$$I^+ + OH^- \longrightarrow IOH \quad (15)$$

$$IOH + I^- \longrightarrow I_2 + OH^- \quad (16)$$

If iodide is not in excess the IOH or its degradation products could account for the slow reaction to form the ferriperoxidase–iodine complex.

The oxidation of oxalate observed by Björkstén could be explained if the iodide oxidation proceeds *via* the formation of an I^+–iron complex which is a sufficiently powerful oxidizing agent to react with oxalate. Such a complex could provide a rationale for the involvement of peroxidases in the catalysis of iodination reactions.

Acknowledgments

The authors are indebted to Dr. J. Critchlow for his helpful discussion and to Dr. M. Evett for assistance with computer programing.

References

Brill, A. S. (1966), *Comp. Biochem. 14*, 447.
Brill, A. S., and Sandberg, H. E. (1968), *Biochemistry 7*, 4254.
Björkstén, F. (1968), *Eur. J. Biochem. 5*, 133.
Björkstén, F. (1970a), *Biochim. Biophys. Acta 212*, 396.
Björkstén, F. (1970b), *Biochim. Biophys. Acta 212*, 407.
Chance, B., De Vault, D., Legallais, U., Mela, L., and Yonetani, T. (1967), *in* Nobel Symposium V, Fast Reactions and Primary Processes in Chemical Kinetics, Stockholm, New York, N. Y., Interscience, p 437.
Cotton, F. A., and Wilkinson, G. (1966), Advanced Inorganic Chemistry, 2nd ed, New York, N. Y., John Wiley and Sons, Inc., p 570.
Critchlow, J. E., and Dunford, H. B. (1972a), *J. Biol. Chem.* (in press).
Critchlow, J. E., and Dunford, H. B. (1972b), *J. Biol. Chem.* (in press).
Critchlow, J. E., ank Dunford, H. B. (1972c), *J. Theor. Biol.* (in press).
Hasinoff, B. B., and Dunford, H. B. (1970), *Biochemistry 9*, 4930.
Hosoya, T. (1968), *Gunma Symp. Endocrinol. 5*, 219.
Keilin, D., and Hartree, E. F. (1951), *Biochem. J. 49*, 88.
Morrison, M. (1968), *Gunma Symp. Endocrinol. 5*, 239.
Nunez, J., and Pommier, J. (1968), *Eur. J. Biochem. 5*, 114.
Paul, K. G., and Stigbrand, T. (1970), *Acta Chem. Scand. 24*, 3607.
Ramette, R. W., and Sandford, R. W. (1965), *J. Amer. Chem. Soc. 87*, 5001.
Roman, R. (1972), Ph.D. Thesis, University of Alberta, Edmonton, Canada.
Roman, R., Dunford, H. B., and Evett, M. (1971), *Can. J. Chem. 49*, 3059.
Shannon, L. M., Kay, E., and Lew, J. Y. (1966), *J. Biol. Chem. 241*, 2166.
Taurog, A. (1970), *Arch. Biochem. Biophys. 139*, 212.
Thomas, J. A., Morris, D. R., and Hager, L. P. (1970), *J. Biol. Chem. 245*, 3129.

Dynamics of Fluoroescent Probe–Cholinesterase Reactions†

Richard T. Mayer and Chester M. Himel*

ABSTRACT: 1-(5-Dimethylaminonaphthalene-1-sulfonamido)-3-*N*,*N*-dimethylaminopropane and 1-(5-dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide are active-site-directed, equilibrium fluorescent probes. They are competitive inhibitors of horse serum cholinesterase (3.1.1.8). The basic group in each probe molecule binds at the anionic site. Subsequent binding of the fluorescent moiety is directed to an adjacent, hydrophobic site. Equilibrium dynamics of interaction of the probe–enzyme complexes were investigated with ammonium salts, organic solvents, guanidine, sodium chloride, and chlorinated hydrocarbon-type insecticides. Ammonium salts compete with the probes for the anionic site. Chlorinated hydrocarbon-type insecticides compete for the hydrophobic site. A series of organic solvents had significant effects on probe–enzyme binding. Active-site-directed, equilibrium fluorescent probes allow study of active-site dynamics.

Fluorescent probes of enzyme systems have spectral responses which reflect the environment of the probe. They can be used to monitor changes in conformation in enzymes and can be designed to be active-site-directed, equilibrium, competitive inhibitors (Himel *et al.*, 1971). The spectroscopy of intrinsic and extrinsic fluorescent probes has been reported [*cf.* Steiner and Edelhoch (1963), Chen (1967a), Chen *et al.* (1969), Edelman and McClure (1968), Stryer (1968)].

As early as 1952, Weber used 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) to introduce a covalently bound fluorescent moiety into proteins. 8-Anilino-

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napthalene-1-sulfonic acid (ANS),[1] an equilibrium fluorescent probe, binds to hydrophobic sites on proteins (Weber and Laurence 1954). Both are nonspecific probes. Their protein binding sites are not well defined.

Baker and coworkers (Baker, 1967) and Shaw and coworkers (Shaw, 1970) have made extensive studies involving the concept of active-site-directed inhibition of enzymes. When applied to a fluorescent probe it can define the area of binding of the probe relative to the active site of the enzyme. Active-site-directed fluorescent probes can be synthesized as reversible or irreversible inhibitors (Himel *et al.*, 1971). Their design can incorporate an energy-transfer bridge, or the fluorescent moiety can be isolated from a stereochemically designed "head." Spectral data then reflect mainly the nature of the site to which the fluorescent moiety is bound.

Haugland and Stryer (1967) reported covalent bonding of a fluorescent anthranylate moiety at the active site of α-chymotrypsin. The fluorescence spectra indicated a highly polar active site. Chen and Kernohan (1967) used dansylamide as an equilibrium inhibitor of the active site of carbonic anhydrase. Spectral data indicated a nonpolar active site. Brand *et al.* (1967) showed the binding of the equilibrium fluorescent probe, tetraiodofluorescein (Rose Bengal), with liver alcohol dehydrogenase. ANS was used by Stryer (1965) to determine the polarity of apomyoglobin and apohemoglobin.

Deranleau and Neurath (1966) prepared danysl-D-tryptophan ethyl ester as a competitive inhibitor of α-chymotrypsin. The ethyl ester moiety bound at, and inhibited the active site. Their spectral data corresponded to binding of the dansyl moiety at a nonpolar site.

This research is concerned with the fluorescence spectra of two, active-site-directed, equilibrium fluorescent probes which are competitive inhibitors of horse serum cholinesterase (3.1.1.8) (ChE). The use of these equilibrium probes allowed study of spectral effects which accompany active-site interactions and the effects of environmental stresses on the enzyme system.

Experimental Section

Instrumentation. Emission, excitation, and absorption spectra were obtained with a G. K. Turner "Spectro 210." This instrument (Turner, 1968) presents corrected emission and excitation spectra. Samples for fluorescence measurements were temperature controlled to 25 ± 0.1°. pH measurements were made at room temperature with a Radiometer Model 26 pH meter standardized with NBS standard buffer solutions. Lifetime of fluorescence measurements were made using a TRW nanosecond flash unit and decay computer coupled to a dual gun ocilliscope. Appropriate filters (Corning) were used to isolate excitation and emission wavelengths (*cf.* Chen *et al.*, 1967a,b).

Chemicals and Solvents. Pure grade 5-dimethylaminonaphthalene-1-sulfonic acid (DnsOH) was from Pierce Chemical Co. It was homogeneous on Eastman tlc film, using benzene–methanol (1:1), and was comparable to that of a highly purified standard. Synthesis of TA and QA followed the procedures of Himel *et al.* (1970).

Anal. TA calcd for $C_{17}H_{25}N_3O_2S$: C, 60.8; H, 7.46; N, 12.50. Found: C, 60.43; H, 7.23; N, 12.27; QA calcd for $C_{18}H_{28}N_3O_2SI$: C, 45.28; H, 5.87; N, 8.80. Found: C, 45.16; H, 5.86; N, 8.73. (Analyses performed by Midwest Microlabs, Indianapolis, Ind.)

Et_4PP, *p,p'*-dichlorophenyl-2,2,2-trichloroethane, *p,p'*-dichlorophenyl-2,2-dichloroethylene, Heptachlor, dieldrin, and Thiodan were of analytical quality and gifts of the Southeastern Water Laboratory, Athens, Ga. Maretin was a gift of the Chemagro Chemical Co. Solvents and reagents were reagent grade or fluorescent quality. All water was double distilled from glass. Buffers were either Tris-HCl (0.05 M) or citrate–disodium hydrogen phosphate (0.1 M). Any pH below or above the capacity of these buffers was obtained with 0.1 N H_2SO_4 or 0.1 N NaOH.

Lyophilized horse serum cholinesterase (ChE) (type IV), bovine erythrocyte acetylcholinesterase (AChE) (type III), electric eel AChE (type III), and three-times recrystallized lysozyme (lot no. 2913-8010) were purchased from Sigma. Four-times recrystallized bovine serum albumin (lot 4710) was purchased from Nutritional Biochemicals. The amount of lysozyme used was determined from $\epsilon_{cm}^{1\%}$ 2.63 and taking the molecular weight to be 14,100 (Sophianopoulos *et al.*, 1962). Bovine serum albumin concentrations were determined in a similar manner using a value of 6.6 for $\epsilon_{cm}^{1\%}$ and a molecular weight of 69,000 (Foster and Sterman, 1956). Amounts of cholinesterase are given as milligrams of enzyme preparation per milliliter. The bovine erythrocyte AChE hydrolyzed 2.9 μmoles of ACh per min per mg. The electric eel AChE hydrolyzed 540 μmoles of ACh per min per mg of preparation. Horse serum ChE hydrolyzed 6.0 μmoles of ACh per min per mg of preparation.

Methods. Fluorometric titrations were performed manually with Hamilton microsyringes. Total volumes of all samples for fluorometric measurements were 2.0 ml. Irradiation of samples containing protein was limited to 5 min or less to avoid photooxidation of the tryptophan and histidine residues in the protein. When mixing was necessary, cells with fitted Teflon stoppers were used. Dissociation constants (K_d) were determined graphically after the methods of Chen and Kernohan (1967), Chen (1967b), and Jun *et al.* (1971).

Cholinesterase activities were determined titrametrically through a modified method of Nabb and Whitfield (1967) using 0.005 N NaOH as the titrant. The titration was at pH 7.3. ChE solutions were made up in double-distilled water without salt additives.

The comparative methods of Parker and Rees (1960) were followed in quantum efficiency calculations. The equation used in calculating quantum efficiency has been given by Turner (1968) and by Fletcher (1967). Quantum efficiencies of probe–enzyme complexes were determined in aqueous systems. DnsOH was used as the quantum yield reference standard with the quantum yield taken as 0.36 in 0.1 M $NaHCO_3$ (Chen, 1966; Himel and Mayer, 1970). All solutions which did not contain protein were purged with special purity nitrogen to retard oxygen quenching. Fluorescence quality sample cells (Hellma) 1.0 cm in path length with Teflon stoppers were used. Where absorption was a problem, 0.3-cm microcells with special cell holders were used (AMINCO). Peak areas were integrated with a Hewlett Packard 3370A electronic digital integrator interfaced with the spectrofluorometer.

[1] The following abbreviations are used: ChE, cholinesterase (3.1.1.8), horse serum acylcholinesterase; AChE, acetylcholinesterase (3.1.1.7) from bovine erythrocytes or eel; ANS, 8-anilinonaphthalene-1-sulfonic acid; DnsOH, 5-dimethylaminonaphthalene-1-sulfonic acid; TA, 1-(5-dimethylaminonaphthalene-1-sulfonamido)-3-*N,N*-dimethylaminopropane; Et_4PP, tetraethylpyrophosphate; QA, 1-(5-dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide; TMA, tetramethylammonium chloride; DMA, dimethylammonium hydrochloride.

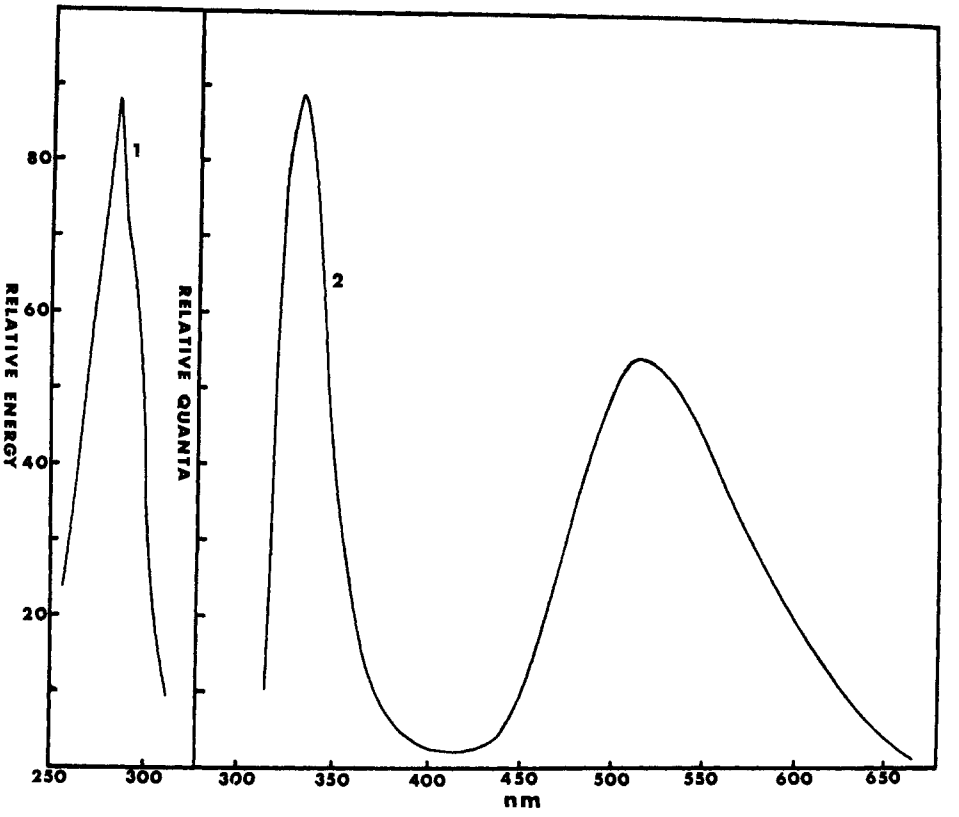

FIGURE 1: Corrected fluorescence spectra of the protonated form of DnsOH (3×10^{-6} M) in 0.1 M citrate–Na_2HPO_4 buffer (pH 2.41). Curve No. 1, excitation spectrum, emission at 335 nm. Curve No. 2, emission spectrum, excitation at 285 nm. (Excitation bandwidth at 2.5 nm and emission bandwidth at 10.0 nm.)

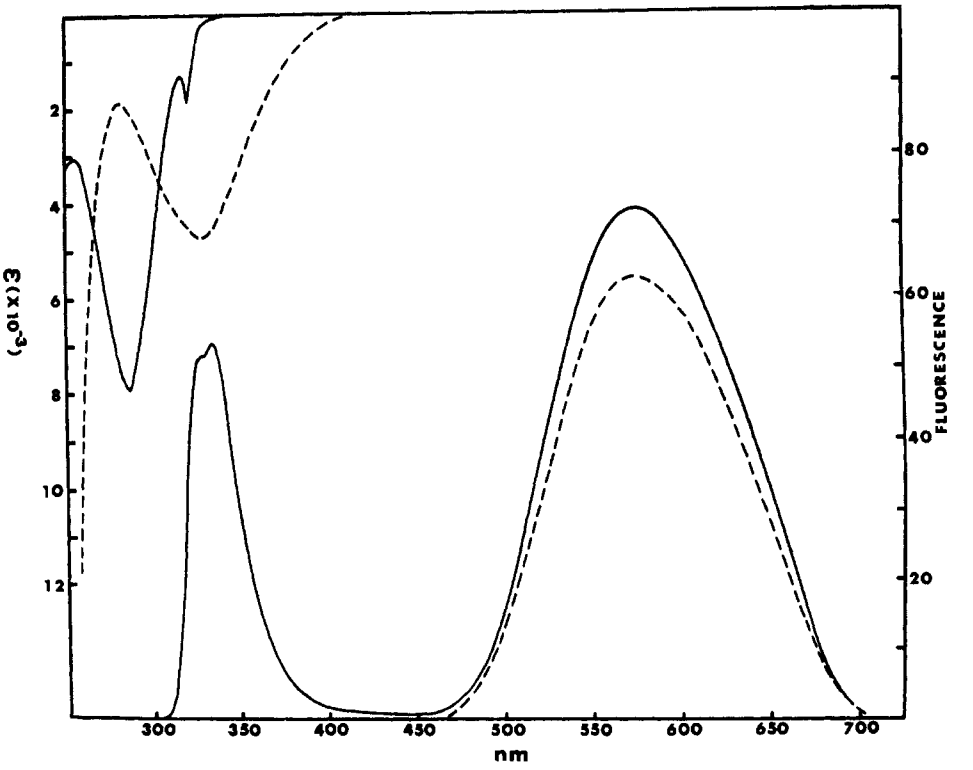

FIGURE 2: pH effects on absorption and emission spectra of aqueous solutions of TA. pH 2.2, 0.1 M citrate (excitation 287.5 nm); pH 9.1, 0.1 M Na_2HPO_4 (excitation 330.0 nm). Absorption and emission measurements were made on 6.6×10^{-5} M and 6.6×10^{-6} M solutions of TA, respectively. (Excitation and emission bandwidths set at 10.0 nm.)

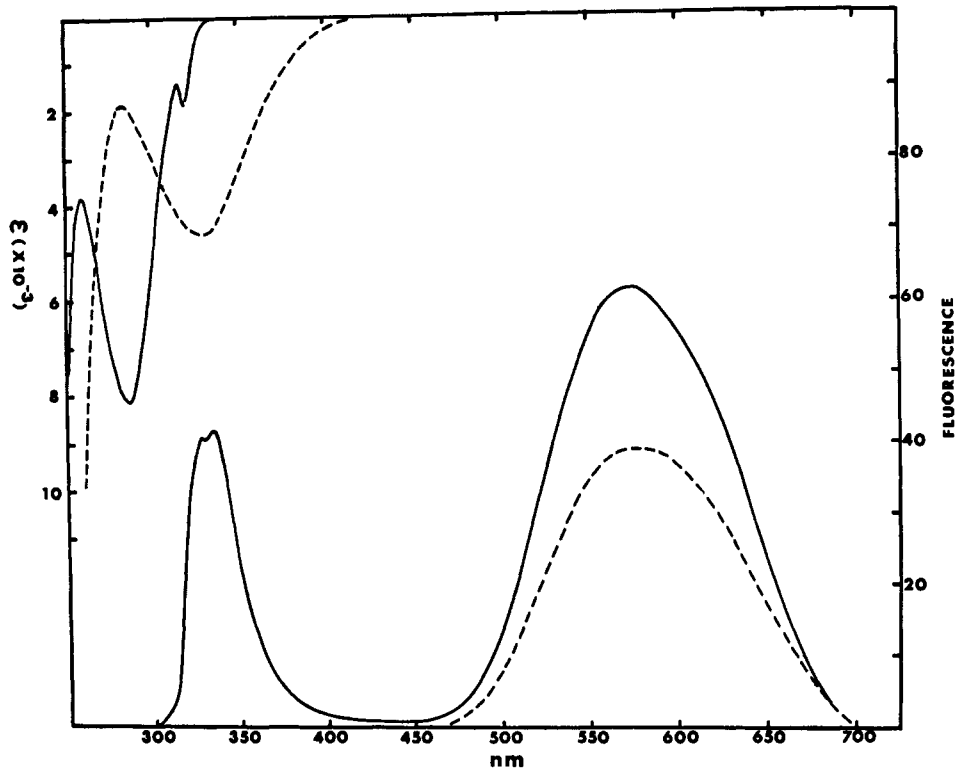

FIGURE 3: pH effects of absorption and emission spectra of aqueous solutions of QA. pH 2.2, 0.1 M citrate (excitation 286.0 nm); pH 9.1, 0.1 M Na_2HPO_4 (excitation 330.0 nm). Absorption and emission measurements were made on 6.6×10^{-5} M and 6.6×10^{-6} M solutions of QA, respectively. (Excitation and emission bandwidths set at 10.0 nm).

Ground state acidity constants (pK_a) were measured *via* absorption techniques and calculated by the method of Klotz and Fiess (1960). The pK_a's reported herein are the averages of the pK_a's determined at several pH values within the transition range.

Equations used for calculating excited state acidity constants $(pK^*)_a$ are those of Weller (1961) given below. $pK_a^* = pK_a - (ch/2.303kT)(\Delta\bar{\nu}) = pK_a - (0.625/T)(\Delta\bar{\nu})$, where c = speed of light, h = Planck's constant, k = Boltzmann constant, T = temperature (°K), and $\Delta\bar{\nu}$ = arithmetic mean of the spectral shifts in the long wavelength absorption and emission of the protonated and deprotonated forms in reciprocal centimeters.

Results

Effects of pH on Absorption, Emission, and Excitation Spectra of Dansyl, TA, and QA. The 1–5 positional relationship of the *N*-dimethylamino (NDA) group and the sulfonic acid moiety makes the spectra of DnsOH unique in the aminonaphthalene series (Himel and Mayer 1970). The emission maximum of the deprotonated excited state was 515 nm in the corrected emission spectrum of DnsOH. Our other spectral data on this compound agree with that of Lagunoff and Ottolenghi (1966) except for the value of the excited state pK_a^*. Protonation of the NDA group has been confirmed by nuclear magnetic resonance (nmr) studies (Whidby *et al.*, 1971). The emission maximum of the corrected emission spectrum of the protonated form was 335 nm. Figure 1 presents the corrected excitation and emission spectra of DnsOH in 0.1 M citrate buffer (pH 2.41). Below a pH of 6, the emission bands of the deprotonated and protonated states are present, a result of protolysis equilibria in the excited state (Weller, 1961).

Figures 2 and 3 show the effect of pH on the spectra of TA and QA. As with DnsOH the short wavelength emission bands are assigned to the protonated forms of the aromatic NDA groups in these molecules. Both of these sulfonamides emit at 330 nm in the protonated form and at 580 nm in the deprotonated forms. The absorption spectra of TA and QA shifts to the blue in going from a deprotonated state to a protonated state.

Variation of the Quantum Yield of DnsOH with pH. The quantum yield of DnsOH was determined over the pH range 0–12.5 as illustrated in Figure 4. It is constant in the ranges 2.0–4.0 and 6.0–10.0 but decreased markedly below pH 2.0 and above 10.0. The effects of pH on the quantum yield of DnsOH were found to be reversible. Change in excitation wavelength from 320 to 285 nm does not effect the quantum yield. The quantum yield is 0.36 in 0.1 N $NaHCO_3$. DnsOH

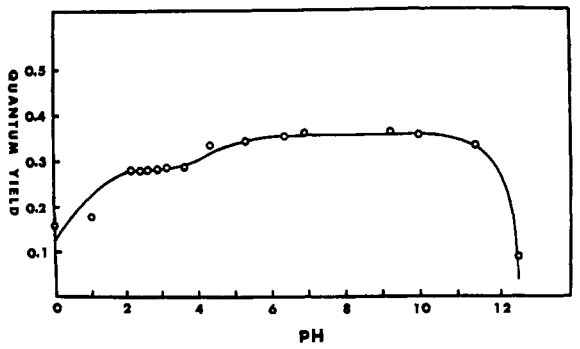

FIGURE 4: Effect of pH on the quantum yield of 3×10^{-6} M solutions of DnsOH. Excitation was at 285 and 320 nm; excitation and emission bandwidths were set at 10.0 nm.

TABLE I: Spectral Characteristics of TA and QA.

Compound	Solvent	Quantum Yield	Excitation (nm)[a]	Emission Max (nm)
TA	Tris-HCl (0.05 M, pH 7.3)	0.05	335	580.0
QA	Tris-HCl (0.05 M, pH 7.3)	0.04	335	580.0
TA	Methanol	0.42	340	530.0
QA	Methanol	0.47	340	535.0
TA	Ethanol	0.69	340	520.0
QA	Ethanol	0.67	340	525.0
TA	Isopropyl alcohol	0.70	340	515.0
TA	Butyl alcohol	0.71	340	515.0
QA	Butyl alcohol	0.66	340	525.0
TA	Cyclohexane	0.71	340	445.0

[a] The excitation wavelengths used are not necessarily the excitation maxima of these compounds in that particular solvent.

TABLE II: Reaction of TA and QA with Several Protein Solutions.[a]

Protein	Concentration	TA Fluorescence Response	QA Fluorescence Response
Horse serum ChE	1 mg of prep/ml	+	+
Bovine erythrocyte AChE	2 mg of prep/ml	−	−
Electric eel AChE	2 mg of prep/ml	−	−
Bovine serum albumin	1×10^{-4} M	−	−
Lysozyme	3.5×10^{-4} M	−	−

[a] Solutions were excited at 340 nm. Tris-HCl buffer (0.05 M, pH 7.3) was used to make up all protein solutions. Probe–protein solutions were also checked for energy transfer from protein tryptophan to the probe by setting the emission wavelength at both 580 and 515 nm and scanning the excitation for the presence of a 280-nm peak. Only the horse serum ChE solutions gave any indication of energy transfer.

is an excellent quantum yield standard (Himel and Mayer, 1970).

Ground and Excited State Acidity Constants. The pK_a and pK_a^* values for DnsOH and several sulfonamides have been reported previously (Klotz and Fiess, 1960; Lagunoff and Ottolenghi, 1966; Whidby *et al.*, 1971). pK_a's were determined by absorption techniques (Klotz and Fiess, 1960) and were found to be 4.45 ± 0.01 for DnsOH, 3.54 ± 0.01 for TA, and 3.60 ± 0.01 for QA. pK_a^*'s (Weller, 1961) were calculated utilizing wavelength shifts in abosprtion and emission spectra. These data gave pK_a^*'s of −9.62 for DnsOH −13.60 for TA, and −13.39 for QA. The pK_a's of TA and QA are approximately 1 pH unit lower than that of DnsOH, but are comparable to the pK_a's of similar dansyl derivatives (Klotz and Fiess, 1960; Lagunoff and Ottolenghi, 1966; Whidby *et al.*, 1971).

Solvent Effects. In aqueous solution TA and QA have low qunatum yields (0.05 and 0.04, respectively) while DnsOH has a relatively high quantum yield (0.36). The quantum yields of TA and QA increase in solvents of low dielectric constant. Table I gives the quantum yields and the emission maxima of TA and QA in several solvents. Both TA and QA exhibited hypsochromic shifts in emission in going from solvents of high polarity to solvents of low polarity; these shifts were accompanied by sharper, narrower, emission peaks. In contrast to emission maxima, absorption maxima demonstrated bathochromic shifts in going from solvents of high polarity to solvents of low polarity.

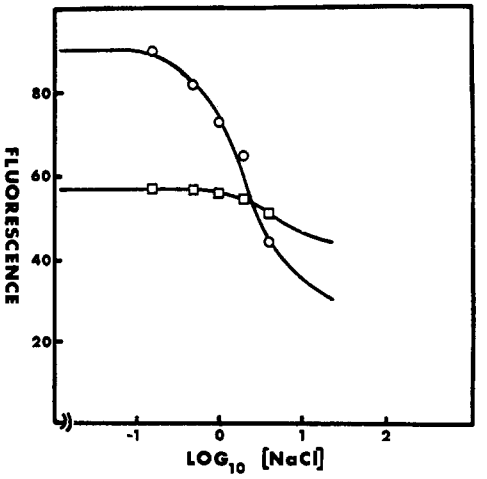

FIGURE 5: Fluorescence quenching of TA and QA by NaCl in 0.05 M Tris buffer solutions (pH 7.3). ○ = TA ($a \times 10^{-5}$ M) △ = QA (2×10^{-5} M). The theoretical half-titrations are taken as being 2.2 M NaCl for Ta and 2.5 M NaCl for QA. (Excitation at 335.0 nm; emission at 580.0 nm; emission and excitation bandwidths set at 10.0 nm).

Electrolyte Effects. Electrolytes decrease the quantum yield of TA and QA. Sodium chloride quenching is illustrated in Figure 5. The theoretical half-titration values of TA and QA with NaCl were given as 2.2 and 2.5 M. The quenching observed upon addition of NaCl to solutions of these compounds follows the Stern–Volmer law (Stern and Volmer, 1919). Quenching constants calculated for NaCl with solutions of QA and TA were 2.8×10^{-2} M^{-1} and 2.2×10^{-1} M^{-1}, respectively. Quenching of DnsOH by NaCl has been reported (Lagunoff and Ottolenghi, 1966). Substitution of their data in the Stern–Volmer equation gave a constant of 7.0×10^{-2} M^{-1} for dansyl.

Lifetime of Fluorescence. The lifetime of fluorescence of 10^{-4} M solutions of TA and QA in 0.05 M Tris buffer solution was 3.6×10^{-9} sec and 3.0×10^{-9} sec, respectively. The lifetime of fluorescence of DnsOH has been reported by Chen *et al.* (1967).

Reactions with Proteins. The fluorescent probes TA and QA showed no fluorescence emission shifts when added in solution to four proteins, including bovine serum albumin. Solutions of the proteins were made up in Tris-HCl buffer and varying amounts of 10^{-6} or 10^{-5} M solutions of the probes added. Criteria used to determine the absence of binding included study of emission spectral shifts and measurement of peak areas. In the peak area measurements, solutions of probe and protein were compared with suitable solution blanks. Data are given in Table II.

Quantum Yields for the Probe–Enzyme Complexes. The quantum yields for the complex of TA–ChE and QA–ChE were 0.45 and 0.43, respectively. The emission maxima of

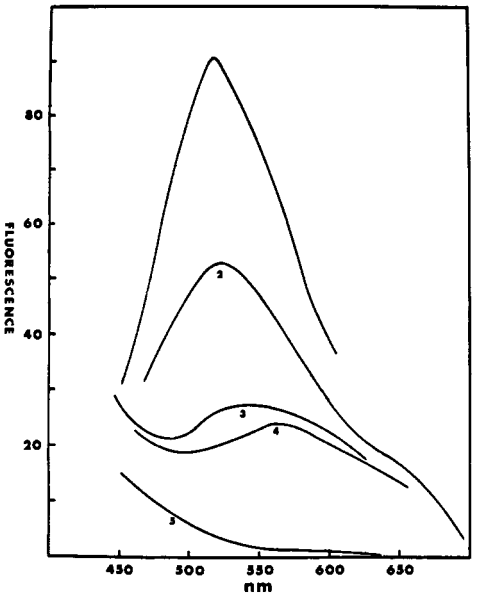

FIGURE 6: Titration of TMA against the TA–ChE complex. Curve 1, TMA = 0.0 M; curve 2, TMA = 0.091 M; curve 3, TMA = 0.457 M; curve 4, TMA = 0.914 M; curve 5 represents a 1-mg/ml solution of ChE preparation without probe. (Excitation was at 340.0 nm; excitation and emission bandwidths were respectively set at 2.5 and 10.0 nm.) All observations of probe–ChE complex fluorescence in this paper were made on solutions containing 1 mg of ChE preparation/ml and 10^{-6} M probe in 0.05 M Tris-HCl buffer (pH 7.3).

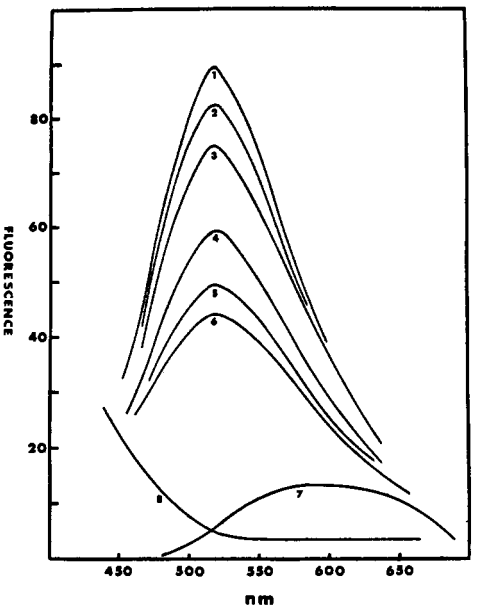

FIGURE 7: Effects of NaCl on the fluorescence of the TA–ChE complex. Curve 1, NaCl = 0.0 M; curve 2, NaCl = 0.046 M; curve 3, NaCl = 0.139 M; curve 4, NaCl = 0.188 M; curve 5, NaCl = 0.565 M; curve 6 represents NaCl concentrations of 1.41 M and 2.26 M; curve 7 is a 10^{-6} M solution of TA in Tris buffer without ChE; curve 8 is a solution of 1 mg/ml of ChE preparation in Tris buffer. (See Figure 6 for instrumental settings.)

the complexes (515 and 525 nm, respectively) indicate a nonpolar binding site for the probes since similar maxima were observed for these compounds in solvents of low polarity (*cf.* Table I).

Competition of Salts with TA and QA for Enzyme Binding Sites. The active sites of AChE and ChE contain an anionic site, an esteratic site, and hydrophobic areas (Augustinsson, 1966; Bergmann, 1955, 1958; Bergmann and Wurzel, 1953; Bracha and O'Brien, 1968, 1970). Himel *et al.* (1970) have shown that TA and QA are reversible competitive inhibitors of horse serum cholinesterase. The anionic site inhibitors, tetramethylammonium chloride (TMA) or dimethylammonium hydrochloride (DMA) (Wilson, 1952), were added to solutions of probe and enzyme. A reversible competition was observed. The data in Figure 6 show that when the anionic site reactant TMA is added to the TA–enzyme complex, the 515-nm emission of the complex is decreased progressively. The fluorescence maximum shifts to 580 nm, the range of free TA in aqueous solution. Increasing the concentration of probe in solution decreased the amount of the λ_{em} shift of the enzyme–probe complex. The converse was also true.

Experiments identical in nature with the titrations of TMA and DMA were carried out with NaCl. As indicated in Figure 7, NaCl does decrease the fluorescence of the TA– and QA–enzyme complexes, however, in contrast to the action of TMA, a residual fluorescence remains at the higher NaCl concentrations.

Reaction of the Probes with Diethylphosphorylated Enzyme. The amount of esteratic site involvement in the binding of the probes by ChE was studied by phosphorylation of the enzyme to block the esteratic site. Solutions of the enzyme were treated with excess Et_4PP to give the diethylphosphorylated enzyme. Varying amounts of the probes were then added and the subsequent evidence of binding noted. Concurrently, a sample of the esteratic site-blocked enzyme was studied by pH-Stat and found to be inactive toward ACh. The inhibited enzyme bound both probes in substantially the same manner as uninhibited enzyme. The insecticide–enzyme complex (Michaelis complex) from bulky phosphate-type insecticides such as Maretin showed competitive interaction with the probes.

Denaturation Studies. The effects of protein denaturants of ChE was determined by (1) ChE reactivity with ACh and (2) the decrease in the fluorescence of the TA–ChE complex. All of the denaturants tested decreased the fluorescence of the complex and shifted the fluorescence response to that of free probe in aqueous media (580 nm). The effects of guanidine and isopropyl alcohol on the TA–ChE complex fluorescence are shown in Figures 8 and 9. As little as 1% isopropyl alcohol had a significant effect on the equilibrium dynamics at the active site of the enzyme.

Data presented in Figures 10 and 11 show the relative effects of organic solvents on the per cent change in activity of ChE toward ACh and the per cent decrease in fluorescence of the ChE–TA complex. The effects on activity and fluorescence parallel each other in magnitude of change and the hierachy of solvents causing the greatest change except in the case of dioxane.

Data with guanidine are given in Figure 12. In the guanidine experiments K_d's were measured fluorometrically for incremental additions of guanidine. It was found that the guanidine did not change the K_d of the TA–ChE complex as long as the complex existed. Loss of active-site activity was complete at 1.57 M guanidine.

Interference by Chlorinated Hydrocarbon-Type Insecticides. Many chlorinated hydrocarbon-type insecticides interfere with the fluorescence of the TA–ChE complex. Figure 13 illustrates the effects of *p,p'*-dichlorophenyl-2,2,2-trichloroethane, *p,p'*-dichloro-2,2-dichloroethylene, Thiodan, dieldrin,

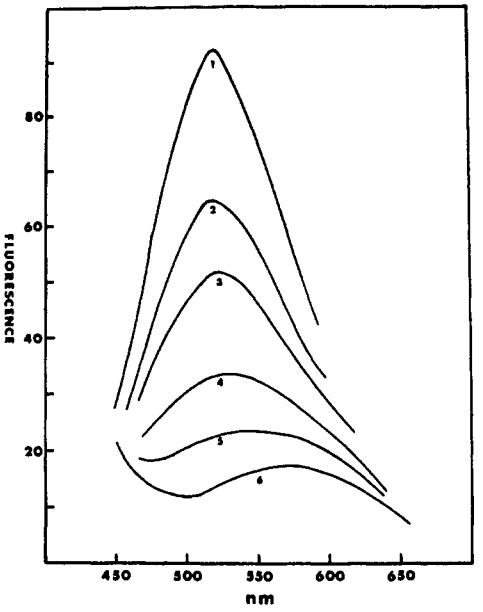

FIGURE 8: Effects of guanidine on the TA–ChE complex. (1) TA = 10^{-6} M, ChE = 1 mg of ChE preparation/ml; (2) guanidine = 0.052 M; (3) guanidine = 0.104 M; (4) guanidine = 0.261 M; (5) guanidine = 0.523 M; (6) guanidine = 1.57 M. (Instrumental settings are the same as given in Figure 6.)

and heptachlor on the probe–enzyme complex. All of the chlorinated insecticides studied decreased the complex fluorescence to some extent, as did a sample of polychlorobiphenyl. Each of the compounds had a major inflection at some point in the titration curve. Previous reports (Chadwick, 1963; Mair-Bode, 1968) that the chlorinated hydrocarbons do not influence the ACh substrate activity of ChE toward ACh were confirmed. Probe–insecticide solutions without ChE were made up in several different solvents. The concentrations in the solutions ranged from 10^{-6} to 10^{-3} M. From spectral observations, we concluded that no reaction occurred between the probes and insecticides.

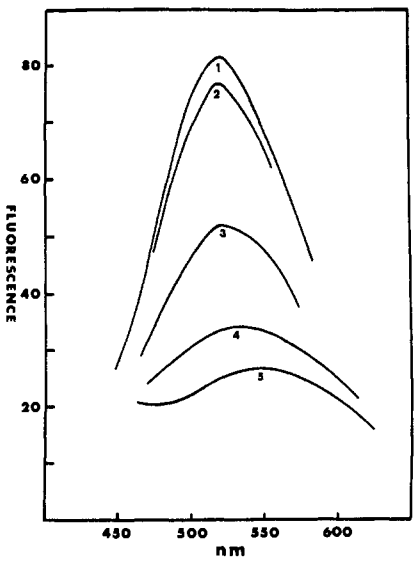

FIGURE 9: Isopropyl alcohol effects on the TA–ChE complex. (1) 0.0% Isopropyl alcohol; (2) 0.5% isopropyl alcohol; (3) 2.5% isopropyl alcohol; (4) 5% isopropyl alcohol; (5) 7.5% isopropyl alcohol. (See Figure 6 for instrumental settings.)

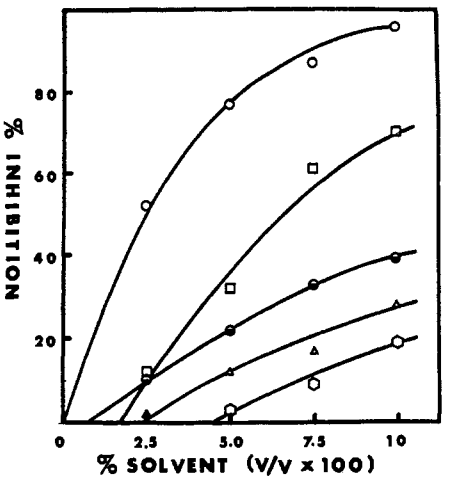

FIGURE 10: The effects of various organic solvents on the hydrolytic activity of ChE. ○ = methanol; ◒ = ethanol; □ = isopropyl alcohol; ○ = *n*-butyl alcohol; △ = dioxane.

Using a sample of purified horse serum ChE (approximately 90% pure), made available by Professor A. R. Main, we observed that 1×10^{-6} M and 2×10^{-6} M solutions of dieldrin quenched the enzyme fluorescence and produced a change in the nature of the emission band.

Discussion

The *N*-dimethylaminoalkyldansylamide (TA) and the trimethylammoniumalkyldansylamide (QA) show spectral parameters similar to other N-substituted dansylamides (Chen, 1967b, Lagunoff and Ottolenghi 1966, Weber, 1952, Chen and Kernohan 1967). The quantum yields of dansylamides are low in aqueous media, a fact that increases their potential utility as fluorescent probes.

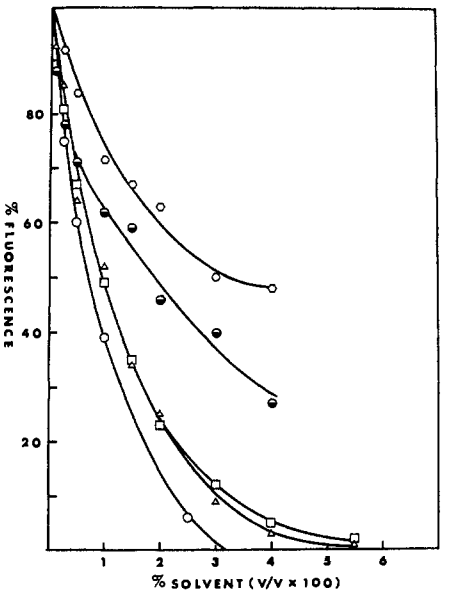

FIGURE 11: Solvent effects on the fluorescence of the TA–CHe complex. ○ = methanol; ◒ = ethanol; □ = isopropyl alcohol; ○ = *n*-butyl alcohol; △ = dioxane. (Excitation and emission bandwidths were fixed at 2.5 nm at 10.0 nm, respectively, λ_{em} set at 515 nm).

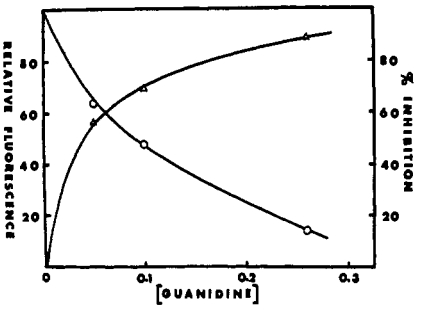

FIGURE 12: Comparison of the effects of guanidine on the fluorescence of the TA–ChE complex (O) and on the activity of ChE (△). (See Figure 11 for instrumental settings.)

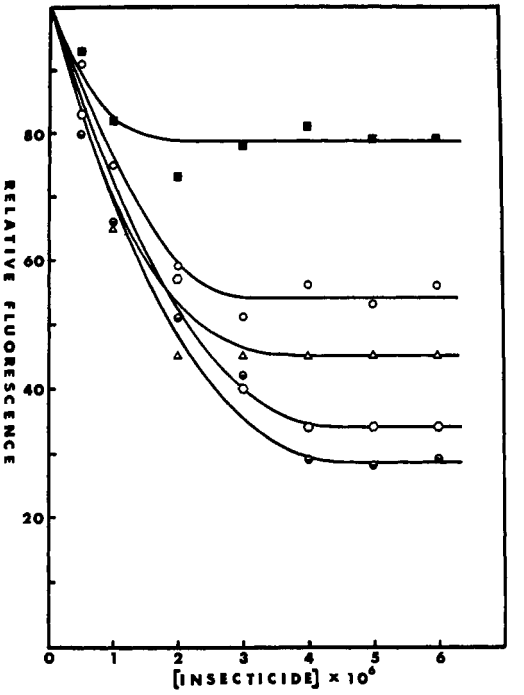

FIGURE 13: The fluorescence response of the TA–ChE complex in the presence of insecticides. ■ = *p,p'*-dichlorophenyl-2,2-dichloroethylene; O = *p,p'*-dichlorophenyl-2,2,2-trichloroethane; △ = heptachlor; ○ = dieldrin; ◒ = thiodan. (See Figure 11 for instrumental settings.)

The shifts in emission, absorption, and excitation maxima and the decrease in pK_a's and pK_a^*'s are associated with changes in the basic character of the aromatic *N*-dimethylamino group. The decreased basicity of the NDA group in these sulfonamides implies that the sulfonamide group has electrophilic properties. The dansylamides have an excited state which is more polar than that of DnsOH. The bathochromic shift of the emission maximum from 515 to 580 nm is favorable, since the emission of complex has little overlap with that of tryptophan or the free probe. Changes in emission maximum, emission peak broadness, and increase in fluorescence intensity can be correlated with similar spectral changes in naphthols and naphthylamines, *cf.* Mataga *et al.* (1956), Mataga and Kaifu (1962), Mataga (1958). Fluorescent probes are characterized by an excited state which is more polar than the ground state (Stryer, 1968).

The absorption spectra of TA and QA undergo bathochromic shifts in going from polar to nonpolar solvents whereas the emission spectra exhibit hypsochromic shifts. The former shifts are normally associated with π^*–n transitions and the latter with π^*–π transitions (Parker, 1968a). Chen (1967b) concluded from fluorescence polarization data that the mechanism of fluorescence in dansylamides stems from π^*–π transitions. The quantum yields of these compounds increase in nonpolar media which is characteristic of π^*–π transitions (Parker, 1968b). D. M. Hercules (personal communication) indicates that bathochromic absorption shifts commonly occur in aminonaphthalene compounds and may represent charge-transfer involvement of the aromatic NDA groups.

The emission maxima of the ChE–TA and ChE–QA complexes are a part of the evidence showing that the fluorescent moiety is bound at a nonpolar area. TA and QA are competitive inhibitors at the active site of ChE, therefore some area of the active site must be involved in the binding of the probes. We have shown that blocking of the esteratic site of ChE with a diethylphosphoryl group does not inhibit binding of TA and QA. The emission maxima of the phosphorylated ChE–TA complex are identical with the ChE–TA complex. Therefore, the fluorescent moiety must be bound in the enzyme (in both cases) at a site or sites other than the esteratic site. Binding of the amino and quaternary groups in TA and QA at the anionic site was indicated by competitive displacement reactions with ammonium compounds known to be competitive inhibitors of the anionic site. Binding of the fluorescent moiety at a hydrophobic site adjacent to the anionic site is postulated on the basis of the following evidence: (1) the polarity measured by the emission maxima indicates binding at a nonpolar site; (2) the probes are competitively displaced by chlorinated hydrocarbon-type insecticides; (3) the chlorinated hydrocarbon-type insecticides do not inhibit the active site of the enzyme; (4) increasing the size of the alkyl group in a series of alcohols increases the displacement reaction of TA from the TA–ChE complex; (5) Christian and Janetzko (1969) have shown the binding of the fluorescent probe 1–8 ANS at a hydrophobic site in ChE, adjacent to the active site.

The presence of hydrophobic site binding of nonsubstrate molecules in cholinesterase enzymes indicates that analogs and homologs of TA and QA could be important in determining the nature of hydrophobic areas adjacent to the active sites in these enzymes.

The idea of hydrophobic area in the vicinity of the active site of cholinesterase is not new. Wilson (1952) initially discussed the importance of noncoulumbic forces involved with complex formation of the anionic site of AChE. Bovine erythrocyte AChE appears to have a hydrophobic area large enough to accommodate six methylene groups (Bracha and O'Brien, 1968, 1970). Similar areas have been postulated for horse serum cholinesterase. In contrast to acetycholinesterases they are visualized as being separate from the anionic site (Brestkin *et al.*, 1964; Abdubakhabov *et al.*, 1968). The findings reported here support a hydrophobic site hypothesis. Bergmann and Segal (1954) calculated the binding energy per methylene group for a series of *n*-alkyl trimethylammonium salts and found an increase of 0.5 kcal mole^{-1} per methylene unit in butyrlcholinesterase over the -0.3 kcal mole^{-1} value for AChE.

In the absence of substantial amounts of organic solvents, chlorinated hydrocarbon-type insecticides do not affect the kinetics of interaction of cholinesterase enzymes with substrate ACh. The competitive removal of TA from the TA–ChE complex indicates that these chlorinated hyrocarbons

bind at and compete for the hydrophobic site occupied by the dansyl moiety. Preliminary data with pure ChE enzyme supplied by Professor A. R. Main indicated spectral changes in tryptophan emission when 1×10^{-6} M solutions of dieldrin were added to the pure enzyme. The effects on the fluorescence of the TA–ChE complex and on the fluorescence emission of tryptophan in pure ChE appear to be the first spectral proof of binding of chlorinated hydrocarbon-type insecticides with cholinesterase enzymes.

Use of aqueous solutions of organic solvents with horse serum ChE can have severe consequences. The capacity of an alcohol to effect a change in activity increases with increased chain length. Herskovits *et al.* (1970) have studied the effects of alcohols as denaturants on a number of proteins. They have reported the effects of increased chain length on effectiveness of an alcohol as a denaturant. Organic solvents and their reactions with ChE's have been reviewed by Chadwick (1963). Professor A. R. Main (personal communication) has indicated that with horse serum ChE the effects observed with any particular solvent on activity vary with the substrate used.

The active-site-directed equilibrium fluorscent probes TA and QA are anionic site, cholinesterase probes. Additional utility is indicated in preliminary work as fluorescent tracers of flyhead ChE's in disc electrophoresis (J. L. Zettler, R. T. Mayer, C. M. Himel, and U. E. Brady, 1971, unpublished data). They have been particularly valuable in providing supplementary data for pH-Stat methods as well as other techniques of cholinesterase analyses.

References

Abduvakhabov, A. A., *et al.* (1968), *Izv. Akad. Nauk. SSSR. Ser. Khim.*, 744.

Augustinsson, K. (1966), *Biochim. Biophys. Acta 128*, 351.

Baker, B. R. (1967), *in* Design of Active-Site-Directed Irreversible Enzyme Inhibitors, New York, N. Y., Wiley.

Bergmann, F. (1955), *Discuss. Faraday Soc. 20*, 126.

Bergmann, F. (1958), *Advan. Catal. Relat. Subj. 10*, 130.

Bergmann, F., and Segal, R. (1954), *Biochem. J. 58*, 692.

Bergmann, F., and Shimoni, A. (1925), *Biochim. Biophys. Acta 9*, 473.

Bergmann, F., and Wurzel, M. (1953), *Biochim. Biophys. Acta 11*, 440.

Bracha, P., and O'Brien, R. D. (1968), *Biochemistry 7*, 1555.

Bracha, P., and O'Brien, R. D. (1970), *Biochemistry 9*, 741.

Brand, L., Gohlke, J. R., and Rao, D. S. (1967), *Biochemistry 6*, 3510.

Brestkin, A. P., *et al.* (1964), *Dokl. Akad. Nauk. SSSR 158*, 880.

Chadwick, L. E. (1963), *in* Handuch der Experimentellen Pharmakologie, Koelle, G. B., Ed., Berlin, Springer-Verlag, pp 741–798.

Chen, R. F. (1966), *Nature (London) 209*, 69.

Chen, R. F. (1967a), *in* Fluorescence Theory, Instrumentation and Practice, Guilbault, G. G., Ed., New York, N. Y., Dekker, p 443.

Chen, R. F. (1967b), *Arch. Biochem. Biophys. 120*, 609.

Chen, R. F., Edelhoch, H., and Steiner, R. F. (1969), *in* Physical Principles and Techniques of Protein Chemistry, New York, N. Y., Academic Press, pp 171–274.

Chen, R. F., and Kernohan, J. C. (1967), *J. Biol. Chem. 242*, 5813.

Chen, R. F., Vurek, G. G., and Alexander, N. (1967), *Science 156*, 949.

Christian, S. T., and Janetzko, R. (1969). *Biochem. Biophys. Res. Commun. 37*, 1022.

Deranleau, D. A., and Neurath, H. (1966), *Biochemistry 5*, 1413.

Edelman, G. M., and McClure, W. O. (1968), *Accounts. Chem. Res. 1*, 65.

Fletcher, A. N. (1967), *J. Mol. Spectrosc. 23*, 221.

Foster, J. F., and Sterman, M. D. (1956), *J. Amer. Chem. Soc. 78*, 3656.

Haugland, R. P., and Stryer, L. (1967), *Conform. Biopolym. Pap. Int. Symp.* 321.

Herskovits, T. T., Gadegbeku, B., and Jaillet, H. (1970), *J. Biol. Chem. 245*, 2588.

Himel, C. M., Aboul-Saad, W. G., and Uk, S. (1971), *Agr. Food Chem. 19*, 1175.

Himel, C. M., and Mayer, R. T. (1970), *Anal. Chem. 42*, 130.

Himel, C. M., Mayer, R. T., and Cook, L. L. (1970), *J. Polym. Sci. 8*, 2219.

Jun, H. W., Mayer, R. T., Himel, C. M., and Luzzi, L. A. (1971), *J. Pharm. Sci. 60*, 1821.

Klotz, I. M., and Fiess, H. A. (1960), *Biochem. Biophys. Acta 38*, 57.

Lagunoff, D., and Ottolenghi, P. (1966), *C. R. Trav. Lab. Carlsberg 35*, 63.

Mair-Bode, H. (1968), *Residue Rev. 22*, 1.

Mataga, N. (1958), *Bull. Chem. Soc. Jap. 31*, 481.

Mataga, N., and Kaifu, Y. (1962), *J. Phys. Chem. 36*, 2804.

Mataga, N., Kaifu, Y., and Koizumi, M. (1956), *Bull. Chem. Soc. Jap. 29*, 115.

Mendel, B., Mundell, D., and Strelitz, I. (1939), *Nature (London) 144*, 479.

Nabb, D. P., and Whitfield, F. (1967), *Arch. Environ. Health 15*, 147.

Parker, C. A. (1968a), *in* Photoluminescence of Solutions, New York, N.Y., Elsevier, pp 378, 431–433.

Parker, C. A. (1968b), *in* Photoluminescence of Solutions, New York, N.Y., Elsvier, pp 28–35.

Parker, C. A., and Rees, W. T. (1960), *Analyst 85*, 587.

Shaw, Elliott (1970), *in* The Enzymes, 3rd ed, Boyer, P. D., Ed., New York, N.Y., Academic Press, pp 91–146.

Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., and Van Holde, K. E. (1962), *J. Biol. Chem. 237*, 1107.

Steiner, R. F., and Edelhoch, H. (1963), *Biochim. Biophys. Acta 66*, 341.

Stern, O., and Volmer, M. (1919), *Physik. Z. 20*, 183.

Stryer, L. (1965), *J. Mol. Biol. 13*: 432.

Stryer, L. (1968), *Science 162*, 526.

Turner, G. K. (1968), *Science 146*, 183.

Weber, G. (1952), *Biochem. J. 51*, 155.

Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56*, *31*.

Weller, A. (1961), *Progr. React. Kinet. 1*, 189.

Whidby, J. F., Leyden, D. E., Himel, C. M., and Mayer, R. T. (1971), *J. Phys. Chem., 75*, 4056.

Wilson, I. B. (1952), *J. Biol. Chem. 197*, 215.