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Recording Spectrophotometric Method for Determination of Dissociation and Phosphorylation Constants for the Inhibition of Acetylcholinesterase by Organophosphates in the Presence of Substrate†

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ABSTRACT: An experimental procedure, based on the use of a recording spectrophotometer, has been developed for determination of affinity, phosphorylation, and bimolecular reaction constants for the inhibition of an esterase by organophosphates *in vitro*. The theoretical background and derivation of the relevant equations are discussed. The method has been applied to a study of the effect of temperature on the inhibition of acetylcholinesterase by paraoxon, and to an investigation of the influence of carbon chain length on the inhibitory potencies of five *O,O*-diethyl (*ω-tert*-butalkyl) phosphates. Results are discussed in terms of the relative contributions of affinity and phosphorylation enhancement to overall inhibitory power.

Studies on the inhibition of cholinesterase by organophosphates have led to the recognition of the formation of a reversible enzyme–inhibitor complex (Aldridge, 1950) that can be related to the overall inhibition process as follows

$$E + PX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EPX \xrightarrow{k_2} EP + X \qquad (1)$$

where E represents the enzyme, PX the organophosphate with its leaving group X, EPX the reversible complex, and EP the covalently phosphorylated enzyme. Despite the implications of reaction 1, in much of the subsequent work kinetic treatments were used which ignored the formation of EPX and yielded a simple bimolecular rate constant (k_i'), suggesting that the reaction is a one-step process of the type

$$E + PX \xrightarrow{k_i'} EP + X \qquad (2)$$

Main and Dauterman (1963) have described a procedure, using a pH-Stat, for the determination of k_i' in the presence of substrate.

The development of more definitive kinetic treatments (Wilson, 1960; Main, 1964) allowed the determination of the dissociation constant, K_d (*i.e.* k_{-1}/k_1), and the phosphorylation constant, k_2, for reaction 1. These two parameters are related to overall inhibitory potency by the expression $k_i = k_2/K_d$, where k_i may conveniently be described as a "bimolecular reaction constant" (Main, 1964) since it is a combination of an equilibrium and a rate parameter and does not have the same meaning as the bimolecular rate constant, k_i'. Only a limited number of organophosphates have been subjected to this affinity *vs.* phosphorylation analysis because of the difficulties inherent in the present experimental procedures and the consequent need to use special reaction vessels (Main and Iverson, 1966; Chiu and Dauterman, 1969a,b; Chiu *et al.*, 1969; Lee and Metcalf, 1972). The difficulties arise due to the desirability of using inhibitor concentrations approaching K_d (Main, 1969a). Consequently, readings have to be taken over

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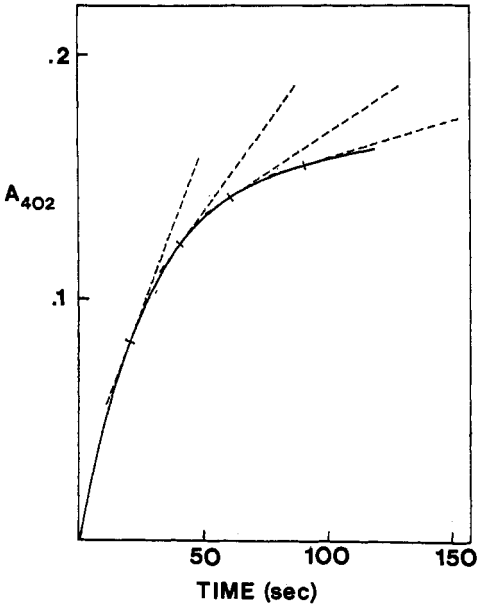


FIGURE 1: Reproduction of recording spectrophotometer trace showing change in absorbance with time during inhibition of acetylcholinesterase (60 units ml^{-1}) by paraoxon (2×10^{-5} M) in the presence of a chromogenic substrate (2 mM *p*-nitrophenyl acetate) at 25° in 0.067 M sodium phosphate buffer at pH 6.9. Tangents may be drawn at convenient points as shown.

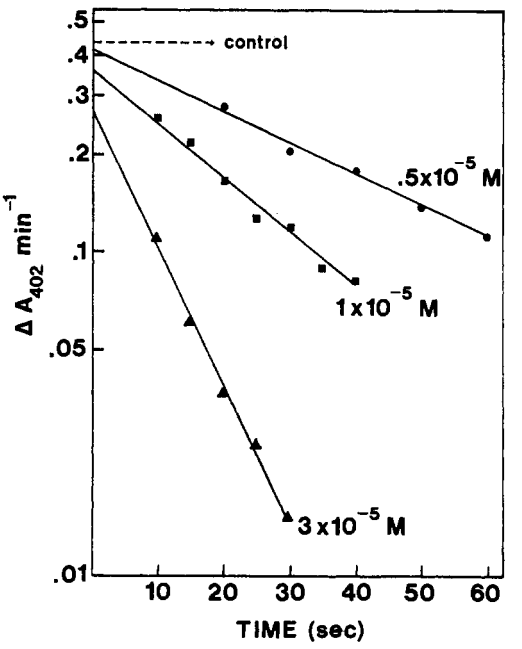


FIGURE 2: Zero-time plot (semilogarithmic) of reaction velocities (ΔA min^{-1}) against time during inhibition of acetylcholinesterase at various concentrations (shown) of paraoxon in the presence of 1 mM *p*-nitrophenyl acetate at 25° in 0.067 M sodium phosphate buffer at pH 6.9. Regression lines were determined by the least-squares method. Broken line shows control value.

a short time interval (usually <1 min) when potent inhibitors are being used.

We report here the development of a recording spectrophotometric method and suitable kinetic treatment, for determining K_d and k_2, which allow the difficulties outlined above to be overcome.

Theory

The procedure is based on the use of a system containing enzyme, inhibitor, and chromogenic substrate in a suitable buffer. Since the chromogenic substrate is present throughout the reaction it serves the dual purpose of providing a means of continuously monitoring the progress of the reaction and of slowing the overall rate by competing with the inhibitor for the catalytic site of the enzyme. This rationale is essentially the same as that proposed by Main and Dauterman (1963) for the determination of k_i' using a pH-Stat. In order to determine K_d and k_2 in the presence of a substrate, however, a modified kinetic treatment must be introduced. The system under consideration may be depicted as

$$\begin{array}{l} \mathrm{E + PX + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EPX} \xrightarrow{k_2} \mathrm{EP + X} \\ \qquad\qquad\quad \underset{k_1'}{\overset{k_{-1}'}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2'} \mathrm{E + products} \end{array} \qquad (3)$$

where S represents the chromogenic substrate. Experimental conditions are chosen so that molar concentrations of inhibitor and substrate are each much greater (>10^5) than that of enzyme-active sites, both the enzyme–substrate and enzyme–inhibitor reactions therefore being pseudo first order. Thus, the rate of reaction along either pathway in reaction 3 is proportional to the concentration of nonirreversibly inhibited enzyme. The rate of generation of the chromophore is therefore directly related to the rate of phosphorylation of the enzyme.

Figure 1 shows a reproduction of a recording spectrophotometer trace obtained from a system of the type depicted in reaction 3. Reaction velocities at various times are given by the slopes of the tangents to the curve (tangents drawn freehand). These may be replotted in semilogarithmic form as a function of time as shown in Figure 2. The data points in Figure 2 show only a very limited amount of scatter (correlation coefficients were greater than 0.99 in each case), indicating that drawing tangents by eye does not appreciably affect the reliability of the data. Since the reaction is first order over most of its course the resulting plots are linear and may be extrapolated to the ordinate to obtain the reaction velocity at zero time for each concentration of inhibitor. We shall refer to this as the zero-time plot and to the ordinate intercepts as the zero-time velocities. O'Brien (1968) has shown that in the case of the reaction between carbamates and cholinesterase, the only event reflected in the zero-time velocity parameter is the formation of the reversible enzyme–inhibitor complex. We have found that this approach is applicable to inhibition by organophosphates and that, as with carbamates, at zero time the process may be treated as fully reversible. Equation 4 may therefore be used (Dixon and Webb, 1964b) to allow for the effect of substrate, where v_0 is the zero-time velocity at a given concentration of inhibitor, K_s is the dissociation constant of the ES complex, and V_m is the maximum velocity for the enzyme–substrate reaction in the absence of inhibitor. Using the Michaelis constant, K_m, as the nearest available approximation of K_s allows the determination of K_d. Equation 4 may be converted to a more convenient

$$v_0 = \frac{V_m[S]}{K_s[1 + ([PX]/K_d)] + [S]} \qquad (4)$$

form by introducing the Briggs–Haldane corrected Michaelis–Menten equation, $V_m = v[1 + (K_m/[S])]$ (see Dixon and Webb, 1964a). Thus eq 4 becomes

$$\frac{v_0}{v} = \frac{K_m + [S]}{K_m[1 + ([PX]/K_d)] + [S]} \quad (4a)$$

which may be rearranged to give

$$K_d = \frac{K_m[PX]}{(K_m + [S])(v_c/v_0 - 1)} \quad (5)$$

where v is now designated v_c, *i.e.*, the velocity of a control reaction carried out in the absence of inhibitor but at the same [S] as in the inhibition reaction.

Derivation of an Expression for the Calculation of k_2. Main (1964) has shown that, under conditions where the reaction between enzyme and inhibitor is first order, the ratio $[EPX]/[E_f]$ is constant, where $[E_f]$ is the concentration of free enzyme. Hence, the *reversible* step in the inhibition pathway of reaction 3 approximates an equilibrium at any instant and therefore

$$k_1[E_f][PX] = k_{-1}[EPX] \quad (6)$$

In contrast to the situation in Main's (1964) derivation, however, allowance must be made for the effect of substrate, the necessary conservation equation being

$$[E_f] = [E]_0 - [EP] - [EPX] - [ES] \quad (7)$$

where $[E]_0$ is the initial concentration of enzyme. Substituting eq 7 into eq 6 and rearranging

$$[EPX] = \frac{([E]_0 - [EP] - [ES])[PX]}{K_d + [PX]} \quad (8)$$

For convenience let

$$[PX]/(K_d + [PX]) = \beta \quad (9)$$

Hence

$$[EPX] = \beta([E]_0 - [EP] - [ES]) \quad (10)$$

From reaction 3, the rate of irreversible inhibition at any time, t, is given by

$$d[EP]/dt = k_2[EPX] \quad (11)$$

Hence, combining eq 10 and 11

$$d[EP]/dt = k_2\beta([E]_0 - [EP] - [ES]) \quad (12)$$

The enzyme–substrate reaction in reaction 3 may be subjected to a similar treatment to that outlined for inhibitor in eq 6–10; thus it can be shown that

$$[ES] = \alpha([E]_0 - [EP] - [EPX]) \quad (13)$$

where

$$\alpha = [S]/(K_m + [S]) \quad (14)$$

Alternating substitution of eq 13 and 10 into eq 12 followed by rearrangement gives

$$d[EP]/dt = k_2\beta([E]_0 - [EP])(1 - \alpha + \alpha\beta - \alpha^2\beta + \alpha^2\beta^2 \ldots) \quad (15)$$

where either [EPX] or [ES] appears only in the nth term. Since α and β are each less than unity, the alternating power series in eq 15 is convergent; as n approaches infinity the term containing [EPX] or [ES] approaches zero and may therefore be neglected. Simplification of the power series (Ayres, 1964) allows eq 15 to be written in the form

$$d[EP]/dt = k_2\beta([E]_0 - [EP])[(1 - \alpha)/(1 - \alpha\beta)] \quad (16)$$

which upon integration between the limits t_1 and t_2 and $[E]_0 - [EP_1]$ and $[E]_0 - [EP_2]$ gives

$$\ln([E]_0 - [EP_1])/([E]_0 - [EP_2]) = k_2\beta[(1 - \alpha)/(1 - \alpha\beta)]\Delta t \quad (17)$$

Returning to eq 7 and since $[E_f]$ is proportional to the velocity of the reaction (v) at any time during the first-order phase

$$\frac{v_1}{v_2} = \frac{[E]_0 - [EP_1] - [EPX_1] - [ES_1]}{[E]_0 - [EP_2] - [EPX_2] - [ES_2]} \quad (18)$$

Equations 10 and 13 may be rearranged to give

$$[EPX] + \beta[ES] = \beta([E]_0 - [EP]) \quad (19)$$

and

$$[EPX] + (1/\alpha)[ES] = [E]_0 - [EP] \quad (20)$$

Subtracting eq 20 from eq 19 and rearranging

$$[ES] = [(\beta - 1)/(\beta - [1/\alpha])]([E]_0 - [EP]) \quad (21)$$

Substituting eq 21 into eq 20 and rearranging

$$[EPX] = \{1 - [(\beta - 1)/(\alpha\beta - 1)]\}([E]_0 - [EP]) \quad (22)$$

Equations 21 and 22 may now be substituted into eq 18 which upon rearrangement and cancellation of constants reduces to

$$v_1/v_2 = ([E]_0 - [EP_1])/([E]_0 - [EP_2]) \quad (23)$$

Substituting eq 23 into eq 17 and designating $\ln(v_1/v_2)$ as ($\Delta \ln v$) (after Main, 1964) gives

$$\Delta \ln v = k_2\beta[(1 - \alpha)/(1 - \alpha\beta)]\Delta t \quad (24)$$

which on rearrangement and replacement of β by $[PX]/(K_d + [PX])$ may be shown to give

$$\frac{\Delta t}{\Delta \ln v} = \frac{K_d}{k_2}\frac{1}{[PX](1 - \alpha)} + \frac{1}{k_2} \quad (25)$$

Equation 25 is identical in form with those developed by Main (1964) and Main and Iverson (1966) and may be ana-

lyzed similarly according to the statistical treatment of Wilkinson (1961) by substituting $(\Delta \ln v)/\Delta t$ for the velocity of the enzyme–substrate reaction and $[PX](1 - \alpha)$ for the substrate concentration. It has thus been shown that the effect of the presence of substrate, on the kinetic treatment of the inhibition reaction, may be allowed for simply by introducing the term $(1 - \alpha)$ into the inhibitor concentration variable as indicated. Finally, rearrangement of eq 25 to eq 26 allows

$$k_2 = \frac{\Delta \ln v}{\Delta t}\left(\frac{K_d}{[PX](1 - \alpha)} + 1\right) \qquad (26)$$

direct calculation of k_2 by inserting the value for K_d as determined by the zero-time method (eq 5).

The essential difference between the transforms discussed above is that the zero-time method (eq 5 and 26), unlike the double-reciprocal approach, does not require the determination of inhibition velocities at several concentrations of inhibitor. An alternative procedure for the double-reciprocal method is to vary the substrate concentration while keeping the inhibitor concentration constant. However, this presents difficulties if there are limitations on substrate solubility over the appropriate concentration range, as was the case in the present study. The zero-time method allows the determination to be carried out using only one concentration of inhibitor and of substrate. Ideally, the highest inhibitor concentration that gives an experimentally accessible reaction profile should be used, thus enhancing the accuracy of the method by ensuring a consistently large change in the variable under observation (Jencks, 1969c).

Experimental Section

Materials. All solvents and reagents were A. R. grade or Laboratory grade. Bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from Winthrop Laboratories was used throughout. The chromogenic substrate was *p*-nitrophenyl acetate. Paraoxon was obtained from American Cyanamid. The *O,O*-diethyl (*ω-tert*-butalkyl) phosphates were synthesized in these laboratories (Bracha and O'Brien, 1968). Organophosphates were tested for purity by thin-layer chromatography on Eastman Chromagram 6060 silica gel sheets using 10% (by volume) water in acetone. Iodination of the chromatograms indicated that each organophosphate contained one component only.

Methods. A Beckman Acta III double-beam recording spectrophotometer was used to monitor the changes in absorbance at 402 nm upon adding 1 ml of a solution of acetylcholinesterase in 0.2 M sodium phosphate buffer to 2 ml of a solution (in a 4-ml cuvet) containing 1.5 × the required final concentrations of *p*-nitrophenyl acetate and the inhibitor in 3.15% aqueous ethanol. The final buffer concentration was thus 0.067 M (pH 6.9) in 2.1% aqueous ethanol. It has been shown that this concentration of ethanol is acceptable in the study of various enzymatic reactions (O'Brien, 1956; Tan and Lovrien, 1972). Concentrations of *p*-nitrophenyl acetate from 1 to 2 mM and of acetylcholinesterase from 10 to 60 units ml^{-1} were found to be most suitable, depending on temperature and on inhibitor potency. Rapid mixing was achieved by use of a 3-ml syringe so that the enzyme solution plus 2 ml of air could be injected, through an aperture in the cuvet housing, directly into the cuvet containing the inhibitor–substrate solution. Controls and K_m determinations for *p*-nitrophenyl acetate were carried out in a similar manner.

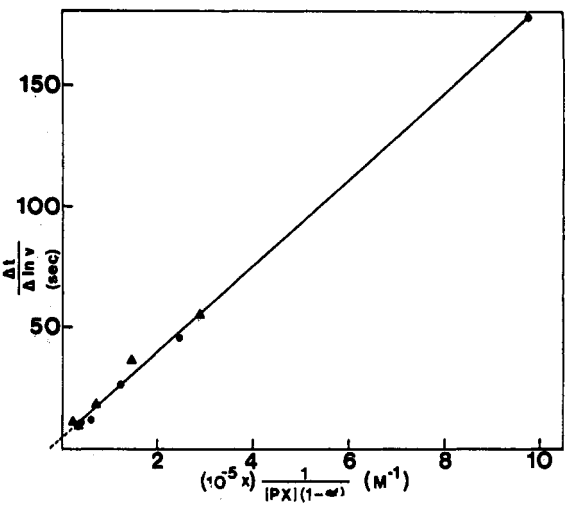


FIGURE 3: Plot showing linear relationship between $\Delta t/\Delta \ln v$ and $1/([PX](1 - \alpha))$ (eq 25) for inhibition of acetylcholinesterase by paraoxon in the presence of 1 mM (●) and 2 mM (▲) substrate (*p*-nitrophenyl acetate) at 25° in 0.067 M phosphate buffer at pH 6.9. The regression line was determined by the least-squares method; correlation coefficient = 0.998. At 1 mM substrate, $\alpha = 0.1855$; at 2 mM substrate, $\alpha = 0.3130$.

For the studies at 5°, enzyme solutions were freshly made using cold buffer and equilibrated at 5° for at least 30 min before use. For determinations at higher temperatures (25 and 37°), enzyme solutions were not always freshly prepared, being stored overnight at 5° in some cases. However, in all cases solutions were equilibrated at the temperature of the experiment for at least 15 min before use. This procedure was considered adequate to eliminate errors which might arise due to interconversion among multiple reversible forms of the enzyme with change in temperature (Main, 1969b).

Results

Semilogarithmic plots of reaction velocities as a function of time for the inhibition of acetylcholinesterase at various concentrations of paraoxon, in the presence of 1 mM *p*-nitrophenyl acetate, are shown in Figure 2. At any concentration of inhibitor ($>0.5 \times 10^{-5}$ M in this case) which gave an ordinate intercept significantly lower than the control value, the zero-time velocity so obtained could be used in eq 5 to calculate K_d (using K_m = 1.25 mM at 5°, 4.39 mM at 25°, and 5.85 mM at 37°). The least-squares method was used to determine the regression lines. The mean K_d value obtained from several determinations was then used to calculate k_2 using eq 26. The alternate approach, *i.e.*, of plotting $\Delta t/(\Delta \ln v)$ *vs.* $1/[PX](1 - \alpha)$ as required by eq 25, was also used and K_d and k_2 were computed by the statistical method of Wilkinson (1961), as described under Theory (*vide supra*). Figure 3 shows this type of modified double-reciprocal plot in which data points were determined at two different values of α. An almost 1.7-fold difference in α, which represents the concentration of available enzyme, does not appear to have a significant effect on the resulting values of K_d and k_2 for the inhibition reaction (the correlation coefficient for the combined data points in Figure 3 is 0.998). This supports the assumption implicit in eq 5 and 25 (which are essentially transforms of each other) that the substrate protects the enzyme in a purely competitive manner from inhibition by the organophosphate. Solubility limitations prevented the extension of this analysis to higher

TABLE I: Effect of Temperature on Dissociation and Kinetic Constants for Inhibition of Acetylcholinesterase by Paraoxon.[a]

Method	Temp (°C)	10^4K_d (M)	k_2 (min^{-1})	$10^{-4}k_i$ (M^{-1} min^{-1})
Zero time	5	0.84 [±0.53]	7.06 [±1.30]	8.46 [±3.78]
	25	0.55 (±0.067)	14.18 (±0.65)	25.88 (±1.97)
	37	0.37 [±0.19]	15.00 [±1.25]	40.66 [±17.49]
Double reciprocal	5	0.94 (±0.15)	8.92 (±1.09)	9.49 (±0.35)
	25	0.47 (±0.24)	13.46 (±4.58)	28.65 (±4.88)
	37	0.33 (±0.038)	13.73 (±1.23)	41.04 (±1.05)

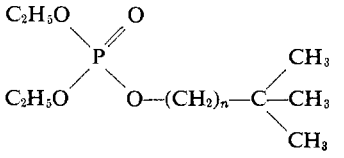
[a] Standard deviations are shown in brackets; standard errors are shown in parentheses.

concentrations of substrate. However, the assumption of purely competitive kinetics is also inherent in the arguments of Main and Dauterman (1963) concerning the determination of the bimolecular rate constant, k_i', for the inhibition of acetylcholinesterase by an organophosphate in the presence of substrate. Main and Dauterman (1963) showed that the values were in excellent agreement with those determined in the absence of substrate. Other authors have also suggested that organophosphates generally react only with the free active center of the enzyme (Dixon and Webb, 1964c).

Table I shows the values for K_d, k_2, and k_i (derived from $k_i = k_2/K_d$), as calculated by both methods, for the inhibition of acetylcholinesterase by paraoxon at various temperatures. The results are in excellent agreement, thus appearing to validate the assumptions upon which the zero-time method is based. The values for K_d and k_2 at 5° differ considerably from those found by other workers. The averages of the values reported by Main (1967) and Chiu *et al.* (1969) are $K_d = 3.85 \times 10^{-4}$ M, $k_2 = 44.65$ min^{-1}, and $k_i = 12.3 \times 10^4$ M^{-1} min^{-1}. While our average k_i is reasonably close to their value, our findings indicate an approximately 4.3-fold higher affinity combined with a (±) 5.6-fold slower phosphorylation step.

The zero-time method was subsequently used to evaluate the kinetic constants for inhibition of acetylcholinesterase by a series of *O,O*-diethyl (*ω-tert*-butalkyl) phosphates. Results are shown in Table II. Except for the addition of the first methylene group the overall pattern is clearly one of increasing inhibitory potency (as reflected in k_i) with increasing length of the butalkyl chain. Examination of the K_d values shows that increasing inhibitory power is accompanied by a monotonic increase in affinity with chain length. In contrast, k_2 values do not show a systematic variation and do not change significantly after the addition of the second methylene group.

TABLE II: Effect of Number of Carbons (m) in Longest Branch of Main Side Chain on Dissociation and Kinetic Constants for Inhibition of Acetylcholinesterase by *O,O*-Diethyl (*ω-tert*-Butalkyl) Phosphates at 25° as Determined by the Zero-Time Method.[a]



n	m	10^4K_d (M)	k_2 (min^{-1})	$10^{-4}k_i$ (M^{-1} min^{-1})
0	2	56.47 [±6.30]	2.80 [±0.47]	0.050 [±0.0028]
1	3	44.40 [±10.62]	1.19 [±0.19]	0.027 [±0.0021]
2	4	11.68 [±3.18]	5.48 [±0.62]	0.47 [±0.075]
3	5	4.38 [±1.91]	4.37 [±0.67]	1.00 [±0.28]
4	6	3.83 [±1.37]	5.38 [±0.57]	1.40 [±0.35]

[a] Standard deviations are shown in brackets.

Discussion

The temperature study (Table I) indicates a decrease in K_d as temperature is raised. This enhancement of affinity may be explained in terms of the endothermic nature of hydrophobic interactions and their consequent increase in stability with increasing temperature up to about 50–60° (Kauzmann, 1959; Némethy and Scheraga, 1962; Jencks, 1969b). The increase in k_2 at higher temperatures reflects the enhancement of the rate of covalent bond formation as expected. The enthalpy of the reversible (K_d) step may be determined from the integrated van't Hoff equation (Laidler, 1958) by plotting ln ($1/K_d$) against $1/T$, the resulting slope being $-\Delta H°/R$. The plot obtained was linear (correlation coefficient >0.999) in accordance with the widely accepted assumption that $\Delta H°$ is constant over the temperature range appropriate to the study of biological systems. Standard free energies and entropies were calculated from the dissociation constants and the enthalpy using the relationships $\Delta G° = RT \ln K_d$ and $\Delta S° = (\Delta H° - \Delta G°)/T$. The collected values are shown in Table III which serves to emphasize the contribution of the entropy term ($T\Delta S°$) to the increase of free energy of formation of the EPX complex with increasing temperature.

The K_d and the k_2 values for the *O,O*-diethyl (*ω-tert*-butalkyl) phosphate series (Table II) are revealing with respect to the relative contributions of the factors influencing inhibitory power. As predicted by Bracha and O'Brien (1968), the increase in inhibitory potency with carbon chain length is

TABLE III: Thermodynamic Parameters for the Formation of the EPX Complex during Inhibition of Acetylcholinesterase by Paraoxon.[a]

Temp (°K)	$\Delta H°$ (cal M^{-1})	$\Delta G°$ (cal M^{-1})	$\Delta S°$ (eu)	$T\Delta S°$ (cal M^{-1})
278.2	5575.8[b]	−5125.5	38.47	10701.3
298.2		−5904.7	38.50	11480.5
310.2		−6354.1	38.46	11929.9

[a] Values were calculated from the dissociation constants determined by the double-reciprocal method (Table I). [b] The van't Hoff plot from which this $\Delta H°$ was determined had a correlation coefficient of 0.9998.

TABLE IV: Effect of Number of Carbons (m) in Longest Branch of Main Side Chain on Standard Free Energy of Binding of O,O-Diethyl (ω-*tert*-Butalkyl) Phosphates to Acetylcholinesterase at 25°.[a]

m	$\Delta G°$ (cal M^{-1})	$\Delta\Delta G°$ (cal M^{-1})
2	−3067.3	
3	−3209.8	−142.5
4	−4001.0	−791.2
5	−4582.2	−581.2
6	−4661.7	−79.5

[a] Calculated from the K_d values shown in Table II.

due largely to an increase in affinity. This is clearly evident among the higher members of the series. It is of interest that the second member of the series (m = 3) is less inhibitory than the first, as shown by the decrease in k_i. This decrease in k_i is due solely to the decrease in k_2 since the expected increase in affinity occurs in all cases as the series is ascended. It is suggested that the apparently anomalous variation in k_2 may be due to the absence of an intervening alkyl chain between the phosphate and *tert*-butyl groups in the first member of the series. Thus, when m = 2 the phosphorus atom is directly linked through one of its oxygens to the tertiary carbon atom of the *tert*-butyl group. The bulky *tert*-butyl group may then promote its own leaving character, much as in the case of alkyl-*N*-nitrocarbonates, in which the strained *tert*-butyl isomer decomposes 10^3 times faster than its *n*-butyl counterpart (Jencks, 1969a).

The m = 4 and higher members of the series have similar k_2 values which, however, are considerably larger than those for the first two members. This may be due to the fact that, with the m = 4 and higher members, there is a discrete ethylene or longer chain between the electrophilic phosphate group and the bulky *tert*-butyl group, a situation somewhat analogous to that existing in the natural substrate, acetylcholine. It seems feasible that unfavorable steric factors might lead to a decrease in k_2 when there is no ethylene or longer chain separating the large terminal moieties. As suggested above, however, this may be partially compensated for by strain when the *tert*-butyl group is directly attached to the oxygen atom.

Comparison of the values for paraoxon and the O,O-diethyl (ω-*tert*-butalkyl) phosphates (Tables I and II) at 25° shows that the greater inhibitory power of paraoxon is due only to a moderate extent to the electron-withdrawing effect of the *p*-nitrophenyl group, as reflected by the larger k_2, and to a much greater extent to increased affinity. Thus, while paraoxon has a k_2 only 2.6-fold greater than that of the most potent member of the diethyl butalkyl phosphate series (m = 6), it has a 7.5-fold smaller K_d. This markedly greater affinity suggests that the *p*-nitrophenyl group contributes strongly to the binding energy.

Standard free-energy differences, calculated from the dissociation constants ($\Delta G° = RT \ln K_d$), are shown in Table IV. Addition of the first methylene group to give the m = 3 compound contributes only −142 cal M^{-1} to the binding energy, in keeping with the suggestion of unfavorable steric interactions (*vide supra*). The second (m = 4) and third (m = 5) methylene groups contribute −791 and −581 cal M^{-1}, respectively, values that are in excellent agreement with those widely accepted for the contribution of methylenes to hydrophobic interactions (Tanford, 1962). The fourth methylene group (m = 6) contributes least of all, supporting the suggestion of a cutoff point due to limited size of a hydrophobic region (Bracha and O'Brien, 1968), which appears to be close to the primary anionic site of the enzyme (Kabachnik *et al.*, 1970).

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