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AGED AND NON-AGED PYRENEBUTYL-CONTAINING ORGANOPHOSPHORYL CONJUGATES OF CHYMOTRYPSIN

PREPARATION AND COMPARISON BY ^{31}P-NMR SPECTROSCOPY*

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Abstract—Homologous pairs of non-aged and aged pyrene-containing phosphoryl conjugates of chymotrypsin were prepared in order to characterize by NMR and optical spectroscopy putative differences in the conformation of non-aged and aged organophosphoryl conjugates of serine hydrolases. Pyrenebutyl-O-P(O)(OC_2H_5)F and pyrenebutyl-O-P(O)(OC_2H_5)Cl were used to obtain the non-aged form pyrenebutyl-O-P(O)(OC_2H_5)-Cht, whereas pyrenebutyl-O-P(O)Cl_2, pyrenebutyl-O-P(O)(*p*-nitrophenoxy)Cl, and pyrenebutyl-O-P(O)(*p*-nitrophenoxy)$_2$ were used to produce the aged conjugate pyrenebutyl-O-P(O)(O^-)-Cht. These ligands bind covalently to the active site of serine hydrolases. The absorption spectra of both the non-aged and aged conjugates fitted approximately a 1:1 stoichiometry of bound organophosphate and enzyme in the non-aged and aged conjugates. Pyrenebutyl-O-P(O)(OC_2H_5)-Cht could be reactivated by pyridine-3-aldoxime methiodide, whereas no reactivation was observed for the similarly treated pyrenebutyl-O-P(O)(O^-)-Cht. The ^{31}P-NMR and reactivation data taken together strongly support the hypothesis that the aged form of the OP–Cht conjugate contains a P—O^- bond. These results provide a partial interpretation for the known resistance of the aged conjugates of serine hydrolases to reactivation.

Many serine hydrolases such as acetylcholinesterase (EC 3.1.1.7; AChE‖), and chymotrypsin (EC 3.4.21.1; Cht) 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].

Phosphorylated serine hydrolases may be reactivated effectively by various nucleophiles (e.g. quaternary oximes) which detach the phosphoryl moiety from the serine hydroxyl of the enzyme [2]. In addition, they may undergo a dealkylation reaction, commonly termed "aging" which converts the inhibited enzyme into a non-reactivatable form [1, 3, 4]. This aging process is particularly pronounced for OP–AChE conjugates in which the OP moiety contains a secondary alkyl group such as isopropyl (e.g. sarin and DFP) or pinacolyl (e.g. soman). Irrespective of the mechanism of aging it is usually accepted that the common denominator is the net loss of an alkyl group from the OP-bound moiety. This loss is depicted in Scheme I:

$$EH + XP(O)(OR')R \xrightarrow{-HX} E\text{-}P(O)(OR')R \xrightarrow{-R'} E\text{-}P(O)(O^-)R$$

where: R = alkyl, aryl, alkyloxy or aryloxy
R′ = alkyl, aryl
X = F, Cl, *p*-nitrophenoxy, dialkylaminoethanethiol

Scheme I

This scheme implies that aging is associated with the introduction of a formal negative charge into the active site of the inhibited enzyme. The observation that a pre-dealkylated anticholinesterase, such as $(C_2H_5O)P(O)(OH)SCH_2CH_2N(C_2H_5)_2$ [5], as well as phosphonic ester dihalides, such as $CH_3P(O)Cl_2$ [6], produce an aged enzyme instantaneously substantiates the hypothesis that ageing indeed involves formation of a negatively charged phosphoryl residue at the active site of the inhibited enzyme. The structure proposed for the aged enzyme (Scheme I) sug-

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‖ Abbreviations: AChE, acetylcholinesterase; Cht, α-chymotrypsin; OP, organophosphorus; DFP, diisopropyl fluorophosphate; DEPC, diethyl phosphorochloridate; TEPP, tetraethylpyrophosphate; DEPF, diethyl phosphorofluoridate; EPDC, ethyl phosphorodichloridate; PBEPF, 1-pyrenebutyl ethyl phosphorofluoridate; PBEPC, 1-pyrenebutyl ethyl phosphorochloridate; PBPDC, 1-pyrenebutyl phosphorodichloridate; PB(pNP)PC, 1-pyrenebutyl *p*-nitrophenoxyphosphorochloridate; PB(pNP)$_2$P, 1-pyrenebutyl bis(*p*-nitrophenoxy) phosphate; PBDEP, 1-pyrenebutyl diethylphosphate; pNP, *p*-nitrophenoxy; DEP, diethylphosphoryl; EP, ethyl hydroxyphosphoryl; DIP, diisopropylphosphoryl; PBEP, 1-pyrenebutyl ethyl phosphoryl; PBP, 1-pyrenebutyl hydroxyphosphoryl; ATEE, *N*-acetyl-L-tyrosine ethyl ester; 3-PAM, pyridine-3-aldoxime methiodide; pNPH, *p*-nitrophenol; HMPA, hexamethylphosphoramide; PB, 1-pyrenebutyl; and dd, double distilled.

gests that the negative charge may impose an electrostatic barrier to nucleophilic attack on the P atom by anionic nucleophiles such as oximes. However, non-enzymatic studies with the corresponding phosphoric acid ester analogs of the assumed aged enzyme [7–9] indicate that, although the negative charge would indeed retard reactivation, it would not do so to an extent which would explain the complete failure to detect any reactivation of aged phosphorylated serine esterases. It is, therefore, unlikely that the electrostatic effect of the negative charge assumed to be present in the aged enzymes is alone responsible for their unusual resistance to reactivation.

A possible explanation for the observed resistance to reactivation may be the occurrence of conformational changes concomitant with aging. Indeed electron spin resonance [10], nuclear magnetic resonance [11, 12], fluorescence spectroscopy [13, 14] and X-ray crystallography [15] have all been used to study the structure of OP conjugates of various serine hydrolases. The parallel use of ^{31}P-NMR and optical spectroscopy permits correlation between structural changes in the substituents attached to the P atom and conformational changes in the active site. We chose, therefore, to synthesize suitable fluorescent OPs which could be used to prepare homologous pairs of aged and non-aged fluorescent OP–Cht conjugates. Cht was selected for this study since it is a well-characterized enzyme whose sequence and three-dimensional structure have been fully worked out [16–18]. Furthermore, it is commercially available in highly purified form in large quantities, allowing for ^{31}P-NMR spectroscopy. Indeed, ^{31}P-NMR spectroscopy has been utilized in studying the aged forms of various OP–Cht conjugates [11, 12, 19]. In these studies, however, the appearance of the P–O$^-$ function in the inhibited enzyme as monitored by ^{31}P-NMR spectroscopy was not correlated with the reactivatability of the phosphorylated enzyme. Pyrene was selected as the fluorophore since it is particularly suitable for detecting conformational changes in proteins [13, 14].

In the following we describe the preparation and subsequent characterization by ^{31}P-NMR of the non-aged and aged conjugates of Cht which were obtained by using several novel pyrenebutyl-containing organophosphorus inhibitors in addition to the two inhibitors previously utilized to obtain the corresponding conjugates of AChE [14]. The ^{31}P-NMR data, taken together with the reactivation studies, provide direct evidence for the presence of a P—O$^-$ bond in the aged conjugate as opposed to the trialkyl-type phosphorus ester structure assigned to the non-aged form (i.e. the reactivatable conjugate). We further present evidence to substantiate the hypothesis that dihalide organophosphates, [R-P(O)Cl$_2$, R = alkyl or alkoxy], are suitable reagents for preparing aged organophosphoryl conjugates of Cht (and presumably of other serine hydrolases). Optical spectroscopic studies carried out with these OP–Cht conjugates show that conformational changes indeed occur concomitantly with the aging process [20].

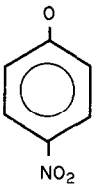
MATERIALS AND METHODS

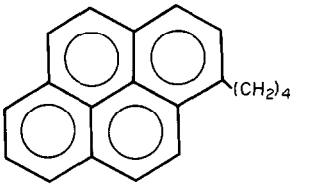
Preparation of ligands. Table 1 lists the names and structures of the various OP ligands employed in this study. Compounds 1, 2, 4 and 5 were obtained from the Aldrich Chemical Co. (Milwaukee, WI) (2 and 4 were redistilled, whereas 1 and 5 were used as obtained). Compounds 3 and 6–8 were prepared by procedures published earlier [14]. The fluorescent ligands 9–11 were prepared as described below:

The starting materials were purchased from the Aldrich Chemical Co. and purified before use by either distillation or recrystallization. The *p*-nitrophenyl-containing fluorescent ligands were prepared according to the general synthetic routes shown in Scheme II.

a. 1-pyrenebutanol + (pNP)P(O)Cl$_2$ ⟶ pyrenebutyl-O-P(O)(pNP)Cl
9. PB(pNP)PC

b. pyrenebutyl-O-P(O)Cl$_2$ + 2 pNPH ⟶ pyrenebutyl-O-P(O)(pNP)$_2$
10. PB(pNP)$_2$P

where: pNP: 

1-pyrenebutyl: 

Scheme II

Pyrenebutyl (pNP) phosphorochloridate [9, PB(pNP)PC]

A cold solution (3°) of 1-pyrenebutanol [14] (2.3 g, 8.4 mmol) in 10 ml of dry dioxane was added dropwise to a stirred solution of (pNP)P(O)Cl$_2$ (2.15 g, 8.4 mmol) in 2 ml of cold dry dioxane. After 24 hr, the solvent was evaporated under reduced pressure (25°/0.001 mm Hg), and the residue was triturated with *n*-hexane. The insoluble material was removed by filtration. Yellow crystals (100 mg) precipitated at 4° (m.p. 98–100°).

^{31}P-NMR (relative to H_3PO_4): δ, −1.8 ppm (C_6H_6).

Mass spectrometry (EI): 493 (M^+, 5%), 256 ($C_{20}H_{16}^+$, 100%), 215 ($C_{16}H_9CH_2^+$, 60%).

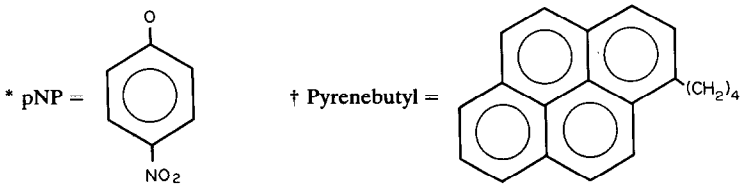
Pyrenebutyl bis-(pNP) phosphate [10, PB(pNP)$_2$P]

Pyrenebutyl-O-P(O)(Cl$_2$) (3.9 g, 0.01 mol) and sodium *p*-nitrophenolate (3.70 g, 0.023 mol) were refluxed in 60 ml of dry benzene for 2 hr. Diethyl ether (100 ml) was added to the cooled mixture, followed by filtration and removal of the precipitate. A viscous red crude product was obtained after evaporation of the filtrate under reduced pressure. The oil was redissolved in CH_2Cl_2 and extracted twice with 10% aqueous NaOH (5°), followed by stripping off the CH_2Cl_2 solution. Pure 10 was obtained as a viscous yellow oil.

^{31}P-NMR (relative to H_3PO_4): δ, −13.40 ppm (CH_2Cl_2).

Table 1. Structures and names of OP ligands

Compound	Name	Chemical structure
1	Paraoxon	$(C_2H_5O)_2P(O)(pNP)$*
2	DEPC	$(C_2H_5O)_2P(O)Cl$
3	DEPF	$(C_2H_5O)_2P(O)F$
4	EPDC	$(C_2H_5O)P(O)Cl_2$
5	DFP	$(isoC_3H_7O)_2P(O)F$
6	PBEPF	Pyrenebutyl-O-P(O)(OC_2H_5)F†
7	PBEPC	Pyrenebutyl-O-P(O)(OC_2H_5)Cl
8	PBPDC	Pyrenebutyl-O-P(O)Cl_2
9	PB(pNP)PC	Pyrenebutyl-O-P(O)(pNP)Cl
10	PB$(pNP)_2$P	Pyrenebutyl-O-P(O)$(pNP)_2$
11	PBDEP	Pyrenebutyl-O-P(O)$(OC_2H_5)_2$



Mass spectrometry (EI): 596 (M^+, 8%), 340 ([M-pyrenebutyl]$^+$, 15%, 256 ($C_{20}H_{16}^+$, 100%), 215 ($C_{16}H_9CH_2^+$, 60%).

Anal. calc. for $C_{32}H_{25}N_2O_8P$: C, 64.43; H, 4.19; N. 4.70. Found: C, 64.15; H. 4.22; H. 4.79.

Pyrenebutyl diethyl phosphate [11, PBDEP]

O,O-Diethylphosphorochloridate (8.0 g, 47 mmol) in dry dioxane was added dropwise to a stirred solution of pyrenebutanol (5.8 g, 21 mmol) and triethylamine (4.70 g, 47 mmol) in 30 ml dioxane at room temperature. The mixture was stirred for 12 days. Following evaporation under reduced pressure (25°, 0.001 mm Hg), the precipitated solid was removed by filtration, redissolved in benzene, and extracted with 10% aqueous NaOH at 5°. After removal of benzene under reduced pressure, 11 was recrystallized from *n*-hexane at −70° (m.p. 37–38°).

^{31}P-NMR (relative to H_3PO_4): δ, −0.70 ppm (C_6H_6).

Mass spectrometry (EI): 410 (M^+, 25%), 256 ($C_{20}H_{16}$+, 43%), 215 ($C_{16}H_9CH_2$+, 100%).

Enzyme source and activity assay

Bovine pancreatic α-chymotrypsin (Type II, 3× crystallized, salt-free and lyophilized) was purchased from the Sigma Chemical Co. (St Louis, MO). The active site concentration of solutions of such preparations was determined by use of cinnamoyl imidazole according to Schonbaum *et al.* [21]. The concentrations found were 78–93% of the theoretical value, assuming a molecular weight of 25,000 and $A_{280}^{1\%} = 20$. Cht activity was monitored by the pH-stat procedure of Cunningham and Brown [22], utilizing *N*-acetyl-L-tyrosine ethyl ester (ATEE) as substrate.

Preparation of OP–Cht conjugates

Large quantities of the OP–Cht conjugate (200–400 mg/batch) were obtained by dropwise addition (over periods of 30–60 min) of a fresh concentrated solution of paraoxon (1), DEPC (2) DEPF (3), EPDC (4) or DFP (5) (0.01 to 0.1 M) in acetonitrile, to a stirred solution of Cht (5 to 10 mg/ml) in double-distilled (dd) water at room temperature. A pH of 7.4 was maintained during the inhibition period by adjusting with 0.02 N NaOH. In the case of PBEPF (6), PBPDC (8), PB(pNP)PC (9) and PB$(pNP)_2$P (10), inhibition was carried out in fresh dilute solutions of both Cht (0.5 to 1 mg/ml) and of the inhibitors (0.2 to 1 mM) due to the limited solubility of the pyrene-OP ligands in water. In no case did the amount of organic solvent in the final inhibition medium exceed 10%. The decrease in enzymic activity was monitored until 40–96% and >98% of the initial enzyme activity was inhibited by the pyrene-containing OP ligands and by the non-fluorescent inhibitors respectively. The solution of the inhibited enzyme was then lyophilized, redissolved in 4 ml of dd water, and loaded onto a Sephadex G-10 column (3 × 60 cm). The column was eluted with dd water at a flow rate of 1.5 ml/min. The main fractions containing the protein were pooled and relyophilized. The dry OP–Cht conjugates were stored over desiccant at −20° until used for either ^{31}P-NMR or optical spectroscopy. Occasionally, samples were dialyzed against either dd water or buffered solutions (5 mM Tris, pH 7.5) for 48 hr at 4° so as to remove traces of free hydrolyzed ligands. Dialysis was carried out in dialysis tubes flat width 25 mm (Thomas Scientific) with a mol. wt cutoff at 12,000.

In control experiments, Cht was first preincubated with the specific non-fluorescent active-site inhibitor DFP (5) which inhibited >99.5% of the enzymic activity. These control samples were then treated with the fluorescent OP inhibitor, gel-filtered, and lyophilized as described above.

Table 2 lists the names and structures of the various OP–Cht conjugates used in this study.

Inhibition of Cht by PBEPF (6)

To a 5-ml solution of Cht (50 nM) in 0.1 M KCl–2 mM Tris, pH 7.8, placed in a thermostated pH-stat titration cell (25°) was added 5–25 μl of a stock solution of the inhibitor (0.26 mM) in acetonitrile to give a final inhibitor concentration of 0.26 to 1.3 μM. At given time intervals, inhibition was terminated

Table 2. Structures of OP–Cht conjugates, RO-P(O)(R')–Cht

Conjugate	Inhibitor*		RO-P(O)(R')–Cht R	R'	Status†
DEP–Cht	Paraoxon	(1)	C_2H_5	OC_2H_5	>75
DEP–Cht	DEPF	(3)	C_2H_5	OC_2H_5	>75
EP–Cht	EPDC	(4)	C_2H_5	OH	<3
DIP–Cht	DFP	(5)	$CH(CH_3)_2$	$OCH(CH_3)_2$	10–15
PBEP–Cht	PBEPF	(6)	pyr‡	OC_2H_5	>75
PBP–Cht	PBPDC	(8)	pyr	OH	<3
PBP–Cht	PB(pNP)PC	(9)	pyr	OH	<3
PBP–Cht	$PB(pNP)_2P$	(10)	pyr	OH	<5

* See Table 1 for molecular structure.
† Percent reactivation after 48 hr in the presence of 0.1 M 3-PAM.
‡ pyr = pyrenebutyl.

by adding 100 μl of substrate (ATEE, 100 mg/ml in dioxane) to produce a final ATEE concentration of 0.016 M. A control experiment demonstrated that addition of 1.3 μM PBEPF to a Cht solution containing 0.016 M ATEE did not cause significant inhibition within the first 2 min. Residual Cht activity was measured immediately upon addition of the substrate, by the pH-stat procedure mentioned above. Straight lines were obtained for the first 2 min. Blanks were treated in the same manner except that the inhibitor solution was replaced by pure acetonitrile for estimation of 100% Cht activity. Under the experimental conditions described above, 90% inhibition of the enzyme was achieved within 15 min. A separate evaluation of the reversible inhibition constant, K_I, was carried out as follows: Various amounts of PBEPF were diluted 100 to 500-fold from a stock solution in acetonitrile (0.1 mM), into 5–10 ml of Cht (0.1 μM) in 2 mM Tris buffer (pH 7.8, 0.1 M KCl, 25°) containing 1–8 mM ATEE. Enzymic activity was measured both before and immediately after addition of the inhibitor. K_I was calculated according to the Hunter–Downs equation as described elsewhere [1].

Reactivation of inhibited Cht

For reactivation experiments, lyophilized samples of OP–Cht (inactivated 70–98% as described above) were dissolved in dd water to yield solutions which were *ca.* 0.4mM in catalytic sites. Aliquots were diluted 100-fold into a reactivation medium (0.1 M Tris, pH 7.0, 25°) containing 0.1 M 3-PAM [23]. At selected time intervals, 20-μl aliquots of the reactivation mixture were diluted into 5 ml of Tris buffer (2 mM, pH 7.4) and assayed as described before. Blanks were treated in the same manner except that the inhibited enzyme was replaced by native Cht. We note that 0.1 M 3-PAM partially stabilized native Cht solutions for periods of 48–72 hr as compared to controls without 3-PAM.

Release of p-*nitrophenol*

The release of *p*-nitrophenol in reactions where PB(pNP)PC (9) and $PB(pNP)_2P$ (10) were allowed to react with either Cht, phosphate buffer or alkali was measured spectrophotometrically at 400 nm.

NMR measurements

^{31}P-NMR spectroscopy at 101.2 MHz was performed with a Bruker WM250 spectrometer coupled to an Aspect 2000 computer. D_2O (20–99%) in the same tube (10 mm diameter) served for field-frequency locking. A power of 1 W was maintained for the continuous broadband heteronuclear proton decoupling so as to avoid internal build-up of heat. Throughout the run, the temperature was maintained at 23 ± 3°. Spectral data were accumulated in the Fourier transform by application of 70° pulses with a spectral width of 16,000 Hz. A delay time of 0.8 sec was utilized between accumulations.

All chemical shifts were recorded from the built-in absolute crystal frequency and assigned to the internal standard, hexamethylphosphoramide (HMPA). The ^{31}P-NMR spectra of the OP–Cht conjugates and the model compounds were recorded in a concentration range of 1–10 mM, assuming mol. wt = 25,000 for Cht; 5K–100K scans were accumulated for each run.

RESULTS

Syntheses

The synthetic routes described under Materials and Methods were selected on the basis of purity and homogeneity of the final product rather than of yield or convenience of starting materials.

Determination of the homogeneity and structure of the newly synthesized fluorescent OP ligands was based on MS, ^{31}P-NMR and UV spectroscopy, as well as upon elementary analysis and TLC. For compounds 9 and 11, the elementary analyses deviated from the theoretical value by more than ±0.4%, for one or more elements. Nevertheless, since ^{31}P-NMR spectroscopy exhibited the presence of only one kind of phosphorus-containing compound, we assumed that the contamination, if present at all, was caused by non-phosphorus-containing molecules whose origin was presumed to be the crystallization medium. We note that 9 was used, only for a single preparation of the corresponding OP–enzyme conjugate.

The stoichiometric release of *p*-nitrophenol (pNPH) from PB(pNP)PC (9) and $PB(pNP)_2P$ (10) after 6 hr reflux in 1 M NaOH provided additional

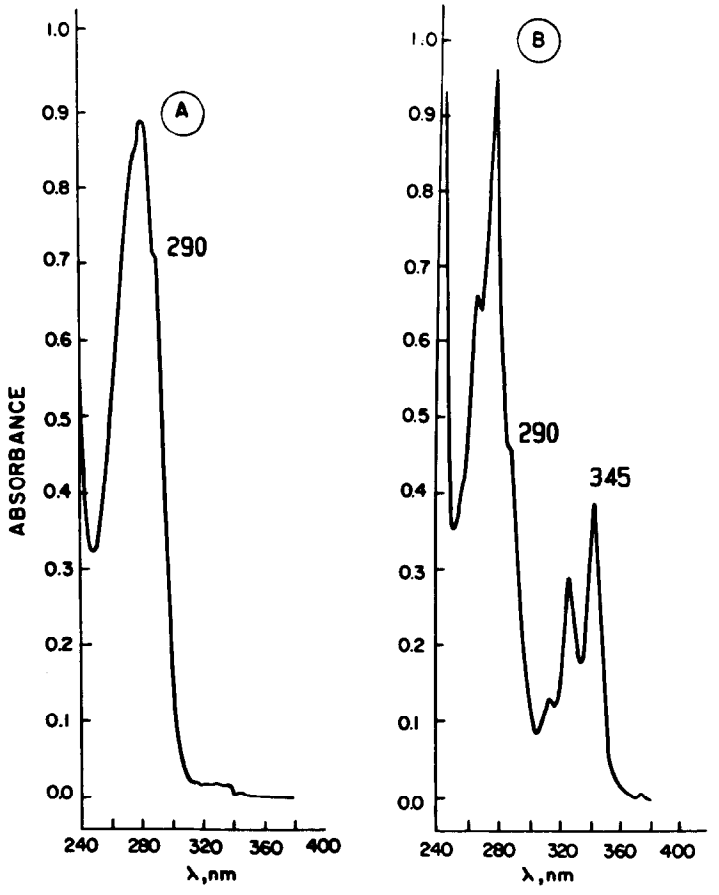


Fig. 1. (A) Absorbance spectrum of Cht (0.5 mg/ml, dd H_2O) which had been treated with DFP prior to incubation with PBEPF. (B) Absorbance spectra of PBP–Cht conjugate (0.3 mg/ml) after unbound fluorophore (PBPDC + hydrolysis products) had been removed on Sephadex G-10. In both cases absorption spectra were measured on conjugates redissolved after lyophilization.

support for the proposed structure of these pNP-containing ligands. Thus, 9 and 10 released 1 ± 0.1 and 2 ± 0.1 equivalents of pNPH respectively. It should be noted that a similar stoichiometry was found for the release of pNPH in the reaction of 10 with Cht, where large quantities of conjugate were prepared for ^{31}P-NMR and optical spectroscopy.

Specificity of binding and stoichiometry of OP–enzyme fluorescent conjugates

The specificity of binding of 6–10 to Cht was demonstrated by employing the same methodology utilized previously for AChE [14]. Thus, Cht was inhibited >99% with DFP to yield DIP–Cht, and this conjugate was exposed to the pyrene-containing OP inhibitors under the same conditions employed for the unmodified native enzyme. The reaction mixture was then passed through a Sephadex G-10 column so as to separate the OP–enzyme conjugate from the unbound fluorescent OP. Pretreatment with DFP prevented almost completely the binding of all the fluorescent OPs to Cht, as can be seen from the representative data displayed in Fig. 1A for PBEPF.

Furthermore, fluorometric measurements indicated that less than 3% of the fluorescence detected upon excitation at 345 nm was non-specific. Thus, the fluorescent OPs appear to interact specifically and exclusively with the active-site serine, and the Sephadex G-10 column provides an efficient tool for separation of the fluorescent OP–Cht conjugate from the free fluorophore.

The stoichiometry of binding was determined from the absorption of the bound pyrene at 345 and 346 nm which are the absorption maxima of pyrene in non-aged and aged pyrene-containing OP–Cht conjugates respectively. Molar extinctions of the chromophore in the aged and non-aged conjugates were determined by measuring the increase in absorption of PBEPF from $3.05 \pm 0.1 \times 10^4$/M/cm and of $BP(pNP)_2P$ from $2.7 \pm 0.15 \times 10^4$/M/cm (both in Ch_3CN:water, 1:11) to $3.5 \pm 0.05 \times 10^4$/M/cm and $4.1 \pm 0.1 \times 10^4$/M/cm, respectively, upon the addition of 1 mM Cht to either PBEPF or $BP(pNP)_2P$ (3–8 μM) in 0.05 M Tris buffer at pH 7–8 (Fig. 2). A similar increase in extinction coefficient ($\varepsilon_{349} = 3.9 \times 10^4$/M/cm) was observed to occur during formation of the analogous pyrene-containing conjugate of AChE [13]. Figure 2 also demonstrates a kinetic correlation between the rates of increase in absorbance at 346 nm (due to conjugation of the pyrene to Cht) and at 400 nm (due to release of pNPH) for the reaction between Cht and

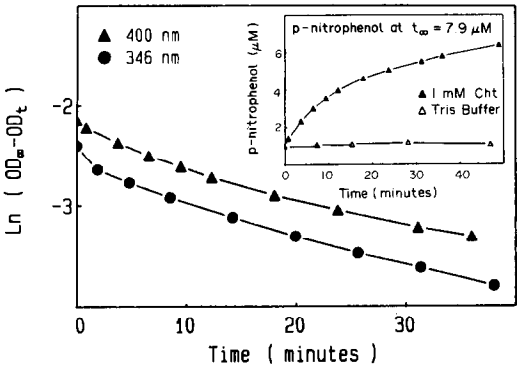


Fig. 2. First-order plot of the increase in absorbance at 400 nm (release of *p*-nitrophenol) and 346 nm (conjugation of pyrene to Cht) for the reaction of 1 mM Cht with 3.5 μM $PBP(pNP)_2P$ containing 9% free *p*-nitrophenol at 25° (0.05 M Tris, pH 8.0). Inset: Time-dependence of the release of *p*-nitrophenol, demonstrating stoichiometric release of 2 equivalents of phenol under the same experimental conditions. The extinction coefficient at 346 nm was calculated to be 4.06×10^4/M/cm after correction for *p*-nitrophenol absorption at this wavelength ($\varepsilon_{346} = 0.58 \times 10^4$/M/cm).

$PB(pNP)_2P$, verifying that the binding of one equivalent of pyrene residue to Cht is accompanied by the release of two equivalents of pNPH. These results substantiate the assumption that $PB(pNP)_2P$ eventually produces the aged form PB-O-P(O)(O^-)-Cht. The increase in molar absorption was accompanied by a shift in the absorption maxima from 343 to 345 nm (PBEPF) and from 349 to 346 nm ($PB[pNP]_2P$) upon completion of the inhibition reaction. The absorption maximum of the aged conjugate prepared from PBPDC was found to be at 346 nm, whereas hydrolyzed PBPDC (i.e. PB-O-P(O)$(OH)_2$) showed a maximum absorption at 343 nm, both in the presence and absence of unmodified Cht. Furthermore, the addition of 2–3 μM PBPDC to a large excess of Cht (> 1.8 mM) caused an immediate increase in the extinction coefficient from $3.4 \pm 0.1 \times 10^4$/M/cm (343 nm) to $4.2 \pm 0.15 \times 10^4$/M/cm (346 nm), which remained constant in the range of 1.8 to 4 mM Cht. These results support the assumption that PBPDC and $PB(pNP)_2P$ produced similar conjugates which differ from the conjugate obtained by modification of Cht with PBEPF [20]. Bound pyrene contributes substantially to the protein absorption at 278 nm and makes a small but significant contribution at 290 nm. Since lyophilization of Cht does not affect significantly the activity/weight ratio of unmodified enzyme under the experimental conditions employed for the preparation of the conjugates, protein concentration of aged and non-aged conjugates was determined by weight.

Table 3 summarizes stoichiometry data calculated for pyrene-containing OP–Cht conjugates using the data obtained from the absorption measurements at either 345 or 346 nm. It was calculated that the non-aged and aged OP–Cht conjugates contained 0.94 ± 0.03 and 0.97 ± 0.05 (mean ± SE, N = 5–8) molecules of bound pyrene per catalytic site. It should be noted, however, that one cannot exclude completely the presence of a small amount of denatured Cht prior to phosphorylation or the occurrence of partial denaturation in the course of phosphorylation either of which might lead to deviations from 1:1 stoichiometry.

Inhibition of Cht by PBEPF (6)

The initial rates of inhibition were found to be pseudo first-order, but the pseudo first-order rate constants were not linearly related to the concentration of the inhibitor. Since all the lines passed through the origin, we assume that a Michaelis complex (or other reversible complex) was formed which was readily dissociated by addition of substrate. This kinetic behavior implies that the inhibition of Cht follows the pathway depicted in Scheme III.

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k'} E_i$$

Scheme III

The mathematical solution for Scheme III is given in Eqn 1:

$$1/k_{obs} = [K_I/k'][1/I] + 1/k' \qquad (1)$$

The double-reciprocal plot (Fig. 3), plotted according to Eqn 1, provided a straight line from which K_I and k' were calculated to be 7.5×10^{-7} M and 0.44/min respectively. The bimolecular rate constant, $k_i = (k'/K_I)$ was calculated to be 5.8×10^5/M/min. The value obtained for K_I shows that PBEPF exhibits a high affinity for Cht. To verify this value, the dissociation constant for the reversible Michaelis complex, K_I, was measured by the Hunter–Downs method, as described by Aldridge and Reiner [1]. This method examines the effect of substrate (S, ATEE) on the reversible inhibition of a given inhibitor (I), and the mathematical expression for the effect of the substrate is shown in Eqn 2.

$$[I]E/(E_o - E) = K_I + K_I[S]/K_m \qquad (2)$$

In Eqn 2, $[I]$ and $[S]$ are the concentrations of inhibitor and substrate, respectively, E_o is the enzyme activity in the absence of inhibitor, and E is the enzyme activity in the presence of inhibitor. K_I and K_m are, respectively, the dissociation constants for the reversible inhibitor–Cht complex and the Michaelis complex. After the addition of PBEPF to the enzyme–substrate mixture, there was an immediate decrease in enzymatic activity which could be monitored for 0.5 to 1 min (during which period no further significant decrease in enzyme activity could be detected). The results of plotting $[I]E/(E_o - E)$ vs $[S]$ (not shown) provided a straight line from which K_I and K_m were calculated. Thus, K_I was found to be 5×10^{-7} M, in good agreement with the value obtained from the kinetic measurements (Fig. 3, Eqn 1). The value of K_m obtained for ATEE from the Hunter–Downs plot (1.3 mM) was found to be in good agreement with the value obtained by the standard Lineweaver–Burk plot (1.2 mM, not shown).

Table 3. Stoichiometry of binding of pyrene-containing OPs to Cht

Conjugate*	% Inhibition of Cht activity	OP conjugate concentration† (μM)	Stoichiometry‡
PBEP–Cht			
I	100	17.0	0.91
II	100	16.4	0.87
III	90	7.1	0.94
IV	99	12.4	1.02
V	97	17.0	0.94
PBP–Cht			
VI	52	9.1	0.98
VII	85	17.0	1.06
VIII	76	12.3	1.07
IX	96	18.4	0.84
X	97	16.4	0.89
XI	85	7.1	0.93
XII	42	15.1	0.96
XIII	90	11.4	0.99

* OP–Cht conjugates were prepared as follows: I–V from PBEPF, VI–X from PBPDC, XI and XII from PB(pNP)$_2$P, and XIII from PB(pNP)PC.

† Determined by weight assuming mol. wt 25,000, and corrected for percent inhibition and for the presence of inactive enzyme measured by active-site titration of unmodified Cht.

‡ The ratio between the optical density measured at 345 nm or 346 nm (non-aged or aged form respectively) divided by the OP–conjugate concentration (†), and the extinction coefficients found for total complete binding of the OP–ligands to Cht as described under Results.

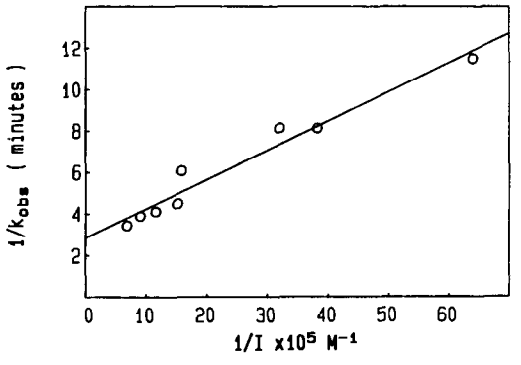


Fig. 3. Evaluation of K_I, k' and k_i for inhibition of Cht by PBEPF in accordance with the double-reciprocal plot presented in Eqn 1. Inhibition was performed in 0.1 M KCl, 2 mM Tris, pH 7.8, at 25°.

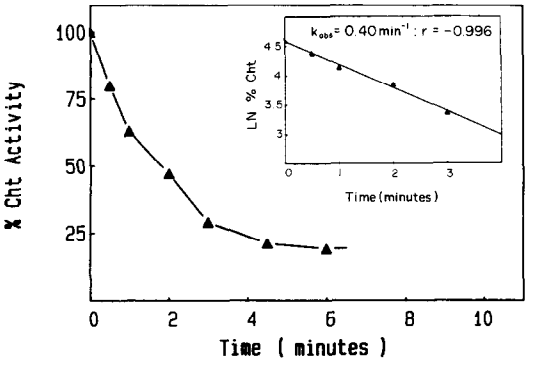


Fig. 4. Time course of inhibition of Cht (0.025 mM) by PBEPF (0.022 mM) at 25° in 0.1 M KCl, 2 mM Tris, pH 7.8; $CH_3CN < 2\%$. Inset: semilogarithmic plot of the inhibition data. The straight line was obtained by the least squares method.

These results imply that PBEPF binds competitively to the substrate-binding site.

PBEPF possesses a chiral center on the phosphorus atom. Since a stoichiometry of 1:1 was established when a slightly sub-stoichiometric amount of this racemic mixture was reacted with Cht (Fig. 4), it may be concluded that both enantiomers react with the enzyme and display a rather similar rate of inhibition. It is also worth noting that, since the initial concentrations of both Cht and PBEPF (2.2 to 2.5 $\times$ 10^{-5} M) were considerably higher than the dissociation constant for the corresponding Michaelis complex (K_I = 5–7.5 $\times$ 10^{-7} M), we assume that the rate of inhibition is controlled by the unimolecular step described in Scheme III (i.e. k'). Indeed, a first-order plot (Fig. 4) provided a straight line with k' = 0.40/min, in good agreement with the value obtained from the double-reciprocal plot in accordance with Eqn. 1.

In view of the poor solubility and low anti-Cht activity of 7–10, accurate quantitative kinetic data based on monitoring the rate of loss of Cht activity are not presented for these compounds.

Reactivation of inhibited Cht

The reactivation of lyophilized OP–Cht conjugates

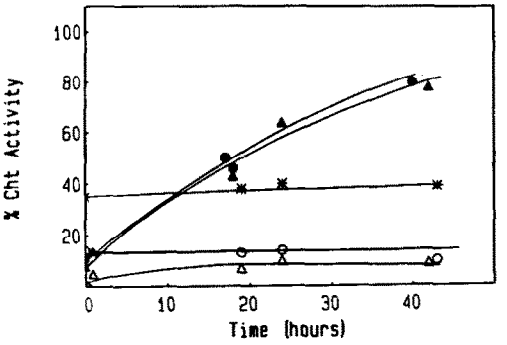


Fig. 5. Reactivation of OP–Cht conjugates by 0.1 M 3-PAM in 2 mM Tris, pH 7.0, at 25°. Key: (●—●) PBEP–Cht; (○—○) PBP–Cht obtained by use of PBPDC; (*—*) PBP–Cht obtained by use of $PB(pNP)_2P$; (▲—▲) DEP–Cht obtained by the use of paraoxon; and (△—△) DIP–Cht obtained by the use of DFP.

was performed with 0.1 M 3-PAM in 2 mM Tris, pH 7.0, at 25°. The activity of the regenerated enzyme was compared to that of a control sample of unmodified Cht similarly incubated in the presence of 0.1 M 3-PAM. Figure 5 illustrates the reactivation profile for PBEP–Cht (obtained from PBEPF), PBP–Cht (obtained by use of either $PB(pNP)_2P$ or PBPDC), DIP–Cht (obtained from DFP) and DEP–Cht (obtained from paraoxon). As expected, PBP–Cht could not be reactivated, irrespective of the inhibitor employed. Thus, aged enzyme was formed either instantaneously (PBPDC) or gradually [$PB(pNP)_2P$], as depicted in Scheme I. Aging of the latter conjugate was expected by analogy with an earlier study in which Cht inhibited by Tris–(pNP)-phosphate was demonstrated to undergo aging concomitantly with release of a stoichiometric amount of *p*-nitrophenol [24].

PBEP–Cht and DEP–Cht could be partially reactivated (*ca.* 75%), whereas in the case of DIP–Cht only 10–15% of the original enzyme activity could be restored. Since reactivation of the OP–Cht conjugates proceeds very slowly, it may be assumed that several side-reactions may occur, such as denaturation of both the inhibited and free Cht, as well as dealkylation to form the aged conjugate of the enzyme as was observed by ^{31}P-NMR measurements in the case of DIP–Cht [12].

To substantiate the results of the reactivation experiments, progressive changes in the reaction mixture PBEP–Cht/0.1 M 3-PAM were also monitored by ^{31}P-NMR spectroscopy. The principal signal, which showed up at −29.15 ppm (Fig. 6), was assigned to the expected reactivation product, namely $PB\text{-}O\text{-}P(O)(OC_2H_5)OH$, which stems from

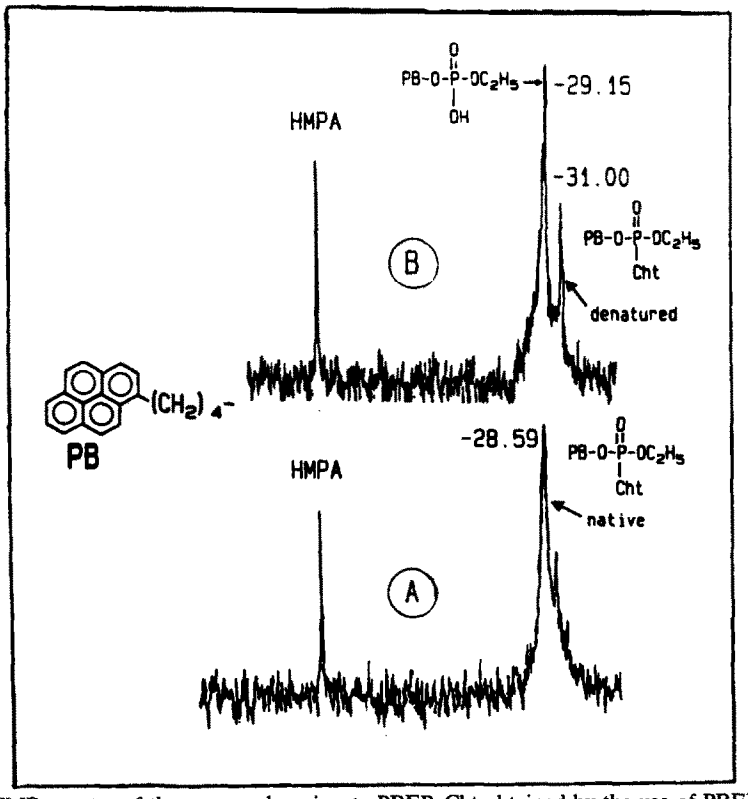


Fig. 6. ^{31}P-NMR spectra of the non-aged conjugate PBEP–Cht obtained by the use of PBEPF, in the presence of 3-PAM (for experimental conditions see Fig. 5). (A) Before initiation of reactivation. (B) After 48-hr incubation with 3-PAM. Negative signs (δ, ppm) indicate an upfield chemical shift relative to HMPA. The main peak at −29.15 ppm (B) represents unbound OP ligand released upon reactivation. The second peak at −31.00 ppm was assigned to the denatured form of PBEP–Cht conjugate.

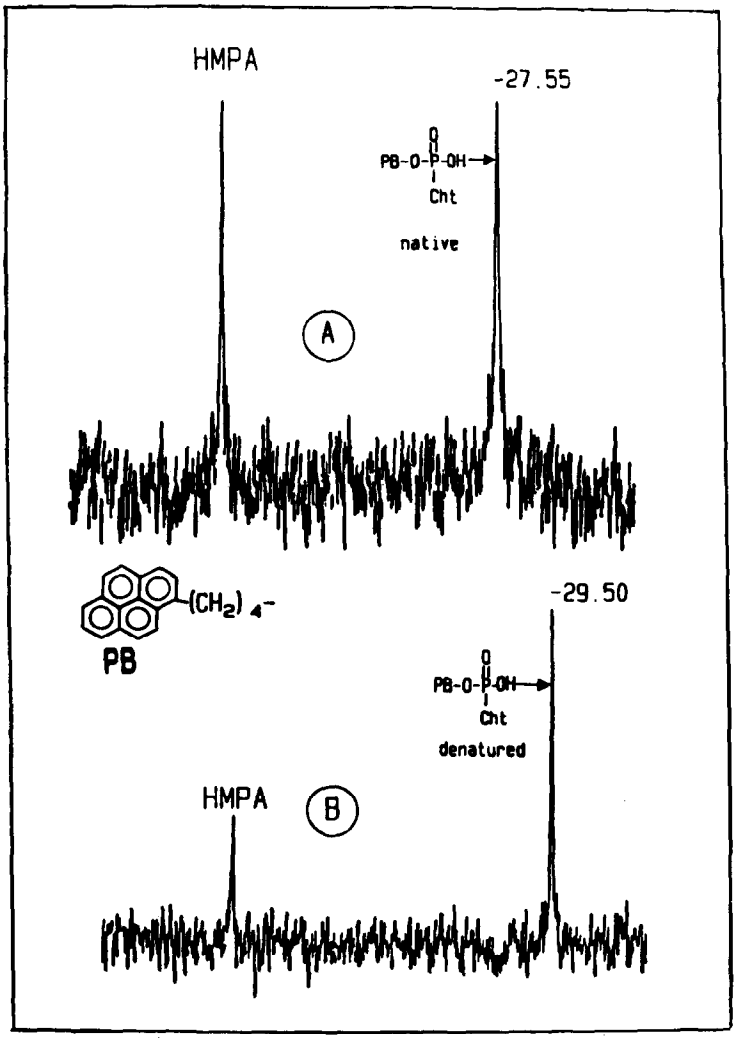


Fig. 7. ^{31}P-NMR spectra of the aged conjugate PBP–Cht, obtained by use of PBPDC. (A) Native conjugate. (B) Conjugate denatured in 6 M guanidine·HCl. Negative signs (δ, ppm) indicate an upfield chemical shift relative to HMPA.

the nucleophilic displacement at the P atom of the phosphorylated enzyme. This assignment was based on comparison of the chemical shift obtained for the hydrolysis product of PB-O-P(O)(OC_2H_5)F (PBEPF), in the presence of 0.1 M 3-PAM (−29.15 ppm), with the signal shown in Fig. 6.

^{31}*P-NMR spectroscopy*

Hexamethylphosphoramide $[CH_3)_2N]_3P(O)$, HMPA, was used as an internal standard throughout this study. The chemical shift of HMPA was 30.50 ppm downfield to external 85‰ phosphoric acid and was well separated from the signals both of the model compounds and of the protein–OP conjugates (Figs 6 and 7). The mean value of the chemical shift of HMPA, as recorded from the built-in absolute crystal frequency, was found to be 8919 ± 3 Hz (N = 60) (or 88.05 ± 0.03 ppm), whereas 85% phosphoric acid absorbed at 57.55 ± 0.03 ppm as related to the same crystal frequency.

The results obtained for HMPA were recorded in sixty different experiments in which the pH ranged between 2 and 9 and the solution contained either 1–4 mM OP–Cht conjugate or a 1–10 mM concentration of the model compound. The chemical shift of HMPA was not sensitive to these variations and remained constant throughout. However, in a number of cases we observed that, in the presence of 6 M guanidine hydrochloride, a very small upfield shift occurred (to 8900 ± 5 Hz).

To correlate ^{31}P-NMR chemical shifts of the aged and non-aged conjugates of Cht with the various substituents attached to the P atom, we first established the ^{31}P-NMR chemical shifts of the model compounds under the same experimental conditions used for the OP–Cht conjugates. The results of these experiments are summarized in Table 4. The addition of 1–4 mM Cht, whether free or OP-inhibited, did not affect the chemical shifts of the model compounds. The results in Table 4 show that replacing one alkoxy group in

Table 4. ^{31}P-NMR chemical shifts of model compounds in water (pH 3–7)

Type	Structure*	Chemical shift† (ppm from HMPA)
Tertiary esters	Pyr-O-P(O)$(OC_2H_5)_2$	−30.54‡
	$(C_2H_5O)_3P(O)$	−30.38
Secondary esters	Pyr-O-P(O)(OC_2H_5)(OH)	−29.42
(monoacids)	$(C_2H_5O)_2P(O)(OH)$	−29.19
Primary esters	Pyr-O-P(O)$(OH)_2$	−25.50§
(diacids)	$(C_2H_5O)P(O)(OH)_2$	−26.15§

* Pyr = pyrenebutyl.
† Negative sign indicates upfield shift relative to HMPA. Error estimate: <0.05 ppm.
‡ In 35% acetonitrile, HMPA shifted upfield to 8849 Hz, and the reported value corrected accordingly.
§ pH >8.

Table 5. ^{31}P-NMR chemical shifts (δ, ppm relative to HMPA) of native and denatured OP–Cht conjugates at pH 3–8*

		Structure R-P(O)(R′)–Cht		δ, ppm (from HMPA)†	
Type	OP used	R	R′	Native	Denatured‡
Triesters	DEPF	C_2H_5O	OC_2H_5	−28.65§	−31.02‖
(non-aged	Paraoxon	C_2H_5O	OC_2H_5	−28.74§	−31.05
	DFP	IsoPrO	OisoPr	−30.13	NM¶
	PBEPF	pyr-O**	OC_2H_5	−28.68	−30.97
Diesters	EPDC	C_2H_5O	OH	27.60	−29.38
(aged)	DFP	IsoPrO	OH	−28.60††	NM
	PBPDC	Pyr-O	OH	−27.50	−29.38
	PB(pNP)PC	pyr-O	OH	−27.55	−29.50
	$PB(pNP)_2P$	pyr-O	OH	−27.50	−29.40

* Results are averages of three to five measurements. Estimated error <0.05 ppm.
† Negative signs indicate an upfield shift relative to HMPA.
‡ Denatured with 6 M guanidine·HCl.
§ pH 3–5.
‖ Assumed to undergo denaturation during preparation of conjugate.
¶ Not measured.
** pyr = pyrenebutyl.
†† ^{31}P-NMR signals with relatively high intensity which accompanied the analogous non-aged preparations and which are assumed to represent the aged form (see Results and Discussion).

the phosphate triester molecule with a negatively charged oxygen, so as to form a diester, caused a significant downfield shift in the ^{31}P-NMR signal (*ca.* 1.2 ppm) relative to the triester model compound. Similar shifts were also observed by van der Drift *et al.* [12] with respect to diisopropylphosphates. The chemical shift is further displaced downfield upon insertion of a second ionized oxygen (diester ——→ monoester). Table 5 summarizes the chemical shifts observed for the various OP–Cht conjugates, and Figs 6 and 7 present ^{31}P-NMR spectra of the OP–Cht conjugates before and after denaturation. The relative chemical shifts of the non-aged (assuming a triester structure) and aged (assuming a diester structure) OP conjugates of Cht were found to be in good agreement with the corresponding ^{31}P-NMR chemical shifts of the model compounds (Table 4). After denaturation, the chemical shifts for both aged and non-aged conjugates moved upfield and became close to the reported values for the tri- and diester model compounds, thus maintaining the relative differences in chemical shifts observed prior to denaturation.

The line width at half height ($W_{1/2}$) was found to range between 6 and 20 Hz for HMPA, for the model compounds and for the denatured conjugates. In contrast, and as might be expected due to the reduced intramolecular segmental mobility of the phosphorus atom in the protein conjugate [25, 26], the undenatured pyrene enzyme conjugates showed $W_{1/2}$ values of 40–150 Hz. No clear correlation, however, could be established between $W_{1/2}$ and changes in either the pH or structure of the OP residue.

A pH-dependent chemical shift profile was observed for the native diethylphosphoryl–Cht conjugate obtained by utilizing either DEPF or

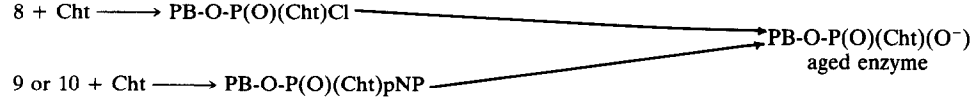
paraoxon. An upfield shift in the ^{31}P-NMR signal with increasing pH (not shown) displayed a titration curve with one titratable group (pK_a 7.1). Similar results were reported for diisopropylphosphoryl–Cht [12]. The small pH-chemical shift dependence (less than 0.2 ppm in the pH range 5–8) observed for the non-aged pyrenebutyl-OP-Cht conjugates did not permit the calculation of the pK_a of the titratable acidic group as was done for the above-mentioned non-fluorescent OP–Cht conjugates. No pH-chemical shift dependence was detected for any of the aged conjugates, whether native or denatured.

DISCUSSION

The pyrenebutyl–OP inhibitors utilized in the present study were found to be suitable for preparation of homologous pairs of aged and non-aged conjugates of Cht. Significant inhibition was obtained within a relatively short time, whether at room temperature or at 4°, with all the pyrene-containing OPs examined. PBEPF, in particular, was found to be a rather powerful inhibitor of Cht, with a bimolecular rate constant (k_i) of 5.8×10/M/min. The value obtained for the binding constant, K_I (0.75 μM), shows that the pyrenebutyl ethyl phosphoryl residue exhibited a high affinity for Cht. The rate of inhibition of Cht by PBEPF was considerably higher than the values reported in the literature for DFP and TEPP [1] but lower than values reported by Lamden and Bartlett [27] for alanine and phenylalanine phosphonates ($k_i = 1.1 \times 10^7$/M/min). Despite the bulkiness of the pyrenebutyl group, visual inspection of a three-dimensional Kendrew skeletal model of Cht constructed in the laboratory of Dr D. R. Davies (Laboratory of Molecular Biology, NIH) [28], showed that PBEPF fitted readily into the active-site pocket of the enzyme, and its high affinity is presumably due to its hydrophobicity, since polyaromatic compounds have been shown to interact hydrophobically with the active site of Cht [29, 30]. The ease with which it fitted, together with the flexibility of the butyl chain, might perhaps explain the lack of stereospecificity in the reaction of the two enantiomers of PBEPF with Cht. Substitution of the OC_2H_5 group by bulky residues such as *p*-nitrophenoxy [PB(pNP)PC, $PB(pNP)_2P$] reduced the anti–Cht potency of the pyrenebutyl-containing OP compounds. Nevertheless, all the fluorescent OP inhibitors that were employed in the present study reacted specifically, and at approximately 1:1 stoichiometry, with the active site of Cht, thus permitting correlation of the aging process with the physicochemical changes in the OP–enzyme conjugates.

Although interpretation of the chemical shifts reported in Table 4 is complex in terms of steric and electronic contributions of the substituents to the degree of bonding to the phosphorus atom [31, 32], it is clear from the ^{31}P-NMR spectra of the model compounds (Table 4), taken together with the results obtained by van der Drift *et al.* [12], that changing the structure of a phosphorus triester to a diester leads to a shift of 1–2 ppm downfield from the triester. The consistency of the downfield shift of the various aged conjugates relative to the reactivatable forms, i.e., the non-aged OP–Cht conjugates, strongly supports the assumption that the aged fluorescent conjugate is formally a diester compound which contains the P—O^- bond at neutral pH. The observation that the conjugates which displayed the diester-type chemical shift could not be reactivated, while the activity of the triester conjugates could be partially restored by 3-PAM, further substantiates the correlation between the formation of a P—O^- and conversion of the phosphoryl enzyme to a non-reactivatable conjugate. Denaturation did not change the relative differences in chemical shift between homologous pairs of aged and non-aged conjugates. It did appear, however, to abolish the interaction of the phosphorus ligand with the protein backbone, inasmuch as the chemical shifts moved upfield and became closer to the reported values for the analogous model compounds. The ^{31}P chemical shift of the triester DIP–Cht, was found to be upfield relative to DEP–Cht and PBEP–Cht (*ca.* 1.3 ppm), as predicted from similar differences observed in the chemical shifts of branched versus unbranched trialkylphosphates [33]. The results are consistent with the empirical concept underlying the utilization of ^{31}P-NMR spectroscopy to distinguish between aged and non-aged OP–enzyme conjugates.

The ^{31}P-NMR data, taken together with the results of the reactivation experiments, provide direct evidence that a P—O^- bond is formed irrespective of the leaving group which is detached concomitantly with aging (Scheme IV):



Scheme IV

Furthermore, these results substantiate previous suggestions [7–9] that the resistance of the aged conjugate to reactivation may be partially ascribed to the electrostatic repulsion of a nucleophile reactivator by the P—O^- bond.

Possible conformational changes around the phosphoryl residue in the aged form which make it less available to the nucleophile reactivator are discussed elsewhere [20].

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