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Free Energy Relationships in the Inhibition of Acetylcholinesterase by Diethyl Phosphates

JAMES A. MAGLOTHIN, WEN-SHERNG CHEN, AND IRWIN B. WILSON[1]

Department of Chemistry, University of Colorado, Boulder, Colorado 80309

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SUMMARY

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Acetylcholinesterase inhibited by diethyl phosphorofluoridate (DEP-enzyme) was reactivated by a number of phenols and hydroxyquinolines. The second-order rate constants for reactivation were divided into the known second-order rate constants for inhibition by the corresponding diethyl phosphoryl derivatives of the reactivators (conjugate inhibitors) to obtain the equilibrium constant in terms of analytical concentrations for the inhibition of the enzyme. These values were multiplied by the known value of the equilibrium constant for the hydrolysis of the DEP-enzyme, ECH (DEP-enzyme), to obtain equilibrium constants for the hydrolysis of the inhibitor, ECH (inhibitor). These values were converted to ECH (inhibitor) in terms of acidic species, ECH (inhibitor-acidic), and it was found that a linear free energy relationship was obeyed with the pK_a of the conjugate acid of the leaving group (reactivator):

$$\log \text{ECH (inhibitor-acidic)} = 13.18 - 0.620\ pK_a$$

Using this relationship, log k_i/k_r vs. pK_a and log ECH (anal.) vs. pK_a plots were calculated for pH 7.0. Since the DEP-enzyme falls on these curves using $pK_a = 13.6$ for the hydroxyl group of serine, it was concluded that there were no very substantial interactions, positive or negative, between the phosphoryl group and the protein. This is not the case with other DEP-enzymes, such as chymotrypsin, which is 4 orders of magnitude more stable. Diethyl phosphorofluoridate is much more stable than esters of oxygen leaving groups, with the same pK_a as HF. Compounds containing a sulfur leaving group are much less stable than oxygen esters of the same pK_a. A number of observations can be explained using the thermodynamic data.

INTRODUCTION

The reaction of tertiary organophosphate and phosphonate esters with acetylcholinesterase is of practical and theoretical interest. The reaction illustrated on the horizontal line in Scheme 1[2] for compounds of the diethyl phosphate series with variable leaving group, X, is a nucleophilic displacement reaction. This reaction is intrinsically reversible and often can be demonstrated to go in both direc-

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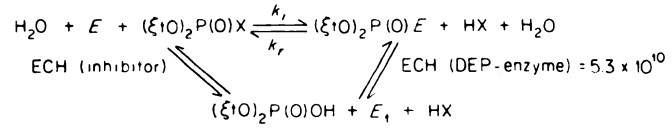
[1] To whom correspondence should be addressed.

[2] The abbreviations used are: ECH, equilibrium constant for hydrolysis; DEP, diethyl phosphoryl-; 2-PAM, 2-pyridinealdoxime methiodide.



SCHEME 1

tions. In almost all cases the equilibrium lies far to the right in favor of the inhibited enzyme, that is, diethyl phosphoryl-enzyme (DEP-enzyme). For example, with enzyme from electric eel and X = F, k_i = 2.3×10^5 M^{-1} min^{-1}, k_r = 10 M^{-1} min^{-1}, and the equilibrium constant k_i/k_r is 2.3×10^4 (1, 2). When HX is choline, no inhibition is obtained, but reactivation still occurs readily. In this case the equilibrium cannot lie far to the right and may actually favor the reactants.

The values of the rate constants and the equilibrium constant given above are for analytical concentrations of the various substances at 25° and pH 7.0 without regard to the distribution of any substance between acidic and conjugate base species. Thus we do not distinguish between X and HX, etc. Therefore the values of these constants depend upon the pH.

The equilibrium constant for hydrolysis of the DEP-enzyme, ECH (DEP-enzyme), has the value 5.3×10^{10} at pH 7.0 and 25° (3). The equilibrium constant for hydrolysis of the inhibitors, ECH (inhibitor), is known only for X = F and X = *p*-nitrophenol. However, since the reactions form a cycle, it is apparent that the three equilibria written in Scheme 1 are not independent and that ECH (inhibitor) can be ascertained if k_i and k_r are measured for an inhibitor and its conjugate reactivator by using the relationship

$$\text{ECH (inhibitor)} = \frac{k_i}{k_r} \times 5.3 \times 10^{10} \qquad (1)$$

This relationship follows from the rule that the product of equilibrium constants around a cycle of reactions is equal to unity.

We also want to know the value of ECH (inhibitor) in terms of the acidic species of the products of hydrolysis. These values can be calculated from the relationship

$$\frac{\text{ECH (inhibitor-analytic)}}{\text{ECH (inhibitor-acidic species)}} = \left(1 + \frac{4.2 \times 10^{-2}}{(\text{H}^+)}\right)\left(1 + \frac{K_a}{(\text{H}^+)}\right) \qquad (2)$$

where 4.2×10^{-2} is the dissociation constant of diethyl phosphoric acid and K_a is the dissociation constant of HX.

It is both interesting and useful to ascertain whether a Brönsted type of linear free energy relationship exists between ECH (inhibitor-acidic species) and K_a, because such a relationship can serve as a basis for understanding the chemical behavior of these and related compounds. Values for k_i for a number of diethyl phosphate esters are known. Several are potent phosphorylating inhibitors, and it is reasonable to suspect that the corresponding leaving groups will be good reactivators of the DEP-enzyme. For example, k_i for diethyl (1-methyl-6-hydroxyquinoline) phosphate is 9.3×10^6 M^{-1} min^{-1}. It is reasonable to guess that ECH for this compound is about the same as for paraoxon (X = *p*-nitrophenol), since the pK_a of both leaving groups is 7. If this is so, the ratio k_i/k_r must be the same for both compounds. For paraoxon k_i is 2.3×10^5 M^{-1} min^{-1} and k_r = 27 M^{-1} min^{-1}, so we estimate that k_r for 1-methyl-6-hydroxyquinoline ion should be 1.1×10^3 M^{-1} min^{-1}. Our measurement yielded the value 1.6×10^3 M^{-1} min^{-1}, which encouraged us to believe that a Brönsted relationship might exist. Apart from its obvious interest for the field of organophosphorus chemistry, a Brönsted relationship is also important for enzymology because it enables calculation of either k_i or k_r when the other is known, even for cases in which one is too small to measure easily. A Brönsted relationship serves as a basis for exploring the relationship between k_i and k_r for various substances having different pK_a values. In this way the effects arising from different pK_a values can be elimi-

nated and in favorable circumstances the effects of other structural and compositional features can be discerned. We therefore undertook a number of measurements of k_r, using hydroxyquinolines and other phenols, and we did in fact find a linear relationship between log ECH (inhibitor-acidic species) and the pK_a of the leaving group.

Many substances, including reactivators, form reversible complexes with the DEP-enzyme. Therefore it may be difficult to reactivate the enzyme under second-order conditions, because the required concentration of reactivator may be very low and the rate of reactivation correspondingly slow. However, there is no need to avoid higher concentrations of reactivator, because the second-order rate constant can be evaluated even when substantial amounts of the reversible complex are formed.

Let us include the formation of a reversible complex between DEP-enzyme and the reactivator in the reactivation process:

$$HX + (\xi tO)_2P(O)E \underset{K_R}{\rightleftharpoons} (\xi tO)_2P(O)E \cdot X \xrightarrow{k_r^\circ} (\xi tO)_2P(O)X + E$$

Since (HX) $\gg E$, the reactivation is pseudo-first-order, with

$$\ln\left(1 - \frac{(E)}{E^\circ}\right) = \frac{-k_i^\circ t}{1 + K_R/(HX)} \qquad (3)$$

where (E) is the concentration of nonphosphorylated enzyme and E° is the total concentration of enzyme. In general the observed pseudo-first-order rate constant is

$$k_r\,(\text{obs}) = k_r^\circ/\left(1 + \frac{K_R}{(HX)}\right) \qquad (4)$$

which takes on the form corresponding to a second-order reaction,

$$\frac{k_r^\circ}{K_R}(HX)$$

when (HX) $\ll K_R$. The second-order rate constant is

$$\frac{k_r^\circ}{K_R}$$

In practice it is convenient to cast Eq. 4 in double-reciprocal form:

$$\frac{1}{k_r\,(\text{obs})} = \frac{1}{k_r^\circ} + \frac{K_R}{k_r^\circ} \cdot \frac{1}{(HX)} \qquad (5)$$

for plotting the experimental data. The slope of the line is the second-order rate constant. If the line does not pass through (or near) the origin, k_r° and K_R can also be evaluated. The reactivators also form reversible complexes with the free enzyme. We have measured the dissociation constant for these complexes, K_I, by using the reactivators as reversible inhibitors of acetylthiocholine hydrolysis. We are thus able to compare K_R with K_I.

We were unable to use *p*-chlorophenol as a reactivator because it rapidly denatured the enzyme. However, we did evaluate its ECH by chemical means, using the forward and reverse rates of the reaction of *p*-chlorophenol with diethyl phosphorofluoridate.

METHODS

Enzyme. Eel acetylcholinesterase was the 11 S form purchased from Worthington Biochemical Corporation.

Acetylcholinesterase activity was determined by the method of Ellman *et al.* (4), with 1.0 mM acetylthiocholine iodide and 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid) at pH 7.0 and 25° in 0.05 M sodium phosphate buffer. The development of absorbance at 412 nm was displayed with a recorder.

Reversible inhibition. The reactivators were studied as reversible inhibitors of the enzyme. Three or more inhibitor concentrations were used, with substrate concentrations varying between 50 μM and 1.0 mM. Double-reciprocal plots of $\bar{v}^{-1}$ vs. $(\bar{s})^{-1}$ were used to evaluate the dissociation constants. The enzyme concentration was about 0.1 nN.

Reactivation of diethyl phosphoryl acetylcholinesterase. Inhibited enzyme was prepared by allowing 1 μN acetylcholinesterase to react with 2 μM diethyl phosphorofluoridate. After 1 hr the solution was diluted 100-fold with 0.1 M NaCl–0.01 M phosphate, pH 7.0. Twenty-five microliters of this dilution were added to 4.0 ml of the same buffer containing the reactivator, and 0.25 ml of this solution was withdrawn periodically and assayed for enzyme activity by addition of 3 ml of assay solution. The reactivator concentrations were chosen so that their range included K_R, the

dissociation constant for the reactivator–DEP-enzyme complex.

The concentration of enzyme was kept low, at about 50 pN, during reactivation, to minimize the formation of conjugate inhibitor and ensure complete reactivation.

Equilibrium constant for reaction of DEP-enzyme and 2-pyridinealdoxime methiodide. Diethyl phosphoryl acetylcholinesterase, 14–102 nN, of known concentration, was allowed to react with 25 or 50 μM 2-PAM. Aliquots of 25 μl were withdrawn for assay of active enzyme by the Ellman method at various intervals up to 5 min.

Equilibrium constant for reaction of diethyl phosphorofluoridate with p-chlorophenol. The equilibrium constant was determined by measuring the rate of reaction in both directions at pH 9.4 (0.01 M borate buffer) at 25°.

For the rate of reaction of fluoride with diethyl (*p*-chlorophenol) phosphate, initial rates of release of *p*-chlorophenol were measured by optical absorbance at 244 nm, using 0.2–0.8 M fluoride and 0.1 mM phosphate ester. Sodium chloride was added to keep the sum of sodium chloride and sodium fluoride equal to 0.8 M.

In the opposite direction, the release of fluoride from 1 mM diethyl phosphorofluoridate was followed with a fluoride electrode at pH 9.4, using concentrations of *p*-chlorophenol from 5 to 10 mM. At the completion of the reaction, the amount of diethyl (*p*-chlorophenol) phosphate that was formed was measured by adjusting the pH to 11 with NH_4OH and extracting the solution with petroleum ether. The petroleum ether was removed with a rotary evaporator, the residue was dissolved in water, and a portion was treated with 0.1 N NaOH to produce *p*-chlorophenol, which was measured spectrophotometrically. The hydrolysis of diethyl (*p*-chlorophenol) phosphate is fairly slow ($k_{OH} = 6.7 \times 10^{-3}$ M^{-1} min^{-1}), but the final concentration could be easily estimated using the Guggenheim method. The initial absorbance was quite low. The rate constants obtained for the release of fluoride were multiplied by the fraction of diethyl phosphorofluoride that ended up as diethyl (*p*-chlorophenol) phosphate to obtain the rate constant for the nucleophilic reaction of *p*-chlorophenol with diethyl phosphorofluoridate.

RESULTS

Reactivation of DEP-enzyme. In studying the reactivation of DEP-enzyme, we plotted our measurements of pseudo-first-order rate constants at different concentrations of reactivator in accord with Eq. 5 to obtain straight lines that could not be construed to pass through the origin. This showed that our concentrations of reactivator were sufficiently high to form large amounts of the reversible complex. Indeed, our highest concentrations were always much higher than the value of K_R obtained from these plots. Thus we could obtain accurate values for K_R, k_r°, and k_r (Table 1).

Dissociation constants of reactivator-enzyme complexes. Dissociation constants for the reversible binding of reactivators with the free enzyme, K_I (Table 1), are smaller than K_R, but in some cases the difference is small, as for compounds 2, 3, 4, and 8. The double-reciprocal plots used for obtaining K_I did not pass through the origin (except for 3-hydroxyphenyltrimethylammonium ion), which indicates that complexes were being formed with the acetyl enzyme, as expected. Although we did not evaluate dissociation constants for the acetyl enzyme carefully, we can say that they are much larger than K_I for all the compounds and therefore also much larger than K_R for compounds 2, 3, 4, and 8.

Equilibrium constant for reaction of 2-PAM with DEP-enzyme. The reaction of 2-PAM and DEP-enzyme does not go to completion if the concentration of inhibited enzyme is high, because the conjugate inhibitor, diethyl (2-PAM) phosphate, that is formed in the reaction is a potent inhibitor (9–11). Actually, this conjugate inhibitor is unstable and converts to the nitrile, so that we had to use a farily high concentration of 2-PAM to get a fast approach to equilibrium with a half-time of less than 0.5 min. Under these circumstances reversible complexes form between 2-PAM and enzyme and between 2-PAM and DEP-enzyme. Since the complex with the free enzyme is the stronger by a factor of about 2, the equilibrium is shifted slightly toward free enzyme and

TABLE 1

Various rate and equilibrium constants

All k_i values are from published data except for that of compound 1, which was remeasured in this work and is higher than the published value by almost a factor of 2 and compound 8, which was evaluated from ECH (anal.) and k_r. The values of k_i for compounds 2, 3, and 4 are from Kitz *et al.* (5); for compound 5, from Froede and Wilson (6); for compound 7, from Aldridge and Reiner (7); for compounds 10, 11, and 13, from Maglothin and Wilson (8); and for compound 12, from Wilson and Rio (2). All the data for compounds 10, 11, 12, and 13 are from the above references. All other data except ECH (enzyme), from Froede and Wilson (3), are from this work. The pK_a assigned to the hydroxyl group of serine in the enzyme is the value for *N*-acetylserinamide.

Leaving group	pK_a	k_i	k_r	k_i/k_r	ECH (anal.)	ECH (acid)	K_I	K_R	k_r°
		$M^{-1}min^{-1}$					M		min^{-1}
1. 1-Methyl-7-hydroxyquinolinium	5.7	2.1×10^8	2.4×10^2	8.8×10^5	4.6×10^{16}	5.2×10^9	5.0×10^{-6}	2.2×10^{-4}	5.3×10^{-2}
2. 1-Methyl-6-hydroxyquinolinium	7.0	9.3×10^6	1.6×10^3	5.8×10^3	3.1×10^{14}	3.7×10^8	3.4×10^{-6}	6.8×10^{-6}	1.1×10^{-2}
3. 7-Hydroxyquinoline	8.6	1.0×10^5	1.1×10^2	9.1×10^2	4.8×10^{13}	1.1×10^8	9.1×10^{-5}	1.9×10^{-4}	2.2×10^{-2}
4. 6-Hydroxyquinoline	8.8	4.1×10^3	17.6	2.2×10^2	1.2×10^{13}	2.8×10^7	6.6×10^{-5}	6.5×10^{-5}	1.2×10^{-3}
5. *p*-Nitrophenol	7.1	2.3×10^5	27	8.5×10^3	4.5×10^{14}	6.0×10^8		2.3×10^{-3}	6.2×10^{-2}
6. *p*-Chlorophenol	9.4			1.7×10^2	9.2×10^{12}	2.2×10^7			
7. 3-Hydroxyphenyltrimethylammonium	8.1	4.0×10^4	17.6	2.3×10^3	1.2×10^{14}	2.6×10^8	2.6×10^{-7}	6.2×10^{-4}	1.1×10^{-2}
8. 2-PAM	8.0	2.2×10^8	4×10^4	5.5×10^3	2.9×10^{14}	6.3×10^8	4.7×10^{-5}	1×10^{-4}	4.0
9. Enzyme (serine)	13.1			1.0	5.3×10^{10}				
10. Thiocholine	7.7	2.7×10^6	1.7	1.6×10^6	8.5×10^{16}	1.7×10^{11}	1.1×10^{-4}	9×10^{-3}	1.5×10^{-2}
11. Dimethylaminoethanethiol	7.9	1.8×10^6	1.0	1.8×10^6	9.5×10^{16}	2.0×10^{11}	1.5×10^{-4}	1.3×10^{-2}	1.3×10^{-2}
12. Fluoride	3.5	2.3×10^5	10	2.3×10^4	1.1×10^{15}	8.3×10^5	3×10^{-4}		
13. Choline	14.0	<0.2	0.036	<5	$<1 \times 10^{11}$	$<2.4 \times 10^5$	4.1×10^{-4}	6.7×10^{-2}	2.4×10^{-3}

$$K = \frac{\text{(DEP-enzyme) (2-PAM)}}{\text{(enzyme) (DEP-2-PAM)}} \cdot \frac{1 + \text{(2-PAM)}/K_I}{1 + \text{(2-PAM)}/K_R} \quad (6)$$

The reversible complexes dissociate during the assay. The concentration of DEP–2-PAM is equal to the concentration of active enzyme, since we started with completely inhibited enzyme.

Six experiments were conducted with two 2-PAM concentrations and four enzyme concentrations. As anticipated, the concentration of active enzyme rose rapidly but did not level off. Instead there was a slow rise in enzyme activity that was linear for 5 min. We attributed this slow rise in enzyme activity to the decomposition of diethyl (2-PAM) phosphate and calculated a half-time of about 12 min for this reaction. This slow rise in active enzyme makes it difficult to obtain precise values from our experiments, but precise values are not needed for our purposes and satisfactory values are easily obtained. We extrapolated the line back to 1 min in order to make a small approximate correction for the decomposition of the conjugate inhibitor. Equilibrium formation of active enzyme ranged from 45% to 80%.

The equilibrium constant for inhibition of the enzyme is $5.5 \pm 0.7 \times 10^3$ and is equivalent to k_i/k_r. The value of ECH (anal.) for diethyl (2-PAM) phosphate was calculated from Eq. 1, and k_i, from the known value of k_r (Table 1).

Equilibrium constant for reaction of p-chlorophenol with diethyl phosphorofluoridate. The equilibrium constant was determined at pH 9.4 (the pK_a of *p*-chlorophenol) by measuring the rate of reaction in both directions. The rate of release of fluoride was greatly increased by *p*-chlorophenol as a result of both nucleophilic reaction and general base catalysis. The portion attributable to nucleophilic reaction was evaluated from the amount of diethyl (*p*-chlorophenol) phosphate produced in the reaction. We found that about 80% of the effect of *p*-chlorophenol was due to nucleophilic reaction, and the remaining 20% is assumed to be general base catalysis. The nucleophilic second-order rate constant was 4.8 M^{-1} min^{-1}.

The reverse reaction of fluoride with diethyl (*p*-chlorophenol) phosphate was slow, and we therefore measured only "initial" rates, corresponding to up to 15% reaction. The second-order rate constant was 4.6×10^{-4} M^{-1} min^{-1}. The equilibrium constant for the reaction at pH 9.4 was 1.0×10^4, which corresponds to ECH (anal.) = 3.1×10^{15} at pH 9.4. We converted this value to ECH (anal.) at pH 7.0 and ECH (acid) (Table 1).

ECH vs. pK_a. With the aid of k_i values from the literature, our measured values of k_r give the equilibrium constant for the phosphorylation of the enzyme, k_i/k_r. Values of ECH (anal.) were calculated from Eq. 1 and converted to ECH (acid) with Eq. 2. Log ECH (acid) is plotted vs. pK_a of the leaving group in Fig. 1 for seven compounds with phenolic leaving groups. A range of about 4 pK_a units is covered, and all points fall within a factor of 2 of the best straight line. The equation of this line obtained by least squares is

$$\log \text{ECH (acid)} = 13.18 - 0.620\ pK_a$$

We also marked other types of compounds on the graph, compounds with fluoride, thiol, and oxime leaving groups. A graph of log k_i/k_r is presented in Fig. 2. Log k_i/k_r is the same as log ECH (anal.) − 10.72. The number 10.72 is the log ECH (anal.) of DEP-enzyme, i.e., log 5.3×10^{10} (Eq. 1). This curve has two linear branches, with slopes of 1.620 and 0.620, that have a

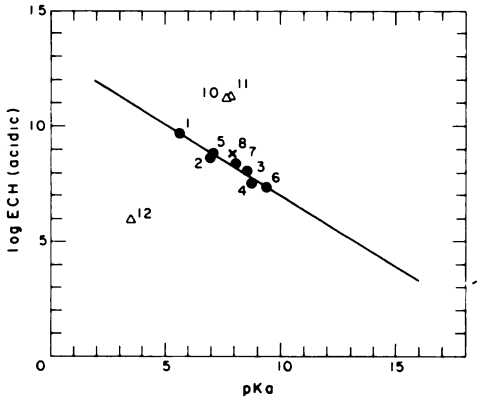


FIG. 1. *Linear relationship between log ECH (acidic species) and pK_a of leaving group for seven diethyl phosphate esters of phenols at 25°*

Other compounds are plotted for comparison. The numbers are the same as in Table 1.

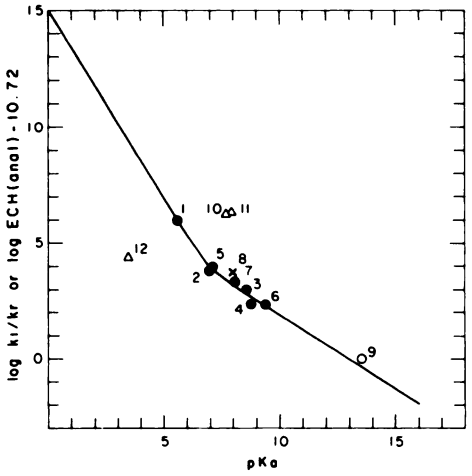


FIG. 2. *Relationship between log ECH (anal.) and pK_a of leaving group for seven diethyl phosphate esters of phenols at pH 7.0 and 25°*

The solid line was calculated from the straight line of Fig. 1. Other compounds, including the enzyme from electric eel, are included for comparison. The numbers are the same as in Table 1.

curved intersection at pH 7.0. This curve was calculated from the linear equation of Fig. 1, and its form is determined only by the linearity of Fig. 1. The seven experimental points, of course, must fit this curve, but it is interesting that the enzyme (pK_a = 13.6) also fits the curve.

DISCUSSION

It is valuable to have a relationship between log ECH and pK_a for oxygen leaving groups of diethyl phosphate esters, because relationships of this kind, especially if linear, are customarily used as a basis for comparing other compounds of a different type and for estimating the value of ECH for other compounds of the same type. The ECH imposes relationships between certain rate constants that are pertinent for an understanding of the reactions of enzymes with these and related compounds. We did find a linear relationship (Fig. 1), although our measurements were restricted to the pK_a range 5.5–9.5, and long extrapolation to other pK_a values, to include alcohols, may be uncertain. In the case of acetate esters, a linear relationship does hold over the range of pK_a, including phenols and alcohols (12), and this, of course, heightens the probability that our relationships also holds for alcohols.

The oxime 2-PAM falls a little high as a leaving group, by about a factor of 4 in ECH. Since our other points all fall within a factor of 2, the difference is probably real, but even so it does suggest that oximes will probably obey approximately the same relationship. On the other hand, it is readily apparent that the fluoride derivative is extremely stable as compared with an oxygen leaving group. It is more stable by a factor of 10^5. The stability of P—F bonds has generally been recognized, but it is only now that we can make a quantitative statement of its extent. Fluoride behaves normally as a leaving group, but it is an especially good nucleophile for phosphorus. Similarly, compounds with a sulfur leaving group do not obey the relationship; they are very much less stable, by a factor of 10^3. In biochemical terminology the P—S bond would be described as a high-energy bond. These results can be rationalized when it is recognized that the increasing stability of the oxygen compounds with pK_a reflects the increasing ability of oxygen to contribute electrons to the *d* orbitals of phosphorus. The stability of P—F bonds has been attributed to an especial ability of fluorine to contribute electrons, and the relative instability of P—S bonds reflects the relative inability of sulfur to contribute electrons.

Thioesters of carboxylic acids are considered high-energy compounds because they are less stable with respect to hydrolysis than analogous oxygen esters, but they are actually more stable than oxygen compounds with the same pK_a. By contrast, the thioesters of diethyl phosphoric acid are decidedly less stable than oxygen compounds, even when compared with compounds with the same pK_a. Thus the P—S bond is very much less stable toward hydrolysis than the P—O bond. This explains the poor nucleophilicity of sulfur toward phosphorus.

Figure 2 shows the curve for log ECH (anal.) − 10.72 or $\log k_l/k_r$ at pH 7.0 calculated from the linear relationship of Fig. 1. The ratio of k_l and k_r for a given enzyme is

fixed by the pK_a for compounds that follow the linear free energy relationship, but the individual values of k_i and k_r may vary widely as factors of steric hinderance and molecular complementarity come into play, depending upon the enzyme. Structural features in the inhibitor which complement the active surface of the enzyme will also provide molecular complementarity in the reactivator, so that the leaving group of a good inhibitor tends to be a good reactivator. But the effect of molecular complementarity may be apparent in only one of the conjugate inhibitor–reactivator pairs, or may be completely obscured because of the very strong dependence of k_i on pK_a. As the pK_a decreases, k_i tends to rise more rapidly than k_i/k_r; the slope on a log-log plot is nearly 2 as compared to 0.62 (5, 13), so that both k_i and k_r tend to increase. This is true down to about pK_a = 7.5, but at lower pK_a things reverse; k_i is no longer so dependent on pK_a, and now k_i/k_r rises rapidly, with a slope of 1.62. Now k_r tends to decrease. The effects of molecular complementarity are superimposed on these general trends. The molecular complementarity of 2-PAM is evident; it is the best reactivator, and at the same time its diethyl phosphoryl derivative is the best inhibitor of the group. It is true that 2-PAM with unbonded electrons on the nitrogen atom adjoining the nucleophilic oxygen enjoys the enhanced reactivity arising from the α effect, but this effect is too small to account alone for the outstanding reactivity of this reactivator and its conjugate inhibitor. There is a marked geometric similarity between 1-methyl-7-hydroxyquinolinium and 2-PAM, which may account for the high reactivity of 1-methyl-7-hydroxyquinolinium as a reactivator, despite its very unfavorable pK_a, and for the high potency of its conjugate inhibitor.

The value of ECH (anal.) for the diethyl phosphoryl enzyme falls on the curve of Fig. 2, using pK_a = 13.6 (14) for the hydroxyl group of serine. Assuming that the curve holds for alcohols as well as phenols, we may conclude that the DEP-enzyme behaves in normal fashion as far as ECH is concerned and that there are no very large interactions between the phosphoryl group and the protein. However, other DEP-enzymes, bovine red cell acetylcholine esterase, horse serum butyrylcholinesterase, and chymotrypsin, are more stable; the last is 10^4 times more stable than the eel enzyme (6). We must conclude that with these enzymes there are increasingly strong stabilizing interactions between the diethyl phosphoryl group and the protein. This conclusion is supported by work with an organophosphate inhibitor containing a spin-labeled nitroxide group, which showed that the label was free to rotate in acetylcholinesterase, but not in chymotrypsin (15, 16). Apparently these stabilizing interactions are largely lost in the transition state with red cell acetylcholinesterase and chymotrypsin because, although the rates of inhibition by diethyl phosphorofluoridate are similar to the eel enzyme, reactivation rates by fluoride are very much smaller (6).

1-Methyl-7 hydroxyquinolinium 2-PAM

SCHEME 2

No inhibition was observed with diethyl phosphorylcholine as an inhibitor, even though reactivation by choline is readily observed and molecular complementarity might lead us to expect that it would be a good inhibitor. The inertness of this compound is also surprising because the thiocholine derivative is such a good inhibitor. Moreover, the enzyme does not discriminate between acetylcholine and acetylthiocholine as substrates. Yet this result is entirely predictable from Fig. 2. Using pK_a = 14 for choline, we find k_i/k_r = 0.25, and since $k_r = 3.6 \times 10^2$ (8), we calculate $k_i = 9 \times 10^3$. Now since $k_i = k_i^\circ/K_I$ and $K_I = 2.3 \times 10^{-3}$ from measurements using the compound as a reversible inhibitor, k_i°, which is the maximum rate of inhibition that can be observed with this compound, comes out to be 2×10^{-5} min^{-1}. At this rate only 0.1% would be inhibited in 1 hr, which is much too small to measure. Actually the rate of spontaneous reactivation is 10 times faster, so that only 10%

inhibition could be approached even in a very long time. Thus our failure to observe inhibition with this compound is readily accounted for by the thermodynamic data and simply comes down to the inability of molecular complementarity to completely overcome a very unfavorable pK_a.

Values of k_i for leaving groups that have no structural features that would obviously suggest molecular complementarity show that inhibition is very sensitive to pK_a for pK_a values greater than about 7. Extrapolation of this general trend from $pK_a = 10$ to $pK_a = 14$ suggests a value for k_i of less than 10^{-4} for diethyl phosphorylcholine. Our value of $k_i = 9 \times 10^{-3}$, calculated for diethyl phosphorylcholine from Fig. 2, therefore reveals a substantial promotion of inhibitory activity arising from molecular complementarity even though it is still too weak an inhibitor to measure. This same argument indicates that the reactivity of choline, as a reactivator, is similarly enhanced by molecular complementarity.

On the other hand, the k_i of the conjugate inhibitor of 3-hydroxyphenyltrimethylammonium ion is not substantially higher than would be expected from its pK_a, and this results would seem to be surprising.

Our experimental points in Fig. 1 are restricted to a limited range of pK_a because it is difficult to find compounds with pK_a below 5 or above 9.5 for which both k_i and k_r can be measured. Reactivation is very difficult for $pK_a < 5$, and for $pK_a > 10$ both reactivation and inhibition are very difficult. Reactivation by choline is an exception, as already noted.

The tertiary hydroxyquinolines have a nitrogen atom that might act as a nucleophile, but there is little doubt that it is the hydroxyl group that is the nucleophile in reactivation, because quinoline ($K_I = 3 \times 10^{-4}$ M) at 1 mM concentration produced only marginal reactivation in several hours.

The ratio k_i/k_r for every compound in this study can be calculated for pH values other than 7.0 once it has been determined for any one of them at the new pH. Similarly, the ratio can be estimated for any oxygen compound of known pK_a; i.e., a curve analogous to Fig. 2 can be constructed for the new pH. A curve analogous to Fig. 2 can also be constructed for any enzyme for which k_i/k_r is known for any compound. Thus curves could be constructed for the bovine erythrocyte enzyme, horse serum butyrylcholinesterase, and bovine chymotrypsin from present knowledge. It is possible to obtain a lot of information from a few measurements. For example, from a single measurement of k_i and k_r for human acetylcholinesterase, the values of k_i/k_r for all compounds could be calculated and the ratio could be estimated with reasonable confidence for any oxygen compound of known pK_a.

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