Table I are of sufficient reliability to be used in equation 6 to describe the course, over an extended

$$k_3[E]t = K_S(1 + [S]_0/K_{P_1} + [I]/K_I) \ln [S]_0/[S]_t + (1 - K_S/K_{P_1})([S]_0 - [S]_t) \quad (6)$$

range, of the α-chymotrypsin-catalyzed hydrolysis of a number of representative specific substrates of this enzyme in the presence of their D-enantiomorphs. With but one exception we wish to defer discussion of the relationships existing between the K_I values and the structures of the various competitive inhibitors listed in Table I until we have had an opportunity to present revised values for the other competitive inhibitors which have been studied in these laboratories.

From a comparison of the K_I and K_S values of the pairs acetyl-D- and L-tryptophanamide, nicotinyl-D- and L-tryptophanamide, acetyl-D- and L-tyrosinamide, nicotinyl-D- and L-tyrosinamide, acetyl-D- and L-phenylalaninamide and nicotinyl-D- and L-phenylalaninamide and a consideration of molecular models of these compounds and of surfaces which were complementary to certain aspects of these models we were led to the hypothesis that the modes of combination of the D- and L-enantiomorphs with the catalytically active site of the enzyme were substantially the same, and because of the parallel behavior of K_I and K_S in the above series it was believed that K_S could be taken as being approximately equal to k_2/k_1.[7,10,23] Substituting the revised values of K_S and K_I for the original values of the above series does not require us to alter the above conclusion. However, with data now available for the first time for trifluoroacetyl-D- and L-tyrosinamide and chloroacetyl-D- and L-tyrosinamide it is clear that our original conclusion is in error in at least one respect. For the series acetyl-, trifluoroacetyl-, chloroacetyl- and nicotinyl-L-tyrosinamide the values of K_S are in the ratio of 32:26:27:12 whereas the corresponding values of K_I for the D-enantiomorphs are in the ratio of 12:20:6.5:9. Thus, with no tendency for parallel behavior of the K_S and K_I values in this series it is clear that unless one resorts to the undesirable practice of introducing additional *ad hoc* hypotheses it must be concluded that (a) the modes of combination of the D- and L-enantiomorphs are significantly different and that the variation of the K_S and K_I values with the structures of the above specific substrates and competitive inhibitors cannot be used to determine whether K_S does or does not approximate k_2/k_1 or, (b) that the modes of combination of the enantiomorphs are substantially the same and that K_S does not approximate k_2/k_1. Because we have at hand a considerable amount of unpublished data which supports the conclusion that K_S does approximate k_2/k_1 for the specific substrates which have been considered in earlier studies conducted in these laboratories[4] we suggest that the first explanation given above is the correct one.

The authors are indebted to Drs. H. T. Huang and D. W. Thomas for the use of previously unpublished data.

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Reactivation of Human Serum Esterase Inhibited by Alkylphosphates[1]

BY IRWIN B. WILSON

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Human serum esterase inhibited by tetraethyl pyrophosphate or diisopropyl fluorophosphate may be reactivated 50% in 15 minutes in the former case and in 3 hours in the latter instance by suitable hydroxamic acids. Hydroxylamine and pyridine also were active. Quaternary ammonium hydroxamic acids are much better reactivators than the tertiary amino or primary ammonium acids. Thus the distinction between quaternary, tertiary and primary nitrogen groups observed in the enzyme still persists in the inhibited enzyme, indicating that the anionic site of the enzyme still functions to bind and orient the reactivating molecules.

Introduction

Certain phosphate esters such as tetraalkyl pyrophosphates, dialkyl *p*-nitrophenyl phosphates, and dialkyl fluorophosphates are potent irreversible inhibitors of the cholinesterases and esterases in general. The development of the theory of enzymic hydrolysis clarified the mechanism of irreversible inhibition and suggested the means whereby reactivation might be achieved. During the course of the enzymatic hydrolysis of carboxylic esters a basic group in what has been termed the esteratic site of the enzyme makes a nucleophilic attack upon the carbonyl carbon of acetylcholine. An acylated enzyme is formed as an intermediate which reacts rapidly with water to yield carboxylic acid and free enzyme.[2] In the case of the irreversible inhibitors the basic group of the esteratic site is phosphorylated, involving the same nucleophilic substitution mechanism,[3,4] but unlike the acylated enzyme this compound reacts only very slowly with water. As a result the enzyme is converted to a dialkylphosphoryl enzyme which in view of the theory is expected to be without activity. It is the slowness with which the phosphoryl enzyme reacts with water which makes these compounds inhibitors rather than substrates.

The theory suggests that esterase might be

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(2) (a) I. B. Wilson, F. Bergmann and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781 (1950); (b) I. B. Wilson, "The Mechanism of Enzyme Action," ed. by W. D. McElroy and Bentley Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 642.

(3) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950).

(4) I. B. Wilson, *ibid.*, **190**, 111 (1951).

reactivated (dephosphorylated) through bimolecular nucleophilic displacement reactions and this is in fact the case.[4-6] Acetylcholinesterase inhibited by tetraethyl pyrophosphate (TEPP) has been reactivated by a number of compounds containing hydroxylamino, amino, pyridino, guanidino, amidino, hydroxy and mercapto groups. Only certain hydroxamic acids were effective when diisopropyl fluorophosphate (DFP) was the inhibitor. Chymotrypsin inhibited by TEPP also has been reactivated with hydroxylamine.[7]

It appeared appropriate to extend these studies to another type of cholinesterase. It is shown in this paper that the human serum esterase inhibited by TEPP or DFP may be reactivated by suitable nucleophilic reagents.

Methods

The human serum esterase used in this work was a white powder, fraction IV-6-3, obtained from the Department of Physical Chemistry, Harvard Medical School.[8] A stock solution contained 50 mg./ml. in 0.02 *M* phosphate buffer at *p*H 7. Under refrigeration, this solution maintained its activity for several weeks. Enzyme activity was measured at 25°, usually with the equivalent of 0.25 mg. of powder, by the manometric procedure in a medium containing 0.1 *M* NaCl, 0.025 *M* $NaHCO_3$, 0.1% gelatin, 0.01 *M* acetylcholine bromide as substrate and gassed with 5% CO_2–95% N_2 (*p*H 7.4). Substrates other than acetylcholine were used where indicated, and in those cases where poor substrates were used much more enzyme was added.

Stock TEPP inhibited enzyme was prepared by adding 0.01 ml. of TEPP solution (9.5 γ/ml.) to 0.200 ml. of stock enzyme. After remaining overnight in the cold the solution was diluted to 2.0 ml. with 0.05 *M* NaCl, 0.05% gelatin at *p*H 7 and stored under refrigeration. This solution was used for two or three days. Stock DFP solution was prepared similarly, but the DFP solution contained 20 γ/ml. and the inhibition was allowed to proceed for 1 hour at 0° before being diluted to 2.0 ml. This stock was used within an hour.

The reactivations were conducted at 25.0° and usually at *p*H 7.0. The reactivator solutions were made up as 0.25 molar in a medium containing 0.03 *M* ethylenediaminetetraacetate acid and 0.06 *M* phosphate buffer. 0.04 ml. of reactivator solution was added to 0.20 ml. of inhibited enzyme stock. The concentration of reactivator during reactivation was thus 0.042 *M*. EDTA was added to guard against contamination by small amounts of heavy metals. EDTA under these conditions does not significantly reactivate the enzyme. In computing percentage reactivation the small activity (2–7%) of the inhibited enzyme was subtracted from both the activity of the reactivated samples and from the uninhibited enzyme activity.

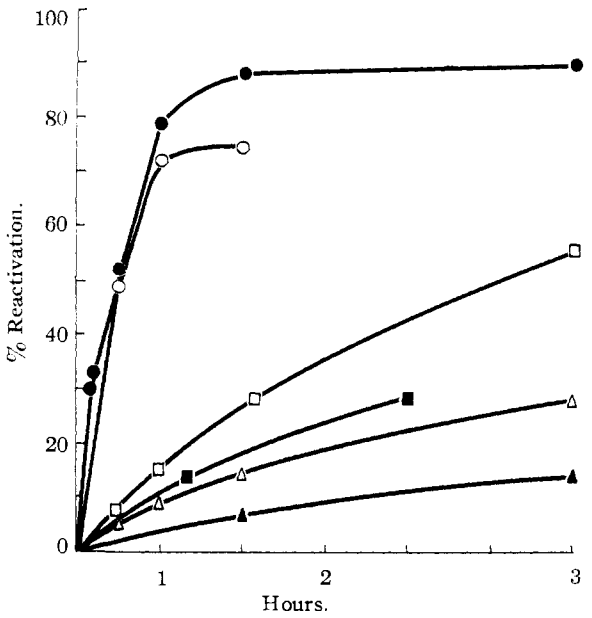


Fig. 1.—Reactivation of TEPP inhibited human serum esterase as a function of time at 25.0° and at *p*H 7. The reactivations are for 0.042 *M* solutions: ●, nicotinhydroxamic acid methiodide; ○, betaine hydroxamic acid (hydrochloride); □, glycine hydroxamic acid; ■, hydroxylamine (hydrochloride); △, nicotinhydroxamic acid; ▲, pyridine.

Results

The properties of the enzyme preparation were characteristic of the serum cholinesterases[9-12]: absence of substrate inhibition, greater activity toward butyrylcholine than acetylcholine, practically no activity toward acetyl-β-methylcholine. The tertiary ester dimethylaminoethyl acetate was hydrolyzed very slowly (Table I).

TABLE I

RATE OF HYDROLYSIS OF DIFFERENT SUBSTRATES BY HUMAN SERUM ESTERASE 25°, *p*H 7.4

The rates are given in micromoles per minute per milligram of enzyme preparation.

Substrates	0.005 *M*	0.01 *M*	0.03 *M*
Acetylcholine (bromide)	0.93	1.08	1.20
Butyrylcholine (tosylate)	2.05	2.20	2.00
Acetyl-β-methylcholine (chloride)	...	0.010	...
Dimethylaminoethyl acetate (hydrobromide)	0.109	0.120	0.185

Reactivation of TEPP Inhibited Enzyme.—Pyridine, hydroxylamine and four hydroxamic acids showed significant reactivating capacity (Fig. 1) at *p*H 7.0 and 25.0°. The two other compounds that were tested, choline and ethyl glycinate, were without activity although both compounds have previously been shown to reactivate TEPP inhibited acetylcholinesterase.[4,5]

Pyridine, nicotinhydroxamic acid and hydroxylamine were very much weaker in reactivating the serum esterase than in reactivating acetylcholinesterase, but the two quaternary hydroxamic acids were about equally active toward the inhibited forms of both enzymes.

If (in the case of nicotinhydroxamic acid methiodide) the initial amount of inhibited enzyme is taken not as 100% but as the maximum amount which is ultimately reactivated (90%), the time course corresponds to first-order kinetics. This observation was not pursued.

The reactivations of the four hydroxamic acids were also compared at *p*H 8.0 (Table II) to offset the large difference in the extent of dissociation which exists at *p*H 7.

(5) I. B. Wilson, *J. Biol. Chem.*, **199**, 113 (1952).
(6) I. B. Wilson and E. K. Meislich, THIS JOURNAL, **75**, 4628 (1953).
(7) L. W. Cunningham, Jr., *J. Biol. Chem.*, **207**, 443 (1954).
(8) We are greatly indebted to Dr. John T. Edsall, Harvard University, for the generous gift of this material.
(9) E. Stedman, E. Stedman and L. H. Easson, *Biochem. J.*, **26**, 2056 (1932).
(10) D. Glick, *J. Biol. Chem.*, **125**, 729 (1938).
(11) D. Nachmansohn and M. A. Rothenberg, *ibid.*, **158**, 653 (1945).
(12) K. B. Augustinsson, *Arch. Biochem.*, **23**, 111 (1949).

TABLE II

REACTIVATION OF TEPP INHIBITED ENZYME AT *p*H 8.0

The reactivations produced by 0.042 *M* solutions at 25.0° are given in percentage. The pK_a's (*p*H of 1/2 neutralized solution at concentration of 0.03 *M*) of the acids at 25° are also included.

	*p*Ka	6′	15′
Nicotinhydroxamic acid methiodide	6.5	31	58
Nicotinhydroxamic acid	8.4	7	11
Betaine hydroxamic acid (hydrochloride)	6.8	..	52
Glycine hydroxamic acid	7.3	..	11

The dependence of reactivation rate on concentration is not linear (Fig. 2) but approaches a zero-order dependence. This is reminiscent of the dependence of enzyme activity with substrate concentration and as in the latter case suggests that a complex may be formed between reactivator and inhibited enzyme.

Reactivation of DFP Inhibited Enzyme.—Only the two quaternary hydroxamic acids and nicotinhydroxamic acid were tested. All reactivated the DFP-inhibited enzyme, but very much more slowly than the TEPP-inhibited enzyme (Table III). Nicotinhydroxamic acid methiodide reactivates DFP-inhibited serum esterase at a faster rate than DFP-inhibited acetylcholinesterase.[6]

TABLE III

REACTIVATION OF DFP INHIBITED ENZYME

The reactivations produced by 0.042 *M* solution at 25° and *p*H 7 (*p*H 8 where indicated) are indicated in percentage.

	1 hr.	3 hr.	25 hr.
Nicotinhydroxamic acid methiodide	48	58	45
Betaine hydroxamic acid (hydrochloride)	35	49	31
Nicotinhydroxamic acid (*p*H 8.0)	14	26	20

If the DFP inhibition is carried out at 25° instead of at 0°, the reactivations which can be obtained are only one-half as great. As with acetylcholinesterase the subsequent reactivation is highly dependent upon the conditions of inhibition. TEPP inhibition does not show this dependence, at least not to any marked extent.

Discussion

As might have been anticipated, human serum cholinesterase can be reactivated from irreversible alkylphosphate inhibition with several of the nucleophilic agents which have been found effective with acetylcholinesterase.[4-6] Like acetylcholinesterase the serum cholinesterase contains an anionic site as well as an esteratic site.[13] The anionic site contributes to the catalytic process by binding and orienting substituted ammonium structures. It was demonstrated with acetylcholinesterase that the anionic site is still functional in the dialkylphosphoryl enzyme (TEPP inhibited enzyme) though less effective in binding ammonium structures than in the normal enzyme. If we assume that the anionic site of the serum enzyme is still active when the esteratic site is phosphorylated, which occurs during inhibition, we should find a specificity pattern in the reactivators

(13) I. B. Wilson, *J. Biol. Chem.*, **208**, 123 (1954).

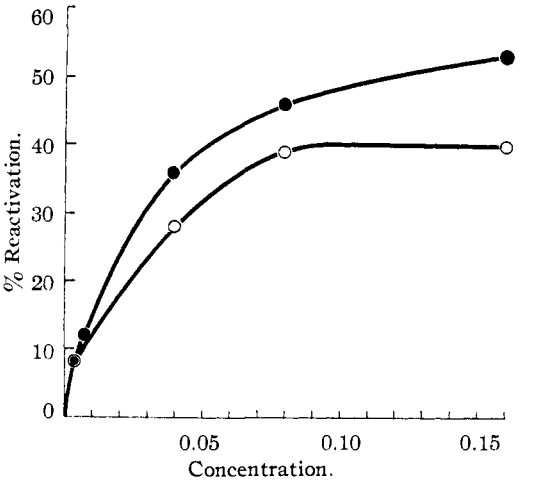


Fig. 2.—Reactivation of TEPP inhibited enzyme at 25.0° and *p*H 7.0 as a function of concentration. The reactivations are for nicotinhydroxamic methiodide (●) in 6 minutes and for nicotinhydroxamic acid (○) in 3 hours.

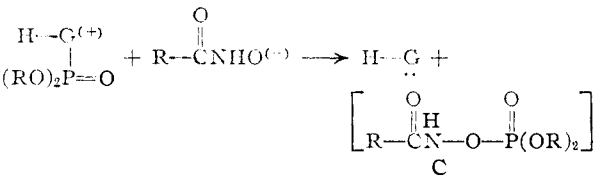
which parallels to a considerable extent the specificity pattern of substrates for the normal enzyme. The comparison of the four hydroxamic acids appears to bear this out since the two quaternary compounds are very much more active than the tertiary nicotinhydroxamic acid and the primary glycine hydroxamic acid. This agrees with the observation that acetylcholine is hydrolyzed much more rapidly than the ammonium ions of dimethylaminoethyl acetate, methylaminoethyl acetate or aminoethyl acetate. Since it is expected from physical organic theory and has been demonstrated that the reactivating species of these compounds are the hydroxamate zwitterions (anion in the case of nicotinhydroxamic acid) and since the acids have different acidities, the comparison was extended to *p*H 8 where in all cases the hydroxamate ion exists in large amounts. Since the base strength, or nucleophilicity toward hydrogen, of the non-quaternary ions is greater, we might expect their nucleophilicity toward phosphorus also to be greater than that of the quaternary anions. That despite this consideration the quaternary hydroxamate ions are the better reagents demonstrates the importance of the specificity characteristics of the anionic site.

The reactivation obtained with the two quaternary hydroxamic acids, which at *p*H 7 are largely hydroxamate ion, increase only slightly when the *p*H is changed from 7 to 8. But the reactivation with the two other hydroxamic acids which at *p*H 7 are largely in the undissociated acid form, increases markedly at *p*H 8. These results are consistent with the proposition that the hydroxamate ions are the reactivators.

The equations for the inhibition and reactivation (illustrated with a hydroxamic acid) are

$$\mathrm{H{-}G + (RO)_2P(=O){-}X \longrightarrow HX + (RO)_2P(=O){-}G}$$

$$\mathrm{(RO)_2P(=O){-}G + H_2O \rightleftharpoons (RO)_2P(=O){-}G^{(+)}H + OH^{(-)}}$$



where H-G represents the esteratic site of the enzyme H being an acidic group and the electron pair (..) representing a basic group, X represents an halogen, dialkylphosphate or *p*-nitrophenyl moiety, etc. In the case of TEPP, R = ethyl and X = diethylphosphate, and in the case of DFP, R = isopropyl and X = fluorine. The resulting inhibited enzymes differ, being in the case of TEPP diethylphosphoryl enzyme and diisopropylphosphoryl enzyme in the case of DFP.

The postulated product C has not been described, but diacetylhydroxylamine, an acyl analog, has been prepared. It is easily hydrolyzed in alkali.[14] If C should be unstable in water and have only a transitory existence, these hydroxamic acids might then be described as catalysts for the hydrolysis of the dialkylphosphoryl enzymes.

The failure to obtain complete reactivation was not investigated since it appeared probable that secondary factors which are not of immediate interest were responsible.

Acknowledgments.—The author wishes to thank Dr. Sara Ginsburg for preparing the hydroxamic acids and to acknowledge the assistance of Miss Carole Quan. He also is pleased to acknowledge indebtedness to Dr. David Nachmansohn for fruitful discussions and for his continuing interest.

(14) R. W. J. Taylor and B. Wilson, "Sidgwick's Organic Chemistry," Clarendon Press, Oxford, 1949.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE COBB CHEMICAL LABORATORY, UNIVERSITY OF VIRGINIA]

Analogs of Nucleotides. I. Theophyllinylalkylphosphonic Acids and Related Compounds[1]

BY JEKISHAN R. PARIKH AND ALFRED BURGER

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ω-(7-Theophyllinyl)-alkylphosphonic acids (I, n = 3, 4, 5) have been prepared in order to explore synthetic routes to purine glycosidylphosphonic acids. In addition to these compounds, several ω-(7-theophyllinyl)-alkanols and the corresponding alkylthiols have been synthesized.

One of the most distinguishing properties of the tumor cell is its ability to glycolyze rapidly under aerobic or anaerobic conditions. Simple nucleotides such as ATP play an important role in this reaction. The same nucleotides also have been recognized as participating in the synthesis of nucleic acids and this in turn is associated with the multiplication of the neoplastic cell. Numerous attempts have been made to interfere with these important biochemical sequences of the tumor cell by altering the structures of the naturally occurring heterocyclic or carbohydrate moieties or both,[2] with the hope that they may act as antimetabolites or form fraudulent nucleotides.[3]

With this in mind, it was decided to prepare for biological screening against neoplastic growth, compounds in which the normal phosphoric acid ester linkage [ROP(O)(OH)$_2$] would be replaced by an analogous phosphonic acid group [RP(O)(OH)$_2$]. Such compounds might be expected to interfere with some biochemical reactions of the tumor cell involving phosphate ester type nucleotides. This report deals with (7-theophyllinyl)-alkylphosphonic acids (I), and analogous compounds which were needed as models for synthetic routes to purine glycosidylphosphonic acids on which we shall report later. Theophylline was chosen as a purine component because of its ready availability, and the unbranched alkyl chain afforded an opportunity to study the introduction of functional groups in the simplest possible manner.

Two routes to (7-theophyllinyl)-alkylphosphonic acids were tried. The first one led from dialkyl ω-halogenoalkylphosphonates and silver theophylline to the desired phosphonate esters. The second method, shown in Scheme 1 (Th = 7-theophyllinyl), consisted of the conversion of ω-(7-theophyllinyl)-alkyl iodides to the corresponding phosphonate esters by the Michaelis–Arbuzov reaction.[4] The latter procedure proved to be more fruitful. The esters were hydrolyzed to the free phosphonic acids I; the derivatives with n = 3, 4 and 5 have been prepared.

$$\text{Th-Ag} + \text{I(CH}_2)_n\text{Cl} \longrightarrow \text{Th(CH}_2)_n\text{Cl} \longrightarrow \text{Th(CH}_2)_n\text{I} \longrightarrow \text{Th(CH}_2)_n\text{P(O)(OC}_2\text{H}_5)_2 \longrightarrow \underset{\text{I}}{\text{Th(CH}_2)_n\text{P(O)(OH)}_2}$$

Scheme 1

A threefold excess of the chloroalkyl iodides was necessary to obtain optimum yields of the ω-(7-theophyllinyl)-alkyl chlorides and suppress the simultaneous formation of ω-bis-(7-theophyllinyl)-alkanes.

Since 4-(7-theophyllinyl)-butyl iodide and its next higher alkyl homolog had become readily available, these compounds also were converted to the corresponding ω-(7-theophyllinyl)-alkanols and alkylthiols. The alkanols were obtained by

(1) This work was supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

(2) G. B. Brown, *Texas Rep. Biol. Med.*, **10**, 961 (1952).

(3) H. E. Skipper, *Cancer Research*, **13**, 545 (1953).

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