in the pulse-chase method, permitting an extrapolation to zero time to obtain a single turnover event, allows one to cancel out kinetic effects arising from the presence of excess cold substrate in the chase media. For the case of FBPase, these effects are a serious hazard owing to its inhibition by substrate at 1 mM concentrations.

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Fluorescent Phosphonate Label for Serine Hydrolases, Pyrenebutyl Methylphosphonofluoridate: Reaction with Acetylcholinesterase†

Harvey Alan Berman* and Palmer Taylor

ABSTRACT: Pyrenebutyl methylphosphonofluoridate reacts with acetylcholinesterase to yield a conjugate with a stoichiometry of one fluorescent label per 80 000 molecular weight subunit. Kinetics of inactivation by the phosphonate and the subsequent reactivation behavior, as well as competition between the fluorescent compound and diisopropyl fluorophosphate, indicate that equivalent active site serines are involved in the labeling reaction. The long wavelength absorption band of the pyrene conjugate exhibits an 8-nm bathochromic shift, whereas its fluorescence emission spectrum shows little change. The shift correlates best with wavelength maxima observed for pyrenebutanol in solvents of high refractive index and is indicative of an association between the pyrene moiety and aromatic residues in the active center of the enzyme. Dynamic fluorescence quenching of the conjugated pyrene moiety by iodide, nitromethane, and thallous ion is markedly reduced when compared with the corresponding quenching constants obtained for pyrenebutanol in solution. Quenching by thallous ion is most efficient and iodide is least efficient when quenching efficiencies for the conjugated fluorophore are considered relative to the free fluorophore. The absorption spectrum of the pyrene moiety overlaps with the emission spectrum of the enzyme tryptophanyl residues and phosphonylation results in a quenching of the tryptophan fluorescence. Propidium, a peripheral site ligand, binds with equal affinity to the phosphonylated and native enzyme. Propidium binding results in 85–90% quenching of pyrene fluorescence. These findings show pyrenebutyl methylphosphonofluoridate to be a specific active site fluorescent label which can serve as a donor and acceptor of fluorescence energy. The conjugated pyrene appears to be buried in a region of high polarizability where it is restricted in contact with solvent and where the dominant electrostatic effect on pyrene would suggest an anionic subsite in the vicinity.

Catalysis by acetylcholinesterase (AChE[1]) shows essential mechanistic features similar to those observed for other serine hydrolases whose crystal structures have been elucidated. This group of enzymes is characterized by a charge relay system which enhances the nucleophilicity of the active site serine (Henderson et al., 1971) and an oxyanion hole which serves to stabilize a tetrahedral transition state and bind certain tetrahedral inhibitors (Robertus et al., 1972). The serine within the active center of AChE is capable of nucleophilic attack upon esters of carbon, sulfur, phosphorus, and boron, forming acyl-enzyme intermediates which differ in the subsequent rate at which they deacylate to give active enzyme and hydrolysis products. Acetylcholine, for example, reacts with AChE to form the acetyl-enzyme intermediate which readily reacts with

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[1] Abbreviations used: AChE, acetylcholinesterase; PBMPF, pyrenebutyl methylphosphonofluoridate; PBMP-AChE, pyrenebutyl methylphosphonoacetylcholinesterase; PBOH, pyrenebutanol; PBA, pyrenebutyric acid; DFP, diisopropyl phosphorofluoridate; 2-PAM, 2-pyridinealdoxime methiodide; BSA, bovine serum albumin.

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water to give acetate and free enzyme, the half-time of this reaction being less than 0.1 ms (Lawler, 1961; Kremzer & Wilson, 1963). Carbamoylated AChE, in contrast, deacylates with a half-time of minutes (Iverson & Main, 1969), whereas the phosphoryl and sulfonyl esterases are virtually unreactive, possessing a half-time of days (Kitz & Wilson, 1962; Greenspan & Wilson, 1970).

Because alkyl phosphates and phosphonates react in an essentially irreversible manner they should serve as convenient handles with which to label the active center of AChE as well as other related serine hydrolases. Phosphonates containing a nitroxide moiety have been employed in electron spin resonance studies of the rotational mobility at the active center of AChE (Morrisett & Broomfield, 1972; Sentjurc et al., 1976). Rosenberry & Bernhard (1971) have described a carbamoylating reagent for AChE that releases the fluorescent moiety, *N*-methylhydroxyquinolinium, which has proven useful for measuring enzyme normality and following transient kinetics. A fluorescent alkyl phosphate, Maretin, which becomes nonfluorescent upon reacting with AChE has also been described (Seitz & Himel, 1977). In contrast, an acylating agent, such as an alkyl phosphonate in which the fluorescent moiety remains covalently attached to the serine, should prove particularly informative since it can probe the intrinsic structure of the macromolecule.

Fluorescent probes that react with AChE with a defined stoichiometry and site specificity should allow study of the topology as well as dynamic behavior of the macromolecule. We describe here an alkyl phosphonate, pyrenebutyl methylphosphonofluoridate (PBMPF), which renders AChE and other serine hydrolases fluorescent. The fluorophore is long lived and possesses excitation and emission bands outside those observed for the protein envelope. The specificity of this ligand for the active site serine is high as might be predicted for a potent alkyl phosphonate. Some of our findings have been presented previously in abstract form (Berman & Taylor, 1976, 1977).

Experimental Procedures

Materials. *d*-Tubocurarine, DFP, 2-PAM, α-chymotrypsin (type 1S), and hog liver carboxylesterase (type I) were products of the Sigma Chemical Co. Propidium diiodide was obtained from Calbiochem, and gallamine triiodide and decamethonium chloride were from K & K Chemicals. Potassium iodide was purchased from G. Frederick Smith Co., thallium chloride from Alfa Inorganics, and spectral quality nitromethane from Eastman Organic Chemicals. Pyrenebutyric acid (Eastman) was recrystallized from ethanol–water mixtures for use in fluorescence studies. Other materials were used without further purification. [^{3}H]DFP (specific activity 25.1 Ci/mol) was a product of New England Nuclear.

Preparation of PBMP–AChE for Spectroscopic Studies. AChE from *Torpedo californica* was purified to apparent homogeneity as previously described (Taylor et al., 1974). All studies were carried out on the 11S or "lytic" AChE species. The purified enzyme in a 10 mM Tris-Cl buffer, pH 8.0, containing 0.1 M NaCl and 0.04 M $MgCl_2$ was allowed to react with a 1.5–3-fold molar excess of PBMPF delivered from a concentrated ethanolic stock solution. The course of reaction was monitored by titrimetric assay of acetylcholine hydrolysis. The reaction was complete within 10 min and the reaction mixture was then immediately passed over a Sephadex G-25 column at 4 °C. Labeled enzyme appeared in the void volume, while most of the unbound fluorophore appeared to be adsorbed to the column. The enzyme was subsequently dialyzed overnight at 4 °C against the above buffer and flash frozen in liquid nitrogen. Structural integrity of the enzyme could be ascertained by measuring the ratio of its absorption at 280 to 252 nm. It was observed that with a freeze-thaw cycle of AChE in low ionic strength buffers this value precipitously decreases. This effect was less pronounced in high ionic strength buffer. Preparations suitable for spectral studies had 280/252 ratios greater than 2.0.

PBMPF Inhibition of Acetylcholinesterase and Its Subsequent Reactivation. Kinetics of inhibition of AChE by PBMPF were monitored by the general procedure of Hart & O'Brien (1973) using *p*-nitrophenyl acetate as a substrate. The reaction medium was 0.1 M sodium phosphate, pH 7.0. Reactivation of the phosphonylated enzyme was carried out either at 4 or 25 °C in 0.01 M Tris-Cl buffer, pH 8.0, containing 0.1 M NaCl and 0.04 M $MgCl_2$. The PBMP–AChE used for these reactivation studies was passed over a Sephadex G-25 column at 4 °C to remove unbound ligand. When spontaneous hydrolysis was measured, 2-PAM (1 mM) was added after the last time interval to ascertain total enzyme susceptible to reactivation. Control enzyme samples were incubated under the same conditions to monitor enzyme instability over long time intervals. Reactivation studies were based on return of esterase activity against 0.125 mM acetylcholine. Enzyme was diluted 200–3000-fold prior to assay.

PBMPF Protection of Labeling of the Enzyme by [^{3}H]DFP. AChE (0.7 mg/mL) was allowed to react with a 1.5 molar excess of PBMPF until the reaction approached completion and then passed over a Sephadex G-25 column to remove unbound label. The PBMP–AChE and a sample of control enzyme were reacted with 200 μM [^{3}H]DFP (specific activity 25.1 Ci/mol) until 98% inhibition was observed for the control enzyme. Both samples were passed over Sephadex G-25 columns. The protein fraction appearing in the void volume was dialyzed overnight at 4 °C, counted by liquid scintillation spectrometry, and analyzed for total protein.

Spectroscopy. Fluorescence measurements were made on a Farrand Mark I spectrofluorometer equipped with a corrected excitation source and a Hamamatsu R818 photomultiplier tube. The emission monochromator–photomultiplier combination was calibrated using $BaSO_4$ (Eastman Kodak white reflectance standard) and Halon (Diano Corp., Mansfield, Mass.), a fluorinated hydrocarbon of nearly constant reflectance (Eckerle et al., 1976). Both materials gave identical calibration curves. Samples were maintained at constant temperature in a four-cell turret housed in a water-jacketed sample compartment controlled with a Haake thermostat. Absorption spectra were recorded on a Cary 16 spectrophotometer using quartz cells of 1-cm path length. For the kinetic studies, temperature was maintained at 22 °C.

Fluorescence Titrations. Fluorescence titrations were carried out using $(1\ cm)^2$ or $(0.3\ cm)^2$ cells at 20 °C as previously described (Taylor & Lappi, 1975). The following excitation and emission wavelengths were employed: tryptophanyl residues, 290/335; pyrene, 348/400; propidium, 535/625 nm. Fluorescence values were corrected for dilution resulting from added titrant, lamp fluctuations, and inner filter effects. Competitive displacement studies were carried out by making incremental additions of competing ligand to solutions of PBMP–AChE and native AChE containing at least a twofold stoichiometric excess of propidium. Titration profiles for PBMP–AChE could be obtained directly by monitoring propidium fluorescence (Taylor & Lappi, 1975) or, indirectly, by measuring return of pyrene fluorescence which is diminished due to quenching by propidium through energy transfer.

Dynamic Quenching. Dynamic fluorescence quenching occurs through random collisional encounters of a fluorophore

(F) and a quenching ligand (Q) that occur during the excited state lifetime of the fluorophore (Stern & Volmer, 1919; Lehrer, 1971). The process shows a linear dependence on concentration of the quenching ligand and is described as a bimolecular reaction involving formation of an encounter complex between F* and Q:

$$F^* + Q \overset{K_Q}{\rightleftharpoons} [F^*Q] \quad (1)$$

In the absence of a static component, dynamic fluorescence quenching is described by the Stern–Volmer equation

$$F_0/F = 1 + K_Q[Q] \quad (2)$$

where F_0 and F denote the fluorescence intensities in the absence and presence of quenching ligand. K_Q is the dynamic quenching constant (M^{-1}) and is determined from the slope of linear plots of F_0/F vs. [Q]. When the fluorescence lifetime (τ_0) is known, the bimolecular quenching rate, k, for collisional deactivation of F* by Q can be obtained through the relationship

$$K_Q = k\tau_0 \quad (3)$$

The quenching rate characteristic of the enzyme-associated fluorophore can be compared with that for the freely diffusing entity to give the relative quenching efficiency, $\gamma = k^b/k^f$, where the superscripts denote bound and free fluorophore. Values of γ are generally less than 1, which reflect the limited accessibility of small quenching molecules to the macromolecular matrix that contains the fluorophore.

For a series of different quenching ligands (Q_1, Q_2, . . .), the following relationships hold for the quenching of fluorescence of the enzyme-associated and free fluorophores:

$$K_1^b = k_1^b\tau_0^b \qquad K_1^f = k_1^f\tau_0^f$$
$$K_2^b = k_2^b\tau_0^b \qquad K_2^f = k_2^f\tau_0^f \quad (4)$$

Subscripts denote different quenching ligands. Taking the ratio of different quenching efficiencies of Q_1 to Q_2 we obtain:

$$\frac{\gamma_1}{\gamma_2} = \frac{K_1^b/K_2^b}{K_1^f/K_2^f} = \frac{k_1^b/k_1^f}{k_2^b/k_2^f} \quad (5)$$

The resultant expression is independent of knowledge of the fluorescent lifetimes τ_0^b and τ_0^f which are characteristic of the bound and free fluorophore. The quantity γ_1/γ_2 reflects the accessibility of F to Q_1 as compared with Q_2 and is controlled by the steric and electrostatic interactions experienced by the quenching ligands. Nitromethane was selected as a reference compound against which to compare the effects of charged quenching ligands.

To minimize effects due to quenching by dissolved oxygen, enzyme samples were prepared from a concentrated stock solution in a buffer equilibrated with nitrogen at 5 °C. All solutions of KI contained sodium thiosulfate (0.1 mM) to prevent formation of the triiodide anion.

Energy Transfer. The efficiency (T) of Förster energy transfer can be expressed by

$$T = 1 - F/F_0 = 1/[1 + (R/R_0)^6] \quad (6)$$

where F and F_0 are the fluorescence intensities in the presence and absence of acceptor, respectively, and R is the distance separating the donor–acceptor pair. The critical transfer distance, R_0, is calculated from

$$R_0 = 9.765 \times 10^3(\kappa^2 n^{-4} QJ)^{1/6} \quad (7)$$

where κ^2 is the dipole–dipole orientation factor for which we assume a value 0.67. The refractive index, n, of the protein matrix is 1.6, and the quantum yield of enzyme emission is 0.121 (Taylor & Jacobs, 1974). The overlap integral, J, is calculated from

$$J = \frac{\Sigma F_d(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda}{\Sigma F_d(\lambda)\Delta\lambda} \quad (8)$$

where F_d is the intensity of the donor emission and ϵ_A is the molar decadic extinction coefficient for the acceptor at wavelength λ.

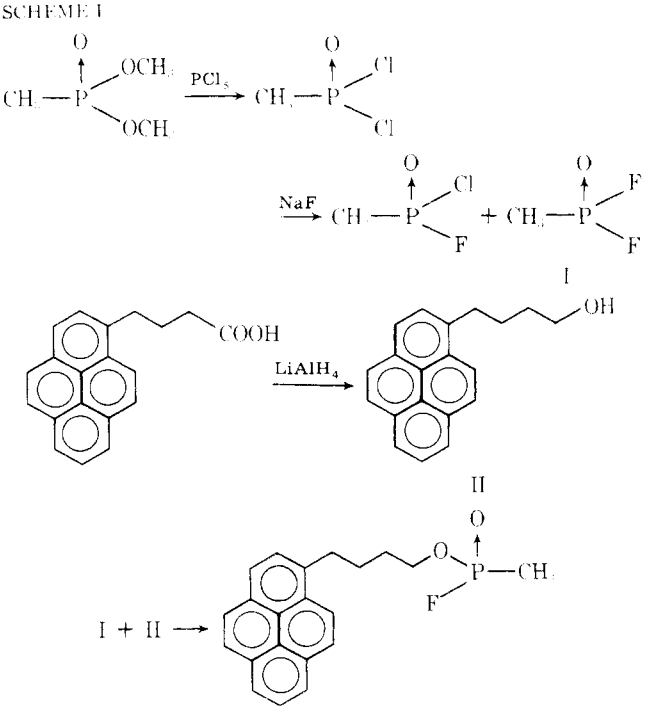
Synthesis. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian HR-220 spectrometer interfaced with a Fourier transform system from Nicolet Technology Corp. Chemical shifts (δ) are reported in parts per million downfield from internal tetramethylsilane and coupling constants (J) in hertz. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Microanalytical elemental analyses were carried out by Galbraith Laboratories, Knoxville, Tenn.

The overall synthesis of PBMPF involved reaction of pyrenebutanol with CH_3POF_2 as shown in Scheme I.

Methylphosphonodifluoridate. Methylphosphonodichloridate, CH_3POCl_2, was synthesized by reaction of dimethyl methylphosphonate with PCl_5 (Voss et al., 1975) or was purchased from Specialty Organics (Irwindale, Calif.). Methylphosphonodifluoridate, CH_3POF_2, was synthesized by reaction of the dichloridate with NaF in tetramethylene sulfone (Tullock & Coffman, 1960). The product was distilled through a Vigreux column (20 × 1 cm) under atmospheric pressure to give a colorless liquid, bp 99–101 °C (lit. 98–99 °C; Dawson & Kennard, 1957).

The course of the phosphonate reactions could be conveniently followed by noting changes in the NMR spectrum of the methyl group. The NMR spectrum for CH_3POCl_2 shows a doublet centered at 2.5 ppm (J_{HP} = 16 Hz). Formation of CH_3POF_2 as well as the higher boiling product of incomplete reaction, CH_3POClF, are detected by the appearance of their characteristic first-order proton spectra, the monofluoridate showing a doublet of doublets centered at δ 2.2 (J_{HF} = 6; J_{HP} = 18), and the difluoridate showing a doublet of triplets centered at δ 1.9 (J_{HF} = 6; J_{HP} = 20).

SCHEME I



Pyrenebutanol. To a stirred suspension of lithium aluminum hydride (2.8 g, 35 mmol; 50% dispersion in oil) and dry THF (50 mL) in a round-bottomed flask equipped with a mechanical stirrer, reflux condenser, and $CaCl_2$ drying tube was added a solution of pyrenebutyric acid (10 g, 35 mmol) in dry THF (125 mL). After stirring for 1 h the reaction was quenched by a sequential addition of H_2O and 15% NaOH, and the heterogeneous mixture was suction filtered. The product was extracted from the filtrate with ether, and the organic layer water washed (2 × 150 mL), dried (Na_2SO_4), and reduced in vacuo to give the crude alcohol that showed a single spot on thin-layer chromotography (yield, 6.4 g (70%)). A portion of this material was treated with activated charcoal, filtered through Celite, and crystallized from mixtures of chloroform and hexane to afford white crystals, mp 75–76.5 °C. Anal. Calcd for $C_{20}H_{18}O$: C, 87.56; H, 6.61; O, 5.83. Found: C, 87.47; H, 6.81; O, 5.61. NMR ($CDCl_3$) δ 1.72 (m, 2 H, γ-methylene), 1.93 (m, 2 H, β-methylene), 3.39 (triplet, 2 H, J = 7 Hz, δ-methylene), 3.7 (triplet, 2 H, J = 7 Hz, α-methylene), 8 (m, 9 H, aromatic). Assignments were made on the basis of spin decoupling experiments. The crude material was dried in vacuo at 40 °C for 24 h and used in the next reaction without further purification.

Pyrenebutyl Methylphosphonofluoridate (PBMPF). A solution of pyrenebutanol (1 g, 3.7 mmol) in chloroform (50 mL) was added dropwise to a 1.5–2-fold molar excess of methylphosphonodifluoridate in a flame-dried round-bottomed flask equipped with a condenser and $CaCl_2$ drying tube. After refluxing for 2–3 h the reaction mixture was cooled to room temperature, water washed (2 × 100 mL), dried, and concentrated in vacuo to give an oil which was chromatographed through a silica gel column using mixtures of chloroform in hexane. The product was crystallized and recrystallized from pentane to give long white needles, mp 64 °C (yield, 0.71 g (54%)). Anal. Calcd for $C_{21}H_{20}O_2PF$: C, 71.18; H, 5.69; P, 8.74; F, 5.36. Found: C, 71.32; H, 5.80; P, 8.88; F, 5.19. NMR ($CDCl_3$) δ 1.6 (doublet of doublets, J_{HF} = 6 Hz, J_{HP} = 18.5 Hz, 3 H, methyl), 1.9 (broad multiplet, 4 H, β,γ-methylenes), 3.4 (triplet, J = 7.2 Hz, 2 H, δ-methylene), 4.2 (broad multiplet, 2 H, α-methylene), 8 (multiplet, 9 H, aromatic); ϵ_M^{341} = 2.2 × 10^4.

Results

Inhibition of AChE by PBMPF and Reactivation of the Phosphonyl-Enzyme. Acetylcholinesterase inhibition by PBMPF proceeds rapidly and can be measured by conventional techniques only when submicromolar concentrations of inhibitor and enzyme are employed (Figure 1a). Inhibition with 0.1 μM PBMPF occurred at 12 times the rate found for an equivalent concentration of paraoxon. When inhibitor is in excess, the observed first-order kinetics are indicative of the existence of a single phosphonylating species. At high concentrations of ligand the reaction is too rapid to measure a limiting rate. Thus, it was not possible to determine equilibrium association and phosphonylation constants for PBMPF interaction (Main, 1964; Hart & O'Brien, 1973).

Reactivation kinetics for phosphorylacetylcholinesterases have been examined extensively (cf. Froede & Wilson, 1971; Aldridge & Reiner, 1972). Since reactivation rates are characteristic of the acyl group, the kinetics should be indicative of the uniformity of the phosphate species. Reactivation at 25 °C in the presence of the nucleophile 2-PAM (1.0 mM) behaves as a first-order process and proceeds to greater than 90% completion (Figure 1b). The overall rate appears to be about one-sixth of the rate at which diisopropylphosphoryl-AChE

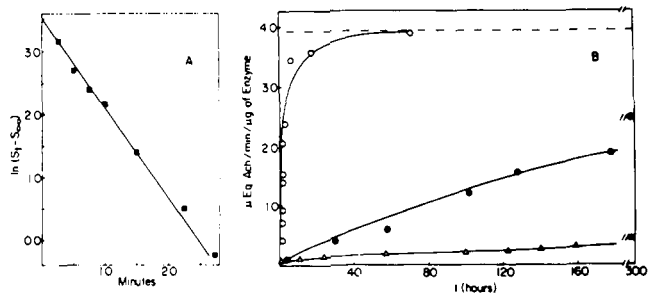


FIGURE 1: Inhibition of acetylcholinesterase by pyrenebutyl methylphosphonofluoridate and subsequent reactivation of the conjugate. (A) Inactivation of AChE. Pyrenebutyl methylphosphonofluoridate (0.26 μM) and *p*-nitrophenyl acetate (1 mM) were added to a cuvette containing 0.1 M sodium phosphate buffer, pH 7.0. After positioning in the light path of the Cary 16, acetylcholinesterase was added to give an estimated concentration of 0.06 μM in active sites and the reaction measured by the increase in optical density at 402 nm. Since the reaction during the initial 2–3 min in the absence of inhibitor is linear, time dependence of inhibition can be estimated from the progressive change in slope on the tracing (Hart & O'Brien, 1973). S_t and S_∞ are the respective slopes at the specified time and in the absence of enzyme; k_i = 9.2 × 10^4 M^{-1} s^{-1}. (B) Reactivation of pyrenebutyl methylphosphonoacetylcholinesterase. Acetylcholinesterase (12 μM in active sites) was inhibited to 95–99% with a 1.5–2.0-fold molar excess of PBMPF and passed over a Sephadex G-25 column at 4 °C to remove unbound ligand. Reactivation was measured in the presence and absence of 1 mM 2-pyridine aldoxime methiodide in a pH 8.0 Tris-Cl buffer containing 0.1 N NaCl and 0.04 M $MgCl_2$. The reactivation was monitored by the return of activity against 0.625 mM acetylcholine chloride after a 200–1000-fold dilution of enzyme from the incubation condition. (O—O) Reactivation with 2-PAM at 25 °C; k_R = 4.2 × 10^{-3} min^{-1}; (●—●) spontaneous hydrolysis at 25 °C; k_R = 7.8 × 10^{-5} min^{-1}; (Δ-- Δ) spontaneous hydrolysis at 4 °C. A_t and A_∞ are the activities at the specified time and after complete reactivation.

can be regenerated (k_R = 4.2 × 10^{-3} min^{-1} vs. 3.0 × 10^{-2} min^{-1}, respectively).

Incubation of PBMP-AChE at 25 °C for 16 h prior to addition of 2-PAM gives rise to the same rate and extent of 2-PAM reactivation. If these conditions are applied to the diisopropylphosphoryl enzyme prior to addition of 2-PAM, aging prevails since less than 5% reactivation can be achieved (cf. Davies & Green, 1956).

Spontaneous reactivation of PBMP-AChE at 25 °C is a far slower process (k_R = 7.8 × 10^{-5} min^{-1}) (Figure 1b). This value corresponds to a rate of 0.4% per h. At 4 °C the rate is considerably slower, less than 0.1% per h and after 2 weeks only 12% spontaneous reactivation was observed. Subsequent addition of 2-PAM resulted in 85% of the original activity returning after 16 h. Thus, aging at 4 °C could not be detected even after prolonged incubation.

In a separate experiment, PBMP-AChE was reactivated with 2-PAM until 83% of the original activity was restored and the sample was immediately placed on a Sephadex G-25 column at 4 °C to remove unbound pyrenebutyl methylphosphonic acid and 2-PAM. The enzyme appearing in the excluded peak showed a 76% reduction in bound pyrene. As a further demonstration of specificity, two preparations of AChE were inhibited with PBMPF until 1.0 and 1.5% of the original activity remained. Incubation with [^{3}H]DFP for an interval required to inhibit completely native enzyme resulted in 2.8 and 3.1% of the incorporated ^{3}H/mg of protein found in the unreacted enzyme.

PBMPF was also found to be an effective irreversible inhibitor of α-chymotrypsin and hog liver carboxylesterase, where 10^{-5} M PBMPF produced complete inhibition of the enzymes within 10 and 15 min, respectively. Assuming $\epsilon_{1\%}^{280}$ = 16.0 for chymotrypsin and ϵ_M^{345} = 4 × 10^4 for the enzyme-associated pyrene, we find the stoichiometric ratio of

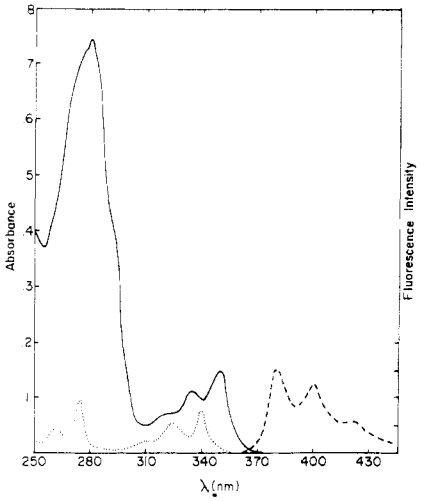


FIGURE 2: The absorption spectrum (—) and the corrected emission spectrum (- - -) for pyrenebutyl methylphosphonoacetylcholinesterase in 0.01 M Tris-Cl containing 0.1 M NaCl and 0.04 M $MgCl_2$, pH 8.0. For the absorption spectrum, the enzyme was present at 4.0×10^{-6} M in subunit concentration and the concentration of the associated pyrene moiety is calculated to be 3.8×10^{-6} M. For the fluorescence spectrum (λ_{ex} = 348 nm) the sample was diluted 20-fold. The absorption spectrum of pyrenebutanol (3.8×10^{-6} M) in water (· · ·) is also shown.

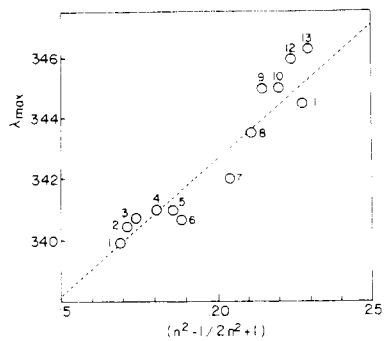


FIGURE 3: Relationship between the position of the O,O absorption maxima and refractive index for solutions of pyrenebutanol in a variety of organic solvents. The abscissa is represented as $[(n^2 - 1)/(2n^2 + 1)]$ where n is the refractive index. The numbers denote the following solvents: (1) methanol; (2) water; (3) acetonitrile; (4) ethanol; (5) hexane; (6) 1-propanol; (7) cyclohexane; (8) chloroform; (9) carbon tetrachloride; (10) dimethyl sulfoxide; (11) benzene; (12) bromobenzene; (13) pyridine. Values for refractive indices are taken from Weast (1977).

pyrene to enzyme to be 0.87 ± 0.1 after labeling and removal of excess reactants by gel filtration and subsequent dialysis.

Spectroscopic Characterization. The absorption spectrum for the pyrene chromophore shows a small but consistent variation upon alteration of its immediate environment. The position of the O,O band in the absorption spectrum of PBMP-AChE (Figure 2) is shifted to 349 nm ($\epsilon = 3.9 \pm 0.1 \times 10^4$ M^{-1} cm^{-1}) from a value of 341 nm observed for solutions of PBOH in H_2O ($\epsilon = 2.2 \pm 0.1 \times 10^4$ M^{-1} cm^{-1}). This bathochromic shift associated with covalent binding of PBMPF is greater than the shifts observed for the corresponding fluorescent conjugate with chymotrypsin (λ_{max} = 345 nm), the pyrenebutyric acid-BSA complex (λ_{max} = 345 nm; Vaughn & Weber, 1970), and the pyrenebutyrate-antipyrenebutyrate antibody complex (λ_{max} = 347 nm; Lovejoy et al., 1977). The position of the absorption spectrum also changes with transfer of PBOH from H_2O to a variety of organic solvents. The spectral shifts correlate more closely with the refractive index, n, of the medium rather than its static dielectric constant. A plot of λ_{max} vs. $(n^2 - 1)/(2n^2 + 1)$ (Figure 3)

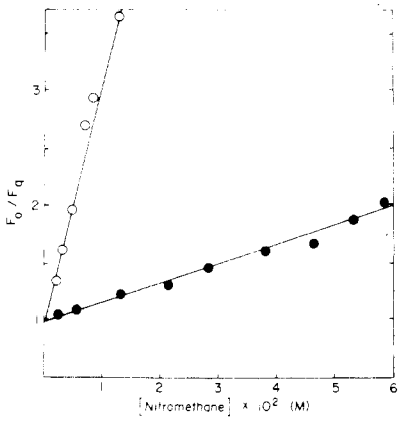


FIGURE 4: Stern-Volmer plots of fluorescence quenching as a function of nitromethane concentration. F_0 and F_q denote the fluorescence intensities in the absence and presence of quenching ligand. (O—O) Pyrenebutanol; (●—●) pyrenebutyl methylphosphonoacetylcholinesterase. All measurements were carried out at 20 °C in 0.01 M Tris-Cl containing 0.1 N NaCl and 0.04 M $MgCl_2$, pH 8.

shows a linear trend and suggests that the spectral shifts originate from the electronic polarizability of the solvent which is responsible for the refractive index (Smyth, 1955). None of the solvents, though, afforded an environment which gave a shift of the magnitude seen for the PBMP-AChE conjugate.

Upon denaturation of PBMP-AChE with 8 M urea, the absorption maximum is shifted from 349 to 345 nm. This hypsochromic shift, which should be compared with the value of 343 nm found for the free ligand in 8 M urea solution, presumably reflects partial unfolding of the protein concomitant with exposure of the ligand to a more aqueous environment.

We assume the molar extinction coefficient for the enzyme-bound pyrene moiety to be 3.9×10^4 M^{-1} cm^{-1}. This appears reasonable since in a variety of organic solvents the pyrene chromophore exhibits extinction coefficients which range from 3 to 4×10^4 M^{-1} cm^{-1}. Furthermore, the extinction coefficient of 3.9×10^4 and an estimate of enzyme concentration from its absorption spectrum results in a pyrene to enzyme stoichiometry between 0.85 and 1.1 for the preparations used for the spectrophotometric studies. Labeling and reactivation studies discussed in previous sections suggest only a single site of phosphonylation per 80 000 molecular weight subunit which can be completely dephosphonylated with the site directed reagent, 2-PAM (Figure 1). Finally, we have measured the stoichiometry of conjugated pyrene to enzyme by comparing the extinction coefficient obtained for the PBMP-AChE conjugate following urea denaturation with that for an admixture of known amounts of pyrenebutanol and AChE in 8 M urea. If the extinction coefficients for the pyrene conjugate and free PBOH in 8 M urea are equal, stoichiometry values between 0.8 and 1.0 are estimated for the conjugates prior to denaturation. Maxima in the fluorescence emission spectra exhibited minimal changes either upon reaction of PBMPF and AChE or changes in solvent environment.

Dynamic Quenching. Quenching of pyrene fluorescence by iodide, nitromethane, and thallous ion was determined for the covalent PBMP-AChE conjugate, noncovalent complexes of pyrenebutyric acid and BSA (PBA-BSA), and the corresponding free fluorophores, PBOH and PBA. The results for quenching of PBMP-AChE and PBOH by nitromethane are illustrated in Figure 4; the values determined for the quenching constants for nitromethane, thallous, and iodide ions are presented in Table I. All entries are the result of at least five de-

TABLE I: Dynamic Quenching of Pyrenebutyl Moiety by Different Quenching Ligands.

Quenching ligand	K_Q (M^{-1}) ± SEM[a]					
	PBMP-AChE	PBOH	PBA	PBA-BSA	$(\gamma_Q/\gamma_{nitromethane})_{AChE}$[b]	$(\gamma_Q/\gamma_{nitromethane})_{BSA}$[b]
I^-	1.6 ± 0.3	85 ± 5	73 ± 12	15 ± 3	0.3	5.5
Nitromethane	34 ± 7	756 ± 77	775 ± 35	28 ± 3	1.0	1.0
Tl^+	47 ± 16	684 ± 66	683 ± 45	32 ± 8	1.6	1.3

[a] Standard errors and mean values were calculated from at least five determinations. [b] γ is the ratio of bimolecular quenching rate constants characteristic of the bound and free fluorophores. ($\gamma_Q/\gamma_{nitromethane}$) relates the quenching efficiency for a particular quenching ligand to that of nitromethane; this is equivalent to the ratio of dynamic quenching constants, K_Q, determined for quenching ligands Q and nitromethane in the situation where the fluorophore is bound relative to those values observed where it is free (see text).

terminations. The fluorescence of the samples showed no variation with time and were unperturbed by stirring, thereby indicating the lack of oxygen quenching during the course of the fluorescence determinations.

Several observations are noteworthy. First, in all cases the quenching of the covalent PBMP-AChE is at least an order of magnitude less efficient than for the free fluorophore, therein reflecting limited accessibility of the quenching ligand to the enzyme-associated fluorophore. Second, the quenching constants determined for PBOH and PBA are virtually identical, demonstrating random diffusional kinetics and that the quenching process is independent of the nature of the alkyl chain. Third, plots of F_0/F vs. [Q] are linear for the three quenching ligands over the entire range of concentrations employed.[2] Deviations from linearity could result from formation of nonfluorescent complexes prior to excitation (static quenching). As noted by Lakowicz & Weber (1973), only a small static component (~3%) is necessary to explain large positive deviations from linearity, such effects occurring at higher concentrations where greater than 80% quenching was observed.

From the data in Table I we can calculate quenching efficiencies of the different ligands for the pyrene moiety conjugated to AChE in relation to the unconjugated, freely diffusing ligand (cf. eq 5). The ratio of quenching efficiency of iodide to that of nitromethane for the AChE conjugated pyrene moiety is 0.4 of the ratio found for the free, unconjugated pyrenebutanol. The ratio of thallous ion to nitromethane quenching is enhanced 1.6-fold for the enzyme-associated fluorophore compared with the free fluorophore. Ranking these values we obtain: Tl^+ > nitromethane > I^-. Thus Tl^+ experiences easier access than I^- to the enzyme-conjugated pyrene moiety when compared with access to free pyrenebutanol. Since the thallous and iodide ions are characterized by comparable effective radii as expressed by their equivalent limiting ionic conductances (Robinson & Stokes, 1959), steric factors are not expected to be grossly different for these charged ligands. Rather, the quenching differences likely reflect electrostatic interactions characteristic of the site of collision, where Coulombic forces enhance the probability for encounter of the pyrene moiety with Tl^+ as compared with I^-. Thus, the quenching order suggests that the pyrene fluorophore resides close to a region of negative charge, an anionic site.

The dynamic quenching for complexes of pyrenebutyric acid and BSA would be a useful reference for studying the influence of electrostatic interactions on measured quenching efficiency. Iodide is shown to be a fourfold more effective quenching ligand for PBA-BSA than is thallous ion, relative to the values

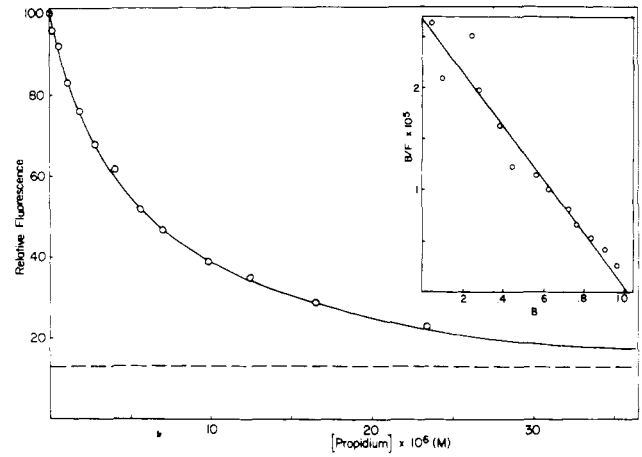


FIGURE 5: Association of propidium with pyrenebutyl methylphosphonoacetylcholinesterase. The ordinate represents relative fluorescence of the pyrene moiety measured at 400 nm following excitation at 349 nm. Incremental additions of propidium were made to a solution of PBMP-AChE (1.3×10^{-6} M in subunit concentration) in 0.01 M Tris-Cl containing 0.1 N NaCl and 0.04 M $MgCl_2$, pH 8. From a Scatchard plot of the same data (inset), we obtain a dissociation constant of 3×10^{-6} M.

obtained for the free pyrenebutyric acid (Table I). Ranking the order we obtain for the PBA-BSA complexes: $I^- \gg Tl^+$ > nitromethane. This order suggests that the PBA probe associates with the protein at cationic sites which are expected to enhance the probability for encounter with the anionic iodide ion. The high and selective binding affinity of serum albumin for organic anions is well recognized (Steinhardt & Reynolds, 1969).

Influence of Peripheral Site Ligands on Pyrene Fluorescence. Propidium diiodide is a fluorescent ligand that exhibits high affinity for native AChE and a stoichiometry of one molecule bound per subunit. Previous data indicate that propidium binds at a site that is spatially distinct from the active center (Taylor & Lappi, 1975). Since its absorption spectrum overlaps with the emission spectrum of the pyrene moiety, the propidium ligand should be capable of effectively quenching pyrene fluorescence through long-range Förster energy transfer (Förster, 1959).

Association of the propidium acceptor with PBMP-AChE is demonstrated by measuring the quenching of donor pyrene fluorescence at different propidium concentrations. In a typical titration (Figure 5) pyrene fluorescence is 87% quenched at equilibrium. That the titration profile approaches a constant value indicates that quenching is related to saturation of propidium sites whereas the free ligand has negligible effect on pyrene fluorescence. Calculation of the propidium dissociation constant from a Scatchard plot of the same data (inset, Figure 5) gives a value of 3×10^{-6} M and a stoichiometry of one ligand bound per subunit. These values are identical with those

[2] For $CuSO_4$ and PBMP-AChE a concave plot was observed in the concentration range between 0.1 and 3 mM. This presumably reflects association of the metal with AChE since divalent cations have a considerably higher affinity for the enzyme.

TABLE II: Parameters Characterizing Energy Transfer from Acetylcholinesterase to Various Acceptors.[a]

Acceptor	$J \times 10^{15}$ (cm^6/mol)	R_0 (Å)	T_{obsd}
Bis(3-aminopyridinium)-1,10-decane[b]	4.21	18.9	0.35
Propidium[c]	10.3	21.9	0.40
Benzoquinonium[b]	18.8	24.4	0.50
Pyrenebutylmethylphosphonate[c]	25.7	25.5	0.53
Propidium and pyrenebutylmethylphosphonate[d]	32.6	26.5	0.64*

[a] Excitation and emission wavelengths were 290 and 333 nm, respectively; t = 20 °C. J is the overlap integral, R_0 the critical transfer distance, and T the observed energy transfer efficiency. [b] Taylor & Jacobs (1974). [c] 0.001 M Tris-Cl, pH 8.0. [d] T_{obsd} represents the total quenching efficiency effected by both ligands simultaneously. The contribution to this process by propidium quenching in the presence of the pyrene label is 23% of the residual PBMP-AChE fluorescence. This value corresponds to 11% quenching of emission of unmodified acetylcholinesterase.

found for the native enzyme at this ionic strength and indicate that phosphonylation of the active site does not appreciably influence the binding of ligands at the peripheral site. Dissociation constants calculated for binding of propidium to diisopropyl phosphorylacetylcholinesterase are also identical with those of native AChE and demonstrate the general feature of these observations.

Gallamine is a nonfluorescent ligand that binds at the peripheral site competitively with propidium (Taylor & Lappi, 1975). Since it does not possess spectral overlap with the pyrene moiety displacement of propidium by gallamine results in enhancement of donor fluorescence (not shown). Equivalent titration profiles are obtained when the propidium fluorescence signal is monitored directly. Gallamine dissociation constants calculated from these data are nearly identical for the native and phosphonylated AChE. For other competing quaternary ligands, however, the enhancement of pyrene fluorescence is not a simple function of propidium dissociation and may reflect changes in the conformational state of the enzyme (Berman, H. A., et al., in preparation).

Tryptophan Fluorescence Quenching. AChE from *Torpedo* contains about 19 tryptophanyl residues per subunit (Taylor et al., 1974). The fluorescence emission at 333 nm upon excitation at 290 nm for PBMP-AChE is markedly reduced from that observed for native AChE due to energy transfer from tryptophan to the pyrene moiety. The efficiency of energy transfer was determined from the excitation spectrum of PBMP-AChE recorded at 400 nm, using the following relationship (cf. Haugland & Stryer, 1967).

$$T = \left(\frac{F_{290}}{F_{348}} - \frac{\epsilon_{290}{}^{a}}{\epsilon_{348}{}^{a}}\right)\frac{\epsilon_{348}{}^{a}}{\epsilon_{290}{}^{d}} \qquad (9)$$

where ϵ^a and ϵ^d are the extinction coefficients of pyrene acceptor and protein donor, respectively, and F_{290} and F_{348} are the fluorescence intensities at the respective wavelengths monitored at 400 nm. Values of the extinction coefficients were 1×10^3, 4×10^4, and 1×10^5 M^{-1} cm^{-1} for $\epsilon_{290}{}^a$, $\epsilon_{348}{}^a$, and $\epsilon_{290}{}^d$, respectively. Using these values the transfer efficiency for sensitized emission is calculated to be 70 ± 3%. From measurement of the enzyme emission at 333 nm for equimolar concentrations of AChE and PBMP-AChE, quenching of tryptophan emission is determined to occur with 53 ± 3% efficiency.

The difference in transfer efficiencies determined from sensitization of acceptor and quenching of donor is reminiscent of the situation observed for carbonic anhydrase and dansylsulfonamide (Chen & Kernohan, 1967). It was suggested that such disparity arises from nonequivalent quantum yields of the individual tryptophanyl residues which likely exist in different microscopic environments.

The association of propidium with AChE and PBMP-AChE is accompanied by quenching of the enzyme fluorescence when excited at 290 nm. Quenching of fluorescence from native AChE occurs at saturation with a transfer efficiency of 40%. For PBMP-AChE, where the fluorescence is 47% of that found for the native enzyme, the transfer efficiency to propidium is 23% of the residual fluorescence. Thus, the additional quenching of PBMP-AChE is equivalent to 11% of the emission for the native enzyme (0.47 × 0.23).

The efficiencies of energy transfer from AChE to a variety of acceptor ligands, the spectral overlap integral between the trytophanyl emission and acceptor, and the corresponding critical transfer distances are presented in Table II. Included in the table are values for bis(3-aminopyridinium)-1,10-decane and benzoquinonium (Taylor & Jacobs, 1974) which are bisquaternary ligands that bind at the active center and at a peripheral locus. A general correspondence between spectral overlap and transfer efficiency is observed.

Discussion

Reaction Kinetics for Pyrenebutyl Methylphosphonofluoridate. The information to be derived from an investigation of AChE structure through fluorescent ligands is in large measure dependent on establishing site specificity and stoichiometry for the associated ligand. PBMPF seems well suited for this approach since it is a potent and selective inhibitor. The kinetics of inactivation and subsequent reactivation with the nucleophile 2-PAM do not reveal heterogeneity in the reaction and hence indicate that the acylating group behaves as a uniform species. Moreover, spontaneous hydrolysis of the conjugate is sufficiently slow to enable one to conduct long term titrations. Finally, the correspondence between the return of activity and the loss of the phosphonyl group as well as the observation that enzyme reactivation is possible after long term exposure precludes appreciable aging of the enzyme. Aging is believed to be a consequence of release of an alkyl group upon alkyl-oxygen cleavage (cf. Aldridge, 1975). Loss of the pyrenebutyl moiety due to aging would result in altered pyrene to enzyme stoichiometry as well as reduced susceptibility of the phosphonylated enzyme to reactivation.

It should be recognized that PBMPF exists as a racemic mixture where one of the enantiomers might preferentially react with the enzyme. A stereospecific reaction between AChE and alkyl phosphates has been shown for compounds containing nitrophenol and thiocholine leaving groups, but as yet have not been shown for fluorophosphonates (Wustner & Fukuto, 1974; Ooms & Boter, 1965; Adie et al., 1956). Since a stereospecific synthesis or resolution of uncharged phosphonate isomers would prove difficult we have not attempted to isolate individual enantiomers. However, the optical rotatory properties of the PBMP-AChE conjugate should be amenable to direct study. The question of reaction stereospecificity will become of more importance in attempting to affirm the precise orientation of the pyrene chromophore.

Using a molar extinction coefficient of 3.9×10^4 M^{-1} cm^{-1} for the bound pyrene moiety, we find a stoichiometry corresponding to one phosphonylation site per 80 000 molecular weight, which is the estimated size for the subunit on the tet-

ramer (Taylor et al., 1974). Although this estimate of stoichiometry is based on an extinction coefficient which can only be indirectly ascertained, the value closely corresponds to the extinction coefficients found for solvents in which the pyrene spectrum shifts to longer wavelengths. The absence of further labeling of PBMP-AChE by DFP, the first-order kinetics of inactivation and reactivation and the correspondence between pyrene quenching and propidium binding are all indicative of unitary stoichiometry with each subunit. Similar estimates of ligand stoichiometry for *Torpedo* acetylcholinesterase have been obtained with carbamoylation of the active site serine, binding of bisquaternary inhibitors and the association of propidium, a peripheral site ligand (Taylor & Jacobs, 1974; Taylor & Lappi, 1975).

Spectral Characteristics of Bound Pyrenebutyl Methylphosphonofluoridate. The bathochromic spectral shift observed for PBMP-AChE exceeds that observed for the pyrene moiety when complexed with other enzymes or for pyrenebutanol in a variety of organic solvents (Figure 3).

That a correlation exists between the position of the O,O absorption band of the pyrene moiety and the solvent refractive index suggests that the spectral shifts originate with the electronic polarizability of the immediate environment. Such a relationship is obtained when solvent reorientation about the excited state is negligible, where the dominant contribution to the spectral shift arises instead from displacement of electrons relative to the atomic nuclei (Bayliss, 1950). This situation is expected for the case of a nonpolar solute immersed in a nonpolar medium (Coggeshall & Pozefsky, 1951; Badger & Pearce, 1951). Hence the site of association of the pyrene moiety with the enzyme is highly polarizable and likely undergoes facile deformation of its electronic milieu typical of a region containing aromatic residues and other electron rich moieties. Furthermore, the presence of water at such a site would be expected to alter the effective polarizability of the environment and hence the position of the absorption maximum. For solutions of pyrenebutyric acid in glycerol–water mixtures, shorter wavelengths were found to correlate with higher water concentrations (Vaughn & Weber, 1970).

Alkylphosphonates containing hydrophobic and particularly aromatic residues are known to be potent inhibitors of AChE (Bracha & O'Brien, 1968; Reiner & Simeon, 1975). Shinitsky et al. (1973) have observed a charge-transfer band that arises upon binding of *N*-methylacridinium to AChE. They proposed that the charge-transfer transition originates from the interaction of a tryptophanyl residue at the binding site with the acridinium moiety. Since *N*-methylacridinium binds selectively at the active center of AChE (Mooser & Sigman, 1974; Taylor & Lappi, 1975), its binding site is likely to overlap with the pyrene moiety.

The dynamic quenching studies provide strong evidence for an occluded active center where collisional contact between the conjugated pyrene moiety and the solvent is minimized. This proposal is consistent with the absorption spectroscopic behavior of the bound pyrene. Thus, the active center of AChE may well reside within a rather narrow cavity. Alternatively, a change in conformation may ensue upon binding of the tetrahedral phosphonate which serves to constrain the bound pyrene moiety.

Collisional quenching of bound pyrene, although restricted presumably by steric limitations, occurs more efficiently with positively charged ions relative to the order observed for the free ligand. A negative subsite within the active center stabilizes the quaternary ammonium portion of substrates such as acetylcholine or inhibitors like edrophonium. On the basis of acetylcholine structure, the anionic subsite in the enzyme–substrate complex is 5 Å removed from the catalytic serine. The potency of phosphates and phosphonates is in part a consequence of their tetrahedral geometry resembling the transition state intermediate in serine hydrolase catalyzed ester hydrolysis (Robertus et al., 1972). The carbonyl oxygen in the case of substrate and the phosphonyl oxygen of the inhibitor would be directed toward the oxyanion hole which serves to stabilize the tetrahedral adduct through hydrogen bonding with amide backbone hydrogens in the peptide chain (cf. Robertus et al., 1972; Taylor & Jacobs, 1974). Moreover, steric constraints within the active site in AChE favor an orientation of the methyl group rather than the pyrenebutyl group directed toward the acyl pocket. Thus, the most probable configuration around the phosphorus in PBMP-AChE is the one for which the pyrenebutyl arm is oriented toward the choline binding locus. Accordingly, microscopic charge densities in this anionic subsite are expected to play a dominant role in enhancing cation quenching of pyrene fluorescence while at the same time acting to diminish contact with anionic ligands.

Energy Transfer. Although the ligands reported in Table II experience microscopically different environments, their quenching efficiencies correlate well with the magnitude of their spectral overlap, suggestive of a relatively uniform distribution of tryptophanyl residues (cf. Badley & Teale, 1969). The presence of 19 tryptophanyl residues per subunit and uncertainties in the donor distribution preclude a precise determination of the spatial relationship between the peripheral and active center sites based solely on studies of tryptophanyl fluorescence.

Nevertheless, relative to the energy transfer efficiency obtained with each acceptor when *individually* bound to the macromolecule, the transfer efficiency from a donor array to two acceptors *simultaneously* bound depends on the distance separating the acceptors. It is instructive therefore to compare quenching of tryptophanyl fluorescence effected by propidium in the native and phosphonylated enzymes, for which two limiting cases can be distinguished.

In the first case the two acceptors (propidium and pyrene moiety) may be considered to communicate with separate and independent classes of tryptophanyl residues; hence, propidium quenching of individual tryptophanyl donors is expected to occur with equivalent efficiencies in native AChE and PBMP-AChE. The other limiting case arises when the two acceptors are in close proximity so that they interact only with identical donor residues and hence compete for the fluorescence emission of the individual donors. The consequence of their proximity is that absolute transfer to the acceptor with the lesser spectral overlap (propidium) is diminished in the presence of the acceptor with the greater spectral overlap (pyrene moiety).

When bound to AChE propidium quenches 40% of the native tryptophanyl fluorescence, while it quenches only 23% of the residual fluorescence in PBMP-AChE. This latter value is equivalent to 11% of the total tryptophanyl fluorescence in unmodified AChE. Since a smaller quantity of tryptophan fluorescence is accessible for transfer to propidium when pyrene is conjugated, the respective sites are not isolated, with respect to energy transfer considerations, and the quenching domains of propidium and pyrene moiety must necessarily overlap. This situation is anticipated for those donor–acceptor pairs for which the critical transfer distance, R_0 (cf. Table II), approaches the radius of the subunit ($r = 38$ Å for AChE subunit; Taylor et al., 1974). With regard to the proximal case, the *maximal* transfer efficiency from any tryptophanyl residue to propidium in the presence of the pyrene label is calculated to be 15% of its original AChE fluorescence; such a value would

occur only for donor–acceptor distances that range from 22 to 25 Å. That 11% of the original AChE fluorescence in PBMP-AChE is quenched upon propidium binding would imply that the additional transfer to propidium arises from all tryptophan donors being situated between 20 and 28 Å from the acceptor, a condition which is rather improbable for a subunit of approximate radius of 38 Å. Hence, directly proximal sites are unlikely and the active center and propidium sites represent discrete regions on the enzyme surface.

The fluorescence properties of the conjugated pyrene moiety and propidium enable one to examine energy transfer by both donor quenching and sensitization of acceptor emission. An examination of energy transfer for a series of fluorescent phosphonate donors in relation to acetylcholinesterase topography will be the subject of a forthcoming study (Berman, H. A., et al., manuscript in preparation).

Since the pyrene fluorophore is associated in a highly polarizable region where access to solvent is limited, it seems probable that the pyrene moiety exhibits little segmental motion independent of the enzyme. The AChE tetramer is 330 000 molecular weight in *Torpedo*, but the native form of the enzyme exists as three tetrameric units connected to a 500-Å filament (Cartaud et al., 1975). The latter appears to be of a collagen composition (Lwebuga-Mukasa et al., 1976; Rosenberry & Richardson, 1977) which suggests a helical arrangement for the tail unit. The high specificity of the pyrene ligand coupled with a lifetime approaching 200 ns should make it amenable to examining motion of the various isolated enzyme forms and perhaps the enzyme associated with the basement membrane fraction (Yguerabide et al., 1970).

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Biochemical and Immunochemical Characterization of Hexosaminidase P†

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ABSTRACT: Hexosaminidase P, the main isozyme of hexosaminidase in pregnancy serum, was isolated and purified 600-700-fold by a two-step purification procedure—affinity chromatography on Sepharose-bound ϵ-aminocaproyl-*N*-acetylglucosylamine, followed by ion-exchange chromatography on DEAE-cellulose. The purified enzyme was subjected to biochemical and immunochemical analysis. Its catalytic property, namely, kinetic behavior, is similar to that of the major isozymes of hexosaminidase, A and B. However, it differs from these isozymes in its electrophoretic mobility and in its apparent molecular weight which is around 150 000 compared with 100 000 of the A and B isozymes. Immunochemical analysis indicates that the P isozyme is antigenically cross-reactive with both A and B isozymes, but it does not contain the A-specific antigenic determinants, and exhibits identical antigenic specificity to hexosaminidase B. Two possible structures are suggested that are compatible with the experimental data: (a) a hexosaminidase B like structure with higher extent of glycosylation; (b) a hexamer of β chain, possibly arranged as three β_2 subunits.

The enzyme *N*-acetyl-β-D-hexosaminidase (hexosaminidase, EC 3.2.1.30) is present in human tissues as two main isoenzymes designated hexosaminidase A and B (Robinson & Stirling, 1968) which are localized in the lysosomal fraction of the cells. However, analysis of the enzyme obtained from different tissues, cells, or body fluids indicated the existence of some minor hexosaminidase isozymic forms such as hexosaminidase C (Poenaru et al., 1973; Braidman et al., 1974a,b; Penton et al., 1975; Reuser & Goljaard, 1976), I_1 and I_2 (Price & Dance, 1972), etc. Another isoenzyme was detected in sera of pregnant women (Stirling, 1971, 1972) and was denoted hexosaminidase P.

The increase in levels of hexosaminidase activity in serum during pregnancy was first observed by Walker et al. (1960). In later reports O'Brien et al. (1970) and Huddlestone et al. (1971) suggested that this elevation in activity is due to an increased hexosaminidase B level. However, later studies have demonstrated that the increase in enzymatic activity is due to the appearance of a novel enzyme in the serum, namely, hexosaminidase P.

During the last few years remarkable progress has been made in the elucidation of the molecular structure of the two major human isozymes, hexosaminidases A and B. Based on experimental evidence concerning various aspects of the enzyme as derived from biochemical studies, somatic cell hybrids (Lalley et al., 1974; Gilbert et al., 1974; Thomas et al., 1974; Gilbert et al., 1975), conversion experiments (Carmody & Rattazzi, 1974; Beutler et al., 1975) and direct chemical analysis, a molecular model was proposed for hexosaminidases A and B (Geiger & Arnon, 1976; Lee & Yoshida, 1976; Beutler et al., 1976). According to this model, both hexosaminidases are built of two subunits, each subunit composed of two S-S-linked identical polypeptide chains. However, whereas hexosaminidase B is composed of four identical chains ($\beta_2\beta_2$), the A isozyme has one β_2 and one α_2 subunit ($\alpha_2\beta_2$) (Geiger & Arnon, 1976).

In the present study we have investigated some of the biochemical and immunochemical properties of hexosaminidase P, in an attempt to describe its structure in molecular terms.

Materials and Methods

Hexosaminidase A and B were purified from human placentae to an apparent homogeneity by the procedure described previously (Geiger et al., 1975; Geiger & Arnon, 1976). The purity of the preparations was established by analytical ultracentrifugation, electrophoresis on polyacrylamide gel in the presence of $NaDodSO_4$,[1] as well as by gel electrophoresis and isoelectric focusing under nondenaturing conditions.

Sera of Pregnant Women. Serum samples were collected from women in the third trimester of pregnancy and 48 h after delivery. Sera used for estimation of enzymatic activity were not pooled and were stored at −20 °C until tested. Sera used for purification of hexosaminidase P were collected, pooled, and stored until used at −20 °C.

Hexosaminidase Assay. Enzyme solution (100 μL, diluted in 0.04 M citrate buffer, pH 4.4) was incubated for 10 min at 37 °C with substrate (200 μL) containing 0.1 mg/mL 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Pierce) and 1 mg/mL bovine serum albumin (Grade A, Calbiochem). The

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; IEF, isolectric focusing.

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