Measurement of the Affinity and Phosphorylation Constants Governing Irreversible Inhibition of Cholinesterases by Di-isopropyl Phosphorofluoridate

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1. A procedure is described for determining the affinity constant K_a and the phosphorylation constant k_p for the inhibition by di-isopropyl phosphorofluoridate of erythrocyte acetylcholinesterase and serum cholinesterase. The procedure depends on the use of a specially designed reaction vessel with which incubation times as short as 1.2 sec. could be obtained at any convenient temperature. 2. The K_a of acetylcholinesterase decreased from $1.58 (\pm 0.22) \times 10^{-3} \text{ M}$ at 5° to $1.17 (\pm 0.10) \times 10^{-3} \text{ M}$ at 25° and the associated change in enthalpy was 2980 cal. 3. The k_p of acetylcholinesterase increased from $11.9 (\pm 0.7) \text{ min.}^{-1}$ at 5° to $40.7 (\pm 1.4) \text{ min.}^{-1}$ at 25° , indicating an activation energy of 9600 cal. The change in entropy associated with K_a was $23.5 \text{ cal. degree}^{-1}$ at 25° . 4. At 5° , the K_a and k_p of serum cholinesterase were $9.95 (\pm 1.10) \times 10^{-6} \text{ M}$ and $11.2 (\pm 0.63) \text{ min.}^{-1}$ respectively. 5. The 150-fold difference in the inhibitory power of di-isopropyl phosphorofluoridate for the two cholinesterases was attributed entirely to differences in affinity.

Aldridge (1950) proposed that the irreversible inhibition of acetylcholinesterase by organophosphates could be represented by the scheme:

where E is acetylcholinesterase (EC type 3.1.1.7 hydrolase), I is an organophosphate inhibitor, $(\text{EI})_R$ is a reversible complex and $(\text{E})_I$ is the irreversibly phosphorylated active site. The individual rate constants are k_{+1} , k_{-1} and k_p .

By studying the time-course of inhibition at various inhibitor concentrations Aldridge (1950) also showed that the reaction was bimolecular and in the presence of excess of inhibitor obeyed an equation of the form:

$$k_i = \frac{2.3 \log(v_1/v_2)}{(t_2 - t_1)i} = \frac{2.3 \Delta \log v}{\Delta ti} \quad (2)$$

where v_1 and v_2 are the measured velocities of substrate reactions catalysed by the esterase remaining uninhibited at times t_1 and t_2 of the inhibition reaction, k_i is the bimolecular velocity constant (Hartley & Kilby, 1952) and i is the concentration of inhibitor.

That affinity was involved in these reactions was clearly recognized and later Aldridge (1954) drew attention to the close analogy that exists between

the substrate and organophosphate inhibition reactions of acetylcholinesterase. His evidence further supported the idea that the structure of an organophosphate was important since the inhibitor must fit the active site in a manner analogous to a substrate.

According to reactions (1) and by analogy with substrate reactions, the constant governing reversible complex-formation, K_a (i.e. k_{-1}/k_{+1}), is a measure of affinity. The following equation was derived from reactions (1) and includes K_a (Wilson, 1960; Main, 1964):

$$k_i = k_p/K_a = \frac{2.3 \Delta \log v}{\Delta t} \left(\frac{1}{i} + \frac{1}{K_a} \right) \quad (3)$$

As was pointed out by Wilson (1960), eqn. (2) is the limiting case of eqn. (3) when $i \ll K_a$, and in that case the k_i measured is approximate. Since rates of inhibition have generally been measured at low inhibitor concentrations, experimental data have generally agreed with eqn. (2).

A linear form of eqn. (3) was used to obtain the K_a and k_p values of malaoxon* and serum cholinesterase but experimental limitations prevented

* Trivial names and abbreviations: malaoxon, *O,O*-dimethyl *S*-(1,2-dicarboethoxyethyl) phosphorothiolate; paraoxon, diethyl *p*-nitrophenyl phosphate; DFP, diisopropyl phosphorofluoridate.

their determination for DFP (Main, 1964). The limitations developed because the determination of K_a and k_p required much higher inhibitor concentrations and shorter incubation periods than were needed to determine the corresponding k_i value. Malaoxon was a relatively poor inhibitor (k_i $1.59 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ at 37° and pH 7.6) with good affinity [K_a $4.82 (\pm 0.49) \times 10^{-4} \text{ M}$]. The shortest incubation times then attainable were about 20 sec., and though this was adequate for malaoxon it failed with DFP, the k_i of which was $4.18 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ (Main, 1964).

Constants comparable with K_a and k_p have been determined for the irreversible inhibition by substituted sulphonates of electric-eel acetylcholinesterase (Kitz & Wilson, 1962) and of chymotrypsin (Gold & Fahrney, 1964). The difficulties encountered in determining K_a and k_p for DFP would not have been encountered by Kitz & Wilson (1962) because the sulphonates used were poor inhibitors with relatively low sulphonation rate constants. On the other hand, some of the sulphonates used by Gold & Fahrney (1964) had relatively high sulphonation rates when allowed to react with chymotrypsin and difficulties were experienced similar to those encountered with DFP (A. M. Gold, personal communication).

An attempt has been made to overcome some of the experimental limitations that prevented determination of K_a and k_p by using a pH-stat and a specially designed inhibition reaction vessel. With this vessel shorter incubation times were obtained with precision and the inhibition was carried out conveniently at lower temperatures. This vessel also permitted a relatively large dilution of the inhibition mixture, which, with substrate protection, served to more or less stop inhibition. This was important at the higher inhibitor concentrations used.

The two cholinesterases used in the present work are inhibited at different rates by DFP (Ooms, 1961). It was decided to determine whether differences in affinity, K_a , or phosphorylation potential, k_p , were responsible. In general, knowledge of K_a and k_p should further an understanding of the relationship between the structure of organophosphates and their inhibitory power.

EXPERIMENTAL

Evaluation of constants

Double-reciprocal plots have been used to present data according to the following linear form of eqn. (3):

$$\frac{\Delta t}{2.3 \Delta \log v} = \frac{1}{ik_i} + \frac{1}{k_p} \quad (4)$$

where $2.3 \Delta \log v / \Delta t$ is considered to be the dependent variable y and i is the independent variable x (Kitz &

Wilson, 1962; Main, 1964; Gold & Fahrney, 1964). Subsequently difficulty was experienced in designing experiments and interpreting results according to eqn. (4). In the present paper a different linear form has been adopted which has overcome some of these difficulties. The equation is:

$$\frac{i \Delta t}{2.3 \Delta \log v} = \frac{i}{k_p} + \frac{1}{k_i} \quad (5)$$

The plot of x against x/y will be linear according to eqn. (5). The intercept on the x/y axis is $1/k_i$.

When $i \ll K_a$, values of $i \Delta t / 2.3 \Delta \log v$ could be measured with relative ease and precision. These values closely approximated $1/k_i$ and fell so near the x/y axis when plotted according to eqn. (5) that they appeared to be on the axis. The extrapolation to determine $1/k_i$ was then negligible. Such points provided a valuable check on the following points, which represented $i \Delta t / 2.3 \Delta \log v$ values determined at much higher (about 100-fold) inhibitor concentrations and under more difficult conditions. The statistically fitted line would be expected to pass close to or through those points that were practically on the x/y axis. Values of $i \Delta t / 2.3 \Delta \log v$ when $i \leq K_a$ could not be used in this way with a double-reciprocal plot. Though the plot of x/y versus x appeared to be the one best suited for evaluating K_a and k_p , it probably would not be as satisfactory as the double-reciprocal plot if only k_i were desired.

One of the questions at issue was whether a reversible complex was formed in significant amounts. If such a complex were formed, $i \Delta t / 2.3 \Delta \log v$ would increase as values of i approached or exceeded K_a . If no reversible complex were formed, then $i \Delta t / 2.3 \Delta \log v$ (i.e. $1/k_i$) would remain constant. In this case, k_i would have a different meaning from the one given in eqn. (3) because the reaction would then proceed by simple interaction of E and I to form $(E)_I$. Although eqn. (2) would fit this reaction scheme, it is important to realize that eqn. (2) also fits reactions (1) when $i \leq K_a$. But only one reaction scheme can hold for a particular system, such as DFP and the cholinesterases. If reactions (1) are assumed to be correct, then the meaning of k_i in eqns. (2) and (3) is identical.

When the results were presented either in a Table or graphically according to eqn. (5) the answer to the question of reversible complex-formation became immediately apparent.

The statistical method of Wilkinson (1961) for calculating K_m and $V_{\max}$, and their standard errors is based on a linear form of the differential Michaelis equation analogous to eqn. (5). By substituting $2.3 \Delta \log v / \Delta t$ for the substrate velocity, and i for the substrate concentration, weighted values of K_a and k_p and their standard errors were calculated. To avoid errors in arithmetic and to speed computation an IBM 1410 computer was used.

The analogy with the Michaelis equation can be carried further. $V_{\max}$ becomes the limiting rate of v when $s \gg K_m$. Similarly k_p is the rate-limiting value of $2.3 \Delta \log v / \Delta t$ when $i \gg K_a$.

Materials

Human serum cholinesterase (EC type 3.1.1.8 hydrolase) and bovine erythrocyte acetylcholinesterase. Partially purified preparations of both esterases were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Stock solutions of each esterase were prepared by dissolving 10000 units

(1 unit catalyses the hydrolysis of $0.062\mu\text{mole}$ of acetylcholine/min. at 37° and pH 7.4) in water, the pH being adjusted to 7.6 with a pH-stat and the solution diluted to 50ml. These solutions were stable for weeks if kept in the cold under a drop of toluene. The solutions were further diluted before use.

DFP. This was obtained from Mann Research Laboratories (New York, N.Y., U.S.A.). A 0.400M stock solution was prepared by diluting 0.833g. to 10ml. with propan-2-ol. Aqueous dilutions were made from this.

Measurement of inhibition rates

Principle. The principle of the procedure is the same as that employed by Aldridge (1950), in which esterase and an excess of inhibitor was incubated for a measured time after which the reaction was stopped by addition of substrate and the residual activity was measured. Determination of K_a and k_p depended on the ability to measure incubation times that were not more than about $4/k_p\text{sec.}$ The desired incubation times were obtained in two ways through the use of an inhibition reaction vessel (Fig. 1). First, inhibition rates were measured at lower temperatures (between 5° and 25°), which lowered k_p and permitted the use of relatively longer incubation times. Values of K_a and k_p at higher temperatures were calculated through the use of the Van't Hoff and Nernst equations respectively. Secondly, the technique of starting and stopping the reaction was refined so that incubation times as short as 1.2sec. could be realized. The reaction was started by tipping and was stopped by pulling sharply on the terminating rod, which immediately flooded the inhibition chamber with substrate. The volumes of esterase and inhibitor were small enough to permit almost instantaneous mixing and large enough to facilitate nearly quantitative transfer on tipping. Addition of substrate stopped the reaction by complex-formation with the free enzyme and diluting the inhibitor 30-fold. The effect of dilution and complex-formation with substrate on the rate of inhibition was calculated from the expression $V_s/V_t(1+s/K_m)$, where V_s is the combined volumes of the substrate and inhibition mixture, V_t the volume of the inhibition mixture, s the substrate concentration and K_m its Michaelis constant. With acetylcholinesterase the protection factor was about 2000 and with serum cholinesterase it was 500. After the terminating rod had been pulled, the contents of the inhibition vessel were transferred to the reaction vessel of a pH-stat and the residual activity was measured.

At the highest inhibitor concentrations significant inhibition in the presence of substrate occurred in spite of dilution and complex-formation. The resulting inhibited progress curves have been used to determine v as follows. Slopes to the curve were constructed at various times after addition to substrate and the logarithms of these were plotted against the corresponding times. Extrapolation of the line obtained to zero gave the required v for the incubation time t . This method is described in detail by Main & Dauterman (1963). The precision attainable with the inhibition reaction vessel in combination with a pH-stat is illustrated in Fig. 2.

Procedure. Substrate reactions were carried out in specially made glass cells, jacketed for temperature control, with an interior capacity of 75ml. The cells opened to the top and were covered by glass lids that joined them at ground-glass interfaces. In the top of each lid were five standard taper $14/20$ 'female' openings. A combined electrode (Radiometer type GK 2025C) entered through one port and a titrant delivery tube through another. From a 'male' joint seated in the centre opening hung a glass bucket containing strips of filter paper soaked in 75%

Fig. 1. Inhibition reaction vessel. The illustration is a cross-sectional drawing of the vessel from the side. The vessel (A, substrate chamber) and rods (B, vapour-block rod, and C, terminating rod) were of glass. The terminating plug (D) was either an 'O' ring type 'male' joint (illustrated) or a cut-down rubber stopper. The vapour block (E) was either a cut-down rubber stopper (illustrated) or a ground-glass 'male' joint. The side-arm port (F) (shown with serum stopper, G) to the side arm was through the main barrel opposite the side arm as shown or on top of the side arm. These various alternative designs did not appear to significantly affect precision although the design illustrated was preferred by those who have used both. The side arm held 0.5ml. of inhibitor solution (H) and the centre well 0.5ml. of esterase solution (I). These were brought to the desired temperature by immersing the flask in a bath to the level indicated (J). The reaction was started by tipping and stopped by pulling on the terminating rod (C), which caused about 10ml. of substrate at 25° contained in the substrate chamber (A) to flood the inhibition mixture. The hole through the rubber stopper (K) was bored so that the terminating rod could slide easily through it. At the same time the fit was tight enough to prevent substrate leaking out when the vessel was shaken. Thus, when the terminating rod was pulled, it was pulled through the stopper, which remained in place. Inhibition reaction times as short as 1.2sec. were achieved as a routine with this vessel. Further details are given in the text.

Fig. 2. Illustration of the precision associated with the inhibition reaction vessel. $\log(\text{Divisions}/10 \text{ min.}) = \log v$; 1 division = $0.1 \mu\text{mole}$. The velocity, v , refers to the rate of acetylcholine hydrolysis at 25° by the acetylcholinesterase remaining uninhibited after reacting with DFP at 20° for time t . The lines were fitted by linear-regression analysis. The slopes give $2.3\Delta\log v/\Delta t$ for each of five DFP concentrations. The DFP concentrations were: $\circ$, 0.5 mM; $\triangle$, 1.0 mM; $\square$, 1.5 mM; $\bullet$, 2.0 mM; $\blacktriangle$, 2.5 mM. The $2.3\Delta\log v/\Delta t$ value at 0.01 mM-DFP was also determined, but this is not illustrated because the time-scale was to 300 sec. and consequently would not fit on this graph. The $[\text{DFP}]\Delta t/2.3\Delta\log v$ values calculated from the results illustrated are given in Table 1 under the 20° column.

(w/v) NaOH, which kept the interior free of CO_2 . Reagents were added through the other ports. The lid was supported on a ring clamp, which could be guided on a rod, thus facilitating opening and covering the cell. A magnetic stirrer was used for mixing.

The pH was maintained at 7.6 with 20mN-NaOH delivered from a 0.5 ml. syringe. The temperature of the substrate reaction was maintained at 25° by circulating water from a Haake control unit.

To begin a run, 30 ml. of 3 mM-acetylcholine substrate was added to the pH-stat cell. The substrate did not contain Mg^{2+} or NaCl as these were found drastically to increase K_m .

The machine was set to titrate and a non-enzymic hydrolysis blank was obtained. With the pH-stat going, the inhibition vessel was clamped upright on a ring stand and 0.5 ml. of acetylcholinesterase containing 50 activity units (1 unit catalyses the hydrolysis of $0.0232 \mu\text{mole}$ of acetylcholine/min. at 25° and pH 7.6) was pipetted into the centre well. This acetylcholinesterase solution was 4 mM with respect to sodium phosphate buffer and 2.6 mM with respect to NaCl. These salts were present in the acetylcholinesterase preparations as received from the supplier. The vapour block and terminating plug assembly were then positioned. The vapour block was used to prevent the vapour from the higher concentrations of DFP from inhibiting the esterase during the preparative and equilibra-

tion phases of the procedure. Freshly prepared DFP solution (0.5 ml.), 4 mM with respect to sodium phosphate buffer, pH 7.6, was pipetted into the side arm through the side-arm port, which was then sealed with a serum stopper. Long-nose 0.5 ml. graduated blow-out pipettes were used. The bottom portion of the vessel was immersed to the shoulder in a bath of the desired temperature. At least 5 min. was allowed for temperature equilibration.

If the inhibition reaction was to be less than 30 sec., the pH-stat was stopped and about 10 ml. of substrate transferred to the substrate chamber of the inhibition vessel. If the incubation time was to be longer, the substrate was placed in the substrate chamber after the inhibition reaction had started and just before it was to be stopped.

To start inhibition, the vessel was removed from the bath and the vapour block was drawn up. The inhibitor was tipped into the esterase solution by quickly inverting the vessel and in the same motion shaking it to mix. As tipping occurred a foot-operated timer, calibrated to 0.1 sec., was started. This left both hands free for manipulating the vessel.

The inhibition reaction was stopped by pulling sharply on the terminating rod, which allowed substrate to flood the inhibition chamber. Timing was stopped as the terminating rod was pulled.

The procedure was completed by pouring the contents into the pH-stat vessel and turning on the pH-stat. Elapsed time between stopping the inhibition reaction and starting the pH-stat was about 10 sec. Rapid titration of the acid liberated by hydrolysis while the pH-stat was off rendered the first 30 sec. of the progress curve unusable. The slope of the linear progress curve that developed after the initial titration of accumulated acid, minus the non-enzymic rate, gave v for the incubation time t . If inhibition in the presence of substrate occurred the method mentioned previously for obtaining v was used.

The procedure with serum cholinesterase was the same except that 15 mM-acetylcholine substrate was used. The various $\log v$ values were plotted against the corresponding values of t to determine if a straight line had been sensibly approximated. The slope of this line, $2.3\Delta\log v/\Delta t$, was obtained by linear-regression analysis. At constant i , $2.3\Delta\log v/\Delta t$ is a first-order rate constant, but it varies with i and so has not been designated as a k value.

The velocity at zero time was obtained by using precisely the same procedure as just described, except that the inhibition vessel was not tipped before the terminating plug was pulled. This procedure was adopted for the following reasons. When the substrate and inhibition mixture was poured into the pH-stat vessel about 2.5% of the liquid was retained in the inhibition vessel. The retained volume was relatively constant ($\pm 15\%$) so that, in effect, the retention error cancelled out when all determinations, including v_0 , were done in the same way.

Other errors may occur that the recommended v_0 procedure can either cancel or detect. For example, the error resulting from the inhibition of esterase by DFP vapour during the preparative and temperature-equilibration phases of the procedure was diagnosed by inspection of v_0 values. The vapour block was introduced as a result. Certain types of pipetting error can also be conveniently cancelled by the v_0 procedure. The 10 sec. lapse between pulling the terminating rod and transferring the contents of the inhibition vessel to the pH-stat also involved the

Table 1. *Affinity and phosphorylation constants K_a and k_p of DFP and erythrocyte acetylcholinesterase at various temperatures and the measurements from which they were calculated*

Rates of inhibition were measured at pH 7.6 in approx. 4 mm-sodium phosphate buffer containing NaCl (1 mM) at the temperature indicated according to the procedure in the text. K_a and k_p and their standard errors were computed by the method of Wilkinson (1961).

Temp. of reaction mixture.....	5°		15°		20°		25°	
	[DFP] (mM)	$10^2 \times \frac{[\text{DFP}]\Delta t}{2.3\Delta \log v}$ (m min.)	[DFP] (mM)	$10^4 \times \frac{[\text{DFP}]\Delta t}{2.3\Delta \log v}$ (m min.)	[DFP] (mM)	$10^5 \times \frac{[\text{DFP}]\Delta t}{2.3\Delta \log v}$ (m min.)	[DFP] (mM)	$10^5 \times \frac{[\text{DFP}]\Delta t}{2.3\Delta \log v}$ (m min.)
	5.0	5.35	10	6.03	2.5	13.5	2.5	8.98
	2.5	3.75	5	3.33	2.0	10.7	2.0	7.74
	2.0	3.00	2.5	1.95	1.5	9.69	1.5	6.54
	1.5	2.55	1.5	1.61	1.0	7.40	1.0	5.52
	1.0	2.13	1.0	1.24	0.5	5.55	0.5	3.93
	0.8	1.89	0.01	0.74	0.01	3.80	0.01	2.54
	0.01	1.27	0.01	0.68				
	0.01	1.41						
Values of constants								
Constant	Units		at..... 5°		15°	20°	25°	
K_a	$10^{-3} \times M$		1.58 ± 0.22		1.38 ± 0.12	1.03 ± 0.18	1.17 ± 0.096	
k_p	min. ⁻¹		11.9 ± 0.7		19.1 ± 0.5	26.8 ± 1.9	40.7 ± 1.4	
k_i	$10^3 \times M^{-1} \text{min.}^{-1}$		7.54		13.8	26.1	34.8	

Table 2. *Affinity and phosphorylation constants K_a and k_p for the reaction of DFP and serum cholinesterase and the data from which they were calculated*

Rates of inhibition were measured at 5° and pH 7.6 in 2 mm-sodium phosphate buffer according to the procedure described in the text. K_a and k_p and their standard errors were computed by the method of Wilkinson (1961).

[DFP] (μM)	$10^6 \times \frac{[\text{DFP}]\Delta t}{2.3\Delta \log v}$ (m min.)
20	2.66
10	1.79
4	1.36
2	0.89
1.25	1.00
1.0	1.00
0.1	0.80

Constant	Units	Values
K_a	$10^{-6} \times M$	9.95 ± 1.10
k_p	min. ⁻¹	11.2 ± 0.63
k_i	$10^6 \times M^{-1} \text{min.}^{-1}$	1.13 ± 0.15

an individual's reflex time. It was therefore a valuable check on the validity of the timed points in these respects as well.

RESULTS

The K_a , k_p and k_i values of erythrocyte acetylcholinesterase and DFP were determined at 5°, 15°, 20° and 25° in 4 mm-sodium phosphate buffer, pH 7.6, containing sodium chloride (1.3 mM) according to the procedure described above.

These values, together with the DFP concentrations used and the corresponding $i\Delta t/2.3\Delta \log v$ values from which the standard errors were calculated, are given in Table 1. The k_i values were calculated by using eqn. (3).

Comparable values for the reaction of serum cholinesterase and DFP at 5° are given in Table 2. The serum cholinesterase preparations did not contain added salts so that the final buffer concentration depended on the sodium phosphate in the DFP solution. The final sodium phosphate concentration of the serum cholinesterase-DFP inhibition mixture was therefore 2 mM.

Fig. 3 illustrates the plot of x versus x/y obtained by using data in Table 1 according to eqn. (5). The slopes give $1/k_p$, the intercepts on the x/y axis give $1/k_i$ and the intercepts on the [DFP] axis give $-K_a$. If no reversible complex of significance were formed, or if the range of inhibitor concentrations used did not approach K_a , then the points would fall on a line paralleling the x axis.

possibility of a small error that the v_0 procedure would cancel.

Certain operations associated with the reflexes of the operator and with mixing the esterase and inhibitor solutions could conceivably have resulted in undetected errors. The v_0 procedure did not involve mixing of esterase and inhibitor, nor did it depend to any significant extent on

To calculate K_a and k_p values of significance it was necessary to use a range of inhibitor concentrations that resulted in significant changes in $i\Delta t/2.3\Delta\log v$. The $i\Delta t/2.3\Delta\log v$ values can vary from approximately $1/k_i$ when $i \ll K_a$ to infinity as i approaches infinity. The variation measured ranged from $1/k_i$ to about $4/k_i$, but half of this would have been adequate for calculating significant K_a and k_p values.

Fig. 3. Result of plotting $i\Delta t/2.3\Delta\log v$ against i according to eqn. (5) in the text. The results are taken from Table 1 for the inhibition of erythrocyte acetylcholinesterase by DFP at four temperatures: ●, 5°; ○, 15°; ■, 20°; ▲, 25°. The lines were fitted by the statistical method of Wilkinson (1961). The experimental design was based on this type of plot. The slopes give the reciprocal of the phosphorylation constant, $1/k_p$. If the results fit a line parallel to the x axis, either no reversible complex is formed or the range of i values does not approach the affinity constant, K_a . The intercept in the x axis is $-K_a$ and on the y axis $1/k_i$, the reciprocal of the bimolecular velocity constant.

The pK_a and $\log k_p$ values obtained at each temperature were plotted against the reciprocal of the absolute temperature as shown in Fig. 4. According to the Van't Hoff and Nernst equations, the points should fall on a straight line and a straight-line relationship was approximated. Extrapolation of these lines permitted rather imprecise estimates to be made of k_p and K_a at higher temperatures. At 37°, k_p by extrapolation was 68 min.^{-1} and K_a was $8.9 \times 10^{-4} \text{ M}$.

DISCUSSION

The results indicate that DFP and the cholinesterases do form reversible complexes of significance. This interpretation is based on the assumption that eqn. (5), with which the results are in reasonable agreement, is actually reflecting reactions (1) and not an alternative scheme for which it might also be a valid model. If this assumption is correct, then the reversible complexes are associated with the affinity between the DFP and the active sites of the cholinesterases. Studies based on a series of carboalkoxy homologues of malaoxon and serum cholinesterase have provided independent lines of evidence that support the conclusion that the reversible complex is associated with affinity (A. R. Main & F. L. Hastings, unpublished work).

At 5° the inhibitory power, k_p , of serum cholinesterase was 150 times that of erythrocyte acetylcholinesterase. This difference was entirely attributable to the different affinities of the two enzymes for DFP. Together with the unpublished study with malaoxon these results lend weight to the conclusion originally reached by Aldridge (1950, 1954) that affinity is an important factor in

Fig. 4. Plot of the affinity constant, K_a , and phosphorylation constants, k_p , for the inhibition of erythrocyte acetylcholinesterase by DFP according to the Van't Hoff equation (a) and the Nernst equation (b). When K_a and k_p cannot be determined experimentally at higher temperatures estimates can be made by extrapolation of the results. For example, k_p at 37° was 68 min.^{-1} and K_a was $8.9 \times 10^{-4} \text{ M}$ by extrapolation of the results shown. Estimates of ΔH and E for the appropriate reactions were also made: ΔH was 2980 cal. and E 9600 cal.

determining the inhibitory power of organophosphates.

The results that Kitz & Wilson (1962) obtained for the reaction of organosulphonates and electric eel acetylcholinesterase at 25° and pH 7.0 may be compared with those obtained with DFP and the erythrocyte and serum cholinesterases under approximately the same conditions. To obtain measurable affinities, the organosulphonates apparently required quaternary ammonium or pyridinium substituents. The best binding was obtained with 1,1'-pentamethylenepyridinium iodide 3-hydroxypyridinium iodide methanesulphonate, which had K_a 2.0×10^{-5} M. This compares with K_a values of 1.17×10^{-3} M and 9.95×10^{-6} M for DFP and acetylcholinesterase at 25° and serum cholinesterase at 5° respectively. Evidently quaternary ammonium or pyridinium substituents are not essential to obtain significant binding since DFP has none. The sulphonation rate constant for this organosulphonate was 0.033 min.^{-1} , which is less than one-thousandth that of DFP and acetylcholinesterase at 25°.

The organosulphonate with the highest sulphonation rate constant (0.30 min.^{-1}) was 3-hydroxyphenyl trimethylammonium iodide methanesulphonate and for this compound the binding (K_a 8.3×10^{-4} M) was about the same as for DFP and erythrocyte acetylcholinesterase. The highest sulphonation rates were then less than one-hundredth that of DFP.

The k_p and K_a values of paraoxon and serum cholinesterase have also been determined by the present procedure and at 5° k_p was $21.3 (\pm 3.0) \text{ min.}^{-1}$ and K_a was $9.09 (\pm 2.04) \times 10^{-5}$ M. This suggests that neither the K_a nor k_p values of DFP are of a different magnitude than for other good organophosphate inhibitors. This is also borne out by the results with the malaoxon homologues.

From the rather meagre results at hand it would appear that phosphorylation rates tend to be higher than sulphonation rates. However, the

possibility of significantly higher sulphonation rates is suggested by the rate constant (3.1 min.^{-1}) measured for phenylmethanesulphonyl fluoride and chymotrypsin by Gold & Fahrney (1964). The presence of quaternary ammonium and pyridinium groups in the sulphonate compounds and their absence from the organophosphates examined makes it difficult to draw useful analogies with respect to affinity.

The enthalpy, ΔH , and activation energy, E , calculated from the results shown in Fig. 4 were 2980 cal. and 9600 cal. respectively. The change in entropy, ΔS , was $23.5 \text{ cal. degree}^{-1}$ at 25°. The question whether or not K_a approximates to a true equilibrium constant becomes significant when thermodynamic studies are to be made. Irreversible-inhibition systems, when interpreted as described in this paper, would seem to lend themselves to such studies.

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