Direct Observation and Elucidation of the Structures of Aged and Nonaged Phosphorylated Cholinesterases by ^{31}P NMR Spectroscopy

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ABSTRACT: ^{31}P NMR spectroscopy of butyrylcholinesterase (BChE), acetylcholinesterase (AChE), and chymotrypsin (Cht) inhibited by pinacolyl methylphosphonofluoridate (soman), methylphosphonodifluoridate (MPDF), and diisopropyl phosphorofluoridate (DFP) allowed direct observation of the OP-linked moiety of aged (nonreactivatable) and nonaged organophosphorus (OP)–ChE conjugates. The ^{31}P NMR chemical shifts of OP–ChE conjugates clearly demonstrated insertion of a P–O^- bond into the active site of aged OP–ChE adducts. The OP moiety of nonaged OP–ChEs was shown to be uncharged. The OP-bound pinacolyl moiety of soman-inhibited and aged AChE was detached completely, whereas only partial dealkylation of the pinacolyl group was observed for soman-inhibited BChEs. This suggests that the latter enzyme reacted with the less active stereoisomer(s) of soman. In the case of soman-inhibited Cht, no dealkylation could be experimentally detected for any of the four stereoisomers of OP–Cht adducts. Results are consistent with the contention that the phenomenon of enzyme-catalyzed dealkylation of OP adducts of serine hydrolases strongly depends on the orientation of both the catalytic His and the carboxyl side chain of either Glu or Asp positioned next to the catalytic Ser. The denatured protein of aged OP–ChE or OP–Cht is a convenient leaving group in nucleophilic displacements of tetrahedral OP compounds despite the presence of a P–O^- bond. This indicates that the unusual resistance to reactivation of the aged enzyme cannot be ascribed to simple electrostatic repulsion of an approaching nucleophile. The broadening of the ^{31}P NMR signal of native OP–ChEs relative to that of OP–Cht is in agreement with the crystal structure of AChE, showing that the active site region of ChEs in solution resides in a deep, narrow gorge.

Organophosphorus (OP)[1] esters inhibit acetylcholinesterases (AChE; EC 3.1.1.7) and butyrylcholinesterases (BChE; EC 3.1.1.8) by forming a stoichiometric (1:1) conjugate between the OP and the enzyme at the active-site serine (Aldrich & Reiner, 1972). In general, OP-inhibited cholinesterases (ChE) can be reactivated by various oxime nucleophiles (Aldrich & Reiner, 1972); however, for certain OPs, the displacement of the phosphoryl moiety from the active site to restore enzyme activity is impossible due to a parallel aging reaction (Hobbiger, 1955; Berends et al., 1959) (Figure 1). Thus, aging of phosphorylated ChEs is defined as the process that converts the inhibited enzyme into a form that can no longer be regenerated by commonly used reactivators. The inability to reactivate aged OP–AChE conjugates renders oxime treatment following intoxication with certain OPs extremely difficult, particularly by the toxic isomers of pinacolyl methylphosphonofluoridate (soman) (Loomis & Salafski, 1963; Heilbroon & Tolagen, 1965). On the basis of experiments with radiolabeled OPs (Berends et al., 1959; Michel et al., 1967) and kinetic studies (Benschop & Keijer, 1966), it was hypothesized that aging of OP-inhibited ChEs is associated with introduction of a formal negative charge into the active site of the inhibited enzyme. However, no direct evidence is available to support this contention.

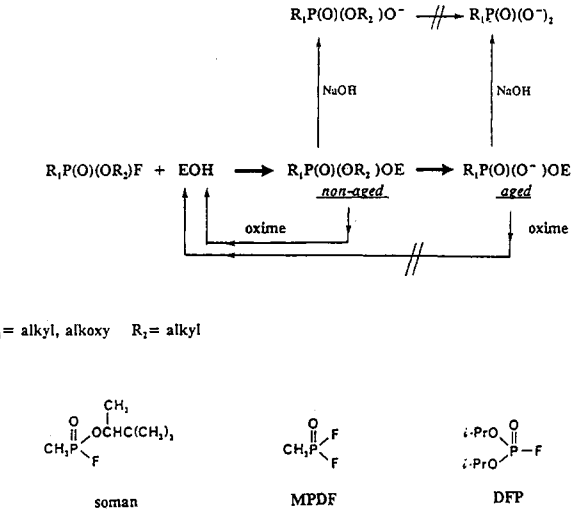

FIGURE 1: Chemical pathway of the inhibition, aging, and NaOH-induced hydrolysis of OP–ChE conjugates obtained by reacting phosphono- and phosphorofluoridates [$R_1P(O)(OR_2)F$] with serine hydrolases (EOH).

^{31}P NMR spectroscopy can provide meaningful information on the structure of the substituents attached to the P atom of phosphorylated proteins. This approach has been utilized successfully to characterize differences between the structure

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[1] Abbreviations: OP, organophosphorus; TcAChE, *Torpedo californica* acetylcholinesterase; FBS-AChE, fetal bovine serum acetylcholinesterase; EqBChE, horse serum butyrylcholinesterase; HuBChE, human serum butyrylcholinesterase; MPDF, methylphosphonodifluoridate; 3D, three dimensional; Cht, chymotrypsin; HMPA, hexamethylphosphortriamide; TMP, trimethyl phosphate; $MP(Pin)_2$, dipinacolyl methylphosphonate; PinMP-OH, pinacolyl methylphosphonic acid; MP-OH, methylphosphonic acid; TIP, tris-isopropyl phosphate; DFP, diisopropyl phosphorofluoridate; DIP-OH, diisopropyl phosphoric acid; MIP-OH, monoisopropyl phosphoric acid; Gdn·HCl, guanidine hydrochloride; P_2S, 2-(hydroxyimino)-1-methylpyridinium methyl methansulfonate; 3-PAM, 3-(hydroxyiminomethyl)-1-methylpyridinium iodide; TMB_4, 1,1′-(trimethylene)bis(4-hydroxyiminomethyl)pyridinium dibromide.

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of the aged and nonaged OP conjugates of various serine proteases (Gorenstein & Findlay, 1976; van der Drift et al., 1985; Gorenstein et al., 1989; Adebodun & Jordan, 1989a,b; Grunwald et al., 1989; Kovach et al., 1993). Recent X-ray crystallographic data on a homologous pair of aged and nonaged OP conjugates of chymotrypsin (Cht; EC 3.4.21.1) were reported to be in excellent agreement with the data obtained by ^{31}P NMR spectroscopy for OP–Cht conjugates in solution (Harel et al., 1991). Thus, it was established that the aged OP–Cht conjugate contains a P–O$^-$ bond which forms close interaction with the N$^{\epsilon 2}$ atom of the catalytic amino acid His 57. Although the structure of AChE from *Torpedo californica* (TcAChE) has been determined by X-ray crystallography (Sussman et al., 1991), the three-dimensional (3D) geometry of its phosphorylated active site has not been reported. The lack of sufficient quantities of purified cholinesterases (ChE) until recently precluded proper utilization of the ^{31}P NMR technique for the determination of the structure of aged and nonaged forms of OP-inhibited ChEs.

In this study we have characterized the OP moiety of phosphorylated ChEs by both direct and comparative ^{31}P NMR spectroscopy. We report here on the elucidation of the structure of the aged and nonaged OP conjugates of AChE and BChE obtained by using soman, methylphosphonodifluoridate (MPDF), and diisopropyl phosphorofluoridate (DFP). This, together with the characterization of a homologous pair of OP–Cht conjugates, permitted speculation on the mechanism of the reactivation and aging of OP–ChEs and offered a partial explanation for the unusual resistance of aged OP-ChEs to reactivation.

MATERIALS AND METHODS

Materials. BChE from either human (HuBChE) or equine (EqBChE) serum was purified by an affinity chromatography technique to be published elsewhere. AChE from fetal bovine serum (FBS) was purified according to De La Hoz et al. (1986). One milligram of pure enzyme contained approximately 11 and 14 nmol of active site of BChE and AChE, respectively, with the following specific activities: HuBChE, 750; EqBChE, 950; and FBS-AChE, 5000 units/mg. Bovine pancreatic α-Cht (type II, 3× crystallized, salt-free, and lyophilized), *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, and ultrapure guanidine hydrochloride (Gdn·HCl) were purchased from the Sigma Chemical Co. (St. Louis, MO). Active site concentration of Cht was found to be approximately 85% of its theoretical value (Schonbaum et al., 1961). DFP, hexamethylphosphortriamide (HMPA), trimethylphosphate (TMP), methylphosphonic acid (MP-OH), diethyl methylphosphonate (DEMP), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), *S*-butyrylthiocholine (BTC), and *S*-acetylthiocholine (ATC) were purchased from the Aldrich Chemical Co (Milwaukee, WI).

Soman and methylphosphonodifluoridate (MPDF) were prepared according to the synthetic principles outlined by Monard and Quinchon (1961). Dipinacolyl methylphosphonate [MP(Pin)$_2$] (Green & Hudson, 1958), monoisopropyl phosphoric acid (MIP-OH), and trisisopropyl phosphate (TIP) were synthesized according to previously published procedures (Auler et al., 1929). Pinacolyl methylphosphonic acid (PinMP-OH) and diisopropyl phosphoric acid (DIP-OH) were obtained by hydrolysis of soman and DFP, respectively, in 0.2 N NaOH. The structure and purity of all phosphorus-containing ligands was confirmed by both ^{1}H and ^{31}P NMR spectroscopy.

Enzymatic Assays. AChE and BChE activity were determined by the method of Ellman et al. (1961), using 0.45 mM BTC and ATC as substrates, respectively. Cht was assayed using the chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Delmar et al., 1979).

Titration of ChEs by Soman and DFP. To approximately 4–180 μM (active site concentration) of a ChE in either 5 mM phosphate or 10 mM Tris buffer, pH 8.0, various amounts of OP inhibitor solution (0.1–2 times enzyme equivalent) were added. The mixtures were incubated at 25 °C. Inhibition was allowed to proceed to completion, and residual enzyme activity was assayed by Ellman's method, as described above. The percentage of residual enzyme activity was plotted against the number of equivalents of OP.

Preparation of OP–ChE Conjugates. Large quantities of OP–ChE conjugates were obtained by adding freshly prepared concentrated solutions (1–5 mM) of either soman (in 50% propylene glycol/water) or DFP (in 50% isopropanol/water) to 10–20 mg of purified ChE in 1–5 mL of 25 mM Tris buffer, pH 8.0. Assuming molecular masses of approximately 65 and 95 kDa for AChE and BChE, respectively, the ratio of the molar concentration of OP to ChE ranged between 1 and 5. The decrease in the enzymic activity was monitored until inhibition was complete. MPDF-inhibited BChE was obtained by sequential addition of 0.1 M MPDF in CH_3CN to a solution of 10 mg of enzyme in 1 mL of 50 mM phosphate buffer, pH 8.0. A total of 25 μL was required to complete the inhibition of enzyme.

To remove traces of phosphorus-containing low molecular weight compounds, the inhibited enzyme was dialyzed against 2 L of 0.1 M Tris buffer, pH 8.0, for 48 h at 6 °C. Finally, volume of the OP–ChE conjugate was reduced to approximately 0.4 mL by ultrafiltration (Millipore Ultrafree-MC, Bedford, MA). The protein content of the concentrated solution (15–30 mg/mL) was determined spectrophotometrically at 280 nm using absorption coefficients of $E^{1\%} = 19$ and 16 for BChE and AChE, respectively.

Preparation of OP–Cht Conjugates. One hundred to 250 μL of either 0.08 M soman in 50% propylene glycol/water or DFP in isoPrOH was added at room temperature to a stirred solution of 100–300 mg of Cht in 4 mL of 0.1 M Tris buffer, pH 7.6. The molar ratio of OP to Cht ranged between 2 and 5. To obtain soman-inhibited Cht that consisted of all four stereoisomers of soman, Cht was dissolved in distilled water, the pH was adjusted to 7.8 with 0.1 N NaOH, and the molar ratio of soman to Cht was adjusted to 1:1. The inhibitor–enzyme mixture was allowed to incubate until >99% inhibition of enzyme activity was obtained. To remove traces of phosphorus-containing low molecular weight compounds, the solution of inhibited enzyme was dialyzed against Tris buffer as described above. To obtain the assumed dealkylated form of soman-inhibited Cht, 4 mL of 1 mM Cht in distilled water was adjusted to pH 8.0 and mixed with 10 μL of 0.65 M MPDF in CH_3CN. Approximately 80% of enzyme activity was inhibited. The pH (6.5) was readjusted to 8.0, and the remaining activity was inhibited with an additional 3 μL of MPDF stock solution. Inhibited enzyme was dialyzed against 0.1 M Tris buffer as described above.

Monitoring the Aging of OP-Inhibited ChEs and Cht. Inhibited ChEs were diluted 1000-fold at selected time intervals into a reactivation medium containing 1 mM of either 2-(hydroxyiminomethyl)-1-methylpyridinium methyl methanesulfonate (P_2S) or 1,1′-(trimethylene)bis(4-hydroxyiminomethyl)pyridinium dibromide (TMB_4) (Gray, 1984) in 50 mM phosphate buffer, pH 8.0. OP–Cht conjugates were diluted 1000-fold into reactivation medium (0.1 M Tris–0.01 M $CaCl_2$, pH 7.8) containing 0.1 M 3-(hydroxyiminomethyl)-

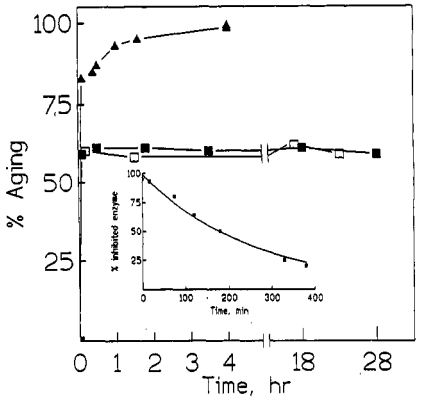

FIGURE 2: Time course of the aging of soman-inhibited EqBChE (□), HuBChE (■) and FBS-AChE (▲) in 50 mM phosphate buffer, pH 8.0, at 25 °C. (Inset) Plot of reactivation of enzyme activity vs time for the nonaged portion of soman-inhibited HuBChE. The line was fitted in accordance with first-order reaction kinetics in presence of 1 mM P_2S.

Table I: ^{31}P NMR Chemical Shifts of Soman-Related OP Moieties of Model Compounds and Phosphonylated ChEs and Cht Obtained with Soman

OP	status	structure	chemical shifts[a]
model compounds			
soman		$CH_3P(O)(OPin)F$[b]	+30.81[c]; +30.45[c]
$MP(Pin)_2$		$CH_3P(O)(OPin)_2$	+29.94[d,e]
DEMP		$CH_3P(O)(OEt)_2$	+31.58[e]
PinMP-OH		$CH_3P(O)(OPin)OH$	+22.27[e]
MP-OH		$CH_3P(O)(OH)_2$	+17.44[f]
OP–enzymes			
PinMP–Cht	nonaged	$CH_3P(O)(OPin)$–Cht	+35.96[g]; +33.02[g]
PinMP–Cht	nonaged	$CH_3P(O)(OPin)$–Cht	+31.57[h]; +31.26[h]
PinMP–BChE	nonaged	$CH_3P(O)(OPin)$–BChE	+31.31[h]
Mp–CHt[i]	aged	$CH_3P(O)(O^-)$–Cht	+27.01[g]
MP–Cht[i]	aged	$CH_3P(O)(O^-)$–Cht	+24.89[h]
MP–BChE	aged	$CH_3P(O)(O^-)$–BChE	+24.68[h]
MP–BChE[i]	aged	$CH_3P(O)(O^-)$–BChE	+27.99[g,j]
MP–BChE[i]	aged	$CH_3P(O)(O^-)$–BChE	+24.69[h]
MP–AChE	aged	$CH_3P(O)(O^-)$–AChE	+24.63[h]

[a] δ, ppm relative to external TMP. Positive signs denote downfield shifts relative to TMP. Unless indicated, the ^{31}P NMR line is a singlet. Error estimate is less than 0.05 ppm. [b] Pin, $-CH(CH_3)C(CH_3)_3$. [c] Center of a doublet, J_{P-F} = 1040 Hz each. [d] Center of three lines. [e] pH 6.0–11.8 and in 0.2 N NaOH. [f] In 0.2 N NaOH. [g] Native conjugate in 0.1 M Tris, pH 8. [h] Unfolded in 0.1 M Tris–6 M Gdn·HCl, pH 8.0. [i] Obtained with MPDF. [j] Chemical shift estimated from the center of a broad peak.

1-methylpyridinium iodide (3-PAM) (Cohen & Erlanger, 1960). To ensure the absence of residual inhibitor, the activity of ChE or Cht freshly added to the corresponding OP–enzyme conjugate was monitored in parallel with the measurement of enzyme activity in the reactivation medium. Controls were treated in the same manner except that (a) the oxime was omitted to enable the detection of spontaneous reactivation, and (b) the inhibited enzyme was replaced by native ChE or Cht. Reactivation was determined after 24 and 72 h of incubation of OP–ChEs and OP–Cht conjugates, respectively.

Kinetics of Reactivation of Nonaged OP–Enzyme Conjugates. Aliquots of either OP–ChE or OP–Cht were diluted at $t = 0$ into the reactivation medium. At selected time intervals, 5–20 μL was diluted into the assay cuvette, and enzyme activity was determined as described above.

Denaturation. Unfolding was carried out in 0.1 M Tris–6 M Gdn·HCl, pH 8.0. In several cases 0.5 M of either TMB_4 or P_2S was added to examine possible acceleration of the displacement of the OP-containing moiety from the unfolded conjugate. Alkaline-induced denaturation and subsequent release of the enzyme-bound OP moiety (see Figure 1) were carried out in the NMR tube: 0.5–4 h prior to the initiation of the ^{31}P NMR data acquisition, 25–40 μL of 2 N NaOH solution was diluted into 0.4–0.5 mL of OP–enzyme conjugate. The pH in the NMR tube was ≈12.

Table II: ^{31}P NMR Chemical Shifts of DFP-Related OP Moieties of Model Compounds and Phosphorylated ChEs and Cht

OP	status	structure	chemical shifts[a]
model compounds			
DFP		$(isoPrO)_2P(O)F$[b]	−13.55[c]
TIP		$(isoPrO)_3P(O)$	−6.30[d]
DIP-OH		$(isoPrO)_2P(O)OH$	−3.97[d]; −4.05[e]
MIP-OH		$(isoPrO)P(O)(OH)_2$	−0.06[f]
OP–enzymes			
DIP–Cht	nonaged	$(isoPrO)_2P(O)$–Cht	−3.55[g]; −6.20[e,h]
DIP–AChE	nonaged	$(isoPrO)_2P(O)$–AChE	−5.99[e]
MIP–Cht	aged	$(isoPrO)P(O)(O^-)$–Cht	−1.09[g]; −3.43[e]
MIP–BChE	aged	$(isoPrO)P(O)(O^-)$–BChE	−1.20[g,i]; −3.35[e]

[a] δ, ppm relative to external TMP. Negative signs denote upfield shifts relative to external TMP. Unless indicated, the ^{31}P NMR line is a singlet. Error estimate is less than 0.05 ppm. [b] isoPr, $-CH(CH_3)_2$. [c] Center of a doublet, J_{P-F} = 973 Hz. [d] pH 6.0–11.8 and 0.2 N NaOH. [e] 0.1 M Tris–6 M Gdn·HCl, pH 8.0. [f] 0.2 N NaOH. [g] Native conjugate in 0.1 M Tris, pH 8. [h] Heat denaturation (50 °C, 0.1 M Tris, pH 8.0) produced a ^{31}P NMR signal at −6.24 ppm. [i] Chemical shift estimated from the center of a broad peak.

NMR Spectrometry Measurements. ^{31}P NMR spectra were recorded with a GN 300WB NMR instrument (General Electric) at 300 (^1H) or 121.65 (^{31}P) MHz for solutions of OPs in $CDCl_3$ or for OP–protein conjugates in aqueous solutions containing 10–20% D_2O. The deuterated solvents also served for field frequency lock. A WALTZ-16 phase modulation program at a rate of 1000 was utilized for continuous ^1H broad-band decoupling to avoid internal build up of heat. Throughout the run, the sample temperature was maintained at 20 °C. Spectral data were accumulated at 30° pulse width, 8-kHz spectral width, and 2-s pulse delay between consecutive scans.

^{31}P NMR chemical shifts were assigned to external 1% TMP in C_6D_6 that was set to 0 ppm. HMPA (26.90 ± 0.03 ppm downfield to TMP) was used as an internal standard to enable normalization of the chemical shifts in case of significant changes in the susceptibility of the tested solution to the ^{31}P NMR signal (Grunwald et al., 1989). Occasionally, phosphoric acid (0.92 ± 0.03 upfield to TMP, at pH 8.0) was also used as internal standard. The ^{31}P NMR spectra of the low molecular weight model compounds were recorded for a concentration range of 1–5 mM. The concentration of OP–ChE and OP–Cht conjugates in the NMR tube was approximately 0.2 and 1 mM, respectively. These estimates were based on protein determination and assuming a molecular mass of 65 (AChE), 95 (BChE), and 25 (Cht) kDa. Depending on the type and concentration of OP used, 100–100 000 transients were accumulated for each run.

Molecular Modeling of Diethylphosphoryl–TcAChE. The modeling of $(C_2H_5O)_2P(O)$–TcAChE was carried out by extracting the structure of the diethylphosphoryl moiety from the X-ray structure of $(C_2H_5O)_2P(O)$–Cht (Harel et al., 1991) and docking it into the active site of TcAChE (Sussman et al., 1991). The resulting adduct was energy optimized as described by Barak et al. (1992).

RESULTS

Titration of ChEs with Soman and DFP. The stoichiometric amount of soman needed to inhibit 100% enzyme activity of either EqBChE or HuBChE was 20–25% higher

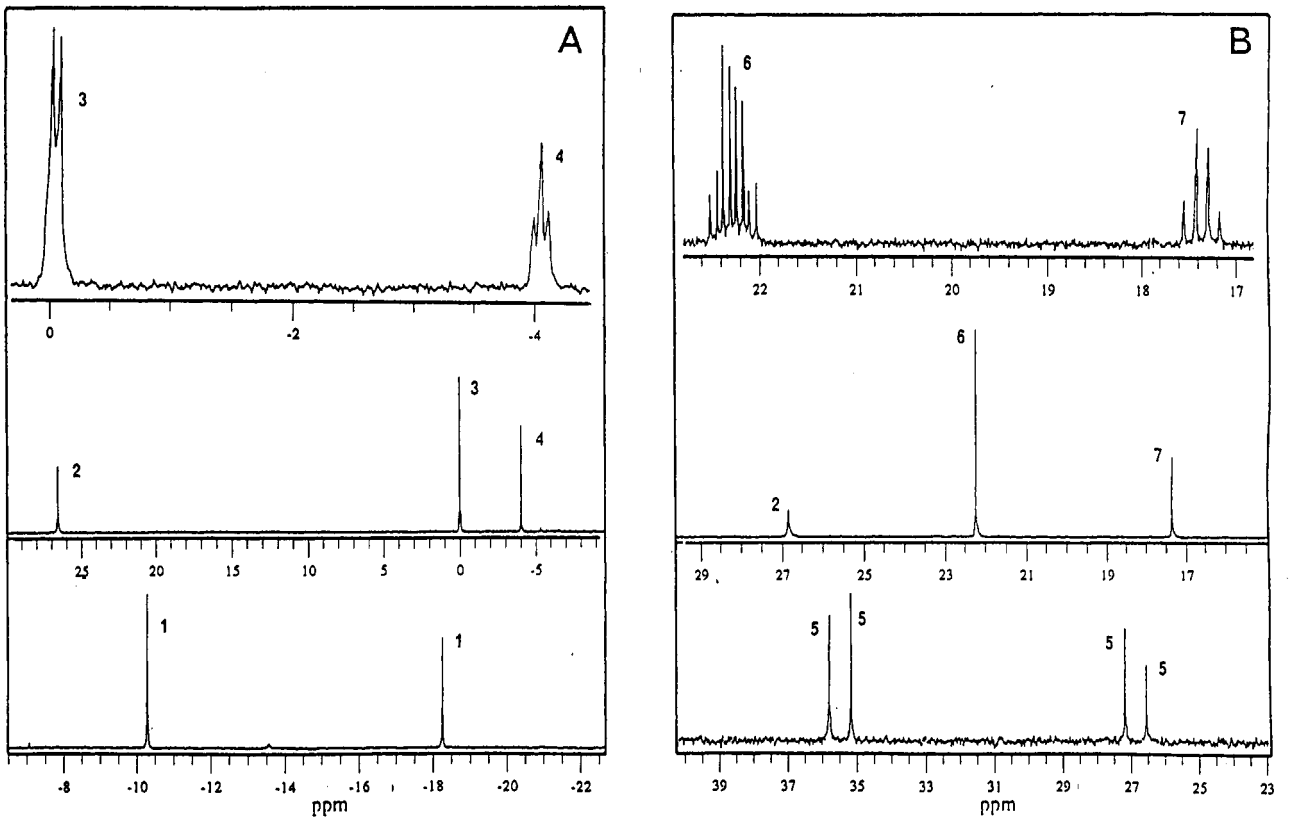

FIGURE 3: ^{31}P NMR spectra (100–5000 transients/spectrum) of 1–5 mM OP model compounds in 0.1 M Tris, pH 8.0. Negative signs indicate an upfield chemical shift relative to external TMP (0 ppm). (Panel A) DFP and its hydrolysis products. (Lower trace) DFP; (middle trace) reconstituted mixture of authentic mono- and diisopropylphosphoric acids in 0.2 N NaOH; (upper trace) normal mode (proton decoupler off) of a mixture of authentic mono- and diisopropylphosphoric acids in 0.2 N NaOH. (Panel B) Soman and its hydrolysis products. (Lower trace) soman; (middle trace) reconstituted mixture of authentic methyl pinacolylphosphonic acid and methylphosphonic diacid in 0.2 N NaOH; (upper mode (decoupler off) of a mixture of authentic methyl pinacolylphosphonic acid and methylphosphonic acid in 0.2 N NaOH. (Lines assignment) **1**, DFP, $[(CH_3)_2CHO]_2P(O)F$; **2**, HMPA (internal standard); **3**, MIP-OH, $[(CH_3)_2CHO]P(O)(O^-)_2$; **4**, DIP-OH, $[(CH_3)_2CHO]_2P(O)O^-$; **5**, soman, $CH_3P(O)[OCH(CH_3)C(CH_3)_3]F$; **6**, PinMP-OH, $CH_3P(O)[OCH(CH_3)C(CH_3)_3]O^-$; **7**, MP-OH, $CH_3P(O)(O^-)_2$.

than that observed for DFP (Raveh et al., 1993). These differences are attributed to variations in the anti-BChE activities of the stereoisomers that constitute the racemic mixture of soman (Keijer & Wolring, 1969). As expected (Benschop & De Jong 1988), the amount of soman required to completely inhibit FBS-AChE was 2-fold higher than that of the achiral DFP. Two equivalents were required to achieve 100% inhibition of 4–10 μM Cht. These results (data not shown) are consistent with the enantioselectivity of Cht for the racemic mixture of soman (Ooms & van Dijk, 1961; Schoene, 1971). However, when the molar concentration of both reactants increased to a millimolar range, ^{31}P NMR spectroscopy revealed that all four stereoisomers of soman were taken up by Cht. The titration curve showed that under these conditions 1 mol soman was sufficient to inhibit >99% of the activity of one active site equivalent of Cht. The titration of ChEs and Cht by the OP ligands substantiated the assumption that the OP–enzyme adducts contain one kind of OP-bound moiety.

Aging of OP Conjugates of ChEs and Cht. Following inhibition of either HuBChE or EqBChE with approximately 1.2-fold stoichiometric excess of soman, 60% of the inhibited enzyme aged within less than 30 min (Figure 2). The remaining 40% could be reactivated completely, even after 28 h of incubation at 25 °C prior to the dilution into the reactivation medium (Figure 2, inset). These results are in agreement with the observations of Keijer and Wolring (1969) who reported (a) a $t_{1/2}$ of approximately 9 and 60 min for the aging of EqBChE inhibited by the two potent stereoisomers of soman, P(–)C(+) and P(–)C(–) and (b) EqBChE inhibited by a third stereoisomer with a P(+) configuration did not undergo detectable aging when incubated 24 h at 25 °C (pH 7.5). In contrast to BChEs, more than 98% of soman-inhibited FBS–AChE was converted to a nonreactivatable form. The time course of the aging of soman-inhibited FBS–AChE displayed a distinct biphasic behavior (Figure 2), and aging was completed only after 120 min of incubation in 50 mM phosphate buffer (pH 8.0) at 25 °C.

No decrease in the ability to reactivate soman-inhibited Cht could be observed even after 96 h of incubation in 0.1 M Tris buffer, pH 8.0, at 25 °C. By adjusting the experimental conditions to enable all four stereoisomers of soman to phosphonylate Cht (see above), the inhibited enzyme could be reactivated only to 72–75% of its original activity. The inability to observe complete reactivation was independent of the time of incubation prior to dilution into the reactivation medium. In contrast to soman-inhibited Cht, no reactivation could be detected after 48 h of incubation of MPDF-inhibited Cht in the presence of 0.1 M 3-PAM. MPDF was expected to produce the aged form $CH_3P(O)(OCht)O^-$ due to rapid hydrolysis of the P–F bond of the phosphonylated enzyme $[CH_3P(O)(OCht)F]$.

DIP–BChE and DIP–Cht could be reactivated only partially (25–65%). Since the reactivation proceeded at slow rates ($t_{1/2}$> 20 h), it was assumed that other side reactions occurred in parallel to the reactivation (e.g., aging, autolysis, denaturation). To convert DIP–BChE and DIP–Cht completely into the aged forms, the inhibited enzymes were either dialyzed

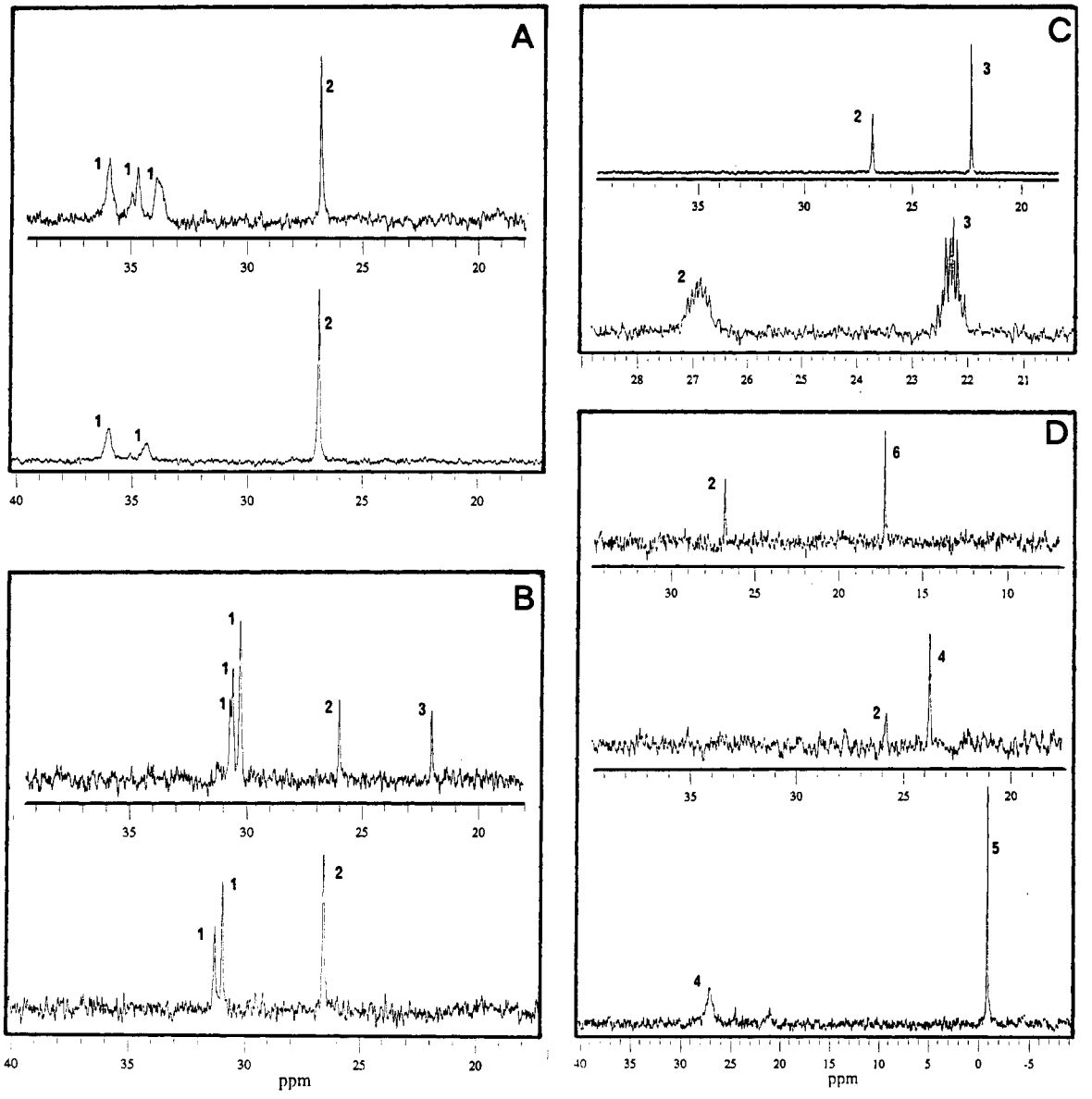

FIGURE 4: ^{31}P NMR spectra of 0.5–1.0 mM OP-Cht conjugates in 0.1 M Tris, pH 8.0. Negative signs indicate an upfield chemical shift relative to external TMP (0 ppm). (Panel A) Soman-inhibited Cht obtained by a soman to Cht molar ratio of 2.2 (lower trace) and 1.0 (upper trace). (Panel B) Soman-inhibited Cht in 0.1 M Tris–6 M Gdn·HCl, pH 8.0. Conjugates obtained by a soman to Cht molar ratio of 2.2. (lower trace) and of 1.0 (upper trace). (Panel C) Release of OP ligand from soman-inhibited Cht in approximately 0.2 N NaOH. (Upper trace) Proton-decoupled mode; (lower trace) normal mode (proton decoupler off). (Panel D) MPDF-inhibited Cht (native, lower trace), its unfolded state in 0.1M Tris–6 M Gdn·HCl, pH 8.0 (middle trace), and following the addition of 0.2 N NaOH into the NMR tube of the native conjugate (pH ≈ 12; upper trace). Lines assignment (for definitions see Table I): **1**, PinMP–Cht; **2**, HMPA (internal standard); **3**, PinMP-OH; **4**, MP–Cht; **5**, phosphoric acid (internal standard); **6**, MP-OH.

at room temperature against 0.1 M Tris buffer, pH 7.0, for 96 h (DIP–BChE) or incubated at 37 °C for 6 days (DIP–Cht).

Reactivation of Nonaged Soman-Inhibited BChE and Cht. The nonaged portion of soman-BChE conjugate was reactivated by 1 mM P_2S at a rate of 0.23 ± 0.02 h^{-1}, in accordance with a single-exponential decay equation (Figure 2). More than 98% of OP-Cht obtained by >2-fold stoichiometric excess of soman could be reactivated by 0.1 M 3-PAM. The data of the time course of reactivation of soman-inhibited Cht were best fitted by a biexponential kinetic equation with the following parameters: 59% reactivated at a rate of 0.072 h^{-1} and 39% at 0.42 h^{-1}. The ratio of the two amplitudes (59/39) may reflect, in part, the relative anti-Cht activity of the two potent epimers P(–)C(±) (Ooms & van Dijk, 1961; Schoene, 1971).

The time course of the reactivation of Cht inhibited by all four stereoisomers of soman could be reasonably described by a triexponential kinetic equation with the following computer-fitted values: 24% at 0.060 h^{-1}, 22% at 0.062 h^{-1}, and 26% at 0.52 h^{-1} (not shown). The inability to obtain full reactivation is attributed to steric hindrance rather than dealkylation (see below).

^{31}P NMR Spectroscopy. (a) ^{31}P NMR Chemical Shifts of Model Compounds. Tables I and II list names, structures, and ^{31}P NMR chemical shifts of soman- and DFP-related OPs, respectively. The characterization of differences between the aged and nonaged OP moieties was based in part on direct identification of the phosphoryl ligand obtained after hydrolysis of the OP–protein adduct in NaOH (Figure 1). Therefore, it was important to determine the pH dependence of the ^{31}P NMR chemical shift of phosphoric and methylphosphonic

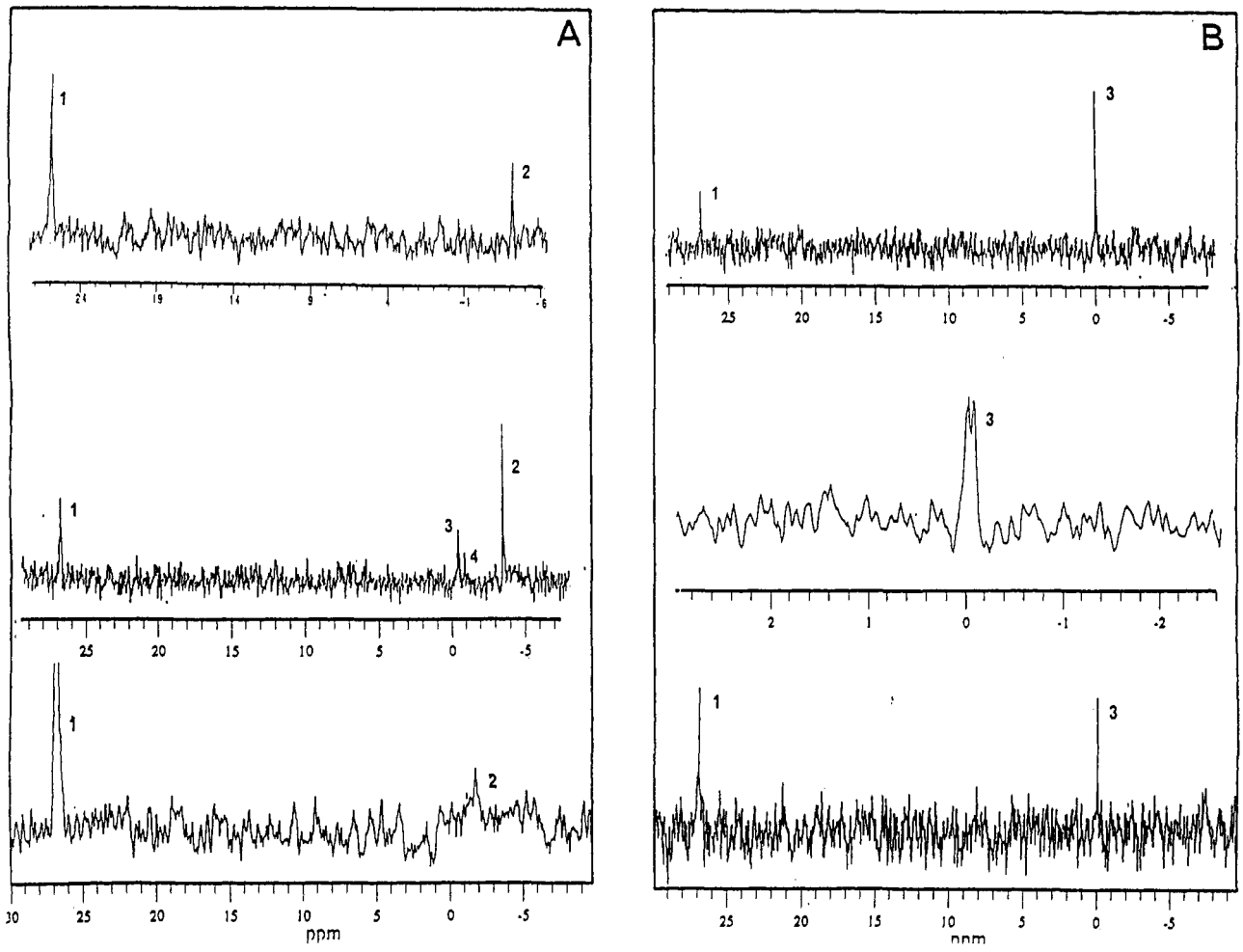

FIGURE 5: ^{31}P NMR spectra of approximately 0.2 mM DFP-inhibited EqBChE in 0.1 M Tris, pH 8.0. Negative signs indicate an upfield chemical shift relative to external TMP (0 ppm). (Panel A, Lower trace) Spectrum of DFP-inhibited and aged BChE after 98 000 scans; (middle trace) spectrum of the same preparation in 0.1 M Tris–6 M Gdn·HCl, pH 8.0, after 22 000 scans; (upper trace) spectrum recorded after dialysis of the unfolded conjugate shown above against Tris buffer and reunfolding in 0.1 M Tris–6 M Gdn·HCl, pH 8.0 (22 000 scans). (Panel B, Lower trace) Spectrum of NaOH-released OP ligand attached to the aged DFP-inhibited BChE (4000 scans); (middle trace) normal mode (decoupler off; 21 000) of the spectrum shown in the lower trace; (upper trace) spiking the sample containing the released OP ligand from the aged conjugate (see lower trace) with monoisopropylphosphoric acid (2000 scans). Lines assignment (for definitions see Table II): **1**, HMPA (internal standard); **2**, MIP–BChE; **3**, MIP-OH; **4**, contamination of phosphate buffer.

acids. Above pH 9, the chemical shifts of the esters were well separated from the mono- and diacid model compounds (Tables I and II). No changes in the ^{31}P NMR spectra could be observed after 5 days of incubation of either the mono- or the diacids in 0.2 N NaOH. This finding validated the reaction pathway depicted in Figure 1 and permitted assignment of the ^{31}P NMR signals of inhibited enzymes to the structure of the OP-bound moiety.

Figure 3 shows differences in both the chemical shifts and line multiplicity of the ^{31}P NMR signals of soman and DFP. The P–F bond couples the ^{31}P NMR line of the achiral DFP into a doublet. In the case of soman, which contains two chiral centers, $CH_3P^*(O)[OC^*H(CH_3)C(CH_3)_3]F$, a doubling of the resonance occurred. The two doublets of the ^{31}P NMR signal of soman originate from two pairs of diastereoisomers, P(–)C(+); P(+)C(–) and P(–)C(–); P(+)C(+). These pairs are distinguishable by their ^{31}P NMR chemical shifts. Due to compensation produced by the opposing chemical environment of the individual constituents, each pair displayed only one doublet. Thus, it is likely that OP adducts of serine hydrolases that show preference for the P(–)C(±) epimers of soman will give rise to two ^{31}P NMR signals. Kovach et al. (1993) have recently demonstrated the formation of two diastereomeric OP-Cht adducts by ^{31}P NMR spectroscopy.

The line multiplicity of the NMR signal (Figure 3, upper traces; proton decoupler off), further demonstrates the applicability of the ^{31}P NMR spectroscopy to determine the nature of the substituents around the P atom.

(b) ^{31}P NMR Chemical Shifts of DFP- and Soman-Inhibited Cht. The ^{31}P NMR chemical shifts of the native and unfolded forms of DIP–Cht, MIP–Cht, and PinMP–Cht (reported here for the first time) were used to assign the ^{31}P NMR chemical shifts of homologous OP–ChE conjugates.

The two NMR signals associated with a fully reactivatable PinMP–Cht, obtained with a 2.2 molar excess of soman over Cht (Figure 4A, lower trace), are consistent with the insertion of the two potent stereoisomers of soman, i.e., P(–)C(+) and P(–) (–) (Ooms & van Dijk, 1966; Schoene, 1971). A third peak appeared in the spectrum of a PinMP-Cht obtained with a 1:1 molar ratio of soman to Cht (Figure 4A, upper trace). This conjugate was demonstrated above to contain also the less active P(+) epimers. Similar multiplicity of the ^{31}P NMR line was observed for the racemic model compound $MP(Pin)_2$ that contains three chiral centers (Table I). Since the P(+)-containing OP–Cht preparation released only the monoacid PinMP-OH (Figure 4C), it is suggested that it did not undergo dealkylation at all.

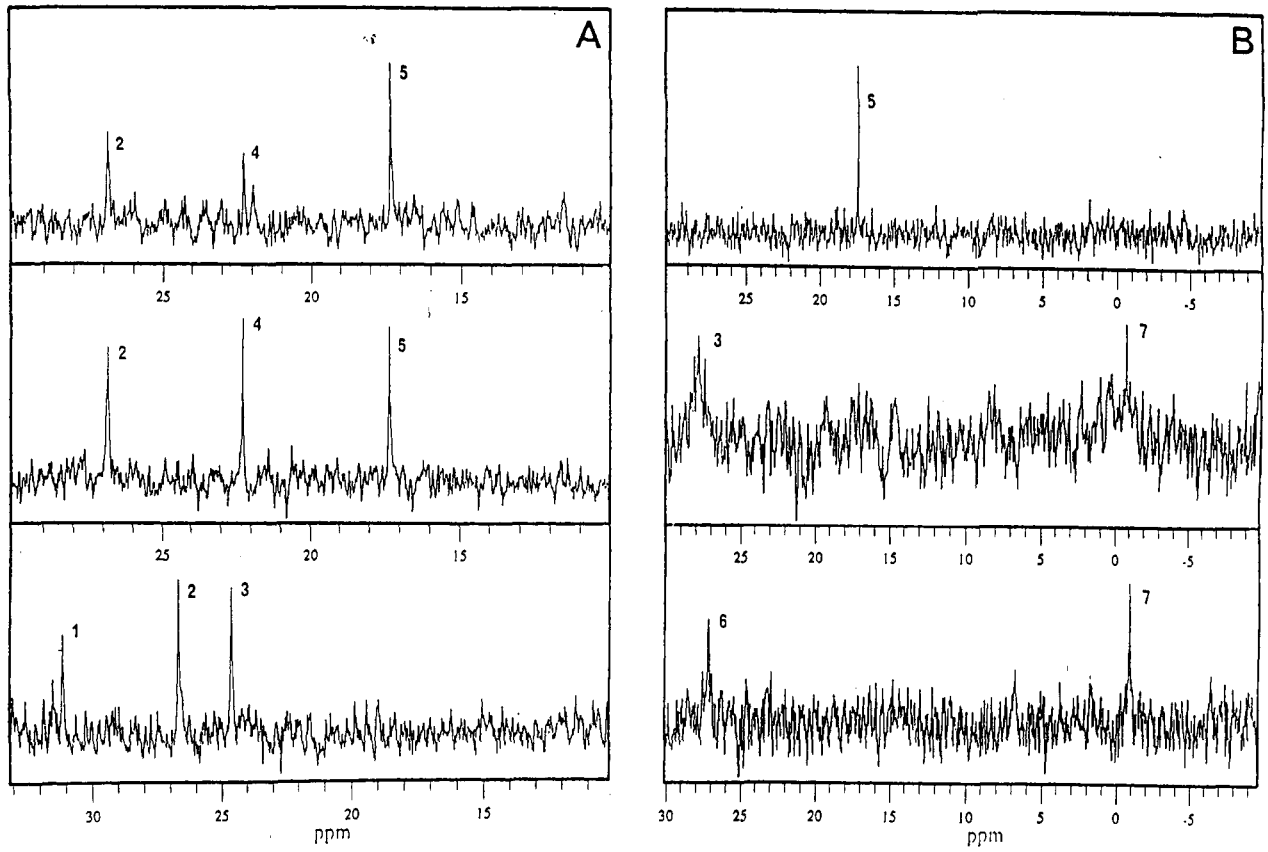

FIGURE 6: ^{31}P NMR spectra of approximately 0.2 mM soman- and MPDF-inhibited BChE in 0.1 M Tris, pH 8.0. Negative signs indicate an upfield chemical shift relative to external TMP (0 ppm). (Panel A) soman-inhibited EqBChE; (lower trace) in 0.1M Tris–6 M Gdn·HCl, pH 8.0 (21 000 scans); (middle trace) NaOH-released OP ligands from a conjugate obtained by reacting 1:1 molar ratio of soman to BChE (25 000 scans); (upper trace) NaOH-released OP ligands from a conjugate obtained by a 5:1 molar ratio of soman-to-BChE (25 000 scans). (Panel B) Spectra of MPDF-inhibited HuBChE and 0.15 mM homologous conjugate of Cht; (lower trace) native MPDF-Cht (21 000 scans); (middle trace) native MPDF-HuBChE (96 000 scans); (upper trace) NaOH-released OP ligand from MPDF-inhibited HuBChE. Lines assignment (for definitions see Table I): **1**, PinMP–BChE; **2**, HMPA (internal standard); **3**, MP–BChE ; **4**, PinMP-OH; **5**, MP-OH; **6**, MP-Cht; **7**, phosphoric acid (internal standard).

When either PinMP-Cht was transferred to 0.1 M Tris-6 M Gdn·HCl, pH 8.0, the NMR peaks narrowed and moved upfield relative to the native enzyme by 1.8–4.7 ppm (Figure 4B). On the basis of previous reports with other OP-Cht conjugates (van der Drift, 1985; Grunwald et al., 1989), these changes were expected upon unfolding of the enzyme that perturbed the environment of the P atom toward that of the model compounds (Table I). Following the addition of NaOH to the native OP–Cht adduct, the broad signals disappeared with the concomitant rise of a single narrow peak which corresponded to the chemical shift of *O*-pinacolyl methylphosphonic acid (PinMP-OH) in 0.2 N NaOH (+22.27 ppm; Figure 4C and Table I). Spiking with an authentic sample of PinMP-OH (not shown) and the multiplicity of the ^{31}P NMR line (Figure 4C, lower trace) confirmed the exclusive release of PinMP-OH. Similar findings were obtained for the least potent P(+)-containing OP-Cht adducts that were incubated 150 h in 50 mM acetate buffer at pH 6.0. Dealkylation could not be detected even after 96 h of incubation at 35 °C (0.1 M Tris, pH 8.0).

To characterize the $CH_3P(O)(O^-)$-containing moiety of Cht (MP–Cht), the difluoridate MPDF was used to instantaneously obtain the aged enzyme. The ^{31}P NMR of the latter conjugate clearly demonstrated that the NaOH-released OP was MP-OH (Figure 4D, upper trace). This enabled assignment of the ^{31}P NMR chemical shifts of the native and the unfolded forms of MP–Cht (Figure 4D, lower trace and middle trace, respectively).

(c) ^{31}P NMR Spectra and Identification of the OP-Containing Moieties of DFP- and Soman-Inhibited ChEs. In marked contrast to OP-Cht conjugates, 0.2 mM adducts obtained by reacting ChEs with either soman, MPDF, or DFP showed a broad ^{31}P NMR signal of low intensity even after accumulation of 100 000 transients/spectrum. However, unfolding in 0.1 M Tris–6 M Gdn·HCl gave rise to an intense narrow signal already detectable after 20 000 scans (Figures 5–7). The ^{31}P NMR chemical shifts of the unfolded form of aged MIP–ChE conjugates (–3.35 ppm; Figure 5A, middle trace) were similar to that observed for aged MIP-Cht in 0.1 M Tris–6 M Gdn·HCl (–3.43, Table II). Following dialysis against 0.1 M Tris buffer, pH 8.0, the ^{31}P NMR peak disappeared and appeared again at the same position upon transfer of the dialyzed solution into 0.1 M Tris–6 M Gdn·HCl (Figure 5A, upper trace). These results demonstrate that the phosphoryl moiety is covalently bound to the enzyme. When the pH of a solution of native aged MIP–ChE conjugates was elevated to ≈12, a new peak with a relatively high intensity was observed after 4000 transients at –0.06 ppm upfield to external TMP (Figure 5B, lower trace). This pointed at the release of $isoPrOP(O)(O^-)_2$ (MIP) from the aged conjugate.

The ^{31}P NMR normal mode (Figure 5B, middle trace) showed a doublet that is consistent with one hydrogen coupling the ^{31}P NMR signal $[(CH_3)_2CHOP(O)(O^-)_2]$. Finally, spiking with authentic $isoPrOP(O)(OH)_2$ (Figure 5B, upper trace) confirmed the assignment of the structure of the aged form to $isoPrOP(O)(O^-)$–ChE. Similar conclusions were

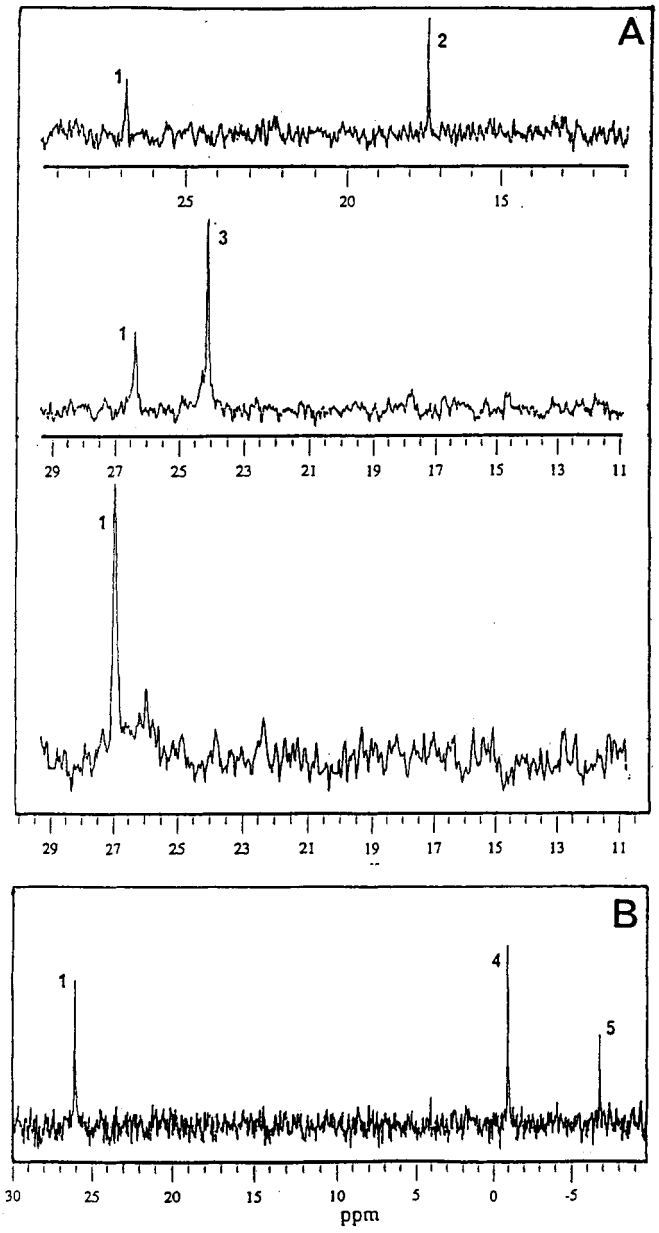

FIGURE 7: ^{31}P NMR spectra of approximately 0.25 mM OP-inhibited FBS–AChE. Negative signs indicate an upfield chemical shift relative to external TMP (0 ppm). (Panel A) Soman–AChE conjugate. (Lower trace) native conjugate in 0.1 M Tris, pH 8.0 (91 000 scans); (middle trace) unfolding in 0.1 M Tris–6 M Gdn·HCl, pH 8.0 (22 000 scans); (upper trace) NaOH-released OP ligand from soman-inhibited AChE (20 000 scans). (Panel B) DFP-inhibited AChE in 0.1 M Tris–6 M Gdn·HCl, pH 8.0 (22 000 scans). Lines assignment (for definitions see Tables I and II): **1**, HMPA (internal standard); **2**, MP-OH; **3**, MP–AChE; **4**, phosphoric acid (internal standard); **5**, DIP–AChE.

reached with MIP–AChE. When the same procedure was applied to the nonaged DIP–ChE conjugates, the exclusive release of $(isoPrO)_2P(O)OH$ (DIP) was demonstrated (not shown). Consequently, $(isoPrO)_2P(O)$–ChE was assigned to the nonaged form.

In the case of soman-inhibited BChEs, unfolding in 0.1 M Tris–6 M Gdn·HCl produced two distinct narrow signals at +31.31 and +24.68 ppm (Figure 6A, lower trace). On the basis of the ^{31}P NMR chemical shifts of the unfolded homologous pair PinMP– and MP–Cht, the following structures were assigned to the partially aged enzyme: PinMP-BChE [+31.31 ppm; $CH_3P(O)(OPin)$–BChE, nonaged] and MP–BChE [+24.68 ppm; $CH_3P(O)(O^-)$-BChE, aged]. Treatment with NaOH led to the release of two OP ligands that were identified unequivocally as PinMP-OH [$CH_3P(O)$-(OPin)(OH), +22.27 ppm] and MP-OH [$CH_3P(O)(OH)_2$, +17.44 ppm] (Figure 6A, middle and upper traces). It should be pointed out that the relative intensity of the NMR signals of the two latter acids did not change over 5 days in 0.2 N NaOH at room temperature. This is consistent with the stability of the authentic individual acids in 0.2 N NaOH. Thus, the two OP acids represent different OP conjugates rather than undergoing chemical modification after being detached from the OP-protein adduct (see Figure 1). Since it appeared that the low intensity signal corresponds to the 40% reactivatable portion of soman-inhibited BChE (Figure 6A, lower trace), it was of interest to examine the effect of the initial soman to BChE ratio on the relative height of the two peaks. When the ^{31}P NMR spectra of two conjugates obtained by 1:1 and 5:1 soman to BChE molar ratios were compared (Figure 6A, middle and upper traces, respectively), the latter showed clear enrichment of the isomers that eventually produced a higher MP-OH to PinMP-OH ratio. These findings are in agreement with the preference of BChE for the potent P(–) epimers of soman that undergo rapid dealkylation compared to the least potent P(+) stereoisomers (Keijer & Wolring, 1969).

To further demonstrate the broadening of the ^{31}P NMR signal of native OP–ChE conjugates relative to OP-Cht adducts, the spectra of aged MP–BChE and aged MP–Cht obtained with MPDF were compared in Figure 6B. As shown, the number of scans required to produce similar peak intensity of equimolar concentration of MP–enzyme conjugates was at least 5-fold higher for MP–BChE (middle trace) than for MP–Cht (lower trace). The structure of MP–BChE was further confirmed by the conversion of the OP-bound moiety to the diacid MP-OH (Figure 6B, upper trace).

Essentially similar observations were made with soman- and DFP-inhibited AChE. The native aged conjugate obtained by inhibition with soman showed a broad-low intensity ^{31}P NMR signal after 91 000 scans (Figure 7A, lower trace). Unfolding in 0.1 M Tris–6 M Gdn·HCl revealed the presence of a methylphosphonyl moiety at 24.63 ppm downfield to TMP (Figure 7A, middle trace). A similar chemical shift was assigned to MP–Cht in 6 M Gdn·HCl (Figure 4D; Table I). Dialysis and reunfolding in Gdn·HCl clearly indicated that the ^{31}P NMR of soman-inhibited AChE originated from a covalently bound OP moiety (not shown). The OP ligand released by NaOH was identified as the methylphosphonic diacid, MP-OH (Figure 7A, upper trace).

The ^{31}P NMR chemical shift of the nonaged DFP-inhibited FBS–AChE in 0.1 M Tris–6 M Gdn·HCl (Figure 7B and Table II) was similar to that assigned to the nonaged DIP–Cht (Table II). These findings suggest that the OP moiety of the former adduct consists of a diisopropylphosphoryl residue.

DISCUSSION

Structure of the OP Moiety of Aged and Nonaged ChEs. The dramatic change in the NMR spectra of the phosphorylated ChEs upon unfolding in 6 M Gdn·HCl not only demonstrated the covalent attachment of the OP residue to the BChE and AChE but also enabled the elucidation of the structure of the OP moiety of aged and nonaged OP–ChEs, by comparing the chemical shifts of the unfolded OP adducts with those of OP–Cht conjugates with known structures. The aged OP–ChE conjugates were shown here to contain a P–O^- bond, whereas the OP of the homologous nonaged form is

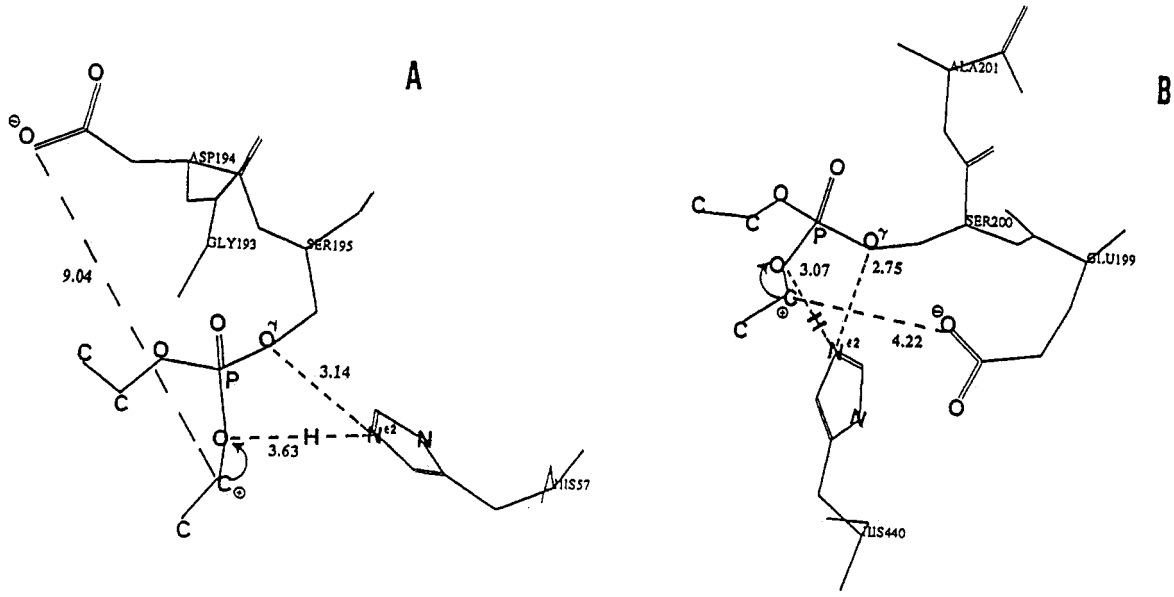

FIGURE 8: Active site residues and phosphoryl moiety of diethylphosphoryl conjugates of Cht and TcAChE. (Panel A) Extracted from the X-ray structure of $(C_2H_5O)_2P(O)$–Cht (Harel et al., 1991). (Panel B) Computer modeling of the diethylphosphoryl moiety into the active site of TcAChE. Dashed lines show interatomic distances (Å) between labeled atoms. ---H--- depicts hydrogen bond between the protonated $N^{\epsilon 2}$ atom of the catalytic His and the oxygen atom of a proximal P–O–ethyl group.

constituted from an uncharged OP triester moiety. This assignment is further supported by the identification of the protein-bound OP ligands released by NaOH (Figure 1).

The denatured form of the aged conjugates could be completely hydrolyzed within 1 h at pH 12. This finding suggests that, in contrast to native aged OP–ChEs or aged OP–Cht, the unfolded protein is the preferred leaving group in a nucleophilic displacement at the P–O^- center. Simple electrostatic repulsion of an approaching nucleophile does not play a major role in the unusual resistance to reactivation of native aged OP adducts of serine hydrolases.

Broadening of the ^{31}P NMR Signal of OP-ChE Conjugates. The ^{31}P NMR signal in a high magnetic field is mainly controlled by the chemical shift anisotropy relaxation mechanism and, to a lesser extent, by the dipole–dipole interactions (Brauer & Sykes, 1984). For native OP-ChEs it is envisaged that the narrow and crowded active site gorge will increase the chemical shift anisotropy of a covalently bound ^{31}P atom, compared to the homologous OP moiety of Cht conjugates that reside close to the surface of the enzyme (Sigler et al., 1968; Harel et al., 1991). Unfolding was expected to decrease significantly the differences between the two OP–enzyme conjugates in terms of (a) chemical shift anisotropy, (b) rotational correlation time, and (c) dipolar interactions. Indeed, the ^{31}P NMR spectrum of both OP-ChE or OP-Cht in 6 M Gdn·HCl showed narrow signals with similar intensity.

It has been recently shown that the active site of TcAChE is structured in a deep, narrow gorge that penetrates 20 Å into the enzyme (Sussman, 1991). Sequence homology between TcAChE and HuBChE enabled the modeling of the 3D structure of the latter enzyme which predicted a similar location for the catalytic region of HuBChE (Harel, 1992) and presumably for that of EqBChE and other AChEs. The apparent broadening of the ^{31}P NMR signal suggests that the active site of ChEs in solution is also located inside a relatively deep and narrow cavity.

Possible Mechanism of Dealkylation of OP-ChEs. On the basis of the mechanism of the dealkylation reaction (Benschop & Keijer, 1966), it has been recently argued that Glu199 (next to the catalytic amino acid Ser200), which is 3.4 Å apart from the charged carbon of the assumed carbonium moiety (Figure 8), may facilitate its rate of dealkylation by means of electrostatic forces operating between the carboxylate side chain and the positively charged carbon (Qian and Kovach, personal communication).

The data on the remarkable stability to dealkylation of the OP moiety of soman-inhibited Cht suggest that comparative analysis of the 3D structure of OP conjugates of Cht and AChE that contain the same phosphoryl moiety might help us to understand the role of a carboxyl side chain in the dealkylation process. Since the 3D geometry of $(C_2H_5O)_2P$-(O)–Cht has been shown by X-ray crystallography (Harel et al., 1991), we chose to model the diethylphosphoryl residue into the known coordinates of TcAChE (Sussman et al., 1991) and to compare interatomic distances between key amino acid side chains and the scissible P–O–alkyl bond. It was assumed that the structural variations in the alkyl residue will not cause significant changes in the relative positioning of the oxygen atom of the P–O–alkyl link.

As shown in Figure 8, the distance between the oxygen atom of a proximal ethoxy group and the $N^{\epsilon 2}$ atom of the catalytic His57 of Cht is 3.63 Å, suggesting that protonation is likely to occur between the corresponding oxygen atom of one or more stereoisomers of soman–Cht adducts and His57, although to different extent than in TcAChE (3.07 Å, His440). In contrast to the close proximity of the carboxyl side chain of Glu199 of TcAChE to the putative charged carbon of the diethylphosphoryl moiety (4.22 Å, Figure 8), the homologous carboxyl side chain of Asp194 in Cht (next to the active site Ser195) is projected >9 Å away from the same carbon. Thus, it can not offer reasonable interaction with the carbonium ion, as might be the case in ChEs. Using the same rationale for OP conjugates formed between the less potent stereoisomer of soman and BChE, it is possible that both His and Glu are projected away from the P–O–C link and that they cannot provide the catalytic machinery for dealkylation to occur.

Finally, it is of interest to point out that OP conjugates of mammalian carboxylesterases (CaE; EC 3.1.1.1), homologous proteins to ChEs that contain, among other conserved sequences, the catalytic triad Ser-His-Glu and a Glu residue next to the catalytic serine (Cygler et al., 1993), do not age readily (Sterri & Fonnum, 1987). It is suggested that, in

addition to the structure of the P–O–alkyl moiety, its rate of detachment strongly depends on the availability of both the imidazole of the catalytic His and the carboxyl side chain to interact in concert with the atoms of the P–O–alkyl bond.

REFERENCES

Adebodun, F., & Jordan, F. J. (1989a) *J. Cell. Biochem. 40*, 249–260.

Adebodun, F., & Jordan, F. J. (1989b) *Biochemistry 28*, 7524–7531.

Aldrich, W. N., & Reiner, E. (1972) *Enzyme Inhibitors as Substrates*, North Holland Publishing Co., Amsterdam, The Netherlands.

Auler, H., Wolf, A., & Helstrom, H. (1929) *Chem. Ber. 62*, 2451–2456.

Barak, D., Ariel, N., Velan, B., & Shafferman, A. (1992) in *Multidisciplinary Approaches to Cholinesterase Functions* (Shafferman, A., & Velan, B., Eds.) pp 195–199, Plenum Press, New York.

Benschop, H. P., & Keijer, J. H. (1966) *Biochim. Biophys. Acta 128*, 586–588.

Benschop, H. P., & De Jong, L. P. A. (1988) *Acc. Chem. Res. 21*, 368.

Berends, F., Posthumus, C. H., Sluys, I. V. D., & Deierkauf, F. A. (1959) *Biochim. Biophys. Acta 34*, 576–578.

Brauer, M., & Sykes, B. D. (1984) *Methods Enzymol. 107*, 36–81.

Cohen, W., & Erlanger, B. F. (1960) *J. Am. Chem. Soc. 82*, 3928–3934.

Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry, M. K., & Doctor, B. P. (1993) *Protein Sci. 2*, 366–382.

De La Hoz, D., Doctor, B. P., Ralston, J. S., Rush, R. S., & Wolfe, A. D. (1986) *Life Sci. 39*, 195–199.

Delmar, E. G., Largman, C., Brodrick, J. W., & Geokas, M. C. (1979) *Anal. Biochem. 99*, 316–320.

Ellman, G. L., Courtney, D., Andres, V., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 1*, 88–95.

Gorenstein, D. G., & Findlay, J. B. (1976) *Biochem. Biophys. Res. Commun. 72*, 640–645.

Gorenstein, D. G., Shah, D., Chen, R., & Kallick, D. (1989) *Biochemistry 28*, 2050–2058.

Gray, A. P. (1984) *Drug Metab. Rev. 15*, 557–589.

Green, M., & Hudson, R. F. (1958) *J. Chem. Soc.*, 3129–3133.

Grunwald, J., Segall, Y., Shirin, E., Waysbort, D., Steinberg N., Silman, I., & Ashani, Y. (1989) *Biochem. Pharmacol.* 19, 3157–3168.

Harel, M., Su, C.-T., Frolow, F., Ashani, Y., Silman, I., & Sussman, J. L. (1991) *J. Mol. Biol. 221*, 909–918.

Harel, M., Sussman, J. L., Krejci, E., Bon, S., Chanal, P., Massoulie, J., & Silman, I. (1992) *Proc. Nat. Acad. Sci. U.S.A. 89*, 10827–31.

Heilbroon, E., & Tolagen, B. (1965) *Biochem. Pharmacol. 14*, 73–77.

Hobbiger, F. (1955) *Br. J. Pharmacol. 10*, 356–359.

Keijer, J. H., & Wolring, G. Z (1969) *Biochim. Biophys. Acta 185*, 465–468.

Kovach, I. M., McKay, L., & Vander Velde, D. (1993) *Chirality 5*, 143–149.

Loomis, T. A., & Salafsky, B. J. (1963) *J. Toxicol. Appl. Pharmacol. 5*, 685–701.

Michel, H. O., Hackley, B. E., Jr., Berkowitz, L., List, G., Hackley, E. B., Gillian, W., & Pankau, M. (1967) *Arch. Biochem. Biophys. 121*, 29–34.

Monard, C., & Quinchon, J. (1961) *Bull. Soc. Chim. Fr.*, 1084–1086

Ooms, A. J. J., & van Dijk, C. (1961) *Biochem. Pharmacol. 15*, 1361–1377.

Raveh, L., Grunwald, J., Marcus, D., Papier, Y., Cohen, E., & Ashani, Y. (1993) *Biochem. Pharmacol. 45*, 2465–2474.

Schonbaum, G. R., Zerner. B., & Bender, M. L. *(1961) J. Biol. Chem. 236*, 2930–2935.

Schoene, K. (1971) *Biochem. Pharmacol. 20*, 2527–2529.

Sigler, P. B., Blow, D. M., Matthews, B. W., & Henderson, R. (1968) *J. Mol. Biol. 35*, 143–168.

Sterri, S. H., & Fonnum, F. (1987) *Biochem. Pharmacol. 36*, 3937–3942.

Sussman, J. L., Harel, M., Frolov, F., Oefner, C., Goldman, A., Toker, L., & Silman, I. (1991) *Science 253*, 872–879.

van Der Drift, A. C. M., Beck, H. C., Dekker, W. H., Hulst, A. G., & Wils, E. R. J. (1985) *Biochemistry 24*, 6894–6903.