentally as $\rho = 2.3(\log v_0 - \log v)/t$ where ($v_0$) is the initial substrate velocity and $v = e$. Since the different enzymes hydrolyze a common substrate and the substrate reactions occur independently, the velocity measured experimentally after inhibition for time $t$ will be the sum of their individual velocities ($\Sigma v$). Assuming, for example, a system containing three different enzymes, it follows that

$$\Sigma v = v^I + v^{II} + v^{III} = e_1 e^{-\rho t} + e_2 e^{-\rho t} + e_3 e^{-\rho t}$$

(1)

where $e$ is the base of the natural logarithms and the properties of the individual enzymes are identified by the superscripts I, II, and III. Equation 1 suggests that the plot of log $\Sigma v$ against $t$ would curve, but for significant curving to occur at least two of the $\rho$ values should differ while the ($v_0$) values should not differ too widely.

For the condition that inhibition follows first order kinetics when $t$ remains constant to hold, the equation derived from a given reaction sequence should express $\rho$ as a constant. Equations were derived for reaction sequences involving two inhibitors, binding to an allosteric site and secondary binding. In each case $\rho$ was expressed as a constant, suggesting that, in general, inhibition will follow first order kinetics. Reversible complex formation preceding phosphorylation of the active site was assumed in each case. The basic scheme is

$$E + I \xrightarrow{k_1} EI \xrightarrow{k_2} E'$$

(2)

where $E$, $I$, $EI$, and $E'$ are the enzyme, inhibitor, reversible complex, and phosphorylated active site, respectively. When $k_4 > k_3$, formation of ($EI$) is controlled by an equilibrium affinity constant: $K_a = k_4/k_3$ and $\rho = k_r/(1 + K_a e)$ (5). When $k_3 = k_r$ the situation is more complex since the solution to the second order differential derived from reaction equation 2 yields an expression containing two exponential terms. It can be shown that one of these disappears when the transient phase of the reaction, in which $E'$ is increasing from 0, is over and then

$$\rho = \left[ k_3 + k_r - \sqrt{(k_4 + k_r + k_3 t^2 - 4k_r k_3 e)} \right] / 2$$

and is constant (30).

Characterization of Log $\Sigma v$ against $t$ Plots

Resolution of Plots into Their Linear Components—A log $\Sigma v$ against $t$ plot typical of those obtained for the inhibition of Strelits horse ChE by amiton at 5° is shown in Fig. 1 on three time scales as Curves 2, A, B, and C. As shown, a preliminary experiment was made to outline the curve so that an adequate number of suitably spaced points could be obtained with the second, more definitive, run. Since the preliminary and final experiments were made at different times and with separately prepared reagents, comparison of the two sets of results gives an indication of reproducibility.

Assume, for the moment, that four forms of ChE were present initially, that they were inhibited irreversibly at significantly different rates, and that, in each case, inhibition followed first order kinetics. The forms were numbered in order of decreasing rates of inhibition, the most rapidly inhibited was ChE I, the next ChE II, and so on. When a finite lower limit of resolution is set for the measurement of substrate velocities, it follows from Equation 1 that the time course of inhibition will resolve into four separate regions. In the first, all forms will be active and then $\Sigma v = v^I + v^{II} + v^{III} + v^{IV}$. After a time $t_1$ the ChE I will be completely inhibited, as defined by the lower limits of resolution, and in the second region which follows $\Sigma v = v^{II} + v^{IV}$. After a further interval ChE II will be completely inhibited and in the third region which follows $\Sigma v = v^{III} + v^{IV}$. When ChE III is completely inhibited, $\Sigma v = v^{IV}$ and in this terminal region the log $\Sigma v$ against $t$ plot will be linear since only one form of the enzyme is active.

The procedure used to resolve the log $\Sigma v$ against $t$ plots began with the terminal phase of inhibition which, for example, is that part of Fig. 1, Curve 2C, between 10,600 and 61,200 sec. The points fell on a straight line, suggesting that only one form of the enzyme, ChE IV, was active. A line was fitted to the points by regression analysis and the slope, $\rho^{IV}/2.3$, and intercept value, log $e^{IV}$, were calculated. The first point (e.g. 10,600 sec) was provisionally selected by inspection with the criterion of linearity. This choice was later confirmed or corrected by the lower time limit of ChE III preceding. The lower time limit was the time at which the activity of a given enzyme form became insignificant commensurate with the lower limit of resolution of the method used to measure velocities. This was
Inhibition of Multiple Cholinesterases

Vol. 244, No. 4

2.5

3-c

C

50000

60000

0

1-0

0

50

100

200

300

40000

50000

60000

FIG. 1. Log $\sum v$ against $t$ plots for the inhibition of Strélita horse ChE by $1 \times 10^{-4}$ M amiton at pH 7.0 in 3 mM sodium phosphate buffer and 5 mM NaCl are shown on three times scales: A, 0 to 60 sec; B, 0 to 3,000 sec; C, 0 to 60,000 sec. Curve 1 (A, B, and C) was obtained with a final ChE concentration of $9 \times 10^{-5}$ M, inhibited at 5°C. Curve 2 (A, B, and C) was obtained with $4.5 \times 10^{-5}$ M ChE, inhibited at 5°C, and includes the results of a preliminary run (0) and a final run ($\triangle$). Curve 3B (m) was obtained with $4.5 \times 10^{-5}$ M ChE, inhibited at 25°C. Curve 5C (A) was obtained with $4.5 \times 10^{-5}$ M ChE, inhibited at 15°C. $\sum v_0$ was measured at 25°C. With Curves 1, 2, and 5, $\sum v$ was measured at 5°C; conditions were otherwise the same as were used for Curve 2, except that 0.5 ml of 1.5 mg per ml of ChE solution instead of the 0.22 ml used to obtain Curve 2 was used. For Curves 2, 3, and 5, $\sum v_0$ was 278.5, 274, and 273 at 25°C, respectively, where 1 unit is 0.01 pmole per min. $\sum v_0$ was 216 at 5°C for Curve 4C and 120 at 25°C for Curve 1. To compare Curves 1 and 4C with Curves 2, 3, and 5, the $\sum v$ values of the former were multiplied by 2.29 and 1.27, respectively.

set at 0.01 pmole per min since lower substrate velocities could not usually be reproduced with adequate precision. This lower limit of $v$ was about 0.4% of $\sum v_0$ and approximated the non-enzymatic butyrylcholine substrate control, which included boiled enzyme.

The $v^I$, components of $\sum v$ values measured at shorter times were calculated by substituting $v^I$, $\rho^I$, and the value of $t$ corresponding to $\sum v$ into the equation, log $v^I = -\rho^I t + \log \rho^I v^I$. To calculate $\rho^I$ and $v^I$, a set of ($\sum v - v^I$) and $t$ values was obtained and log ($\sum v - v^I$) was plotted against $t$. The points approximated a straight line in the time region where the relationship, $v^I = \sum v - v^I$, held and then curved upward. A line was fitted by regression analysis to the points on the straight portion of the curve and, as before, the first point was selected provisionally by inspection. The $\rho^I$ and $v^I$ values were calculated from the fitted line. To limit the random errors which increased as ($\sum v - v^I$) decreased, the lowest value of ($\sum v - v^I$) permitted in the calculation of $\rho^I$ and $v^I$ was not less than one-tenth of $v^I$.

The $\rho$ and $v_0$ values of ChE II and ChE I were successively calculated by a logical extension of this treatment. In summary, the method involved obtaining provisional estimates of the slope and intercept values which were then used to define more closely the time ranges proper to the resolution of each form of ChE. If necessary, terminal points were then either added or excluded and the slopes and intercept values were again calculated by regression analysis to obtain a better estimate.

To permit resolution with reasonable precision, from 25 to 50 appropriately spaced $\sum v$ determinations were necessary. Each resolved curve contained at least six points and more were frequently used. Exceptions are noted.

At 25°C the log $\sum v$ against $t$ plots for the inhibition of Strélita horse ChE by DFP and amiton were resolved into three components by the procedure described. The results obtained with one concentration of DFP are illustrated in Fig. 2 where it is possible to contrast the resolved plots with the parent log $\sum v$ against $t$ plot. A barely significant fourth component (ChE IV) with a $v_0 = 0.36\%$ of $\sum v_0$ (Fig. 1, Curve 3B) was also present. The resolved ChE III plots shown in Fig. 2 and in Fig. 4B included a small correction for this component.

At 5°C, the log $\sum v$ against $t$ plots obtained for the inhibition

Fig. 2. The log $\sum v$ against $t$ plots resolved from it, representing ChE I ( ), ChE II ( ), and ChE III ( ), for the inhibition of $4.5 \times 10^{-5}$ M Strélita horse ChE by $5.60 \times 10^{-3}$ M DFP at 25°C, pH 7.0. The first part of the curve is shown on an expanded time scale in the inset. The straight lines were fitted by regression analysis. Their slopes and intercept values on the ordinate gave the $\rho$ and $v_0$ values of each of the forms of ChE. $\sum v_0$ was 272 at 25°C with 0.22 ml of 1.5 mg per ml of ChE solution where 1 activity unit is 0.01 pmole per min.
of Strelitz horse ChE by DFP and amiton were resolved into four components, indicating four forms of ChE. Human IV-6-3 ChE log $\sum v$ against $t$ plots obtained with amiton at $5^\circ$ were resolved into three components. A fourth component with a $v_0 = 0.4\%$ of $\sum v_0$ was also present.

Effect of Inhibitor Concentration on $v_0$ and $\rho$—Log $\sum v$ against $t$ plots were obtained with various concentrations of amiton and DFP at $5^\circ$ and $25^\circ$ with Strelitz horse ChE. At a given temperature and ChE concentration, the extrapolated plots of the resolved components corresponding to each form of ChE tended to pass through a common intercept on the log $v$ axis regardless of inhibitor concentration. The example of ChE I inhibited by various concentrations of DFP is shown in Fig. 3. The related ChE II and ChE III plots are shown in Fig. 4. The $v_0$ values calculated from the intercepts are given in Tables I, II, and III. The results in Table III were obtained with human Fraction IV-6-3 ChE.

Neither the inhibitor used nor its concentration significantly affects $v_0$. This was consistent with Equation 1 which predicted that, when $t = 0$, $v_0$ would be independent of the inhibitor used and of its concentration. Since $v = e^t$, changes in $v_0$ will reflect changes in the concentration of a given form of ChE.

The $\rho$ values associated with each form of ChE are also given in Tables I, II, and III, together with the standard deviations associated with their estimation. The results indicated that the $\rho$ values of a given form of ChE increased with increasing inhibitor concentration. This also was consistent with a multiple enzyme interpretation of the curving log $\sum v$ against $t$ plots.

Limits of Resolution as Reflected by Two Inhibitors and by Inhibitor Concentration—In Fig. 5, the log $\sum v$ against $t$ plots of amiton and malaoxon obtained with Strelitz horse ChE at $25^\circ$ are compared. The malaoxon plot appeared to be linear to $90\%$ inhibition. The amiton plot began to curve significantly after $75\%$ inhibition. Attempts were made to resolve both plots and the results are given in Table II. The amiton plot resolved into three components, with a suggestion of a fourth, shown in Fig. 1, Curve SB. The intercept values agreed well with those of the more extensive study with DFP. With malaoxon a ChE III intercept value was obtained which agreed with that of amiton, but the ChE I and II components could not be resolved directly. An estimate was made of the $\rho^I$ and $\rho^{II}$ values by a simple iterative process in which the $v_0^I$ and $v_0^{II}$ values obtained with DFP were used. These results suggested that the relationships of the $\rho$ values may vary widely.

![Fig. 3. ChE I. The resolved first order rate plots are shown of Strelitz horse ChE I inhibited at $25^\circ$ by the following concentrations of DFP: $1.12 \times 10^{-4}$ m ($\Delta$), $5.60 \times 10^{-5}$ m ($\bigcirc$), $1.12 \times 10^{-5}$ m ($\bullet$), $1.68 \times 10^{-5}$ m ($\blacktriangle$), and $2.24 \times 10^{-5}$ m ($\blacksquare$). The lines were fitted by regression analysis. The ordinate intercepts gave $v_0^I$ and the slopes, $\rho^I/2.3$. The calculated values are given in Table II. The percentage inhibition is of $v_0^I$, which was $272 \pm 3$ at $25^\circ$, pH 7.0, where 1 unit is $0.01 \mu$ mole per min. The final ChE concentration was $4.5 \times 10^{-4}$ M.](image)

![Fig. 4. A, ChE II. The resolved first order rate plots are shown of Strelitz horse ChE II inhibited at $25^\circ$ by the following concentrations of DFP: $1.12 \times 10^{-4}$ m ($\bullet$), $5.60 \times 10^{-5}$ m ($\bigcirc$), $1.12 \times 10^{-5}$ m ($\blacktriangle$), and $2.24 \times 10^{-5}$ m ($\blacksquare$). The lines were fitted by regression analysis. The ordinate intercepts gave $v_0^I$ and the slopes, $\rho^I/2.3$. The calculated values are given in Table II. The percentage inhibition is of $v_0^I$, which was $272 \pm 3$ at $25^\circ$, pH 7.0, where 1 unit is $0.01 \mu$ mole per min. The final ChE concentration was $4.5 \times 10^{-4}$ M.](image)
**TABLE I**

*First order rate constants (\(\rho\)) and intercept values (\(v_0\)) of multiple forms of Strelitz horse ChE inhibited by amiton and DFP at 5°, pH 7.0*

The \(\rho\) and \(v_0\) values were calculated by regression analysis. Inhibition occurred in 3 mM sodium phosphate-5 mM NaCl. One velocity unit is 0.01 \(\mu\)moles per min at 25°, pH 7.0; \(\rho\) is \(\text{min}^{-1} \pm \text{S.D.}; i\) is inhibitor concentration.

<table>
<thead>
<tr>
<th></th>
<th>ChE I</th>
<th>ChE II</th>
<th>ChE III</th>
<th>ChE IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\rho^I)</td>
<td>(v_0^I)</td>
<td>(\rho^II)</td>
<td>(v_0^II)</td>
<td>(\rho^III \times 10^a)</td>
</tr>
<tr>
<td>Amiton and 4.5 × 10^{-8} M ChE</td>
<td>5.05 ± 0.02</td>
<td>200</td>
<td>0.30 ± 0.04</td>
<td>25.4</td>
</tr>
<tr>
<td>4 × 10^{-7} M ChE</td>
<td>1.61 ± 0.24</td>
<td>206</td>
<td>1.89 ± 0.30</td>
<td>26.7</td>
</tr>
<tr>
<td>1 × 10^{-6} M ChE</td>
<td>27.1 ± 2.1</td>
<td>217</td>
<td>2.35 ± 0.09</td>
<td>24.6</td>
</tr>
<tr>
<td>5 × 10^{-7} M ChE</td>
<td>3.8 ± 1.4</td>
<td>190</td>
<td>2.84 ± 0.57</td>
<td>22.2</td>
</tr>
<tr>
<td>1 × 10^{-5} M ChE</td>
<td>37.3 ± 2.5</td>
<td>195</td>
<td>2.84 ± 0.57</td>
<td>22.2</td>
</tr>
<tr>
<td>4 × 10^{-5} M ChE</td>
<td>40.5 ± 1.1</td>
<td>198</td>
<td>2.84 ± 0.57</td>
<td>22.2</td>
</tr>
<tr>
<td>Average (v_0) ± S.D.</td>
<td>198 ± 11</td>
<td>24.7 ± 1.5</td>
<td>16.9 ± 2.0</td>
<td>28.5 ± 1.2</td>
</tr>
</tbody>
</table>

\(v_0^I\) S.D. = \[v_0^I = \sum (v_0 - (v_0^III + v_0^IV)) = 204 ± 4\]

DFP and 4.5 × 10^{-8} M ChE

<table>
<thead>
<tr>
<th></th>
<th>ChE I</th>
<th>ChE II</th>
<th>ChE III</th>
<th>ChE IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\rho^I)</td>
<td>(v_0^I)</td>
<td>(\rho^II)</td>
<td>(v_0^II)</td>
<td>(\rho^III \times 10^a)</td>
</tr>
<tr>
<td>2.24 × 10^{-4} M ChE</td>
<td>25.5 ± 0.9</td>
<td>196</td>
<td>3.38 ± 0.25</td>
<td>24.6</td>
</tr>
<tr>
<td>Amiton and 9.0 × 10^{-8} M ChE</td>
<td>23.3 ± 3.4</td>
<td>189</td>
<td>2.1b</td>
<td>20b</td>
</tr>
<tr>
<td>Average (v_0) ± S.D.</td>
<td>198 ± 11</td>
<td>24.7 ± 1.5</td>
<td>16.9 ± 2.0</td>
<td>28.5 ± 1.2</td>
</tr>
</tbody>
</table>

\(v_0^I\) S.D. = \[v_0^I = \sum (v_0 - (v_0^III + v_0^IV)) = 204 ± 4\]

\(\sum v_0\) was measured with 10 mM NaOH and was 1.20 \(\mu\)moles per min compared with 2.78 ± 0.04 \(\mu\)moles per min with 1.5 × 10^{-4} M ChE. To permit comparisons, the \(v_0\) values were multiplied by 2.29.

* Calculated with three points.

---

**TABLE II**

*First order rate constants (\(\rho\)) and intercept values (\(v_0\)) of multiple forms of Strelitz horse ChE inhibited by DFP, amiton, and malaoxon at 5°, pH 7.0*

The \(\rho\) and \(v_0\) values were calculated by regression analysis. Plots from the results from which some of these values were calculated are shown in Figs. 3 and 4. The final ChE concentration was 4.5 × 10^{-8} M and 3 mM sodium phosphate-5 mM NaCl was present. One velocity unit is 0.01 \(\mu\) mole per min at 25°, pH 7.0; \(\rho\) is \(\text{min}^{-1} \pm \text{S.D.}; i\) is inhibitor concentration.

<table>
<thead>
<tr>
<th></th>
<th>ChE I</th>
<th>ChE II</th>
<th>ChE III</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\rho^I)</td>
<td>(v_0^I)</td>
<td>(\rho^II)</td>
<td>(v_0^II)</td>
</tr>
<tr>
<td>DFP</td>
<td>1.12 × 10^{-4} M ChE</td>
<td>5.88 ± 0.16</td>
<td>229</td>
</tr>
<tr>
<td>5.60 × 10^{-4} M ChE</td>
<td>25.1 ± 0.8</td>
<td>233</td>
<td>2.89 ± 0.12</td>
</tr>
<tr>
<td>1.12 × 10^{-5} M ChE</td>
<td>43.9 ± 2.4</td>
<td>221</td>
<td>4.81 ± 0.22</td>
</tr>
<tr>
<td>1.68 × 10^{-5} M ChE</td>
<td>54.7 ± 1.7</td>
<td>217</td>
<td>5.69 ± 0.39</td>
</tr>
<tr>
<td>2.24 × 10^{-5} M ChE</td>
<td>67.0 ± 6.9</td>
<td>224</td>
<td>5.69 ± 0.39</td>
</tr>
<tr>
<td>Average (v_0) ± S.D.</td>
<td>227 ± 7.5</td>
<td>30.2 ± 2</td>
<td>21.2 ± 0.8</td>
</tr>
<tr>
<td>(v_0^I) S.D. = [v_0^I = \sum (v_0 - (v_0^III + v_0^IV)) = 221 ± 2]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Amiton | 1 × 10^{-5} M ChE | 63 ± 5 | 222 | 1.47 ± 0.15 | 29.8 | 0.36 ± 0.03 | 20.9 |

Malaoxon | 1 × 10^{-8} M ChE | 3.50a | 221a | 2.34a | 30.2a | 0.39 ± 0.03 | 20.9 |

a Calculated by iteration with the average \(v_0^I\) and \(v_0^II\) values obtained with DFP.
TABLE III

First order rate constants (p) and intercept values (v0) of multiple forms of human IV-6-3 ChE inhibited by various concentrations of amiton at pH 7.0, 5°.

The p and v0 values were calculated by regression analysis. The final ChE concentration was 1.01 × 10⁻⁸ M in 3.3 mM sodium phosphate buffer. One velocity unit is 0.01 pmole per min at 25°, pH 7.0; p is min⁻¹ ± S.D.; i is inhibitor concentration.

<table>
<thead>
<tr>
<th>i</th>
<th>CHE I</th>
<th>CHE II</th>
<th>CHE III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pI</td>
<td>pII</td>
<td>pIII</td>
</tr>
<tr>
<td></td>
<td>v0I</td>
<td>v0II</td>
<td>v0III</td>
</tr>
<tr>
<td>1.07 × 10⁻⁴</td>
<td>5.4 ± 0.3</td>
<td>233</td>
<td>28.5</td>
</tr>
<tr>
<td>8.0 × 10⁻⁴</td>
<td>28.7 ± 2.0</td>
<td>257</td>
<td>27.0 ± 0.3</td>
</tr>
<tr>
<td>1.33 × 10⁻⁴</td>
<td>36.1 ± 3.1</td>
<td>205</td>
<td>27.3 ± 0.4</td>
</tr>
<tr>
<td>2.0 × 10⁻⁴</td>
<td>45.1 ± 2.4</td>
<td>233</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>2.67 × 10⁻⁴</td>
<td>67 ± 1</td>
<td>216</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Average v0 ± S.D.</td>
<td>231 ± 15</td>
<td>26.9 ± 2.4</td>
<td>10.0 ± 1.8</td>
</tr>
</tbody>
</table>

depending on the inhibitor and the ChE forms in question. Thus the ratio (pI/pII) was 43 for amiton compared with 1.5 for malaoxon while the (pII/pIII) ratios were 4 and 6, respectively. The log ∑v against t plot of human Fraction IV-6-3 ChE inhibited by 1 × 10⁻³ M malaoxon to 82% of completion was also linear, as shown in Fig. 5.

A similar effect was observed at low inhibitor concentrations. Comparison of the p values in Tables I, II, and III indicated that in some instances their ratios decreased significantly with decreasing inhibitor concentration and this too resulted in a relative straightening of the log ∑v against t plots. In Table I, for example, (pI/pII) changed from 5.4 at 1 × 10⁻⁶ M amiton to 14.3 at 1 × 10⁻⁵ M amiton. From Table II, (pII/pIII) changed from 5 at 5.6 × 10⁻⁶ M DFP to 2.8 at 1.12 × 10⁻⁵ M DFP. At this latter ratio the validity of the v0 estimate tended to be marginal because of the long extrapolation as shown in Fig. 4B.

Evidence of Reversibility

Effect of Inhibition Reaction Temperature—The terminal phases of the inhibition of Strelitz horse ChE by 1 × 10⁻⁴ M amiton at 5°, 15°, and 25° gave v0 values of 28.5, 8.1, and 1, respectively. The plots are shown in Fig. 1, Curves 2A (5°), 5C (15°), and 8B (25°).

Since all substrate velocities were measured at 25°, the v0 values should have been independent of temperature had the relative concentrations of the various ChE forms remained constant. Although v0 varied, the v0 values did not, suggesting that the variation was not due to ChE inactivation. The v0 values of the other forms of ChE also changed, although not so dramatically. Giving v0 at 5° first followed by the 25° value, the results were: v0IV, 28.6 ± 1.2; v0I, 16.9 ± 2.0; v0II, 21.2 ± 0.8; v0III, 24.7 ± 1.5; v0V, 30.2 ± 2; v0I, 204 ± 4, 221 ± 2. Since the v0 values were not constant with temperature and ChE inactivation did not occur, the results suggested that the forms of ChE were interconvertible and that their relative concentrations depended on temperature.

Similar results were obtained when AcChE was inhibited by amiton at 25° and 5°. The log ∑v against t plots are compared in Fig. 6A. The amiton concentration at 5° was 10 times that of 1 × 10⁻⁴ M amiton (○) with that of 1 × 10⁻⁴ M malaoxon (●) for the inhibition of 4.5 × 10⁻³ M Strelitz horse ChE at 25°. The solid lines were calculated from the p and v0 values characterizing the various forms of ChE as given in Table II, with Equation 1. The broken straight line was calculated by regression analysis with the points of less than 90% inhibition by malaoxon. The first part of the amiton curve is shown on an expanded time scale in the inset. The ∑v values were then 175 units at 25° with 30 ml of 15 mM acetylcholine. The ∑v values were multiplied by 1.58 to facilitate comparison with the results obtained with Strelitz horse ChE.
Inhibition of Multiple Cholinesterases

Vol. 244, No. 4

2.01
1.5
zl
8

FIG. 6.
A, effect of temperature on the log \( \sum v \) against \( t \) plots of bovine erythrocyte AcChE inhibited by 5 \( \times 10^{-4} \) M amiton at 5° (■) and 5 \( \times 10^{-4} \) M amiton at 25° (□). \( v_0 \) was 207 at 25°, pH 7.0, where 1 unit is 0.01 \( \mu \)mole per min, with 3 mM acetylcholine when inhibition was with 5 \( \times 10^{-4} \) M amiton at 25°. The comparable \( \sum v \) for inhibition with 5 \( \times 10^{-4} \) M amiton at 5° was 210. The \( \sum v \) values are compared on the basis, \( \sum v_0 = 100 \).

B, effect of solution age on the log \( \sum v \) against \( t \) plots obtained with 2 \( \times 10^{-4} \) M amiton at 5° and bovine erythrocyte AcChE. One plot (○) was obtained with a preparation 1-day-old while the other (△) was 7-days-old. The inset is on an expanded time scale. \( v_0 \) was 217 and 218 units, respectively, at 25°, pH 7.0, with 50 ml of 30 mM acetylcholine, 1% v/v, in n-butyl alcohol and 0.3 ml of a solution containing 1 mg of erythrocytic protein per ml. The final salt concentrations were approximately 4 mM sodium phosphate and 6 mM NaCl. The \( \sum v \) values are compared on the basis, \( \sum v_0 = 100 \).

at 25° so that the initial rate of inhibition at 5° was higher than that at 25°. Despite this, the 5° curve leveled off at a significantly higher point, indicating a shift in the relative concentrations of the ChE forms.

Effect of ChE Concentration—The effect on the log \( \sum v \) against \( t \) plot of diluting Strelitz horse ChE 5-fold is illustrated in Fig. 1 where Curve 1, A, B, and C, may be compared to Curve 2, A, B, and C, each obtained with 1 \( \times 10^{-3} \) M amiton at 5°. Both curves were resolved and the results are given in Table I. Five-fold dilution increased \( v_0 \) from 0.285 to 0.379 \( \mu \)mole per min, or by 32%. Inspection of Fig. 1 clearly suggested that this difference was significant. The other \( v_0 \) values also changed; \( v_0^{IV} \) increased by about 14%, \( v_0^{II} \) decreased by 19%, and \( v_0^I \) decreased by about 8%. The \( v \) values did not change significantly, suggesting that dilution affected the concentration but not the kinetic characteristics of the ChE forms.

The effect of dilution was observed first with human Fraction IV-6-3 ChE, where it appeared to be more pronounced than with horse ChE.

The effect of dilution was consistent with the hypothesis of reversibility suggested by temperature effects. It suggested further that ChE I would be larger than ChE IV into which it appeared to dissociate, possibly directly or possibly through ChE II and III.

Effect of Substrate Temperature—The curves shown in Fig. 7A were traced from Radiometer pH-stat recorder charts. They are the initial portion of progress curves. Substrate velocities were calculated from the subsequent, linear portion, only part of which is shown. The first five curves, from the left, were obtained with Strelitz horse ChE inhibited by 1 \( \times 10^{-5} \) M amiton at 5° for various times before being added to substrate at 25°. The initial sharp rise of Curves 2, 3, 4, 5, 7, and 8 reflected the
addition of alkali to raise the pH of the substrate solution to the region of the pH set for titration. This was followed, in Curves 2, 3, 4, and 5 by a marked lag in the rate of addition and then recovery to a final steady rate. This “lead in” period to a steady rate occurred almost invariably when Strelitz ChE was inhibited at 5° followed by velocity measurements in substrate at 25°.

Inhibition at 25° and other ChE preparations were not characterized by a lead in period as shown, for example, by Curves 9 and 10 (Fig. 7A).

Except at higher velocities, the lead in period was relatively constant, about 3 to 4 min, regardless of the inhibitor or its concentration. This and the regularity with which it occurred suggested that the lead in period might reflect a shift in the relative concentrations of the ChE forms, brought about by the sudden change in temperature, and possibly dilution. To test the role of temperature, substrate velocities were measured at 5°, eliminating the temperature change. This also eliminated the lead in period as shown by Curves 6, 7, and 8, Fig. 7A, which were typical of those obtained with velocity measurements with substrate at 5°.

A shift in the relative concentrations of the ChE forms would alter the log \( \sum v \) against \( t \) plots and this occurred as shown in Fig. 7B where the 25° and 5° substrate plots are compared. To identify, at least tentatively, the nature of the shift, an attempt was made to resolve the 5° log \( \sum v \) against \( t \) plots. The \( v_0 \) of a particular ChE, obtained with 5° substrate, is given first, followed by the comparable value obtained at 25° substrate: \( v_0^{IV} \), 3, 28.5; \( u_0^{IV} \), 9.5, 16.9; \( v_0^{I} \), 24.7, 29.4; \( v_0^{I} \), 223, 204. By this analysis, the net shift was largely from ChE IV to ChE I, since \( v_0^{IV} \) decreased by 25.5 and \( v_0^{I} \) increased by 28. ChE II and III also appeared to alter, although not so dramatically. Velocity measurements at 5° in the ChE IV region (\( t = >3590 \) sec) were very low, \( v_0 = <2.75 \) when \( \sum v = 215 \pm 4 \). The value of \( v_0^{IV} \) was estimated by assuming that \( v_0^{IV} \) with 25° substrate was the same with 5° substrate. The plot is shown in Fig. 1, Curve 4C.

It would seem that, although the velocity measured at 25° reflected inhibition of ChE IV, actually it was not ChE IV, but primarily ChE I that catalyzed the substrate reaction. The results also suggested that ChE IV hydrolyzed 30 mM butyrylcholine at perhaps one-tenth the rate of ChE I.

The log \( \sum v \) against \( t \) plot of native horse serum inhibited by \( 1 \times 10^{-5} \) M amiton at 5° is also shown in Fig. 7B, where, in one case, velocity was measured with substrate at 25° and in the other it was measured at 5°. The substrate temperature did not in this case affect the shape of the log \( \sum v \) against \( t \) plot. This was consistent with the results obtained with Strelitz horse ChE since the progress curves of native horse serum were not characterized by a lead in period when inhibition was at 5° and velocity was measured at 25°, suggesting that the rate of reversibility of the ChE forms in the native serum was less than that characterizing the Strelitz preparations.

**Rates of Reversibility**—The rates at which the concentration shifts reflected by the changed \( v_0 \) values occurred were measured in two directions: one when the solution temperature was dropped suddenly from 25° to 5°, indicating the rate at 5°, and the other when the temperature was raised suddenly from 5° to 25°, indicating the rate at 25°. Sudden cooling was effected by pipetting a relatively small volume (0.22 ml) of Strelitz horse ChE (1.5 mg per ml), initially at 25°, with previously cooled thick walled 0.5-ml measuring pipettes and transferring quickly to the relatively massive side arm of an inhibition reaction vessel at 5°. After sitting for various times, 0.22 ml of \( 2 \times 10^{-5} \) M amiton in the other side arm was tipped in. Inhibition was allowed to occur for 200 sec and the \( \sum v \) was measured. At first \( \sum v \) increased with time as the quickly inhibited ChE I, and to some extent II, was converted to the less readily inhibited ChE III and, principally, IV. As shown in Fig. 8, \( \sum v \) then remained constant after 250 min, indicating that a new concentration equilibrium of the ChE forms had been reached. Essentially the same procedure was used to estimate the rate of reversibility at 25°. The inhibitor was then \( 2.24 \times 10^{-4} \) M DFP and the inhibition time was 30 sec. Fig. 8 shows that approximately 5 min were required to reach the new equilibrium at 25°. This result is in essential agreement with the 3- to 4-min range suggested by the lead in period. Evidently the rate of reversibility, as well as the relative concentrations of the ChE forms, was affected by temperature. To reach equilibrium at 5° required about 280 min and was 60 times slower than at 25°.

The rates of reversibility of horse serum ChE, bovine erythrocyte AcChE, and human Fraction IV-6-3 ChE were apparently different. The log \( \sum v \) against \( t \) plots shown in Fig. 6B suggested that erythrocyte AcChE preparations had not reached equilibrium after standing for 1 day at 5°. However, plots obtained after 7 days remained reasonably constant thereafter, suggesting an equilibration time of several days. Native horse serum also exhibited an equilibration time of several days at 5°, as indicated by comparison of the 4-day and 14-day log \( \sum v \) against \( t \) plots in Fig. 7B.
Inhibition of Multiple Cholinesterases

TABLE IV

Phosphorylation ($k_\text{p}$) and binding ($K_a$) constants of multiple forms of Streitz horse ChE, human IV-6-3 ChE, and one form of erythrocyte AcChE inhibited by DFP and amiton

The $K_a$ values were calculated by regression analysis with Equation 3. Similarly, the $k_p$ values were calculated with Equation 4. $k_1 = k_2/K_a$. The $\rho$ and $i$ results from which the $K_a$ and $k_p$ values were calculated are given in Tables I, II, and III.

<table>
<thead>
<tr>
<th>ChE No.</th>
<th>$K_a$</th>
<th>$k_p$</th>
<th>$k_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFP and Streitz horse ChE at 25°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.64 ± 0.7 × 10⁻⁴</td>
<td>145 ± 8</td>
<td>5.5 × 10⁴</td>
</tr>
<tr>
<td>II</td>
<td>7.70 ± 1.34 × 10⁻⁴</td>
<td>7.85 ± 0.57</td>
<td>1.0 × 10⁴</td>
</tr>
<tr>
<td>III</td>
<td>2.0 ± 0.5 × 10⁻⁴</td>
<td>0.96 ± 0.10</td>
<td>4.8 × 10³</td>
</tr>
<tr>
<td>Amiton and Streitz horse ChE at 5°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7.6 ± 1.2 × 10⁻⁴</td>
<td>48.8 ± 2.2</td>
<td>6.5 × 10⁴</td>
</tr>
<tr>
<td>II</td>
<td>1.24 ± 0.12 × 10⁻⁵</td>
<td>3.5 ± 1.1</td>
<td>2.8 × 10⁵</td>
</tr>
<tr>
<td>III</td>
<td>0.4 ± 0.10 × 10⁻⁷</td>
<td>6.9 ± 0.4 × 10⁻⁹</td>
<td>7.3 × 10⁴</td>
</tr>
<tr>
<td>IV</td>
<td>3.2 ± 1.5 × 10⁻⁷</td>
<td>2.8 ± 0.3 × 10⁻³</td>
<td>8.8 × 10⁴</td>
</tr>
<tr>
<td>Amiton and human IV-6-3 ChE at 5°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.54 ± 0.28 × 10⁻⁵</td>
<td>90 ± 8</td>
<td>5.8 × 10⁶</td>
</tr>
<tr>
<td>II</td>
<td>2.47 ± 0.43 × 10⁻⁶</td>
<td>3.43 ± 0.73</td>
<td>1.4 × 10⁶</td>
</tr>
<tr>
<td>III</td>
<td>1.15 ± 0.10 × 10⁻⁶</td>
<td>0.23 ± 0.005</td>
<td>2.0 × 10⁵</td>
</tr>
<tr>
<td>Amiton and AcChE at 5°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.8 ± 0.5 × 10⁻⁴</td>
<td>157 ± 27</td>
<td>5.6 × 10⁴</td>
</tr>
</tbody>
</table>

See Equation 6 and Fig. 9.

---

Binding ($K_a$) and Phosphorylation ($k_p$) Constants

$K_a$ and $k_p$ of Multiple Forms of ChE—The $\rho$ and $i$ values given in Tables I, II, and III were used to calculate the $K_a$ and $k_p$ values characterizing inhibition of the multiple forms of Streitz horse ChE and human IV-6-3 ChE by DFP and amiton.

Inhibition was assumed to occur by reaction Equation 2, assuming that $k_1 \gg k_2$ and therefore $K_a = k_{-1}/k_1$ (30).

The $K_a$ values ± standard deviation were calculated by regression analysis with the equation

$$\rho = -\left[\frac{\rho}{i}ight] K_a + k_p$$ (3)

where $K_a$ is the slope.

The $k_p$ values ± standard deviation were calculated by regression analysis with the equation

$$\frac{i}{\rho} = \frac{i}{k_{-2}} + \frac{1}{k_1}$$ (4)

where $1/k_2$ is the slope. The bimolecular velocity constant $k_1$ was calculated from the $K_a$ and $k_p$ values since $k_1 = k_2/K_a$ (5).

The results are given in Table IV. Plots of the $\rho$ and $i$ data according to Equations 3 and 4 were, with one exception, linear. Inhibitor concentrations were selected primarily with the intent of determining the $K_a$ and $k_p$ values of the predominant ChE I. Consequently, they were not always optimal for estimating the same constants of the other forms of ChE since their $K_a$ and $k_p$ values differed so greatly. This explains in part the poor precision associated with the $K_a$ values of ChE III and IV inhibited by amiton at 5°. In this instance, the inhibitor concentrations used were higher than the $K_a$ values. The standard deviation indicated a lack of precision, but not necessarily a lack of accuracy. The $K_a$ values were probably of the order indicated, between 10⁻⁵ and 10⁻⁶ M. The $k_p$ values were, for the same reason, relatively precise since $\rho$ approached 1 as $i = >K_a$.

The $\rho$ against $i$ plot for Streitz horse ChE I and amidon at 5° is shown in Fig. 9. The plot curved significantly at amidon concentrations of less than 1 × 10⁻⁵ M, although it appeared to be linear at concentrations greater than 1 × 10⁻⁵ M.

The curve was consistent with a simplified reaction scheme involving binding to an allosteric site, as follows:

$$E + I \rightleftharpoons IE; IE + I \rightleftharpoons IEI; IEI \rightarrow E'$$ (5)

$$k_1$$
where binding to the active site is governed by \( K'_{at} \) and binding to the allosteric is governed by \( K_a \). The following equation was derived from these reactions

\[
\frac{1}{\rho} = \frac{1}{k_i} \left[ \frac{K_{ad} K_{at}}{i} + 1 \right] + \frac{K'_{at}}{k_i} \tag{6}
\]

\( K_{st} \) was estimated to be about \( 2 \times 10^{-8} \text{ M} \) from the results in Table I, with Equation 6. When \( i \\approx (K'_{at} K_{ad}/i) \), Equation 6 reduced to Equation 4. The results were consistent with Equation 6 and tentatively suggested the existence of an allosteric site on ChE. Evidence of similar curving with AcChE was not obtained although the possibility was explored.

Other assumptions, also based on the presence of an allosteric site, will give equations with the same form as Equation 6. The detailed interpretation of the slope and intercept values in terms of the binding constants involved must therefore be treated with reserve.

\( K_a \) and \( k_z \) of AcChE Inhibited by Amiton at 5°—The \( K_a \) and \( k_z \) values are given in Table IV. They were calculated with nine values of \( \rho \) obtained at seven different inhibitor concentrations which ranged from \( 2 \times 10^{-4} \text{ M} \) to \( 2 \times 10^{-2} \text{ M} \). The \( \rho \) values ranged from 0.99 to 63.5 min\(^{-1} \) and \( i/\rho \) ranged from 2.02 to \( 10^{-5} \text{ M} \) min to 3.22 \( \times 10^{-6} \text{ M} \) min. The sum of the \( v_0 \) values of the ChE II and ChE III components varied from 8.7% to 17.9% of \( \Sigma v_0 \), depending on the age of the AcChE preparation. For example, with a preparation 9 days old and \( 1 \times 10^{-4} \text{ M} \) amiton, \( \rho^1 = 31.1 \text{ min}^{-1} \), \( \rho^{11} = 0.14 \text{ min}^{-1} \), and \( \rho^{111} = 0.023 \text{ min}^{-1} \) when \( v_0^{11} = 6.6\% \) and \( v_0^{111} = 10.4\% \) of \( \Sigma v_0 \).

**Discussion**

The results, taken together, provided reasonably strong support for the hypothesis that the curving log \( \Sigma v \) against \( t \) plots reflected a multiple enzyme system. Similar curving has been obtained with other inhibitors including the carbamates neostigmine and eserine and with electric eel AcChE (31) and with seven dialkylphenylphosphate analogues.8

Resolution of the log \( \Sigma v \) against \( t \) curves obtained with amiton and DFP at 5° indicated that the horse and human serum ChE preparations studied contained four forms of ChE. More may have been present but, if so, they were beyond the present limits of resolution.

Inhibition of Strelitz horse ChE by amiton and DFP at 5° revealed significant differences in the kinetic properties of the ChE forms. Differences between rates of phosphorylation (\( k_z \)) and inhibition power (\( k_{iz} \)) tended to be intermediate between \( k_z \) and \( k_{iz} \), and the time-dependent nature of these effects argued strongly in favor of a reversible system, but one in which the rate of reversibility could vary widely, depending on temperature and the nature of the enzyme preparation.

The effect of temperature and of dilution, their reproducibility, and the time-dependent nature of these effects argued strongly in favor of a reversible system, but one in which the rate of reversibility could vary widely, depending on temperature and the nature of the enzyme preparation.

Inhibition of bovine erythrocyte AcChE by amiton at 5° resulted in curved log \( \Sigma v \) against \( t \) plots which resolved into three components with a suggestion of a fourth. However, the intercept values, which varied from day to day and from preparation to preparation, at first seemed to cast doubt on a multiple enzyme interpretation of these components. Later, when the reversible nature of Strelitz horse ChE preparation was recognized, a re-examination of the results clearly indicated that the multiple forms of AcChE were also reversible and that the intercept variations could be attributed to this and to the effect of temperature on their relative concentrations.

Previous studies concerned with the resolution and characterization of the multiple forms of ChE and AcChE have been based primarily on electrophoretic and chromatographic methods. They have indicated that serum ChE (7, 12-15, 19, 23, 32) and AcChE (16, 17) are aggregates of subunits and there is further evidence which indicates that agglomeration from subunits is a reversible phenomenon (18, 24). The present results are consistent with this picture, and indicate further that the different forms of aggregation may be characterized by profoundly different kinetic properties, insofar as inhibition by certain organophosphates is concerned. In other words, the degree of agglomeration appears to influence the kinetic properties of the various multiple forms.

The present methods (5, 28) have resulted in a relatively definitive differentiation of the multiple forms of serum ChE. This has permitted, on the one hand, their kinetic characterization by organophosphorus inhibitors and, on the other, a description of their interconvertible nature and its dependence on temperature and enzyme concentration.

**References**

18. GRAFUS, M. A., AND MILLER, B. D., Biochemistry, 6, 1034 (1967).