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CHARACTERIZATION BY NMR AND FLUORESCENCE SPECTROSCOPY OF
DIFFERENCES IN THE CONFORMATION OF NON-AGED AND AGED
ORGANOPHOSPHORYL CONJUGATES OF AChE

MIDTERM REPORT

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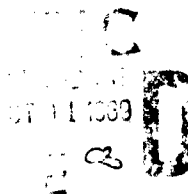
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<p>The objective of this project is to detect and characterize, by NMR and fluorescence spectroscopy, differences in the conformation of non-aged and aged organophosphoryl (OP) conjugates of acetylcholinesterase (AChE) and chymotrypsin (ChT). During the period covered by this report, we developed a synthetic procedure to obtain a novel OP which contained the fluorophore, 2-hydroxynaphthol and showed that it could be employed as a covalent inhibitor of both AChE and ChT.</p> <p>We have employed the circular dichroism technique to demonstrate enhancement of the conformational stability of aged OP conjugates of ChT relative to homologous non-aged conjugates. Circular dichroism also revealed conformational differences between aged and non-aged pyrenebutyl-containing OP conjugates of <u>Torpedo</u> AChE in their native state.</p>				
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FOREWORD

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This work has been carried out with the collaboration of J. Grunwald, Y. Segall, N. Steinberg, C.-T. Su, J.L. Sussman, M. Harel and E. Roth.

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OBJECTIVES

1. General

The objective of this project is to characterize, by NMR and fluorescence spectroscopy, conformational differences between aged and non-aged organophosphoryl conjugates of acetylcholinesterase (AChE) and chymotrypsin (Cht). Such differences may help to explain the unexpected resistance of the aged forms to commonly employed reactivators.

2. Specific aims

During the period covered by this report (September 1987-August 1988), the principal lines of research were as follows:

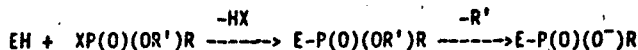
- a. Preparation of stoichiometric aged and non-aged organophosphoryl conjugates of Cht and comparison of their conformational stability.
- b. Synthesis of novel 2-naphthol-containing fluorescent organophosphates.
- c. Preliminary studies of the inhibition of Torpedo AChE by pyrene-containing fluorescent organophosphates.
- d. Preparation and X-ray studies of organophosphoryl conjugates of Cht in the crystalline state.

BACKGROUND

Many serine hydrolases such as acetylcholinesterase (AChE), chymotrypsin (Cht) and trypsin are inhibited irreversibly by organophosphorus (OP) esters. Inhibition is achieved by formation of a stoichiometric (1:1) covalent conjugate with the active site serine (1).

The powerful acute toxicity of these compounds is attributed primarily to their irreversible inhibition of AChE (2). Although poisoning by certain OP's can be treated effectively by various oxime reactivators which detach the phosphoryl moiety from the serine hydroxyl of the enzyme (3, 4), for other OP's, reactivation may be hampered by a parallel competing reaction which transforms the inhibited AChE into an "aged" form which can no longer be regenerated by the commonly used reactivators (5-7). This aging process is particularly pronounced for OP-AChE conjugates in which the OP moiety contains a secondary alkyl group (1), as is the case for conjugates produced by use of diisopropyl phosphorofluoridate (DFP), isopropyl methylphosphorofluoridate (sarin) and pinacolyl methylphosphorofluoridate (soman). The inability to regenerate aged organophosphoryl-AChE conjugates with such oximes renders therapy of intoxication by such OP's notoriously difficult (8, 9).

Although aging may occur by either acid (10, 11) or base catalysis (12, 13), it is usually accepted that the common denominator is the loss of an alkyl group from the bound OP moiety, as depicted in Scheme 1.



where : R= alkyl, aryl, alkyloxy or aryloxy

R'= alkyl, aryl

X= F, Cl, p-nitrophenoxy, dialkylaminoethanethiol

SCHEME 1

It has been suggested that the negative charge resulting from the aging reaction imposes an electrostatic barrier to nucleophilic attack on the phosphorus atom by the oximate anion of the reactivator (14). However, kinetic studies with model phosphate ester analogs of the assumed aged and non-aged conjugates indicate that the negative charge would retard reactivation by no more than 50-100 fold (15), whereas, in fact, reactivation of aged enzyme is not experimentally detectable (7, 11).

We postulated that the observed resistance to reactivation might result from a conformational change in the enzyme occurring concomitantly with aging, and that such a conformational change might be sought by use of suitably designed organophosphate probes in combination with an appropriate physicochemical technique. Fluorescent probes are particularly suitable for detecting changes in the microenvironment of the moiety to which they are attached (e.g. hydrophobicity, microviscosity, chirality and local pH). We have thus been able to provide experimental evidence for the conformational changes postulated, both for AChE and Cht, by use of suitable fluorescent OP's designed and synthesized for this purpose (16, 17). In parallel, for Cht, we were able to establish, by use of ³¹P-NMR spectroscopy, that the phosphate moiety in the conjugates under study was in the form of a triester and a diester in the non-aged and aged conjugates, respectively, as predicted (18). It was hoped to thus clarify and

illuminate the structural relationship between the phosphoryl residue and the protein backbone of aged serine esterases relative to their non-aged counterparts.

Although our eventual interest is in understanding the active site geometry of the aged OP-AChE conjugate relative to its non-aged counterpart, as already mentioned, part of this study was carried out on Cht, which, like AChE and other serine hydrolases, is readily inhibited by DFP and other OP's. We felt that studies of this type would provide very useful information since Cht is a well-characterized enzyme whose sequence and three-dimensional structure have been fully elucidated (19). Furthermore, Cht is commercially available in highly purified form in large quantities. This renders various experimental approaches readily accessible, thus allowing information concerning the aging process to be directly obtained for OP-Cht conjugates, as well as allowing us to evaluate the feasibility of applying the same approaches to AChE itself.

Most of our fluorescence spectroscopy studies to date utilized pyrene-containing OP conjugates of AChE and Cht. Although much valuable information was obtained by use of this fluorophore, it suffers from various drawbacks; in particular, it is relatively insensitive to its environment with respect to both its absorption and emission wavelengths. In this report we will describe the synthesis and initial characterization of a new class of fluorescent OP's in which the fluorophore is 2-naphthol (20). This probe displays an excited state ionization constant very different from that in the ground state, and is known to be highly conformation-sensitive when bound to proteins. It is, therefore, a promising candidate for detecting aging-associated conformational changes, as will be discussed below.

In this report we will also present data obtained by an experimental approach which provides direct evidence that aging is accompanied by an enhanced conformational stabilization of the OP-enzyme conjugate both in the immediate vicinity of the active site and in the protein as a whole, in line with data presented earlier for serum cholinesterase by Masson and Coasdue (21). The approach adopted involves monitoring changes in the circular dichroism (CD) spectrum of the aged and non-aged OP conjugates as a function of increasing guanidine hydrochloride concentration. We will present evidence in support of this contention for two homologous pairs of aged and non-aged conjugates of Cht, and will describe the initial characterization of a similar pair of OP conjugates of Torpedo AChE, which we intend to utilize in analogous experiments.

X-ray crystallography is the only experimental approach currently available which can yield direct information concerning the three-dimensional structure of a protein and for thereby detecting conformational changes which may occur. Examination of homologous aged and non-aged conjugates by this method may yield direct information as to the putative aging-induced conformational change. In the case of a protein for which the three-dimensional structure has already been solved, this may, in principle, be most readily achieved by diffusing suitable OP's into crystals of the native enzyme, to yield modified crystals isomorphous with the solved, native crystal (For a description of this approach see under Results and Discussion, Section 3). Initiation of the use of this experimental approach for aged and non-aged OP derivatives of Cht will be described below.

TECHNICAL APPROACH1. Synthesis of naphthol-containing organophosphates

The synthetic routes are depicted in Schemes 2-6 (see Results section).

a. Synthesis of 1-(2-hydroxynaphthyl)acetamidoethylamine (VI): To a stirred solution of 5.6 g (27.7 mmol) of 2-hydroxy-1-naphthaleneacetic acid (HNAA, Aldrich) in 150 ml ethyl acetate was added 6.0 g (29 mmol) of solid dicyclohexyl carbodiimide. The reaction was allowed to proceed at room temperature, with continuous stirring, for 3 h. After the precipitated by-product, dicyclohexyl urea, was filtered off, the solution was treated with activated charcoal. After refiltration, the solvent was removed by roto-evaporation. The resulting lactone (I) was purified by recrystallization from n-hexane, yielding 3.4 g. The purity of the product was examined by TLC, which revealed a single spot, and by ¹H-NMR, which gave the expected pattern. The m.p., 103-104°C, was in agreement with the literature value of 102-103°C (20, 22).

The lactone (I) was reacted with ethylenediamine to yield VI (see Scheme 3) by the following procedure. Compound I (3 g, 16.3 mmol) was dissolved in 200 ml of ethanol; an excess of ethylenediamine (3.3 g, 55 mmol) was then added; the reaction was then allowed to proceed with stirring, for 2 h at room temperature, with protection from light. The solvent was removed by roto-evaporation, followed by evaporation at high vacuum (0.2 mm Hg) so as to remove residual ethylenediamine. The resulting solid was washed with CHCl₃ and dissolved in a minimum volume of methanol. The pH of the solution was adjusted to 3-4, by adding methanol saturated with gaseous HCl, and was cooled in an ice bath. The precipitate which formed at this stage was removed by filtration, and the solvent was evaporated on a roto-evaporator. The remaining solid was identified as the desired product, VI, by ¹H-NMR.

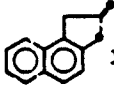
b. Synthesis of O-ethyl O-p-nitrophenyl N-(2(2-hydroxynaphthyl)acetamido)ethylamino phosphoramidate (E(pNP)NPA, VII): O-ethyl O-p-nitrophenyl phosphorochloridate (III) was prepared as follows: A solution of p-nitrophenol (4.17 g, 30 mmol), in 25 ml of dry ether, was added dropwise to a solution of ethyl dichlorophosphate (Aldrich, 4.9 g, 30 mmol) and triethylamine (3.03 g, 30 mmol) in 30 ml of dry ether at 0°C. After addition of the p-nitrophenol, stirring was continued for 5 h at room temperature. The triethylamine hydrochloride which precipitated was removed by filtration, and the filtrate was evaporated under reduced pressure, first with a water pump (24 mm Hg) and then using an oil pump (0.2 mm Hg). The residue was obtained as a light brown viscous oil (5.19 g) which was characterized by ³¹P-NMR (relative to H₃PO₄). δ , -2.7 ppm (neat).

To prepare the final product, E(pNP)NPA (see Scheme 3), a solution of III (3.8 g, 14 mmol) in 20 ml of dry acetonitrile was added dropwise to a solution of VI (3.6 g, 14 mmol) and 2.5 g triethylamine in 350 ml of dry acetonitrile. Stirring at 40°C was required to achieve partial dissolution of VI so as to initiate the reaction. Incubation was continued overnight, with stirring, at room temperature. The solvent was removed by roto-evaporation, and the residue was applied to a silica column (2.5x40 cm) from which it was eluted with chloroform-ethanol (100:5, v/v). The

fractions containing the desired product still contained traces of the p-nitrophenolate ion. In order to remove this contamination, the product was reeluted through a small silica column (2.5x15 cm) yielding 0.8 g of a light yellow viscous oil which solidified after drying under reduced pressure (m.p., 67-71°C, after softening at 58-62°C).

¹H-NMR (CDCl₃, relative to TMS): δ, 8.14 (2H, d, J = 4.5 Hz), 7.92 (1H, d, J = 4.25 Hz), 7.77 (1H, d, J = 4.125 Hz), 7.68 (1H, d, J = 4.50 Hz), 7.45 (1H, m), 7.26 (1H, d), 6.70 (1H, m), 4.08 (2H, m), 4.00 (2H, s), 3.31 (2H, m), 3.13 (2H, m), 1.24 (1H, t, J = 6.25 Hz).

³¹P-NMR (relative to H₃PO₄): δ, 4.19 ppm (acetone D₆)

Mass spec. (EI) 472 ((M-H)⁺, 10%); 473 (M⁺, 8%); 184 ( 100%).

Anal. calcd. for C₂₂H₂₄N₃O₇P: C, 55.80; H, 5.08; N, 8.87. Found: C, 55.73; H, 4.38; N, 8.20.

c. Synthesis of bis-O-p-nitrophenyl N-(2(2-hydroxynaphthyl-acetamido)ethylamino phosphoramidate ((pNP)₂NPA) (XI): This compound was prepared according to the route outlined in Scheme 6. Bis-p-nitrophenyl phosphorochloridate (XII) was prepared by adding a mixture of p-nitrophenol (5.4 g, 39 mmol) and triethylamine (3.94 g, 39 mmol) in 50 ml of dry acetonitrile, dropwise, to a stirred solution, at 0°C, of p-nitrophenyl phosphorodichloridate (VIII, 10 g, 39 mmol) in dry acetonitrile (50 ml). The reaction was continued, with stirring, for 4 h at room temperature, at which stage the reaction had gone to completion as judged by ³¹P-NMR. XII was not separated from the reaction mixture due to its susceptibility to hydrolysis, but was directly reacted as such with VI by adding 50 ml of the above reaction mixture dropwise to a stirred solution of VI (3 g, 12.1 mmol) and triethylamine (1.2 g, 12.0 mmol) in 30 ml acetonitrile at 0°C. The mixture was stirred at 0°C for 1 h, and for an additional 4 h at room temperature. The solvent was removed under reduced pressure, and the residue was applied to a silica column (2.4x50 cm) and eluted with CHCl₃. The fractions containing the product were identified both by TLC and by ³¹P-NMR (relative to H₃PO₄): δ, -13.2 ppm (CDCl₃). The product obtained was not completely pure since, due to its susceptibility to hydrolysis, it contained some free p-nitrophenol.

2. Preparation of stoichiometric aged and non-aged OP-Cht conjugates

Two sets of homologous pairs of aged and non-aged conjugates of Cht were prepared for the physicochemical studies outlined in the present report. One pair consisted of diethylphosphoryl-Cht - (C₂H₅O)₂P(O)-Cht, and monoethylphosphoryl-Cht - (C₂H₅O)P(O)(O⁻)-Cht. These were abbreviated as DEP-Cht and MEP-Cht, respectively. These two conjugates were obtained by reacting Cht with diethyl-(p-nitrophenyl)phosphate (paraoxon) and ethyl bis(p-nitrophenyl) phosphate, respectively. The first conjugate was obtained with concomitant release of one equivalent of p-nitrophenol, and could be almost fully reactivated by 3-hydroxyiminomethyl-1-methylpyridinium iodide (3-PAM, ref. 18 and see below). The second conjugate was obtained with concomitant release of 2 equivalents of p-nitrophenol, and was totally resistant to oxime reactivation. The second pair, in which both conjugates contained the pyrene chromophore, consisted of 1-pyrenebutyl ethylphosphoryl-Cht (PBEF-Cht) and 1-pyrenebutylhydroxyphosphoryl-Cht (PBP-Cht). These two conjugates were prepared by reacting Cht with 1-pyrenebutyl ethyl phosphorofluoridate (PBEPF) and 1-pyrenebutyl phosphorodichloridate (PBPDC), respectively.

PBEPF and PBPCD were synthesized as described previously (16). Paraoxon was purchased from Sigma (St. Louis, MO), and ethyl bis(p-nitrophenyl) phosphate was synthesized by reacting ethyl phosphorodichloridate with 2 equivalents of p-nitrophenol.

Large quantities of DEP- and MEP-Cht (200-400 mg) were obtained by dropwise addition of a fresh concentrated solution of the appropriate OP (0.01-0.1 M) in acetonitrile to a stirred solution of Cht (5-10 mg/ml) in 50 mM sodium phosphate buffer, pH 7.4, at room temperature. For preparation of PBEP- and PBPC-Cht, inhibition was carried out in fresh dilute solutions in double-distilled water of both Cht (0.5-1 mg/ml) and of the inhibitors (0.2-1 mM), due to the limited solubility of the latter in water. The reaction was performed at room temperature, and a pH of 7.4 was maintained by addition of 0.02 N NaOH (18). In no case did the concentration of organic solvent in the final inhibition medium exceed 5%. The decrease in enzymic activity was monitored spectrophotometrically (23) until >98% inhibition had been achieved. Greater than 99.9% inhibition was ensured by subsequent addition of 10 mM diisopropyl phosphorofluoridate (DFP) to the reaction mixture, and incubation for a further 30 min. The solution was then dialyzed against double distilled (dd) water, lyophilized, redissolved in ca. 2 ml of dd water, and loaded onto a Sephadex G-10 column (3x60 cm). The column was eluted with dd water at a flow rate of 1.5 ml/min, and the main protein fractions were pooled, dialyzed and relyophilized. The dry OP-Cht conjugates were stored over desiccant at -20° until employed.

3. Circular dichroism measurements

Circular dichroism (CD) spectra were recorded with a Jasco J-500C spectropolarimeter. The absorption anisotropy factor, g_{ab} , is defined by $g_{ab} = (\epsilon_1 - \epsilon_2)/\epsilon$ where ϵ_1 and ϵ_2 are the extinction coefficients for left- and right-handed circularly polarized light respectively, and ϵ is their average.

4. Enzyme assays

AChE activity was measured either by the Ellman procedure (24), using acetylthiocholine as substrate, or by a microtiter procedure based upon it (25). Cht activity was measured either titrimetrically, using acetyl-N-tyrosine ethyl ester as substrate (26), or spectrophotometrically, employing succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (23).

5. Preparation and chemical modification of crystals of γ -chymotrypsin

γ -Cht was crystallized essentially according to Segal et al. (27) from concentrated $(NH_4)_2SO_4$ in 0.02 M sodium cacodylate, pH 5.6, at room temperature. Well-formed tetragonal crystals appeared within a month, and crystals of suitable size, i.e., 0.2x0.2x0.6 mm, were employed either directly for crystallographic measurements or for chemical modification.

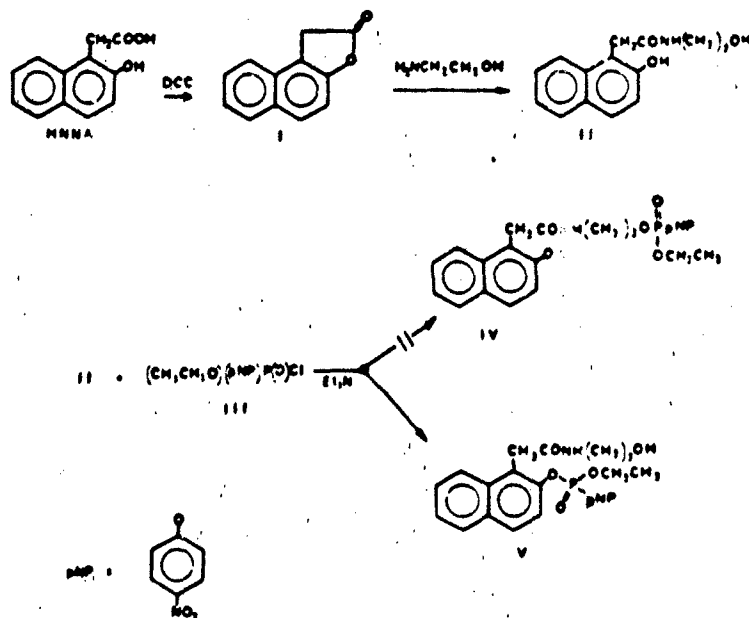
Crystals of DEP-Cht were prepared by prolonged soaking of crystals of γ -Cht in a solution of 0.4 mM paraoxon in 65% saturated $(NH_4)_2SO_4$, 0.4% dioxane, 0.01 M sodium cacodylate, pH 5.6, at room temperature, with weekly refreshing of the inhibitor solution. The extent of inhibition was assessed by dissolving crystals in 0.0025 N HCl and assaying the solution for Cht activity and protein content as compared to control crystals.

6. X-ray data collection

X-ray data were collected under cryogenic conditions which prolong the crystal lifetime almost indefinitely (28). This was achieved by shock-cooling the crystals to -110°C and collecting the X-ray data at this temperature using an apparatus in which a low-temperature attachment had been grafted onto a Rigaku APC5-R rotating anode diffractometer, permitting cooling of the crystal by a stream of boiled N_2 gas during data collection without impeding the movements of the diffractometer. This permitted collection of an entire data set from a single crystal.

RESULTS AND DISCUSSION1. Synthesis of novel 2-naphthol-containing organophosphates

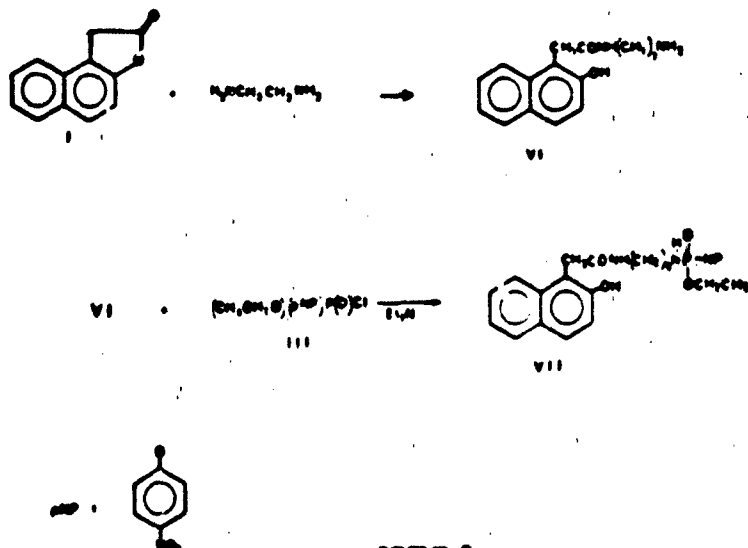
In the grant application, we proposed to embark on the synthesis of a new family of fluorescent OP's in which the fluorophore would be 2-naphthol; this choice was based on the reported spectroscopic properties of probes based on aromatic hydroxyl derivatives, which exhibit excited state ionization constants very different from those observed in the ground state (29), a fact which is reflected in their fluorescence. Thus the rate of proton transfer which such probes undergo is sensitive to the availability of proton acceptors and donors, as well as to their pK_a ; their fluorescence, in protein conjugates, should be highly conformation-sensitive (20), and particularly suitable for detecting the putative conformational changes involved in aging. The synthetic approach which we have adopted is based on the above-mentioned work of Laws et al. (20), in which the lactone of 2-hydroxy-1-naphthalene acetic acid (HNAA) was used as a reagent for attachment of the 2-naphthol probe to proteins. In our case, HNAA was initially used in a coupling reaction with an appropriate amino alcohol side arm, to yield an adduct which could then be coupled with a suitable activated OP. Indeed, in the initial phase of this project, HNAA was coupled, via an aminoethanol extension arm (II), to a phosphorochloridate (III), so as to obtain the desired fluorescent OP (IV), as shown in Scheme 2.



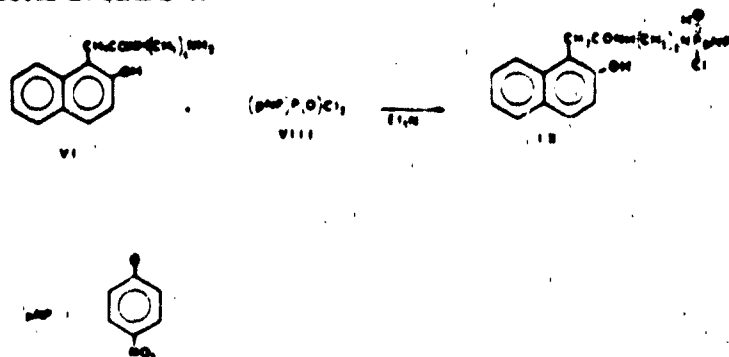
SCHEME 2

However, as is also depicted in Scheme 2, reaction occurred preferentially with the aromatic hydroxyl group, leading to formation of an O-aryl ester (V), rather than to the expected ligand (IV). So as to obtain a suitable

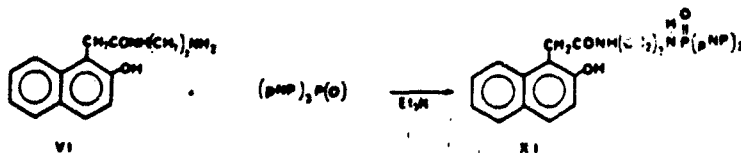
naphthol-containing OP of the general type represented by IV, we decided to employ ethylenediamine as the sidearm. We thus hoped to direct phosphorylation to the amino group (Scheme 3, VI), since it is a stronger nucleophile than the aromatic hydroxyl residue. This reaction (Scheme 3) resulted in an almost exclusive regioselective phosphorylation, to yield the desired product, VII.



Compound VII, O-ethyl O-p-nitrophenyl N-(2-hydroxynaphthylacetamido)ethylenamino phosphoramidate (E(pNP)NPA), was expected to form a non-aged conjugate with AChE and other serine hydrolases. Before proceeding to a detailed study of the kinetics and stoichiometry of the inhibition process, we concentrated on the synthesis of the homologous OP which could be used for production of the homologous aged OP-enzyme conjugates. Our first attempt was to prepare the chloride analog of E(pNP)NPA, as depicted in Scheme 4.

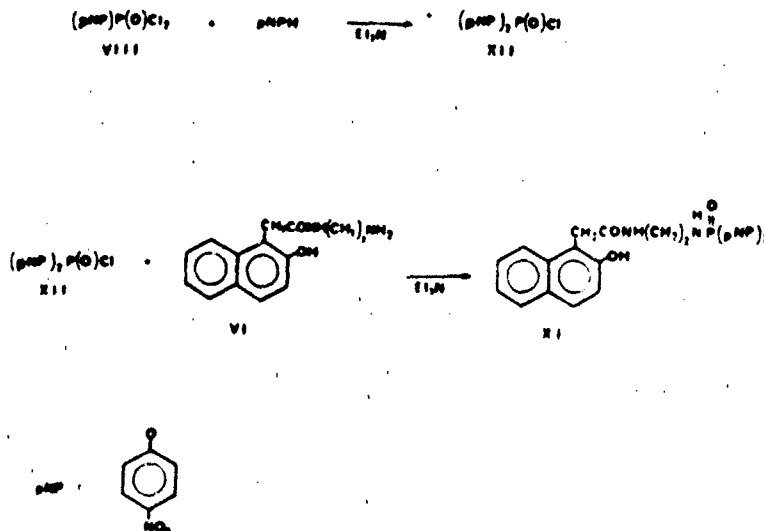


Separation of IX from the reaction mixture proved difficult since the chloridate is very sensitive to hydrolysis. So as to overcome this problem, we decided to replace the chloride by the p-nitrophenoxy (pNP) group, and, thereby, to obtain bis-(O-p-nitrophenyl) N-(2(2-hydroxynaphthyl-acetamido)ethylamino)phosphoramide ((pNP)₂NPA) (XI). We attempted to obtain this compound by the procedure outlined in Scheme 5.



SCHEME 5

The yield of the desired product (XI) obtained by this procedure was not satisfactory. Another synthetic approach was, therefore, adopted. This involved synthesis of bis-(p-nitrophenyl)phosphorochloridate (XII), and its reaction with VI to yield the expected product, XI, as shown in Scheme 6.



SCHEME 6

Due to the instability of the intermediate, XII, it was not isolated from the reaction mixture; instead, after the first reaction had gone to completion, as indicated by monitoring its progress by ^{31}P -NMR spectroscopy, one equivalent of VI was added to the reaction mixture. XI, prepared by this pathway, was isolated by chromatography on silica gel, and characterized by ^1H and ^{31}P -NMR.

Although we were not yet satisfied with the purity of the new fluorescent OP's, we tested their anti-AChE and anti-Cht activity so as to obtain preliminary data pertinent to the envisaged large-scale preparation of the corresponding OP-Cht conjugates. We thought that these initial data would also help us to evaluate progress in improving the homogeneity of these ligands. The following bimolecular rate constants for the inhibition of Cht and Electrophorus AChE were calculated from the initial slopes of curves obtained by plotting the log of the percent of the residual enzymic activity vs. the time of reaction of the esterase with the OP:

	Cht	AChE (eel)
Z(pNP)NPA	300	900
(pNP) ₂ NPA	8300	450

Rate constants are $\text{M}^{-1}\text{min}^{-1}$.

It should be mentioned that the semi-logarithmic plot of percent residual activity vs. time deviated from a straight line, and only 70-90% of enzymic activity (depending on the source of enzyme and the experimental conditions) was inhibited, despite the presence of a large excess of inhibitor over enzyme in the inhibition medium. Since both inhibitors are slowly hydrolyzed under the experimental conditions employed, and in view of our observation that both slow, spontaneous reactivation and aging occurred, more experiments will be necessary in order to clarify the mechanism(s) underlying the inhibition patterns displayed by the two OP's. It should be emphasized that the relatively low potencies of both ligands as inhibitors of AChE and Cht tend to complicate calculation of the individual rate constants for the various processes which occur simultaneously during the time required to achieve significant inhibition. These processes include spontaneous hydrolysis of the inhibitor, as well as spontaneous reactivation and aging of the inhibited enzyme. More experiments are underway to clarify the above. In particular we will place emphasis on the ultra-purification of the two novel fluorescent OP's so as to confirm the inhibition profiles obtained so far.

2. Comparative studies on the conformational stability of aged and non-aged conjugates of Cht

One reason for the resistance to reactivation displayed by aged OP conjugates of serine hydrolases might be the formation of a hydrogen bond (or bonds) as a result of the aging reaction, e.g., between the newly

formed negative charge on the phosphate group and the active-site histidine, which, in turn, could lead to stabilization of the structure of the protein; this might find expression in an increased resistance to denaturation. Evidence for such an increased stability was presented by Masson & Gosdud (21), who used the transverse urea gradient technique of Creighton (30) to demonstrate an increased resistance to unfolding of an aged OP conjugate of human serum cholinesterase. We decided to study this putative conformational stabilization more directly by monitoring the circular dichroism (CD) spectra of homologous pairs of aged and non-aged OP conjugates of Cht as a function of the concentration of the denaturing agent guanidine hydrochloride (Gu.HCl). Two sets of conjugates were studied: a) 1-pyrenebutyl ethylphosphoryl-Cht (PBEP-Cht; non-aged) and 1-pyrenebutyl hydroxyphosphoryl-Cht (PBP-Cht; aged). The preparation of these fluorescent OP conjugates and their characterization by ³¹P-NMR and fluorescence spectroscopy was earlier carried out and reported in the framework of Grant No. DAWD-17-83-G-9548, and published (17, 18). b) diethylphosphoryl-Cht (DEP-Cht; non-aged) and monoethylphosphoryl-Cht (MEP-Cht; aged). As will be apparent in the following, the pyrene-containing pair of OP conjugates permitted comparison of changes in the active-site region in the course of denaturation, while the second set of conjugates permitted monitoring of changes in the CD spectrum which could be ascribed principally to the contribution of the peptide bonds of the polypeptide backbone.

Figs. 1 and 2 compare the CD spectra of PBEP-Cht and PBP-Cht in the range of 0-6 M Gu.HCl. As we have already reported (17), there is a significant difference in the CD spectra of the two conjugates in their native state, in the region of the pyrene absorption band, at ca. 350 nm, which indicates a significant difference in the geometry of the OP group in the active site pockets of the two conjugates. Upon increasing the Gu.HCl concentrations much lower than required for total inactivation of the enzyme (2-3 M). Reactivation studies carried out with 3-PAM (18, 31) demonstrated that the ability of PBEP-Cht to undergo reactivation is not impaired as long as total denaturation has not been achieved. All conformational transitions observed above 310nm, and at Gu.HCl concentrations below 2 M, thus appear to result from different orientations of the pyrene probe within the active site rather than from disruption of the site itself. There is, however, a marked difference between the tendencies of PBEP-Cht and PBP-Cht to undergo such orientational changes. The non-aged conjugate undergoes more such reorientations, the first occurring at a Gu.HCl concentration as low as 0.3 M; in contrast, the first such reorientation observed for PBP-Cht occurs at only 0.7 M Gu.HCl. If one monitors the changes in ellipticity at 230 nm, one again sees a clear difference between the two conjugates. The midpoint of transition for PBEP-Cht lies at ca. 3 M Gu.HCl, whereas that for PBP-Cht lies at ca. 4 M Gu.HCl. The

CD data thus suggest that the aging reaction produces both a local stabilization of the pyrene chromophore within the active site and a more general stabilization of the protein as a whole (Fig. 5A).

Figs. 3 and 4 show the corresponding dependence of the CD spectrum on Gu.HK concentration for DEP-Cht and MEP-Cht. In the case of this pair of conjugates, the CD spectrum can be ascribed almost entirely to the polypeptide backbone, as is shown by the fact that the only major band is at ca. 230 nm. Again, the aged form, MEP-Cht, is significantly more resistant to denaturation than the non-aged form, DEP-Cht. Comparison of Figs. 3 and 4 reveals that this increased resistance is similar to that seen for the pyrene-containing pair of conjugates, as is to be expected if the principal contribution to stabilization comes from an interaction of the negatively charged oxygen on the phosphate atom with a positively charged group in its vicinity. (Fig. 5B).

3. Preparation of non-aged and aged OP conjugates of Cht in the crystalline state

X-ray crystallography is, at present, the only way to directly elucidate the three-dimensional structure of a protein. Comparison, by X-ray crystallography, of aged and non-aged OP conjugates of Cht and, subsequently, of AChE, would thus yield direct information, at the molecular level, regarding the conformational change underlying the aging process. In the case of AChE, although we now possess a crystalline form suitable for X-ray studies (32), the three-dimensional structure of the enzyme has still to be worked out. In the case of Cht, the structure is well known (19). It was shown earlier by Sigler & Skinner (33) that α -Cht in the crystalline state could be almost fully inhibited by diisopropyl phosphorofluoridate, although these authors did not carry out a high-resolution study so as to elucidate the geometry of the OP-inhibited active site. The advantage of such a procedure, as opposed to crystallization of a preformed OP-enzyme conjugate, is that, if successful, it permits preparation of OP-Cht conjugates isomorphous in crystal structure with the unmodified enzyme. Since, as already mentioned, the three-dimensional structure of Cht itself is already known, the structures of the corresponding conjugates can be readily solved. Thus, for example, Rings et al. (34) have recently adapted this approach to study the conformational changes induced upon inactivation of Cht by 5-benzyl-6-chloro-2-pyrone; it has not, however, yet been adopted for seeking conformational differences between non-aged and aged OP-Cht conjugates. Obviously, the same OP's used to produce the homologous pair of aged and non-aged conjugates, DEP- and MEP-Cht, in solution, as was done above for the CD studies, could be similarly utilized to produce the corresponding crystalline conjugates by soaking the Cht crystals in them under suitable experimental conditions. Such an approach was initiated over a year ago, in collaboration with the group of Dr. Joel Sussman in the Dept. of Structural Chemistry at the Weizmann Institute. We began by producing crystals of β -Cht suitable for X-ray diffraction studies by crystallization from ammonium sulfate (27). We were then able to show that such crystals can be inhibited >80% by paraoxon, without damage to their structure, by exposure to the OP, for about 1 month, in the same $(\text{NH}_4)_2\text{SO}_4$ solution used for crystallization; they thus yield a DEP-Cht crystal isomorphous with that of the unmodified, native protein.

X-ray data for β -Cht were collected out to 1.9 \AA resolution from a single native crystal, using the shock cooling procedure (28), and with data collected at -150°C . The crystals are of the same form as previously

reported (27), with space group $P4_22_1$, and the unit cell at low temperature is $a=b=69.1\text{\AA}$, $c=96.4\text{\AA}$ (at room-temperature $a=b=69.7\text{\AA}$, $c=97.4\text{\AA}$). The native structure was refined by a least-squares procedure, starting from the room-temperature coordinates (35). The R-factor converged to 24.5%.

As for the native enzyme, a single crystal of DEP-Cht was used to collect the entire 1.9\AA data set at -150°C . The unit cell dimensions were $a=b=67.0\text{\AA}$, $c=95.8\text{\AA}$, a 0.9% increase in cell volume over the low-temperature native cell volume. The DEP-Cht structure was refined starting from the refined low-temperature native coordinates. The current R-factor of the conjugate is 21.9%. The $F_{\text{der}}-F_{\text{nat}}$ map has its highest peak within bonding distance of the active-site Ser 195 hydroxyl oxygen and it permitted fitting of the DEP moiety to the difference electron density. Comparison of the two structures, using an Evens & Sutherland computer graphics system showed that the distance between Ser 195 O γ and His 57 N ϵ 2 was reduced from 3.43 \AA in the native enzyme to 3.05 \AA in the conjugate. This result indicates that binding of the OP group at the active site induces a sizeable conformational change. In the DEP conjugate, the His 57 side chain is also in proximity to the OP moiety. Thus the distance between His 57 N ϵ 2 and one of the carbons of the DEP group is only 4.05 \AA . This observation provides direct experimental evidence in support of the hypothesis that N ϵ 2 of the imidazole moiety participates in the aging reaction by stabilizing a carbonium ion intermediate.

4. Preliminary studies on the inhibition of Torpedo AChE by pyrene-containing fluorescent organophosphates

During the final quarter of the period covered by this report, we initiated work on AChE from Torpedo californica. We consider the Torpedo enzyme more suitable than Electrophorus AChE for continuation of our studies on conformational differences between aged and non-aged OP-AChE conjugates. This is because the complete amino acid sequence of Torpedo AChE is available as a result of the cloning and sequencing studies of Palmer Taylor and his associates (36, 37), and because the recent crystallization of a dimeric form of Torpedo AChE in our *c.m.* laboratory, to yield crystals of a quality suitable for X-ray crystallography (32), makes it likely that the three-dimensional structure of the Torpedo enzyme will become available. Accordingly, we intend to use, in our spectroscopic studies, the same highly purified preparation of Torpedo G $_2$ AChE which we are using to obtain crystals of the enzyme (32). So far we have inhibited this AChE preparation with both PBEPF and PBPDC, adopting a procedure similar to that used for preparation of the corresponding OP-Cht conjugates, and removing the excess free ligand from the conjugates by gel filtration on a Biogel P-4 column. Both of the OP's serve as efficient inhibitors of Torpedo AChE. PBEPF-AChE, the conjugate obtained by use of PBEPF, could be reactivated >90% by 1 mM pyridine-2-aldoxime methiodide (2-PAM) at pH 7.0, thus showing that, as expected, it does not undergo aging. Torpedo AChE similarly reacted with PBPDC was, again as expected, completely resistant to reactivation by 2-PAM, indicating that the aged conjugate, PBP-AChE, had been obtained. We are now about to initiate studies on the conformational stabilization of the aged vs. the non-aged conjugate, similar to those performed for Cht. in which we will monitor the CD spectra of the two conjugates as a function of guanidine concentration.

SUMMARY AND CONCLUSIONS

1. A synthetic procedure was developed to attach the fluorescent probe, 2-hydroxy-1-naphthalene acetic acid, via an ethylenediamine extension arm, to an organophosphoryl residue capable of inhibiting AChE. The expected OP was obtained, after considerable synthetic efforts, and was found to serve as a covalent inhibitor of both AChE and Cht. In view of the complex kinetic behavior observed, further purification will be necessary prior to interpretation of the data.
2. Two pairs of non-aged and aged stoichiometric OP-Cht conjugates were prepared and the conformational stabilities of these pairs were compared by measuring their susceptibility to guanidine denaturation by monitoring changes in their circular dichroism spectra. For both pairs the aged conjugate was found to be substantially more stable than the corresponding non-aged form.
3. An OP conjugate of crystalline γ -Cht was prepared by diffusing paraxon into crystals of the native enzyme, to yield an OP conjugate, diethylphosphoryl-Cht, isomorphous with the native Cht crystal. This permitted ready elucidation of its three-dimensional structure in which it was possible to visualize the diethylphosphoryl moiety within the active site. Similar preparation of a corresponding aged conjugate should enable us to accurately determine the conformational changes underlying aging at the atomic level.
4. Non-aged and aged stoichiometric fluorescent OP conjugates of Torpedo AChE were prepared by its reaction with appropriate pyrenebutyl-containing OP's. Preliminary circular dichroism measurements reveal significant differences between the two conjugates which are broadly similar to those observed for the corresponding Cht conjugates.

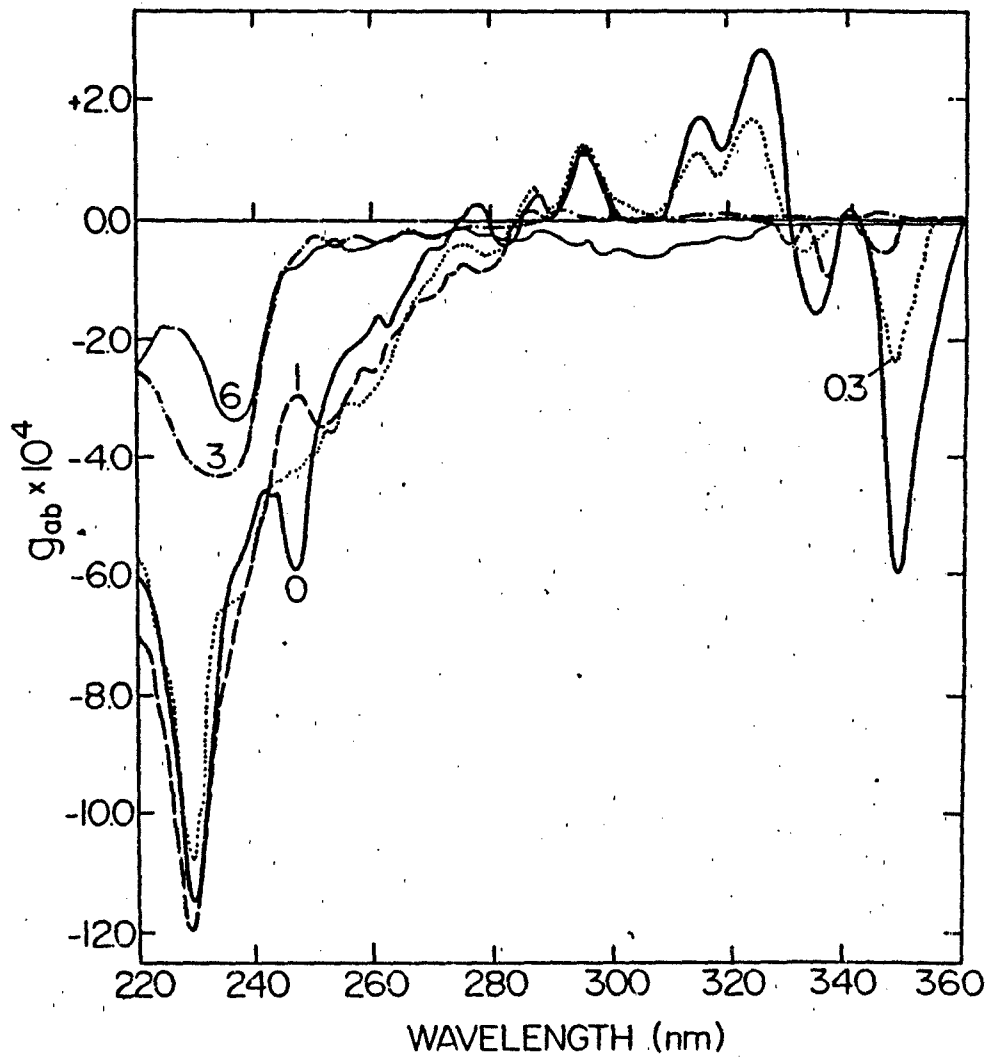


Figure 1: Circular dichroism spectra of PBEP-Cht as a function of Gu.HCl concentration. The concentration of the conjugate was 4×10^{-5} M. The numbers adjacent to each curve denote the relevant Gu.HCl concentration.

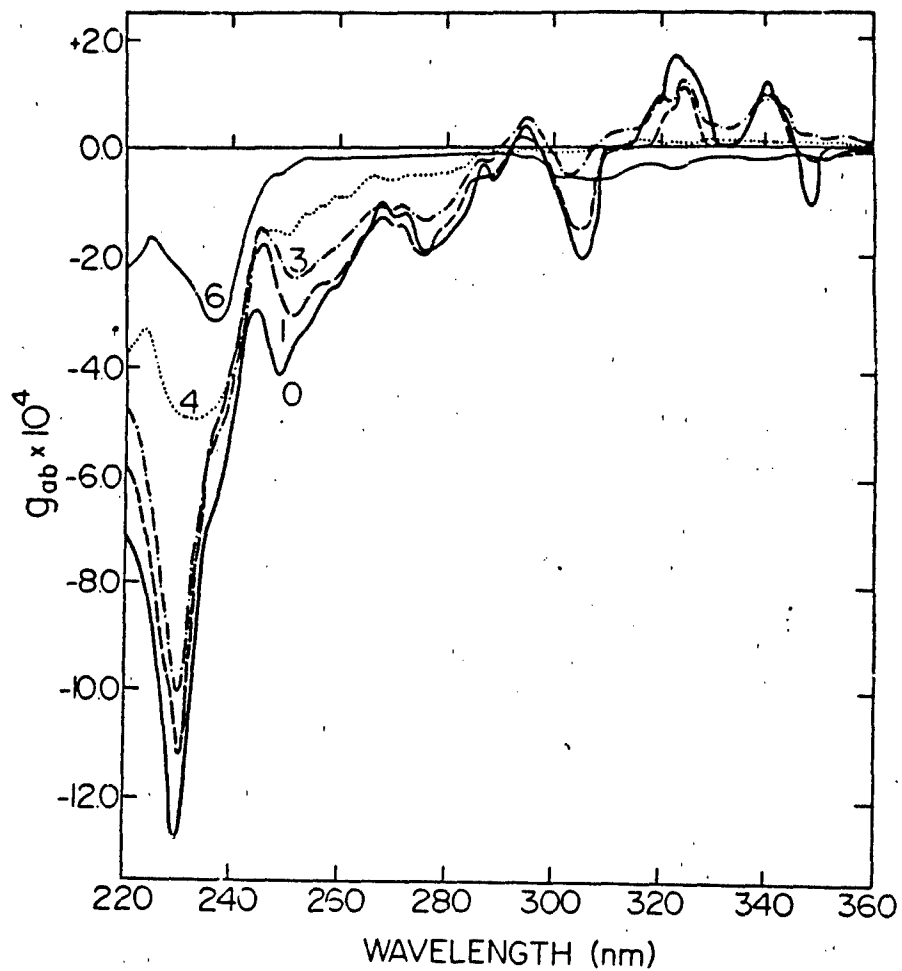


Figure 2: Circular dichroism spectra of PBP-Cht as a function of Gu.HCl concentration. The concentration of the conjugate was 4×10^{-5} M. The numbers adjacent to each curve denote the relevant Gu.HCl concentration.

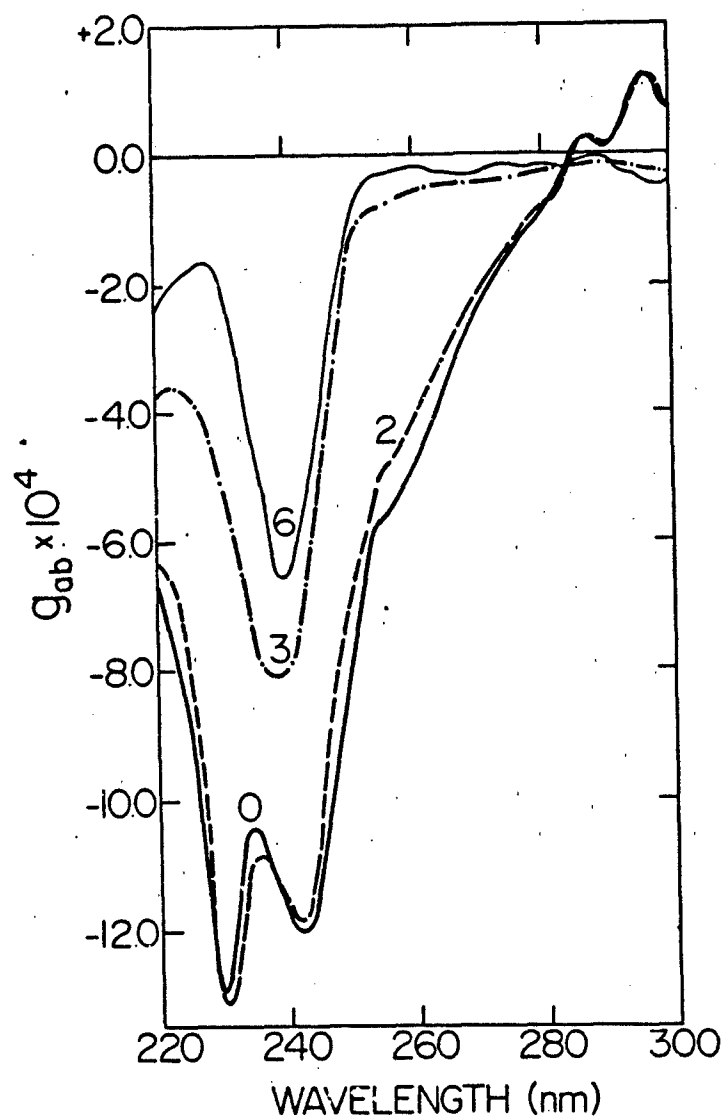


Figure 3: Circular dichroism spectra of DEP-Cht as a function of Gu.HCl concentration. The concentration of the conjugate was 4×10^{-5} M. The numbers adjacent to each curve denote the relevant Gu.HCl concentration.

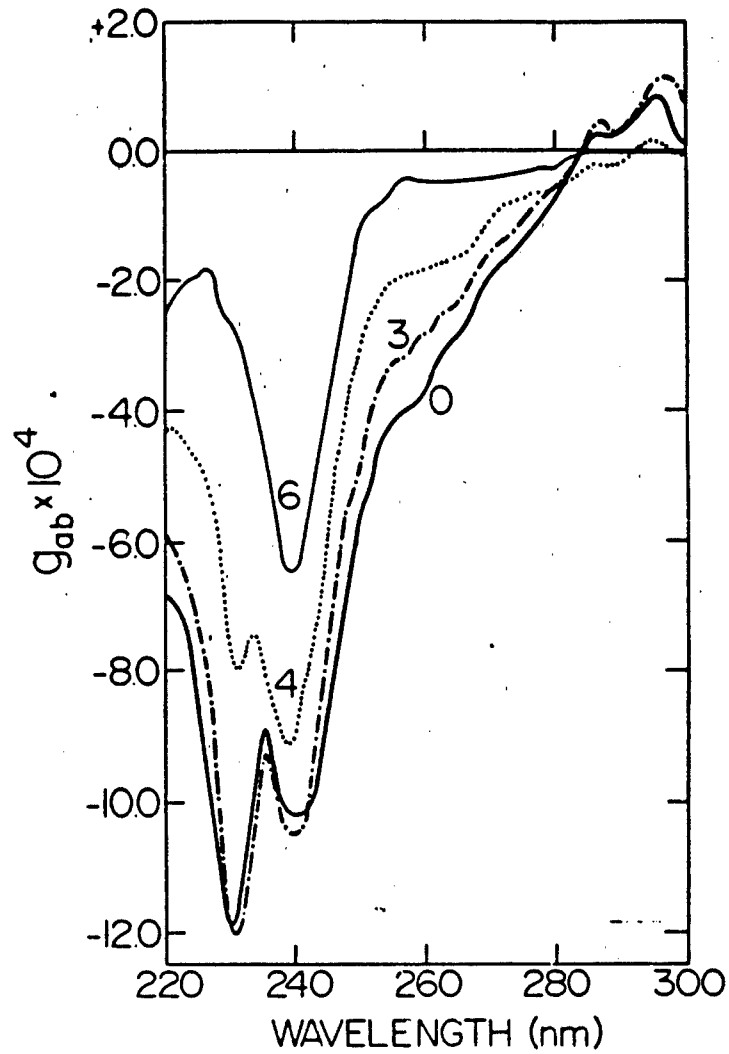


Figure 4: Circular dichroism spectra of MEP-Cht as a function of Gu.HCl concentration. The concentration of the conjugate was 4×10^{-5} M. The numbers adjacent to each curve denote the relevant Gu.HCl concentration.

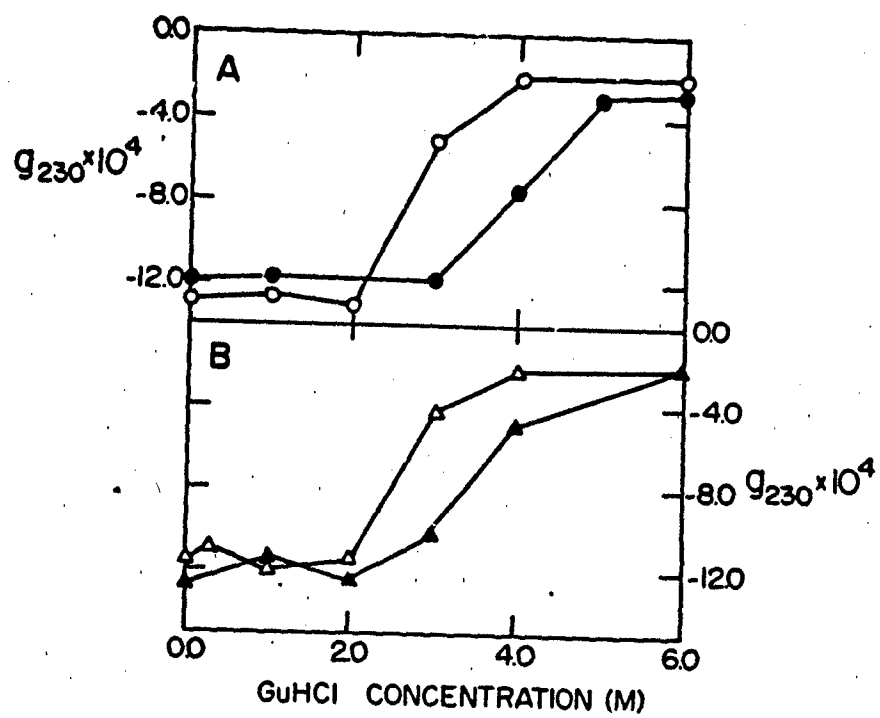


Figure 5: Dependence of the absorption anisotropy factor at 230 nm, g_{230} , on Gu.HCl concentration, for DEP-Cht and MEP-Cht (Fig. 5A) and PBEP-Cht and PBP-Cht (Fig. 5B).

PBEP-Cht, \triangle
 PBP-Cht, \blacktriangle
 DEP-Cht, \circ
 MEP-Cht, \bullet

REFERENCES

1. "Enzyme Inhibitors as Substrates", (1972) Aldridge, W.N. and Reiner, E., North-Holland Publishing Co., Amsterdam, The Netherlands.
2. Handbuch der Experimentellen Pharmakologie, Vol. XII, "Cholinesterase and Anticholinesterase Agents", (1963) Koelle, G.B., Springer-Verlag, Berlin, West Germany.
3. Molecular Complementariness as Basis for Reactivation of Alkyl Phosphate-Inhibited Enzyme, (1958) Wilson, I.B., Ginsburg, S. and Qian, C., Arch. Biochem. Biophys. 77, 286-296.
4. Chemical Reactivation of Phosphorylated Human and Bovine True Cholinesterases, (1956) Hobbiger, F., Brit. J. Pharmacol. 11, 295-303.
5. Reactivation of Phosphorylated Acetylcholinesterases by Pyridinium Oximes and Related Compounds, (1960) Hobbiger, F., Pitman, M. and Sadler, P.W., Biochem. J. 75, 363-372.
6. Development of Resistance of Alkyl-Phosphorylated Cholinesterase to Reactivation by Oximes, (1958) Michel, H.O., Fed. Proc. 17, 275.
7. The Chemical Basis of the "Aging Process" of DFP-Inhibited Pseudocholinesterase, (1959) Berends, F., Posthumus, C.H., Sluys, I.v.d. and Deierkauf, F.A., Biochim. Biophys. Acta 34, 576-578.
8. Antidotal Action of Pyridinium Oximes in Anticholinesterase Poisoning: Comparative Effects of Soman, Sarin and Neostigmine on Neuromuscular Function, (1963) Loomis, T.A. and Salafsky, B., J. Toxicol. Appl. Pharmac. 5, 685-701.
9. Toxogonin in Sarin, Soman and Tabun Poisoning, (1965) Heilbronn, E. and Tolagen, B., Biochem. Pharmacol. 14, 73-77.
10. Dealkylation as a Mechanism for Aging of Cholinesterase after Poisoning with Pinacolyl Methylphosphonofluoridate, (1965) Fleisher, J.H. and Harris, L.W., Biochem. Pharmacol. 14, 641-650.
11. Ageing and Dealkylation of Soman-Inactivated Eel Cholinesterase, (1967) Michel, H.O., Hackley, B.E., Berkowitz, L., List, G., Hackley, E.B., Gillilan, W. and Pankau, M., Arch. Biochem. Biophys. 121, 29-34.

12. Spontaneous Reactivation of Acetylcholinesterase Inhibited with Para-Substituted Phenyl Methylphosphonochloridates, (1972) Hovanec, J.W. and Lieske, C.N., *Biochemistry* 11, 1051-1056.
13. Reactivation and Aging of Diphenylphosphoryl Acetylcholinesterase, (1975) Maglothin, J.A., Wine, P. and Wilson, I.B., *Biochim. Biophys. Acta* 403, 370-387.
14. Reactivation by Pyridinium Aldoxime Methochloride (PAM) of Inhibited Cholinesterase Activity in Dogs after Poisoning with Pinacolyl Methylphosphonofluoridate (Soman), (1967) Fleisher, J.H., Harris, L.W. and Murtha, E.F., *J. Pharmacol. Exp. Therap.* 156, 345-351.
15. The Reactivity of Phosphate Esters. Reaction of Diesters with Nucleophiles, (1970) Kirby, A.J. and Younas, M., *J. Chem. Soc. (B)*, 1165-1172.
16. Novel Pyrene-Containing Organophosphates as Fluorescent Probes for Studying Aging-Induced Conformational Changes in Organophosphate-Inhibited Acetylcholinesterase, (1982) Amitai, G., Ashani, Y., Gafni, A. and Silman, I., *Biochemistry* 21, 2060-2069.
17. Conformational Differences between Aged and Non-Aged Pyrenebutyl-Containing Organophosphoryl Conjugates of Chymotrypsin as Detected by Optical Spectroscopy, (1989) Steinberg, N., Van der Drift, A.C.M, Grunwald, J., Segall, Y., Shirin, E., Haas, E., Ashani, Y. and Silman, I., *Biochemistry* 28, 1248-1253.
18. Aged and Non-Aged Pyrenebutyl-Containing Organophosphoryl Conjugates of Chymotrypsin: Preparation and Comparison by ³¹P-NMR Spectroscopy, (1989) Grunwald, J., Segall, Y., Shirin, E., Waysbort, D., Steinberg, N., Silman, I. and Ashani, Y., *Biochem. Pharm.* 38, 3157-3168.
19. The Structure of Chymotrypsin, (1971) Blow, D.M., in "The Enzymes" (P.D. Boyer, Ed.), 3rd Edition, vol. 3, pp. 185-212.
20. A Covalent Fluorescent Probe Based on Excited-State Proton Transfer, (1979) Lews, W.R., Posner, G.H. and Brand, L., *Arch. Biochem. Biophys.* 193, 88-100.
21. Evidence that the Conformational Stability of 'Aged' Organophosphate-Inhibited Cholinesterase is Altered, (1986) Masson, P. and Gosslue, J.-L., *Biochim. Biophys. Acta* 869, 304-313.

22. The Chemistry of 1-Naphthaleneacetic Acid Derivatives, (1951) Ogata, Y., Okano, M. and Kitamura, Y., *J. Org. Chem.* 16, 1588-1592.
23. A Sensitive New Substrate for Chymotrypsin, (1979) DelMar, E.G., Largman, C., Brodrick, J.W. and Geckas, M.C., *Analyt. Biochem.* 99, 316-320.
24. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity, (1961) Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M., *Biochem. Pharmacol.* 7, 88-95.
25. Microtiter Assay for Acetylcholinesterase, (1987) Doctor, B.P., Tokar, L., Roth, E. and Silman, I., *Analyt. Biochem.* 166, 399-403 (1987).
26. The Influence of pH on the Kinetic Constants of α -Chymotrypsin-Catalyzed Esterolysis, (1956) Cunningham, L.W. and Brown, C.S., *J. Biol. Chem.* 221, 287-299.
27. Substrate Binding Site in Bovine Chymotrypsin A. A Crystallographic Study Using Peptide Chloromethyl Ketones as Site-Specific Inhibitors, (1971) Segal, D.M., Powers, J.C., Cohen, G.H., Davis, D.R. and Wilcox, P.E., *Biochemistry* 10, 3728-3738.
28. Macromolecular X-Ray Data Collection on a Rotating Anode Diffractometer, (1987) Hope, H., Frolow, F. and Sussman, J.L., *Rigaku J.* 4, 3-10.
29. Fast Reactions of Excited Molecules, (1961) Meller, A., *Prog. React. Kinet.* 1, 187-214.
30. Electrophoretic Analysis of the Unfolding of Proteins by Urea, (1979) Creighton, T.E., *J. Mol. Biol.* 129, 235-264.
31. Studies on the Reactivation of Dimethylphosphorylchymotrypsin, (1960) Cohen W. and Erlanger, B.F., *J. Am. Chem. Soc.* 82, 3928-3934.
32. Purification and crystallization of a dimeric form of acetylcholinesterase from *Torpedo californica* subsequent to solubilization with phosphatidylinositol-specific phospholipase C, (1988) Sussman, J.L., M. Harel, M., Frolow, F., Varon, L., Tokar, L., Puterman, A.H. and Silman, I., *J. Mol. Biol.* 203, 821-823 (1988).
33. The Reaction of Diisopropylfluorophosphate with crystals of γ -Chymotrypsin, (1963) Sigler, P.B. and Skunner, H.C.W., *Biochem. Biophys. Res. Comm.* 13, 236-240.

34. Inactivation of Chymotrypsin by 5-Benzyl-6-chloro-2-pyrons: ¹³-NMR and X-ray Diffraction Analysis of the Inactivator-Enzyme Complex, (1987) Rings, D., Seaton, B.A., Gelb, M.H. and Abeles, R.H., *Biochemistry* 24, 64-68.
35. Refined Crystal Structure of γ -Chymotrypsin at 1.9 Å Resolution, (1981) Cohen, G.H., Silverton, E.W. and Davies, D.R., *J. Mol. Biol.* 148, 449-479.
36. Primary Structure of *Torpedo californica* Acetylcholinesterase Deduced from its cDNA Sequence, (1985) Schumacher, M., Camp, S., Mautet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S., Friedman, T. and Taylor, P., *Nature* 319, 407-409.
37. Primary Structures of the Catalytic Subunits from Two Molecular Forms of Acetylcholinesterase, (1985) MacPhee-Quigley, K., Taylor, P. and Taylor, S., *J. Biol. Chem.* 260, 12185-12189.