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Equilibrium Constants and Relationships in the Inhibition of Serinesterases by Organophosphates

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SUMMARY

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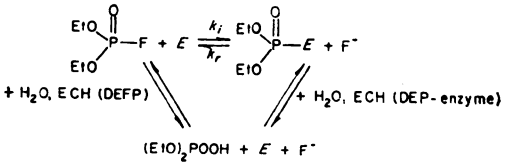
The equilibrium constants for the reactions of diethyl phosphofluoridate and eel acetylcholinesterase, bovine red cell acetylcholinesterase, horse serum butyrylcholinesterase, and bovine α-chymotrypsin were measured. The values obtained were 2.3×10^4, 3×10^5, 6.4×10^6, and 6×10^8, respectively, in terms of analytical concentrations at pH 7.0 and 25°, with water activity as unity. These were converted to equilibrium constants for the hydrolysis of the diethylphosphoryl-enzyme derivative: 5.2×10^{10}, 4×10^9, 2×10^8, and 2×10^6, respectively. These values enable one to calculate the equilibrium constant for the reaction of these enzymes with any diethyl phosphate ester whose equilibrium constant for hydrolysis is known. If the rate constant for inhibition is also known, the rate constant for reactivation of the inhibited enzyme can be calculated. These calculations were made for diethyl *p*-nitrophenyl phosphate and the conjugate reactivator *p*-nitrophenol and checked experimentally for the eel enzyme.

INTRODUCTION

Certain types of organophosphorus compounds containing good leaving groups are remarkably potent inhibitors of acetylcholinesterase and other serinesterases. As such they have commanded the attention of enzymologists and pharmacologists. These compounds have found limited use in medicine (treatment of glaucoma) and in war (nerve gases) but have been widely used as insecticides. The reaction illustrated in Scheme 1 with diethyl phosphofluoridate (DEFP)[2] is intrinsically reversible, although in some cases it may be carried out in an essentially unidirectional manner, either in the forward direction, as inhibition, or in the reverse direction, as reactivation.



SCHEME 1

Fluoride was first demonstrated to be a reactivator by Heilbronn (1), and the equilibrium constant for inhibition, K (DEFP), was evaluated as 2.3×10^4 by Wilson and

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[2] The abbreviations used are: DEFP, diethyl phosphofluoridate; DEP-, diethylphosphoryl-.

Rio (2) from measured values of k_i = 2.3×10^5 M^{-1} min^{-1} and k_r = 10 M^{-1} min^{-1} for enzyme from electric eel, using K(DEFP) = k_i/k_r. These values are for analytical concentrations at pH 7.0.

The value of the equilibrium constant for the hydrolysis (ECH) of the diethylphosphoryl-enzyme would be helpful in understanding this class of reactions as well as the enzyme active site. In cases when other data are available the ECH for DEP-enzyme, ECH(DEP-enzyme), can be used for predicting rate constants or for evaluating other equilibrium constants. It is appearent from Scheme 1 that if the ECH for the phosphofluoridate, ECH (DEFP), were known, it would be possible to calculate the ECH for DEP-acetylcholinesterase:[3]

$$\text{ECH (DEP-acetylcholinesterase)} = \text{ECH (DEFP)} \times (2.3 \times 10^4)^{-1}$$

In an attempt to evaluate ECH (DEP-acetylcholinesterase) Wilson and Rio assumed that the ECH (DEFP) written in terms of the undissociated acids was approximately the same as ECH for fluorophosphoric acid, which was known. This assumption has since proved to be surprisingly and very greatly in error. The measured value ECH (DEFP) written in terms of the undissociated acids (water = unit activity) is 8.3×10^5, and in terms of analytical concentrations[4] at pH 7.0, 1.2×10^{15} (3). Using this latter value, we can evaluate

$$\text{ECH (DEP-acetylcholinesterase)} = 5.2 \times 10^{10}$$

for enzyme from electric eel in terms of analytical concentrations at pH 7.0.

In comparing the inhibition and reactivation of other related enzymes it would be valuable to know their corresponding ECH values. We have undertaken to make these determinations, which we report in this paper.

In using ECH (DEP-acetylcholinesterase) to calculate the value of k_r for p-nitrophenol as a reactivator, we obtained a surprising result. The conjugate inhibitor for p-nitrophenol is diethyl p-nitrophenyl phosphate (paraoxon), and its rate constant for inhibition of eel enzyme is known; it is coincidentally the same as for the phosphofluoridate, $k_i = 2.3 \times 10^5$ M^{-1} min^{-1}. The ECH for paraoxon is also known, 6.7×10^{14} (analytical concentrations at pH 7.0) (3). Thus we can calculate

$$k_r = \frac{\text{ECH (DEP-acetylcholinesterase)}}{\text{ECH (paraoxon)}} \cdot k_i = \frac{5.2 \times 10^{10}}{6.7 \times 10^{14}} 2.3 \times 10^5 = 18 \text{ M}^{-1} \text{ min}^{-1}$$

This k_r value is given in analytical concentrations. Since at pH 7.0 somewhat less than half the p-nitrophenol is ionized (pK_a = 7.1), the k_r value for the p-nitrophenolate *anion* is greater than 40 M^{-1} min^{-1}. This value is remarkably high: it is 4 times higher than the value for fluoride. Yet fluoride is recognized as a potent nucleophile toward phosphorus.[5] The relative values for p-nitrophenolate anion and fluoride "ought" decidedly to be the other way around. For example, fluoride reacts with isopropyl methylphosphonofluoridate (sarin) with a rate constant of about 40 M^{-1} min^{-1}, calculated from the rate of racemization of one of the isomers of sarin (5). On the other hand, the rate constant for the reaction of p-nitrophenolate anion with sarin is only about 1 M^{-1} min^{-1}. Wherever comparisons can be made, fluoride is a distinctly better nucleophile toward phosphates and phosphonates than p-nitrophenol; yet our calculations show just the opposite with respect to DEP-acetylcholinesterase. It therefore appeared important to determine k_r (p-nitrophenol) experimentally.

MATERIALS AND METHODS

DEFP was synthesized from dichlorophosphate according to Saunders and Stacey (6). Paraoxon was synthesized from the chloride as described by Andrews *et al.* (7) as modified by Ginsburg *et al.* (8). p-Nitrophenol was purchased from Eastman Organic Chemicals and recrystallized

[3] The product of the equilibrium constants around any cycle, as in scheme 1, is equal to 1.

[4] Equilibrium constants written in terms of analytical concentrations are pH-dependent.

[5] The P—F bond is unusually stable (3), but fluoride behaves normally as a leaving group (4).

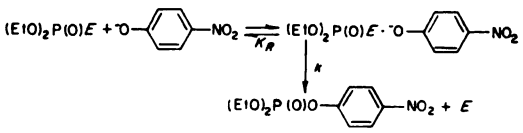
from dilute HCl. Benzoyl-L-tyrosine ethyl ester, butyrylthiocholine, and acetylthiocholine were purchased from Calbiochem. Butyrylcholinesterase (horse serum), α-chymotrypsin (bovine), and acetylcholinesterase (eel) were purchased from Worthington. Bovine erythrocyte acetylcholinesterase was purchased from Sigma. All other reagents were analytical grade from various sources. Water was double-distilled.

The enzymatic activities of acetylcholinesterase and butyrylcholinesterase were determined according to Ellman *et al.* (9) in 0.05 M Tris-HCl, pH 8.0. α-Chymotrypsin activity was determined according to Hummel (10). Inhibition of the enzymes and reactivation were carried out in Tris buffer at pH 7.0 and 25°, using a range of concentrations of DEFP and paraoxon for inhibition and a range of concentrations of fluoride and *p*-nitrophenol for reactivation. For reactivation, concentrated enzyme was completely inhibited with minimal concentrations of DEFP or paraoxon and diluted extensively (40–400-fold) for reactivation.

RESULTS

Reactivation of DEFP-inhibited eel enzyme (DEP-acetylcholinesterase) by *p*-nitrophenol was readily observed. However, our results were not highly reproducible, in that the extents of reactivation obtained after an extensive period of time varied from 80% to 100% in eight measurements, and only 70% reactivation was obtained in one case. We used five different concentrations. In plotting the data for the progressive recovery of enzyme activity as a pseudo-first-order reaction we used the final extent of reactivation to calculate the fraction of activity recovered at any time. Straight lines were obtained in semilogarithmic plots of the data normalized in this way, and apparent pseudo-first-order rate constants were evaluated. To obtain k_r, these constants in turn were divided by the factor 1 plus the ratio of nonreactivated activity to reactivated activity. For 80% reactivation this factor is 1.25. This treatment is proper for cases in which the reaction reaches an equilibrium short of completion (which is not our case) or when the inhibited enzyme deteriorates. At any rate the correction is not large for our purposes, and our duplicate measurements and evaluations of k_r at each concentration (one triplicate) agreed within about 10%.

The pseudo-first-order constants were not proportional to the reactivator concentration (R) but were consistent with the postulate that *p*-nitrophenol forms a reversible complex with the inhibited enzyme,



SCHEME 2

yielding the rate equation

$$k_{obs} = \frac{k}{1 + K_R(R)^{-1}}$$

$$k_{obs}^{-1} = k^{-1} + k_r^{-1}(R)^{-1}$$

where the second-order rate constant is

$$k_r = k(K_R)^{-1}$$

The data plotted as k_{obs}^{-1} vs. $(R)^{-1}$ yielded a straight line (Fig. 1) with the following values for k and K_R :

$$k_r = \frac{6.2 \times 10^{-2}\ \text{min}^{-1}}{2.3 \times 10^{-3}\ \text{M}} = 27\ \text{M}^{-1}\ \text{min}^{-1}$$

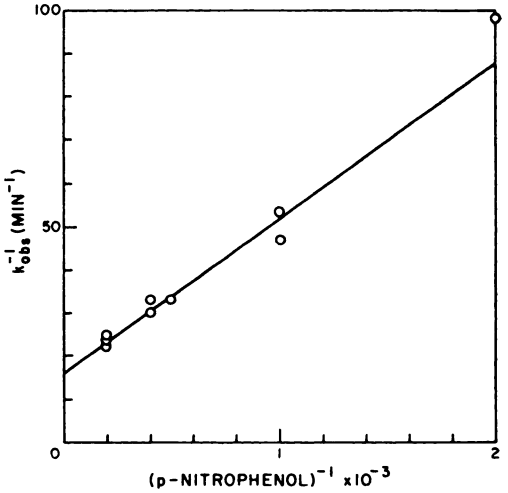


FIG. 1. *Reactivation of DEP-acetylcholinesterase (eel) with p-nitrophenol*
k_{obs} is the pseudo-first-order rate constant.

This value agrees with the calculated value of 18 M^{-1} min^{-1}, since a factor of 1.5 is not unreasonable for the summation of errors in all the experimental numbers that go into the calculation and comparison.

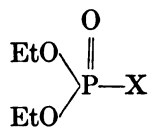
The data for inhibition of the different enzymes by diethyl phosphofluoridate and for reactivation of the inhibited enzyme by fluoride are presented in Table 1. The inhibition experiments appeared to offer no difficulty. Good straight lines were obtained in semilogarithmic plots for pseudo-first-order reaction rates. These pseudo-first-order rate constants were proportional to the concentration of inhibitor, indicating a second-order reaction, and second-order rate conconstants, k_i, were evaluated.

Reactivation rate measurements were again troublesome, in that 87–96% reactivation was obtained with bovine erythrocyte acetylcholinesterase, and 65–81% (mostly above 70%), with horse serum butyrylcholinesterase. Chymotrypsin reactivation was too slow to follow to completion, since only about 20% reactivation was obtained in 2 days with 0.5 M fluoride, and 35% in 100 hr. The chymotrypsin reactivation constant was calculated on the assumption that 100% recovery would be obtained. In general the reactivation rate constants should be regarded as only approximate.

DISCUSSION

Examination of Table 1 shows that there are large differences in the rates of reaction and the equilibrium constants for the different enzymes. The values of ECH (DEP-enzyme) vary by a factor of 20,000, which is of course the same variation as in the equilibrium constant for the reaction with DEFP. Since eel enzyme and chymotrypsin have nearly the same amino acid sequence about the active serine, as do all serinesterases that have been examined (14), it is clear that the enormous differences in ECH (DEP-enzyme), K (DEFP), and K (paraoxon) are a consequence of differences in the three-dimensional structure of the protein in the region of the active site. The differences in ECH (DEP-enzyme) and K(inhibitor) must reflect differences in the interaction of the diethylphosphoryl group with the enzyme and differences in the solvation energy of introducing this group into the microenvironment of the active site.

It is also evident from Table 1 that although the equilibrium constants for the reaction of DEFP, K(DEFP), with the enzymes vary widely, they all lie far to the right (i.e., toward completion). We might inquire whether this should be expected. However, it will be better to examine this question by first changing the form of the question. If we know the ECH for any inhibitor of the form



where X is any leaving group, we can calculate the equilibrium constant for the inhibition reaction. Thus

$$K \text{ (paraoxon)} = K \text{ (DEFP)} \frac{\text{ECH (paraoxon)}}{\text{ECH (DEFP)}}$$

$$= K \text{ (DEFP)} \frac{6.7 \times 10^{14}}{1.2 \times 10^{15}}$$

$$= K \text{ (DEFP)} \times .56$$

These values have been listed in Table 1. These equilibria also lie far to the right and involve the transfer of a diethylphosphoryl group from p-nitrophenol to the hydroxyl group of the active serine residue. We might wonder whether this reaction should go so completely. It would therefore be interesting if we could arrive at some estimate of what the equilibrium constant would be if the serine residue were not part of the active site. We have very little guidance in this quest. We might expect an approximate relationship of the form

$$\log K = \alpha \, \Delta pK_a$$

for the transfer of a diethylphosphoryl group from p-nitrophenol to an anion with a different pK_a :

$$(EtO)_2P(O)O\phi NO_2 + RO^- \overset{K}{\rightleftharpoons} (EtO)_2P(O)OR + {}^-O\phi NO_2$$

TABLE 1

Equilibrium and kinetic constants for the reaction of serinesterases with DEFP and paraoxon

Enzyme	DEFP				Paraoxon		
	k_i	k_r	K (DEFP)	ECH (DEP-enzyme)	k_i	k_r	K (paraoxon)
	M^{-1} min^{-1}					M^{-1} min^{-1}	
Eel acetylcholinesterase	2.3×10^5	10	2.3×10^4	5.2×10^{10}	2.3×10^5	18, 27[a]	1.4×10^4, 8.2×10^{3}[a]
Bovine red cell acetylcholinesterase	1.8×10^5	0.6	3×10^5	4×10^9	4×10^{5}[b]	2.2	1.8×10^5
Horse butyrylcholinesterase	8.6×10^6	1.3	6.4×10^6	1.9×10^8	6.7×10^{4}[c]	1.8×10^{-2}	3.7×10^6
Bovine chymotrypsin	6.1×10^4	1×10^{-4}	6×10^8	2×10^6	2.5[d]	7×10^{-9}	3.7×10^8

K (DEFP) was calculated as the ratio of the measured values of k_i and k_r. ECH (DEP-enzyme) was calculated from ECH (DEFP) = $1.2 \times 10^{15} \div K$ (DEFP). K (paraoxon) was calculated from K (DEFP), ECH (paraoxon) = 6.7×10^{14}, and ECH (DEFP) = 1.2×10^{15}, using ECH (paraoxon) $\times$ K (DEFP) $\div$ ECH (DEFP), except for 8.2×10^3, which was measured in this work. The values of k_i for paraoxon were taken from the references shown in the footnotes and corrected by the factor 0.4 to change from 37° to 25°. The values of k_r for *p*-nitrophenol were calculated using $k_r = k_i \div K$ (paraoxon), except as noted.[a]

[a] The second value was measured in this work.

[b] Value obtained with sheep red cell enzyme by Aldridge and Davison (11).

[c] From ref. 12.

[d] From ref. 13.

Such a relationship holds for acetate esters (15), and such a relationship has been proposed by Bromilow *et al.* (16) for dialkyl phosphate esters with $\alpha = 1.16$. This value of α predicts that the inhibitory reaction of paraoxon and enzyme would have only a small analytical equilibrium constant of about 10 at pH 7.0. Probably this value of α is not valid for such an extreme case as an alkoxide anion of serine. In fact, measurements with diethylphosphorylcholine indicate that α would have to exceed 1.35. We have therefore calculated the equilibrium constant for the reaction in which OH^- ($pK_a \approx 16$) replaces RO^- in the above equation:

$$(EtO)_2P(O)O\phi NO_2 + OH^- \rightleftharpoons (EtO)_2P(O)OH + {}^-O\phi NO_2$$

This value is obtained by dividing the product of the ionization constant of *p*-nitrophenol (10^{-7}) and the ECH of paraoxon written in terms of acidic species (7.4×10^8) by the ion product of water (10^{-14}). The result is 7×10^{15}. This result would require α to be about 1.75 rather than 1.16. If we use this value of α and take the pK_a of the active serine as 13, we get 3×10^{10} for the transfer of a diethylphosphoryl group from *p*-nitrophenol to a serine alkoxide anion. This latter value translates to 6×10^4 in terms of analytical concentrations at pH 7.0, which is similar to the measured values for acetylcholinesterase. This calculation is too uncertain a basis on which to make any firm conclusion, but it does indicate that the intrinsic properties of serine may be sufficient to account for the large values of K (DEFP) and K (paraoxon). We are, of course, left with the problem of why these values are so different for the different enzymes.

We do have some information on the reversible interaction of a diethylphosphoryl group with the active site of the ell enzyme, which suggests that this group is not well accommodated. The diethylphosphoryl esters of choline, ethanolamine, and the corresponding thiols are somewhat less readily bound as reversible inhibitors than are the alcohols and thiols. Since the hydroxyl group of choline does not contribute appreciably to binding (judged by the fact that ethytrimethylammonium ion and propyltrimethylammonium ion reversibly inhibit the eel enzyme about as well as choline), it would appear that the diethylphosphoryl group is not well accommodated and at best contributes little toward binding.

On the other hand, it is true that diisopropyl phosphofluoridate and paraoxon are reversibly bound by cholinesterases (17–19), but still it does not appear that interaction of the diethylphosphoryl group with the protein contributes very much toward stabilizing DEP-acetylcholinesterase. However, it would appear that such interactions must be involved in the stability of the other DEP-enzymes. This discussion is consistent with the results of spin labeling studies of acetylcholinesterase, butyrylcholinesterase, and chymotrypsin, in which it was shown that the nitroxide radical incorporated into the phosphoryl group of the inhibited enzymes is relatively free to rotate in the cholinesterases but is not free to rotate in chymotrypsin (20, 21).

The measured value for k_r (*p*-nitrophenol), 27 M^{-1} min^{-1}, for eel acetylcholinesterase, is in agreement with the predicted value of 18 M^{-1} min^{-1} for the eel DEP-enzyme. Our measurements also showed that a reversible complex is formed between *p*-nitrophenol and the DEP-enzyme. The greater nucleophilicity of *p*-nitrophenol over fluoride in reactivating the eel enzyme is not general (Table 1). The calculated values for k_r (*p*-nitrophenol) are very low, much smaller than the measured values for fluoride for two of the enzymes. The high nucleophilicity of *p*-nitrophenol as a reactivator of the eel enzyme is probably a consequence of the binding of this substance by the DEP-enzyme. If the binding should be at least partially in a productive mode, i.e., one in which the nucleophilic oxygen is near and directed toward the phosphorus atom of the DEP-enzyme, then the high reactivity would be explained. It is reasonable to assume that this is the case.

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