A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H., and Coppola, J. C. (1968), *Brookhaven Symp. Biol. 21,* 24.
McClure, W. O., and Neurath, H. (1966), *Biochemistry 5,* 1425.
Morgan, P. H., Robinson, N. C., Walsh, K. A., and Neurath, H. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 3312.
Petra, P. H. (1970), *Methods Enzymol. 19,* 459.
Petra, P. H. (1971), *Biochemistry 10,* 3163.
Petra, P. H., and Neurath, H. (1971), *Biochemistry 10,* 3171.
Piras, P., and Vallee, B. L. (1967), *Biochemistry 6,* 348.
Quiocho, F. A., and Lipscomb, W. N. (1971), *Advan. Protein Chem. 25,* 1.
Quiocho, F. A., McMurray, C. H., and Lipscomb, W. N. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2850.
Quiocho, F. A., and Richards, F. M. (1966), *Biochemistry 5,* 4062.
Reeck, G. R., and Neurath, H. (1972), *Biochemistry 11,* 3947.
Riordan, J. G., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6,* 3609.
Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2,* 1460.
Robinson, N. C., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12,* 420.
Sokolovsky, M., and Riordan, J. F. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9,* 239.
Uren, J. R. (1971), *Biochim. Biophys. Acta 236,* 67.
Uren, J. R. (1972), Ph.D. Thesis, University of Washington.
Uren, J. R., and Neurath, H. (1972), *Biochemistry 11,* 4483.
Uren, J. R., Walsh, K. A., and Neurath, H. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 3989.
Vallee, B. L., and Riordan, J. F. (1968), *Brookhaven Symp. Biol. 21,* 91.
Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1960), *J. Biol. Chem. 235,* 64.
Whitaker, J. R., Menger, F., and Bender, M. L. (1966), *Biochemistry 5,* 386.
Yamasaki, M., Brown, J. R., Cox, D. J., Greenshields, R. N., Wade, R. D., and Neurath, H. (1963), *Biochemistry 2,* 859.
Yanari, S., and Mitz, M. A. (1957), *J. Amer. Chem. Soc. 79,* 1154.

Equilibrium Constants for the Phosphorylation of Acetylcholinesterase by Some Diethyl Phosphorothiolates and Phosphates†

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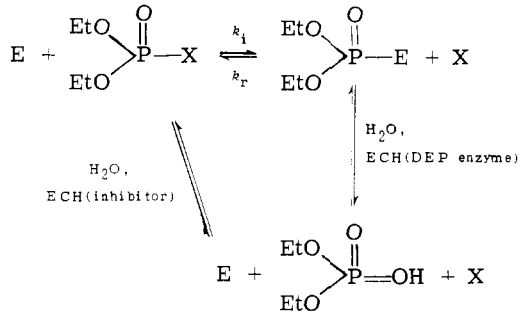
ABSTRACT: Two classes of organophosphorus inhibitors of acetylcholinesterase, phosphorothiolates and phosphates, are compared in terms of their reversible binding with the enzyme, phosphorylating activities, and free energies of hydrolysis. *O,O*-Diethyl *S*-[2-trimethylammonium)ethyl] phosphorothiolate iodide, *O,O*-diethyl *S*-[2-(dimethylammonium)ethyl] phosphorothiolate hydrogen oxalate, *O,O*-diethylphosphorylcholine iodide, and *O,O*-diethyl *O*-[2-(dimethylammonium)ethyl] phosphate hydrogen oxalate display comparable reversible binding affinities for eel acetylcholinesterase, having dissociation constants ranging from 1 to 4×10^{-3} M. The dissociation constant for *O,O*-diethyl *O*-(3,3-dimethylbutyl) phosphate is somewhat greater, 1.1×10^{-2} M. The magnitudes of these dissociation constants are consistent with current knowledge of the factors contributing to molecular complementarity in this system. The phosphorothiolates are potent phosphorylating agents, but no phosphorylating ability is detected for any of the phosphates after removal of active impurities by brief exposure to alkaline solution. The different phosphorylating activities of the phosphorothiolates as compared to the phosphates are consistent with the large differences in the pK_a's of their leaving groups. Rate constants are also obtained for the reverse reaction, the reactivation of the inhibited enzyme by the leaving group. The forward and reverse rate constants allow calculation of the pertinent equilibrium constants for phosphorylation and also the free energies of hydrolysis of the inhibitors. The diethyl phosphorothiolates have free energies of hydrolysis of −23 kcal/mol at pH 7.0 and 25° whereas the values for the diethyl phosphates are ≥−7. The phosphorothiolates are less stable than some similar compounds having leaving groups with even lower pK_a's which suggests that it is appropriate to speak of these compounds as "high-energy compounds" or compounds with a high group transfer potential.

Certain types of organophosphorus compounds are highly potent inhibitors of acetylcholinesterase and other serine esterases. The reaction here illustrated for a diethyl phosphate on the horizontal line is intrinsically reversible although the reaction may in some cases be carried out in an essentially unidirectional manner, either in the forward direction as inhibition, or in the reverse direction as reactivation. In the forward direc-



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tion the enzyme is a nucleophile and X is the leaving group whereas in the reverse direction X is the nucleophile and the enzyme is the leaving group. Thus for every inhibitor there is a potential conjugate reactivator and for every reactivator there is a potential conjugate inhibitor. The diethylphosphoryl enzyme (DEP enzyme)[1] is analogous to the normal acyl-enzyme formed during the hydrolysis of esters but unlike the latter does not hydrolyze readily. Phosphorothiolates such as $(EtO)_2P(O)SC_2H_4N(CH_3)_3^+$ (I), $(EtO)_2P(O)SC_2H_4N(CH_3)_2H^+$ (II), and related substances are very potent inhibitors of acetylcholinesterase, yet the oxygen analogs, phosphates such as $(EtO)_2P(O)OC_2H_4N(CH_3)_3^+$ (III) and $(EtO)_2P(O)OC_2H_4N(CH_3)_2H^+$ (IV), are weak inhibitors (Tammelin, 1957). The trialkyl phosphate $(EtO)_2P(O)OC_2H_4C(CH_3)_3$ (V) and similar compounds are reported to be good inhibitors (Fukuto, 1957; Bracha and O'Brien, 1970).

A priori these results are surprising because the phosphorothiolates and phosphates would appear to enjoy similar molecular complementarities to the enzyme. For example, even though acetylcholine and acetylthiocholine have different preferred conformations, at least in the crystalline state (Shefter and Mautner, 1969) (the former being trans and the latter gauche relative to the choline *c–c* axis), these compounds are equally good substrates. Moreover, since it has been known for many years that choline is a reactivator of the DEP enzyme, eq 1 tells us that diethylphosphorylcholine must be an inhibitor. This anomaly in the behavior of the phosphates (III, IV) relative to the phosphorothiolates (I, II) has been so striking that some investigators have coined a name, the "thiolo effect," to apply to this phenomenon (Bracha and O'Brien, 1968). One explanation advanced for the "thiolo effect" is that the phosphates in distinction to the phosphorothiolates might not bind well to the enzyme (Bracha and O'Brien, 1968). It appeared interesting to further investigate these compounds, the two phosphorothiolates and the two phosphates, in terms of the cycle of reaction 1. In eq 1, the inhibition and reactivation reactions are represented as bimolecular reactions with second-order rate constants. However, it is necessary to expand this representation for these compounds in order to include the formation of reversible addition complexes. Thus

$$\begin{array}{l} E + (EtO)_2P(O)SC_2H_4N(CH_3)_3^+ \underset{K_I}{\rightleftharpoons} \\ \qquad E\cdot(EtO)_2P(O)SC_2H_4N(CH_3)_3^+ \\ \qquad \swarrow\ k_i^0 \quad k_r^0\ \searrow \\ E\text{–}P(O)(EtO)_2\cdot HSC_2H_4N(CH_3)_3^+ \overset{K_R}{\rightleftharpoons} \\ \qquad E\text{–}P(O)(EtO)_2 + HSC_2H_4N(CH_3)_3^+ \end{array} \qquad (2)$$

The equilibrium constant for the hydrolysis of the diethylphosphoryl enzyme, ECH (DEP acetylcholinesterase), is known; its value is 5.3×10^{10} at pH 7.0 in terms of analytical concentrations (Froede and Wilson, 1974). The reversible reaction sequence of eq 2 can be carried out in a unidirectional manner either in the forward direction by reacting inhibitor and enzyme or in the back direction by reacting inhibited enzyme with reactivator. The rate expression in either direction has the form of a rectangular hyperbola; the second-order rate constant for inhibition is given by k_i^0/K_I and for reactivation by k_r^0/K_R (see later). The ratio of the second-order rate constants is the equilibrium constant for the reaction. In this work we have used the two phosphates and the two phosphorothiolates as inhibitors, and their leaving groups, the alcohols and thiols, as reactivators. We have thus evaluated K_I, K_R, k_i^0, k_r^0, $k_i = k_i^0/K_I$, $k_r = k_r^0/K_R$, and the equilibrium constants for the hydrolysis of the inhibitor, ECH (inhibitor), and for the phosphorylation of the enzyme.

Equation 2 in more general symbols becomes

$$E + I \underset{K_I}{\rightleftharpoons} E\cdot I \underset{k_r^0}{\overset{k_i^0}{\rightleftharpoons}} E'\cdot R \overset{K_R}{\rightleftharpoons} E' + R \qquad (2a)$$

where I is any inhibitor, E·I is the reversible noncovalent complex between enzyme and inhibitor, E′ is the inhibited enzyme (DEP enzyme), R is the reactivator (leaving group of an inhibitor or potential inhibitor, X in eq 1), and E′·R is the reversible noncovalent complex between inhibited enzyme and the reactivator. For every inhibitor there is a potential conjugate reactivator, R, which is the leaving group of the inhibitor and similarly for every reactivator (nucleophile) there is a potential conjugate inhibitor. Whether a potential inhibitor or reactivator can be experimentally demonstrated to act in the expected manner depends upon whether the values of k_i^0 and k_r^0 are sufficiently large.

The appropriate kinetic equations for the reaction of enzyme and inhibitor are

$$E^0 = [E] + [E\cdot I] + [E'] = [\varepsilon] + [E'] \qquad (3)$$

where E^0 is the total enzyme concentration, E is free enzyme, E·I is enzyme–inhibitor complex, E′ is inhibited enzyme (DEP enzyme), and ε is the sum of free enzyme and enzyme–inhibitor complex. The symbol ε is introduced because it represents the measured enzyme activity during the course of inhibition when the assay is carried out by diluting an enzyme aliquot with substrate solution so that the reversible complex, E·I, dissociates completely. With the aid of eq 3 and the equilibrium expression defining the dissociation constant K_I, we obtain for the rate expression

$$\frac{d[\varepsilon]}{dt} = -k_i^0[E\cdot I] = -\frac{k_i^0(\varepsilon)}{1 + (K_I/[I])} = -k_i(\text{obsd})[\varepsilon] \qquad (4)$$

which integrates to

$$\ln([\varepsilon]/E^0) = -k_i(\text{obsd})t \qquad (5)$$

Similarly for reactivation

$$E^0 = [E'] + [E'\cdot R] + [\varepsilon] = [\varepsilon'] + [\varepsilon] \qquad (3a)$$

and we obtain

$$\frac{d[\varepsilon']}{dt} = -\frac{k_r^0}{1 + (K_R/[R])}[\varepsilon'] = -k_r(\text{obsd})[\varepsilon'] \qquad (6)$$

which integrates to

$$\ln([\varepsilon']/\varepsilon_0') = -k_r(\text{obsd})t \qquad (7)$$

Here ε' represents the sum of DEP enzyme and reactivator–DEP enzyme complex and ε_0' is the value of ε' at $t = 0$. If inhibition is complete at $t = 0$, $\varepsilon_0' = E^0$. Again ε represents measured enzyme and consists of free enzyme and any reversible complexes that might form as, for example, between free enzyme and reactivator. Note that in the limit of low concen-

[1] Abbreviations used are: DEFP, diethyl phosphorofluoridate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DEP enzyme, diethylphosphoryl enzyme.

trations of inhibitor or reactivator with respect to the appropriate dissociation constants eq 4 and 6 become second-order expressions.

Finally the constants were evaluated by measuring k_i(obsd) for various values of [I] and also k_r(obsd) for various values of [R] using eq 5 and 7. The relationships defining k_i(obsd) in terms of [I] (eq 4) and defining k_r(obsd) in terms of [R] (eq 6) were put in reciprocal linear form

$$\frac{1}{k_i(\text{obsd})} = \frac{1}{k_i^0} + \frac{K_I}{k_i^0}\frac{1}{[I]} \quad (8)$$

and

$$\frac{1}{k_r(\text{obsd})} = \frac{1}{k_r^0} + \frac{K_R}{k_r^0}\frac{1}{[R]} \quad (9)$$

In order to evaluate K_I and k_i^0 it is necessary to use an inhibitor concentration at least equal to K_I. This may require very rapid measurements depending upon the value of k_i^0. Without special techniques, it will be difficult to make the measurement if k_i^0 values are greater than 20/min because in such cases the half-time for inhibition will be less than 2 sec. The phosphorothiolates turned out to have k_i^0 values close to 3×10^3 min^{-1} and special equipment—a mechanical rapid mixer-sampler—had to be used. With data for the above constants at hand, we are in a position to discuss why the diethyl phosphates, the oxygen analogs, are poor inhibitors (in this work no progressive inhibition at all was observed with these compounds) and we are also able to discuss our results in relationship to the findings of other workers with different but closely related compounds.

Experimental Section

Materials. Eel acetylcholinesterase was purchased from Worthington Biochemical Corp. as the lyophilized preparation ECHP 1 JA, nominal specific activity 1076 units/mg, and was used without further treatment or purification. Diethyl phosphorofluoridate (DEFP)[1] was synthesized from diethyl phosphorochloridate (Aldrich) by the method of Saunders and Stacey (1948). *O,O*-Diethyl *S*-[2-(dimethylammonium)ethyl] phosphorothiolate hydrogen oxalate (II) and *O,O*-diethyl *S*-[2-(trimethylammonium)ethyl] phosphorothiolate iodide (I) were gifts from Ayerst Laboratories. *O,O*-Diethyl *O*-[2-(dimethylammonium)ethyl] phosphate hydrogen oxalate (IV) and *O,O*-diethylphosphorylcholine iodide (III) were synthesized by the method of Tammelin (1957) and were recrystallized from methyl acetate. *O,O*-Diethyl *O*-(3,3-dimethylbutyl) phosphate (V) was a gift from Dr. R. D. O'Brien. Choline chloride was recrystallized twice from ethanol. 2-Dimethylaminoethanethiol hydrochloride (Eastman) was recrystallized twice from ethanol under nitrogen gas. Acetylthiocholine iodide was prepared by the reaction of *S*-[2-(dimethylamino)ethyl] thioacetate (Eastman) with methyl iodide in ether and was recrystallized from ethanol. A 10 mM working solution of thiocholine iodide was prepared by the basic hydrolysis of acetylthiocholine iodide under nitrogen followed by neutralization of the excess base with hydrochloric acid. Phenyl acetate was distilled prior to use. All other reagents were analytical grade from various sources. Water was distilled from a quartz still. The buffer used for all enzyme determinations was 0.1 M NaCl–0.01 M phosphate (pH 7.0), 25°.

Enzyme solutions which were maintained for times exceeding a few minutes were stabilized by the addition of 0.5% bovine serum albumin (Sigma).

Enzyme Assays. Unless stated otherwise, acetylcholinesterase activity was determined by the method of Ellman *et al.* (1961) with 1.0 mM acetylthiocholine and 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at pH 7.0 and 25° using 0.05 M phosphate buffer. The development of absorbance *vs.* time was displayed with a recorder.

Rates of Inhibition of Acetylcholinesterase by Phosphorothiolates. Varying amounts of 1×10^{-4} M phosphorothiolate stock solution were added to 1.0 ml of acetylcholinesterase ($\sim 10^{-8}$ M) to give final inhibitor concentrations of $0.5–2.0 \times 10^{-7}$ M corresponding to $[I] \ll K_I$; 10-μl aliquots were assayed for residual enzyme activity after various incubation times by dilution into 3.0 ml of the assay solution in glass cuvets. The increase in absorbance at 412 nm with time was followed spectrophotometrically. This technique with eq 5 yields the pseudo-first-order rate constants from which the second-order rate constants were calculated.

Resolution of Kinetic Parameters K_I, k_i^0, for the Inhibition of Acetylcholinesterase by Phosphorothiolates. The use of the rapid mixer-sampler has been described previously (Reid and Wilson, 1971). To start the inhibition reaction, 0.5-ml volumes of inhibitor (0.1–0.008 M) and enzyme ($\sim 10^{-7}$ M) solutions were forced from syringes in 1.00 sec by a constant-speed motor and slip clutch assembly. The solutions were mixed in an eight-jet chamber (4 μl volume) and passed through a stainless steel capillary, which functioned as the reaction vessel. Incubation times were varied from 16 to 70 msec by using capillaries of appropriate lengths. The reaction was quenched by having the outflow from the reaction capillary discharge at high velocity into a rapidly stirred solution of 100 ml of 5 mM acetylthiocholine in the assay medium; 3 ml of the quenched solution was quickly withdrawn and assayed for residual acetylcholinesterase activity by the Ellman method. Slightly nonlinear recorder traces were obtained at very high inhibitor concentrations despite the large dilution and high substrate concentration because there was still a sufficiently high inhibitor concentration to produce slow inhibition during the assay. In these instances a correction was made to obtain the enzyme activity at the time of dilution (Main, 1967).

Phosphorylation of Acetylcholinesterase by Diethylphosphorylcholine Iodide. Aliquots of 0.1 M compound III (after various recrystallizations and/or base treatments) and $\sim 10^{-8}$ M enzyme (10 μl) were assayed for enzyme activity by addition to 3.0 ml of the assay medium after varying incubation times.

Reactivation of Diethylphosphorylacetylcholinesterase by the Thiols. Since thiocholine iodide and 2-dimethylaminoethanethiol are very sensitive to oxidation, all thiol solutions were prepared in a nitrogen atmosphere from nitrogen-purged buffers, and exhaustive precautions were taken to exclude air during all stages of the experiment. The required volume of acetylcholinesterase ($\sim 10^{-6}$ M) was phosphorylated by the addition of a sufficient volume of freshly prepared diethyl phosphorofluoridate solution (DEFP) (10^{-5} M in buffer) to give a final DEFP concentration of 2×10^{-6} M. After incubation for 0.5–1 hr at room temperature, 10-μl portions of the inhibited enzyme were added to 10 ml of each reactivation mixture ($10^{-3}–10^{-2}$ M thiol) in polypropylene test tubes that were fitted with serum stoppers. Aliquots of this solution were assayed for acetylcholinesterase activity by addition to 2.9 ml of phenyl acetate solution (1.6 mM in pH 7 buffer) in quartz cuvets. The change in absorbance at 269.5 nm with time was followed spectrophotometrically. Since thiols catalyze the hydrolysis of phenyl acetates (Whitaker, 1962) small corrections were made to the observed rates, as indicated by controls. The thiol content of solutions of reactivators and inhibitors was checked by reacting an aliquot with 0.3 mM DTNB (Ellman's reagent) and determining the absorbance at 412 nm. The molar absorb-

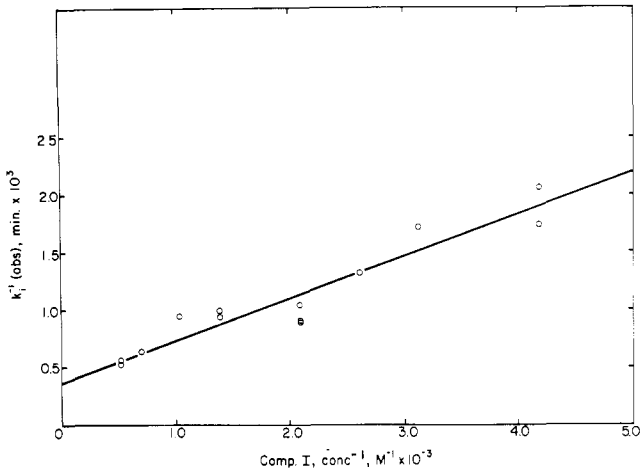

FIGURE 1: Phosphorylation of acetylcholinesterase by *O,O*-diethyl *S*-[2-(trimethylammonium)ethyl] phosphorothiolate iodide at high inhibitor concentrations, pH 7.0, 25°, μ = 0.1. Rapid mixer-sampler data in accordance with eq 8. Results are reported in Table I.

ance index for 5-thio-2-nitrobenzoate under our assay conditions was found to be 1.40×10^4 M^{-1} cm^{-1}.

Reactivation of Diethylphosphorylacetylcholinesterase by Choline and 2-Dimethylaminoethanol. Since reactivation half-times for these alcohols were found to be much longer than for the corresponding thiols, more extensive dilutions of the DEP enzyme into the reactivation solutions were made to eliminate any possibility of further phosphorylation by residual DEFP. Aliquots of the DEFP-inhibited acetylcholinesterase (10 μl) described above were added to 60 ml of buffer (0.05% bovine serum albumin), and 10 ml of this dilution was quickly mixed with 10 ml of the appropriate concentration of reactivator to start the reactivation. Samples of 2.8-ml volume were removed periodically and were assayed by the addition of 30 μl of 0.1 M acetylthiocholine and 0.1 ml of 10 mM DTNB. Reactivator concentrations varied from 10^{-3} to 10^{-2} M for choline and from 6.7×10^{-3} to 0.5 M for 2-dimethylaminoethanol. In all reactivation studies the maximum recoverable activity was checked by using 10^{-4} M pyridine-2-carbaldoxime methiodide as a reactant. We also used controls for the spontaneous reactivation of DEP enzyme.

Reversible Inhibition of Acetylcholinesterase by Dihylphosphorylcholine and Diethyl 2-(Dimethylaminoethyl)Phosphate. Following various treatments to remove traces of contaminants we observed no phosphorylation of the enzyme with compounds III and IV. We obtained the kinetic parameters for reversible inhibition of acetylcholinesterase by these compounds using acetylthiocholine as substrate. The concentration of substrate was varied from 5×10^{-5} to 1.0×10^{-3} M at several fixed inhibitor concentrations, ranging from 1.0×10^{-3} to 1.0×10^{-2} M for compound III and from 2.0×10^{-3} to 1.5×10^{-2} M for compound IV.

Results

Phosphorylation of Acetylcholinesterase by Compounds I and II. Using low concentrations of inhibitor, from (0.5 to 2.0) $\times 10^{-7}$ M, [I] $\ll K_I$, where inhibition is slow, we find that the reaction is second order for both compounds I and II. This is shown by the fact that values of k_i(obsd) for different concentrations of inhibitor, obtained according to eq 5, are proportional to the concentration of inhibitor; plots of k(obsd) *vs.* [I] are linear and pass through the origin. The second-order rate constants are $k_i = 2.6 \times 10^6$ M^{-1} min^{-1} for compound I and $k_i = 1.5 \times 10^6$ M^{-1} min^{-1} for compound II.

TABLE I: Reaction Parameters for the Phosphorylation of Eel Acetylcholinesterase at pH 7.0, 25°, μ = 0.1.

Compound	K_I, M $\times 10^3$	k_i^0, min^{-1}	k_i, M^{-1} min^{-1}
Phosphorothiolates[a]			
I	1.0	2.7×10^3	2.7×10^6
II	1.0	1.8×10^3	1.8×10^6
Phosphates[b]			
III	2.3	$<1.5 \times 10^{-4}$ [c]	$<7 \times 10^{-2}$
IV	4.4	$<1.5 \times 10^{-4}$ [c]	$<4 \times 10^{-2}$
V	11		$<2 \times 10^{-1}$

[a] The data for the phosphorothiolates were obtained at high concentrations using the rapid mixer-sampler. These k_i values agree with the values obtained by ordinary techniques at low inhibitor concentrations, $k_i = 2.6 \times 10^6$ and 1.5×10^6, respectively. [b] No measurable progressive inhibition (phosphorylation) was observed with these compounds. The values of K_I were obtained from reversible inhibition of acetylthiocholine hydrolysis. [c] These values are the maximum rates that might have escaped observation. They correspond to 1% inhibition/hr.

The values of k_i(obsd) obtained at much higher inhibitor concentrations using the rapid mixer-sampler are not proportional to [I] and were plotted as reciprocals according to eq 8, as illustrated in Figure 1 for compound I. Straight lines were obtained for compounds I and II. These lines do not pass through the origin and therefore indicate that a reversible complex is formed. The slopes of these lines, the reciprocals of the second-order rate constants, yielded values for these constants (Table I) that are in good agreement with those obtained from the measurements using ordinary techniques at low inhibitor concentrations. For both phosphorothiolates we were able to employ concentrations of inhibitor both above and below measured dissociation constants, ensuring that a significant fraction of the active enzyme was indeed reversibly complexed with inhibitor in the concentration range studied. In the double reciprocal plot, this means that the extrapolation from the points corresponding to the highest inhibitor concentrations to the y intercept, and consequently the uncertainties for k_i^0 and K_I, are minimized.

Reactivation of Diethylphosphorylacetylcholinesterase. The reactivation process is considered to follow eq 2 and eq 7 and the second-order rate constant corresponding to [R] $\ll K_R$ is given by

$$k_r = k_r^0/K_R \qquad (10)$$

Dephosphorylation of acetylcholinesterase is a relatively slow process for the reactivators used in this study; hence, it is not feasible to measure k_r directly by using very low concentrations of reactivator. Instead we used eq 9.

Pseudo-first-order plots (eq 7) for the reactivation of diethylphosphory acetylcholinesterase by the thiols were linear up to about 1–2-hr incubation, after which a slow decrease in slope was observed. For example, on one line a recovery of 74% was expected at 1.5 hr on the basis of earlier points but only 70% was observed. We therefore used the strictly linear portion of the plots for evaluating k_r(obsd). Secondary plots of k_r^{-1}(obsd) *vs.* [R]$^{-1}$ made in accordance with eq 9 (actually eq 11, see later) were linear and did not pass through the origins, which indicates that a reversible complex is formed in

TABLE II: Reaction Parameters for the Reactivation of Diethylphosphorylacetylcholinesterase, pH 7.0, 25°, μ = 0.1.

Reactivator	K_R, M × 10^3	k_r^0, min^{-1}	k_r, M^{-1} min^{-1}
Thiocholine	9.0	1.5×10^{-2}	1.7
Dimethylaminoethanethiol	13	1.3×10^{-2}	1.0
Choline	67	0.24×10^{-2}	0.036
Dimethylaminoethanol	11[a]	2.1×10^{-4}	0.020
H_2O		2.3×10^{-4} [b]	

[a] From inhibition of the reactivation of DEP enzyme by pyridine-2-carbaldoxime methiodide. [b] The observed first-order rate constant for spontaneous reactivation. No reactivation was observed with 3,3-dimethylbutanol.

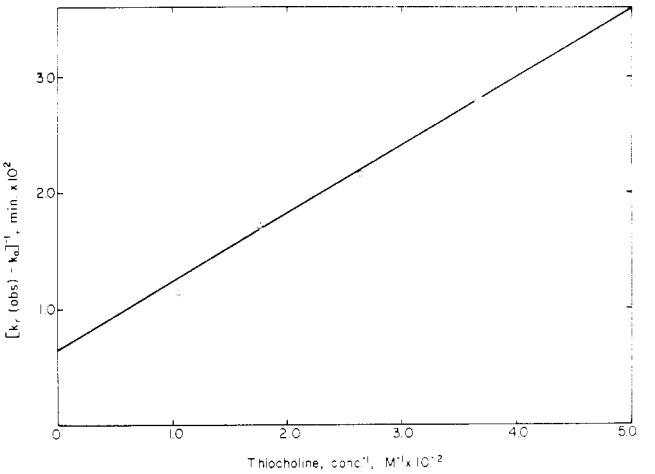

FIGURE 2: Reactivation of diethylphosphorylacetylcholinesterase by thiocholine iodide, pH 7.0, 25 °, μ = 0.1 (eq 9). Results are reported in Table II.

both cases. The values for the reactivation constants obtained from these plots are listed in Table II. The results obtained with thiocholine are shown in Figure 2.

The reactivation of diethylphosphorylacetylcholinesterase by choline has been described previously (Wilson, 1952). This determination was repeated to allow evaluation of the reactivation constants under the same conditions employed in the other aspects of this work. The values obtained for k_r^0 and K_R are listed in Table II.

It has been reported that 2-dimethylaminoethanol is not a reactivator of diethylphosphorylacetylcholinesterase (Wilson, 1952). In confirmation we found that the observed recovery rate was about the same as that found in the absence of reactivator even with concentrations as high as 0.5 M 2-dimethylaminoethanol.

DEP enzyme recovers spontaneously (water is the nucleophile) in a first-order process at the slow rate of $k_0 = 2.2 \times 10^{-4}$ min^{-1}. This rate can be significant relative to the rate of reactivation obtained with very slow reactivators.

Correcting for spontaneous reactivation merely involves substituting $k_{obsd} - k_0$ for k_{obsd} and $k_r^0 - k_0$ for k_r^0 in eq 9 to yield

$$\frac{1}{k_r(\text{obsd}) - k_0} = \frac{1}{k_r^0 - k_0} + \frac{K_R}{k_r^0 - k_0}\frac{1}{[R]} \quad (11)$$

However a question arises concerning the interpretation of k_r^0.

We have used the symbol k_r^0 as indicated in eq 2 but in actuality k_r^0 determined by eq 11 above will also contain the constant for spontaneous reactivation of the complex, if it is not zero. The problem is, "Shall we assume that the complex between DEP enzyme and a reactivator can recover spontaneously as well as by reaction with the reactivator or shall we assume that it can recover only by reaction with the reactivator?" This question is not important when the rate of reactivation by the nucleophile is large compared to the rate of spontaneous reactivation. In our work, except for reactivation by dimethylaminoethanol, this question affects the value of k_r^0 only for choline. In our calculations we assumed that the complex between reactivator and DEP enzyme was subject to little or no spontaneous reactivation. Evidence that substituted amines can substantially reduce spontaneous reactivation is furnished by the observations that these ions can prevent reactivation of DEP enzyme by hydroxylamine and other nucleophiles and also can almost completely abolish the spontaneous recovery of carbamyl enzyme derivatives. In an incomplete study we have shown that tetraethylammonium ion, trimethylammonium ion, and di-*n*-propylmethylammonium ion at 0.1 M concentrations do substantially reduce spontaneous reactivation of DEP enzyme (>50% reduction for the third).

Using dimethylaminoethanol as a reactivator in the concentration range 0.05–0.5 M we found that the rate of recovery was essentially unchanged from the rate of spontaneous reactivation. One possible explanation is that dimethylaminoethanol is unable to bind to the DEP enzyme. This explanation seemed unlikely because this compound inhibits substrate hydrolysis by inhibiting both acetylation and deacetylation. Moreover choline and the two related thiols do bind to the DEP enzyme. Nonetheless, we took the trouble to demonstrate the binding of dimethylaminoethanol by showing that it inhibits the reactivation of the DEP enzyme by 5×10^{-6} M pyridine-2-carbaldoxime methiodide to an extent which depends upon its concentration and approaches complete inhibition. In this way we obtained a dissociation constant of 0.011 M for the dimethylaminoethanol–DEP enzyme complex.

The most probable situation is that the binding of dimethylaminoethanol substantially reduces the rate of spontaneous reactivation of DEP enzyme. Therefore we conclude that the observed rate of recovery in the presence of dimethylaminoethanol is largely due to dimethylaminoethanol acting as a nucleophile.

Inhibition of Acetylcholinesterase by Diethyl Phosphate Oxygen Esters, III and IV. Diethyl (choline iodide) phosphate obtained by us using the method of Tammelin (1957) showed significant phosphorylating activity when added to acetylcholinesterase; however, this anticholinesterase activity decayed under the conditions of assay (pH 7.0, 25°) with a half-time of 25 min. Since this high rate of deactivation at neutral pH was completely inconsistent with the slow rate of hydrolysis of this ester (Tammelin, 1958), it was deemed likely that most, if not all, of the antiacetylcholinesterase activity of our preparation was due to a contaminant. Two more recrystallizations yielded a product that barely inhibited the enzyme. Treatment with 0.04 M NaOH for 10 min completely eliminated progressive inhibition of the enzyme, supporting this conclusion. No decrease of pH of a 0.10 M solution of III was observed under these conditions, indicating that very little hydrolysis of the compound had occurred. Very little hydrolysis would be expected from the published rate constants (Tammelin, 1958). The melting points of the crude material and the second and third recrystallization products were all 98–99° (uncorrected) (lit. 100° (Tammelin, 1957)), and the nuclear magnetic reso-

nance spectrum of each sample was consistent with that anticipated from the structure of III.

III was found to be a competitive inhibitor for the hydrolysis of acetylthiocholine by acetylcholinesterase. Linear Lineweaver–Burk plots were obtained using five concentrations of inhibitor and five concentrations of substrate. The dissociation constants reported in Table III were determined from secondary plots of the slopes and intercepts of the double reciprocal plots *vs.* [I] using the relationship

$$\frac{1}{v} = \frac{1}{k\mathrm{E}^0}\left[1 + \frac{(I)}{K_\mathrm{I}'\left(1 + \frac{k_4}{k_3}\right)}\right] + \frac{K_\mathrm{m}}{k\mathrm{E}^0}\left[1 + \frac{[I]}{K_\mathrm{I}}\right]\frac{1}{[\mathrm{S}]} \quad (12)$$

where K_I is the dissociation constant for the enzyme–inhibitor complex, K_I' is the dissociation constant for the acetyl-enzyme–inhibitor complex, k_3 is the rate constant for acetylation, and k_4 for deacetylation, and the other terms have their usual meaning (Krupka and Laidler, 1961; Wilson and Alexander, 1962). The K_m for acetylthiocholine was found to be 6.7×10^{-5} M under the conditions of the experiments. The value of k_4 is substantially smaller than k_3.

The K_I for *O,O*-diethyl *O*-[2-(dimethylamino)ethyl] phosphate hydrogen oxalate was determined in essentially the same manner. A 0.02 M solution of the ester was brought to pH 12, held for 15 min at room temperature (again no pH change was observed), and then returned to pH 7.0 with HCl. After this treatment no progressive inhibition of acetylcholinesterase was observed. However, this compound is a weak reversible inhibitor. Linear Lineweaver–Burk plots were also obtained using this preparation with the substrate acetylthiocholine. Although there was evidence for noncompetitive inhibition as indicated by small increases in the intercept with increasing concentration of IV, the ester was found to be a predominantly competitive inhibitor of acetylcholinesterase with a K_I of 4.4 mM.

Similar results were obtained with the carbon analog of compound III, diethyl (3,3-dimethylbutyl) phosphate (V). The phosphate at first showed some phosphorylating ability but again after brief treatment with dilute alkali, no progressive inhibition could be obtained. The carbon analog of choline (3,3-dimethylbutanol) used as a reactivator did not produce measurable reactivation. Again both compounds, the ester and the alcohol, were found to be reversible inhibitors. As anticipated there is a sizable difference in the binding of 3,3-dimethylbutanol and choline (Table III), a difference that can be accounted for on the basis of an anionic subsite in the active site of the enzyme.

The phosphorothiolates, I and II, are so active that it is unlikely that the observed activity could be caused by an impurity since an impurity present to the extent of 1% would have to have the enormous value for k_i of 10^8. Tetraethyl pyrophosphate, which is a likely candidate for the impurity in compounds III, IV, and V, has a k_i of 10^6. Even so we checked the activity of compound I by subjecting it to alkaline hydrolysis of pH 10 and other pH at 25° and 0.15 ionic strength using a pH Stat, and noting that the rate of decrease of its activity as an inhibitor accurately coincided with its rate of hydrolysis. We found $k_{\mathrm{OH}^-} = 44\ \mathrm{M}^{-1}\ \mathrm{min}^{-1}$ which is in agreement with the published value (Tammelin, 1958).

TABLE III: Dissociation Constants for Complexes of Acetylcholinesterase with Phosphorothiolates, Phosphates, and Leaving Groups, pH 7.0, 25°, $\mu = 0.1$.

Compounds	K_I, M × 10^3	K_I'',[b] M × 10^3
Phosphorothiolates		
I	1.0[a]	
II	1.0[a]	
Phosphates		
III	2.3	
IV	4.4	50
V	11	65
Leaving groups		
Thiocholine	0.11	1.0
Dimethylaminoethanethiol	0.15	
Choline	0.41	3.7
Dimethylaminoethanol	2.1	2.8
3,3-Dimethylbutanol	7.5	55

[a] These values were obtained with the rapid mixer-sampler. [b] K_I'' is the apparent constant for binding to the acetyl enzyme intermediate, $K_\mathrm{I}'(1 + k_4/k_3)$ in eq 12, and is obtained from the variation of the intercept in a Lineweaver–Burk plot.

Discussion

We found no measurable progressive inhibition of acetylcholinesterase by the phosphate esters III, IV, and V once they had been treated with alkali. This technique to remove traces of a potent inhibitor from relatively alkali stable compounds has been used previously (Aldridge and Davison, 1952). By contrast the phosphorothiolate esters I and II are very potent progressive inhibitors. All five compounds reversibly bind to the enzyme and the phosphate esters accordingly are weak reversible competitive inhibitors. It was previously recognized that compound III might be a reversible inhibitor (Tammelin, 1958).

Chiu and Dauterman (1970) found that the reaction of a compound similar to our compounds I and II, Tetram, the oxalate salt of Amiton (*O,O*-diethyl *S*-[2-(diethylamino)ethyl] phosphorothiolate), with bovine erythrocyte acetylcholinesterase was described by the kinetic parameters $K_\mathrm{I} = 1.8 \times 10^{-4}$ M, $k_\mathrm{i}^0 = 126\ \mathrm{min}^{-1}$, and $k_\mathrm{i} = 7 \times 10^5$ at 5°, pH 7.6. Their results are fairly similar to our findings with compounds I and II, but, of course, their enzyme and their conditions are different. However, usually there is not a large difference in k_i between the two enzymes and this seems to be true in this case also. On the other hand, Aharoni and O'Brien (1968) found very different values with Amiton and bovine erythrocyte acetylcholinesterase at 25°, pH 7.4. They obtained $K_\mathrm{I} = 7.2 \times 10^{-6}$ M and $k_\mathrm{i}^0 = 6.7\ \mathrm{min}^{-1}$. These data are cited in support of the argument that the "thiolo effect" derives from the greater affinity of phosphorothiolates as compared to the affinity of phosphates for acetylcholinesterase. Our data show that this is not the case for enzyme obtained from electric eel, since the dissociation constants, K_I, are about the same for all four compounds, phosphorothiolates I and II and phosphates III and IV. We can explain the "thiolo effect" in terms of previous observations which show that k_i is extremely dependent upon a good leaving group for leaving groups with $pK_\mathrm{a} > 7$ (Ashani *et al.*, 1972; Aldridge and Davison, 1952). The pK_a of thiocholine is about 7.7 (Heilbronn, 1958) and that of 2-dimethylaminoethanethiol (SH) can be assumed to be about the same as that of 2-diethylaminoethanethiol, 7.9 (Bracha and O'Brien, 1968), whereas the pK_a of choline is about 13.9 and the pK_a of the cation of 2-

TABLE IV: Equilibrium Constants and Free Energies of Hydrolysis of Various Organophosphates.

Compound	pK_a of Leaving Group	K(phosphorylation)[b] (anal. conc)	ECH[a] (anal. conc)	ECH (acid. sp.)	$\Delta F°'$(KpM) (anal. conc)	$\Delta F°$ (acid. sp.)
I	7.7	1.5×10^6	8.1×10^{16}	1.6×10^{11}	−23	−15
II	7.9	1.5×10^6	8.0×10^{16}	1.7×10^{11}	−23	−15
III	13.9	<2	$<1 \times 10^{11}$	$<2 \times 10^5$	>−15	>−7
IV	14	<2	$<1 \times 10^{11}$	$<2 \times 10^5$	>−15	>−7
DEFP[c]	3.5	2.3×10^4	1.2×10^{15}	8.3×10^5	−21	−8
Paraoxon[c]	7.1	1.3×10^4	6.7×10^{14}	7.4×10^8	−20	−12
$(EtO)_2PO$–En			5.3×10^{10}		−15	

[a] pH 7.0. [b] Equilibrium constant for the reaction with the enzyme evaluated as k_i/k_r. [c] Froede and Wilson (1973).

dimethylaminoethanol (OH) can be assumed to be about the same. Thus the leaving groups of the two classes of compounds differ in pK_a by 6 pK_a units and we can expect that there will be an enormous difference in the rates of inhibition. Extrapolation of the log k_i–pK_a linear locus covering the pK_a range 7–10 to pK_a = 14 suggests that k_i for the phosphate esters will be about 10^{-6} M^{-1} min^{-1}, if there is no rate enhancement arising from molecular complementarity. Even though the extrapolation is "long" and is based upon aryl hydroxyl groups rather than alkyl hydroxyl groups, there is a factor of 10^4 between the estimated rate constant and the smallest rate constant that we would have been able to discern (based on a rate of inhibition of 1%/hr) and therefore it is safe to explain the inertness of the phosphate esters by the high pK_a values of the leaving groups.

The thiols are much better reactivators than the alcohols, even though ionized thiols are poorer nucleophiles toward phosphorus than ionized hydroxyl, possibly because at pH 7 there is 10^6 times as much ionized thiol. However, we do not know whether the ionized or un-ionized group is the actual nucleophile in this enzymic reaction or even whether the active species may not depend upon the particular nucleophile. It is possible, for example, that in the case of the thiols it is the ionized group and in the case of the alcohols the un-ionized group. If it should be the ionized hydroxyl group of choline that is the actual nucleophile, the rate constant would have the robust value of 3×10^5 M^{-1} min^{-1} for that species of reactant (all our rate constants are given for analytical concentrations at pH 7.0 and not for species concentrations). Simple nucleophiles usually react 1–5 times poorer with DEP enzyme than they do with sarin (isopropyl methyl phosphonofluoridate) or somewhat faster than they do with diethyl phosphorofluoridate. On this basis of comparison the two thiols and choline are much better reactivators than would have been anticipated, which probably reflects the expected molecular complementarity between these compounds and the enzyme.

We note that the alcohols and thiols are bound more poorly by DEP enzyme than by free enzyme or acetyl enzyme. Similarly the diethyl phosphate esters are more poorly bound to the free enzyme than the alcohols and thiols from which they are derived. These findings are in general agreement with previous observations that DEP enzyme does not accommodate substituted amines as readily as free enzyme.

We can calculate the equilibrium constants for the hydrolysis, ECH, of the phosphorothiolates from the data we have at hand. The second-order rate constants k_i and k_r have been obtained in this study and their ratio gives the equilibrium constant for the phosphorylation reaction (Table IV). Since ECH (DEP enzyme) is known, and since the product of the equilibrium constants in any cycle of reactions is equal to 1, ECH (I) and ECH (II) can be calculated from eq 1. Since we can set only upper limits for k_i (III) and k_i (IV) we can set only upper limits for ECH (III) and ECH (IV). These values are given in Table IV and we see that the phosphorothiolates are far less stable thermodynamically than the phosphates. They are considerably less stable than paraoxon or DEFP even though the latter compounds have more acidic leaving groups. This comparison is more pronounced in the ECH values calculated in terms of the acidic species, and suggests that the P–S linkage might be considered a "high-energy bond" or perhaps it would be better to say that these compounds have a high group transfer potential.

In this work we have used the general observation that there is a very strong dependence of phosphorylation rate on the pK_a of the conjugate acid of the leaving group, for p$K_a \geq 7$, to explain the vast superiority of two particular phosphorothiolates (I and II) over the analogous phosphates in phosphorylating acetylcholinesterase. Since these pK_a values differ by 6 units, a very large difference is expected. We have not answered a somewhat different and more difficult question. "Will phosphorothiolates be better inhibitors than phosphates having leaving groups with the same pK_a?" Compound I is a very good inhibitor and better than would be expected for a phosphate ester with a leaving group having a pK_a of 8. But in this instance another explanation is readily available. The structure of the leaving group (thiocholine) is especially appropriate for this enzyme so that molecular complementarity promotes thiocholine as a leaving group. On the other hand, molecular complementarity in diethylphosphorylcholine is not sufficient to overcome the burden of an intrinsically poor leaving group. This is in sharp contrast to substrates (acetate esters) where the pK_a of the leaving group is unimportant when molecular complementarity is involved, as in acetylcholine and acetylthiocholine hydrolysis.

References

Aharoni, A. H., and O'Brien, R. D. (1968), *Biochemistry 7,* 1538.

Aldridge, W. N., and Davison, A. N. (1952), *Biochem. J. 51,* 62.

Ashani, Y., Wins, P., and Wilson, I. B. (1972), *Biochim. Biophys. Acta 284,* 427.

Bracha, P., and O'Brien, R. D. (1968), *Biochemistry 7,* 1545.

Bracha, P., and O'Brien, R. D. (1970), *Biochemistry 9,* 741.

Chiu, Y. C., and Dauterman, W. C. (1970), *Biochem. Pharmacol. 19,* 1857.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Feather-

stone, R. M. (1961), *Biochem. Pharmacol. 7,* 88.
Froede, H. C., and Wilson, I. B. (1973), *J. Amer. Chem. Soc. 95,* 1987.
Froede, H. C., and Wilson, I. B. (1974), *Mol. Pharmacol.* (in press).
Fukuto, T. R. (1957), *Advan. Pest. Control Res. 1,* 147.
Heilbronn, E. (1958), *Acta Chem. Scand. 12,* 1492.
Krupka, R. M., and Laidler, K. J. (1961), *J. Amer. Chem. Soc. 83,* 1445.
Main, A. R. (1967), *Proc. Conf. Struct. React. DFP Sensitive Enzymes, 1966,* 129.
Reid, T. W., and Wilson, I. B. (1971), *Biochemistry 10,* 380.
Saunders, B. C., and Stacey, G. J. (1948), *J. Chem. Soc.,* 695.
Shefter, E., and Mautner, H. G. (1969), *Proc. Nat. Acad. Sci. U. S. 63,* 1253.
Tammelin, L.-E. (1957), *Acta Chem. Scand. 11,* 1340.
Tammelin, L.-E. (1958), *Ark. Kemi 12,* 287.
Whitaker, J. R. (1962), *J. Amer. Chem. Soc. 84,* 1900.
Wilson, I. B. (1952), *J. Biol. Chem. 199,* 113.
Wilson, I. B., and Alexander, J. (1962), *J. Biol. Chem. 237,* 1323.

Effects of Phosphate on the Dissociation and Enzymic Stability of Rabbit Muscle Lactate Dehydrogenase†

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ABSTRACT: Frontal gel chromatography and sedimentation studies have been used to establish that rabbit muscle lactate dehydrogenase dissociates essentially completely into dimeric species (molecular weight ~70,000) in 0.2 *I* acetate–chloride (pH 5.0). Tetramer–dimer conversion, which occurred within a minute of adjusting the pH of an enzyme solution from 7 to 5, was reversed by restoring the solution to neutral pH, provided that exposure to the acidic environment was restricted to less than 4 hr at 4°. However, despite this re-formation of tetramer, some irreversible changes in enzyme structure were indicated by the recovery of only 70% of the original activity after exposure of the enzyme to pH 5 for 4 hr. Inclusion of phosphate (>30 mM) in the acetate–chloride solutions of rabbit muscle lactate dehydrogenase prevented any detectable dissociation into dimer; this increased stability of tetramer is allied with essentially complete retention of enzymic activity. Similar stabilizing effects were observed with lactate dehydrogenase solutions in which the phosphate was replaced by either reduced or oxidized nicotinamide adenine dinucleotide. Kinetic studies at pH 7 have established a pronounced activating effect of phosphate on the enzymic conversion of lactate to pyruvate. Under the same conditions the lactate dehydrogenase was shown to be tetrameric in the presence and absence of phosphate. Thus, although phosphate shares with the pyridine nucleotides the ability to stabilize the quaternary structure of rabbit muscle lactate dehydrogenase, the specific nucleotide-binding sites are not involved in these phosphate interactions unless phosphate and coenzyme can be accommodated simultaneously on the same sites.

In studies of the dissociation of lactate dehydrogenase (EC 1.1.1.27) by either sodium dodecyl sulfate or urea, Di Sabato and Kaplan (1964, 1965) observed that inclusion of pyridine nucleotides or phosphate in the dissociating medium led to increased recovery of enzymic activity on removal of the denaturant. Furthermore, some material with the sedimentation coefficient of tetrameric (native) LDH[1] was observed in the denatured samples that exhibited pronounced protection of enzymic activity (Di Sabato and Kaplan, 1964). Although these studies suggested a probable link between the effects of pyridine nucleotides and phosphate on the dissociation and enzymic stability of LDH, the situation is clouded by the relatively poor recovery of activity (<50%) and by the known tendency for denatured proteins to aggregate. More definitive evidence for the protective role(s) of pyridine nucleotides and phosphate clearly requires the selection of milder conditions for disruption of the quaternary structure of LDH. For this purpose the use of neutral buffers of moderate to high ionic strength (Millar, 1962; Hathaway and Criddle, 1966; Griffin and Criddle, 1970; Bartholmes *et al.,* 1973) or of acidic conditions (Deal *et al.,* 1963; Anderson and Weber, 1966; Jaenicke and Knof, 1968) seems plausible. Under the former conditions a rapidly established dimer–tetramer equilibrium is believed to operate, but there are also reports that dispute not only the rate of the dissociation reaction (Cho and Swaisgood, 1973) but also its existence (Wieland *et al.,* 1963; Jaenicke and Knof, 1968; Anderson, 1969; Mire, 1969). Because of the seemingly variable dissociation behavior of different LDH preparations at neutral pH, we have explored the acidic region for suitable conditions.

In previous studies a pH of 2.0–2.6 has been used for disruption of the tetrameric structure of LDH (Deal *et al.,* 1963; Anderson and Weber, 1966; Jaenicke and Knof, 1968). However, we have found essentially complete dissociation of rabbit muscle LDH into dimers in 0.2 *I* acetate–chloride (pH 5.0), conditions that were without effect on the beef heart enzyme. Rabbit muscle LDH has therefore been studied under these milder

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[1] Abbreviations used are: LDH, lactate dehydrogenase; NADH and NAD^+, reduced and oxidized nicotinamide adenine dinucleotide.