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# Inactivation of Electric Eel Acetylcholinesterase by Acylation with *N*-Hydroxysuccinimide Esters of Amino Acid Derivatives†

Shmaryahu Blumberg* and Israel Silman

ABSTRACT: A series of *N*-hydroxysuccinimide esters of substituted amino acids has been examined as a potential class of acylating agents for covalent modification of acetylcholinesterase from the electric eel. Such esters inactivate acetylcholinesterase, and those which contain bulky aromatic groups are particularly effective. Thus, the *N*-hydroxysuccinimide ester of *N*-acetyl-*p*-(2,4-dinitroanilino)-L-phenylalanine at 25 $\mu$M causes, under appropriate conditions, 50% inactivation of the enzyme within 1 min and 96% inactivation after 20 min. Certain quaternary ammonium salts accelerate the inactivation process, some retard it, and others have little effect. Pyridine-2-aldoxime methiodide fails to reactivate the enzyme modified sequentially with diethyl fluorophosphate and the active ester, indicating that inactivation by the ester results from modification at a site distinct from the active site serine. The rate of inactivation is greatly enhanced upon raising the pH from 7.0 to 8.9, suggesting the modification of an amino acid side chain with a high $pK_a$. Deacylation of the inactivated enzyme by 0.5 M hydroxylamine regenerates activity at a rate indicative of the modification of a tyrosine residue. Absorption spectra of the modified enzyme before and after deacylation with hydroxylamine show that almost complete inactivation is achieved by covalent reaction of one molecule of reagent per catalytic subunit of acetylcholinesterase. The experimental results are interpreted to indicate inactivation of acetylcholinesterase by acylation of a single tyrosine residue near the active site of the enzyme.

Anticholinesterase agents such as organophosphates, carbamates, and sulfonyl halides are generally believed to exert their effects by forming a stable covalent bond with the serine in the active site of the enzyme (Aldridge & Reiner, 1972). We wish to describe the action on acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AcChE)[1] of a different class of acylating agents, *N*-hydroxysuccinimide esters of amino acid

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[1] Abbreviations used are: AcChE, acetylcholinesterase; ONSu, succinimido-oxy; $Nbs_2$, 5,5′-dithiobis(2-nitrobenzoic acid); 2-PAM, pyridine-2-aldoxime methiodide; PTMA, phenyltrimethylammonium; MAC, *N*-methylacridinium; PMI, pyridine methiodide; DEFP, diethyl fluorophosphate; Dnp, 2,4-dinitrophenyl; DnpNH, 2,4-dinitroanilino; DMF, *N,N*-dimethylformamide; Moc, methyloxycarbonyl; DapA, 4-dimethylaminophenylazo; Dns, 5-dimethylaminonaphthalene-1-sulfonyl (dansyl). All other abbreviations are according to IUPAC–IUB rules; see, for example: (1972) *J. Biol. Chem. 247,* 977.

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derivatives. Such esters, generally employed for peptide syntheses, have been successfully utilized for chemical modification of tyrosine residues near the active sites of neutral proteases, leading to dramatic increases in activity and to changes in the specificity of these enzymes (Blumberg & Vallee, 1975; Holmquist et al., 1976).

We demonstrate below that several *N*-hydroxysuccinimide active esters of amino acid derivatives act as efficient inactivators of AcChE. One particular ester, Ac-Phe(4DnpNH)-ONSu, was found to inactivate the enzyme by reacting specifically with an amino acid residue distinct from the catalytic site serine, most likely with a tyrosine residue near the active site of the AcChE molecule.

## Materials and Methods

Acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid) ($Nbs_2$) and pyridine-2-aldoxime methiodide (2-PAM) were obtained from Sigma Chem. Co. Decamethonium bromide was from ICN Pharmaceuticals, Inc., propidium diiodide from Calbiochem, Tensilon from Roche Laboratories, and gallamine triethiodide from K & K Laboratories Inc. Phenyltrimethylammonium iodide (PTMA), obtained from B.D.H.Chemicals, was recrystallized from ethanol before use. *N*-Methylacridinium perchlorate (MAC) and pyridine methiodide (PMI) were obtained as previously described (Shinitzky et al., 1973; Wilson & Silman, 1977). Diethyl fluorophosphate (DEFP) was a gift from Mr. G. Amitai.

AcChE was the 11S form of the enzyme from the electric eel, *Electrophorus electricus*, purified by affinity chromatography as described previously (Dudai et al., 1972).

*N-Hydroxysuccinimide Esters of N-Acetylamino Acids.* Ac-Ala-ONSu, Ac-Val-ONSu, Ac-Phe-ONSu, Ac-Trp-ONSu, Ac-Tyr(Bzl)-ONSu, Ac-Tyr(Dnp)-ONSu, and Ac-Phe(4DnpNH)-ONSu were prepared as previously described (Blumberg & Vallee, 1975).

*N-Methyloxycarbonylamino Acid Derivatives.* Moc-Phe, mp 78–80 °C (from ether/petroleum ether), Moc-Phe($4NO_2$), mp 116–118 °C (from ethanol/water), and Moc-Phe(4DapA), mp 188 °C (dec), were prepared by reacting methyl chloroformate with Phe, Phe($4NO_2$), or Phe(4DapA), respectively; the latter was prepared by Mr. I. Jacobson by diazotization of Phe($4NH_2$) and reaction with *N,N*-dimethylaniline (Benderly, 1972). Moc-Phe(4DnpNH), mp 160–161 °C (from ethanol/water), and Moc-Phe(4DnsNH), mp 100–140 °C (softening) (from ethyl acetate/petroleum ether), were obtained from Moc-Phe($4NO_2$) by catalytic reduction to Moc-Phe($4NH_2$) on $Pd/BaSO_4$ followed by reaction with 1-fluoro-2,4-dinitrobenzene and dansyl chloride, respectively. Details of the synthesis of one of these derivatives are given below.

*Moc-Phe(4DapA).* Phe(4DapA) (4 g, 12.4 mmol) was dissolved in 26 mL of 0.5 N NaOH (13 mmol) and the solution was cooled in ice. NaOH (8 mL of 2 N) and methyl chloroformate (1.2 mL, 15.5 mmol) were added with stirring in four equal portions at 5-min intervals. The solution was stirred for an additional 30 min while keeping the pH at 9–10 by adding 2 N NaOH. The solution was filtered and the pH was brought to 3.5 by adding 2 N HCl. The precipitate which formed was filtered and washed thoroughly with water. The product was dried in a desiccator over $P_2O_5$ and NaOH pellets: yield 3.9 g (80%). Purification of the material was achieved by dissolution at pH 9.0 with NaOH and precipitation by acidification to pH 3.5 with HCl: mp 188 °C (dec). Anal. Calcd for $C_{19}H_{22}N_4O_4$: C, 61.61; H, 5.99; N, 15.13. Found: C, 61.57; H, 6.06; N, 14.92.

*N-Hydroxysuccinimide Esters of N-Methyloxycarbonylamino Acids.* Moc-Phe-ONSu (mp 136–137 °C), Moc-Phe(4DnsNH)-ONSu (mp 211–212 °C), Moc-Phe(4DnpNH)-ONSu (mp 161–162 °C), and Moc-Phe(4DapA)-ONSu (mp 173–175 °C) were prepared from the corresponding carboxyl-free derivatives by condensation with *N*-hydroxysuccinimide in the presence of *N,N′*-dicyclohexylcarbodiimide, in dioxane or DMF, according to the general procedure of Anderson et al. (1964). The products were crystallized from 2-propanol or 2-propanol/DMF. Details of the synthesis of one of these compounds are given below.

*Moc-Phe-ONSu. N,N′*-Dicyclohexylcarbodiimide (2.02 g, 10 mmol) was added to a solution of Moc-Phe (2.23 g, 10 mmol) and *N*-hydroxysuccinimide (1.2 g, 10.4 mmol) in 50 mL of dioxane with cooling. The reaction mixture was kept at 4 °C overnight and the precipitate of *N,N′*-dicyclohexylurea was filtered and washed with dioxane. The filtrate and washings were concentrated in vacuo to yield an oil which crystallized on adding 2-propanol. The product was filtered and recrystallized from 2-propanol: mp 136–137 °C; yield 60%. Anal. Calcd for $C_{15}H_{16}N_2O_6$: C, 56.25; H, 5.04; N, 8.75. Found: C, 56.20; H, 5.30; N, 8.75.

The purity of all the newly synthesized methyloxycarbonyl derivatives was confirmed by thin-layer chromatography on silica gel sheets (chloroform:2-propanol:acetic acid, 90:10:4). Elementary nitrogen analyses of all compounds were within acceptable limits.

It should be noted that *N*-hydroxysuccinimide esters of *N*-acetylamino acid derivatives and of *N*-methyloxycarbonylamino acid derivatives may display different chemical reactivity. This is because active esters of the *N*-acetyl derivatives can undergo cyclization to oxazolones which may also serve as acylating agents (Goodman & Levine, 1964; Blumberg & Vallee, 1975), whereas active esters of the *N*-methyloxycarbonyl derivatives cannot undergo such a conversion.

*AcChE activity* was assayed by the method of Ellman et al. (1961), in 0.1 M NaCl–0.01 M Tris–0.01% gelatin–1 mM $Nbs_2$, pH 8.0, 25 °C, using 3.1 mM acetylthiocholine iodide as substrate. The reaction was followed by recording the change in absorbance at 412 nm using a Gilford 2400-S recording spectrophotometer thermostated at 25 °C.

*Absorbance* at a single wavelength and *spectra* were measured using a Ziess PMQ-II and a Cary 118 spectrophotometer, respectively.

*Enzyme concentration* was determined from the absorbance at 280 nm using $A_{280}^{1\%} = 17.6$ (Dudai et al., 1972), mol wt per catalytic subunit 82 000 (Dudai & Silman, 1974).

*Chemical modification* of AcChE was performed routinely on 100-μL samples of enzyme at an active site concentration of ca. $2 \times 10^{-7}$ M, at 25 °C, in various media of different pH and ionic strengths. Most experiments were carried out at pH 7.9 in either 0.18 M NaCl–0.016 M Tris, 1.62 M NaCl–0.016 M Tris, or in 0.082 M sodium phosphate, as indicated below. Reactions were initiated by the addition to the reaction mixture of 2 μL of active ester reagent (0.6–50.0 mM), dissolved in DMF, and the course of inactivation was followed by first diluting 10-μL samples 200-fold into 0.1 M NaCl–0.01 M Tris–0.01% gelatin, pH 8.0, and then taking 30-μL aliquots of the diluted enzyme for assay in a 2.5-mL volume of the assay mixture. Modification with DEFP was performed in the same manner as modification with the active esters except that the reagent was dissolved in ethylene glycol.

## Results

*Acylation of AcChE by N-Hydroxysuccinimide Esters.* A series of *N*-hydroxysuccinimide esters of *N*-acetylamino acids was previously shown to acylate neutral proteases and thereby

TABLE I: Inactivation of Electric Eel AcChE by *N*-Hydroxysuccinimide Esters.[a]

| *N*-Hydroxysuccinimide ester of | Reagent (mM) | Residual act. (%) at ionic strength[b] 1.62 M | 0.18 M |
|---|---|---|---|
| Ac-Ala | 1.0 | 80 | 85 |
| Ac-Val | 1.0 | 66 | 85 |
| Ac-Phe | 1.0 | 54 | 81 |
| Ac-Trp | 1.0 | 46 | 74 |
| Moc-Phe | 1.0 | 34 | 69 |
| Ac-Tyr(Bzl) | 0.5 | 36 | 69 |
| Moc-Phe(4DapA) | 0.05 | 44 | 84 |
| Moc-Phe(4DnsNH) | 0.05 | 29 | 67 |
| Ac-Tyr(Dnp) | 0.05 | 36 | 79 |
| Moc-Phe(4DnpNH) | 0.05 | 10 | 53 |
| Ac-Phe(4DnpNH) | 0.025 | 4 | 27 |

[a] Acylation of AcChE was carried out in 0.016 M Tris, pH 7.9, 25 °C; containing 1.62 M NaCl or 0.18 M NaCl. [b] Residual activity after 30 min of incubation.

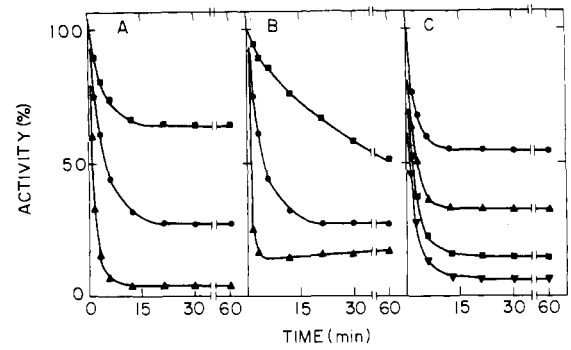


FIGURE 1: Kinetics of inactivation of electric eel AcChE by Ac-Phe(4DnpNH)-ONSu. (A) Ionic strength dependence: inactivation was carried out using 25 $\mu$M reagent in 0.016 M Tris, pH 7.9, containing 0.02 M NaCl (■), 0.18 M NaCl (●), or 1.62 M NaCl (▲). (B) pH-dependence: inactivation was carried out using 25 $\mu$M reagent in 0.016 M Tris–0.18 M NaCl at pH 7.0 (■), pH 7.9 (●), and pH 8.9 (▲). (C) Dependence on active ester concentration: inactivation was carried out in 0.082 M sodium phosphate, pH 7.9, using reagent concentrations at 12 $\mu$M (●), 25 $\mu$M (▲), 50 $\mu$M (■), and 100 $\mu$M (▲). All inactivations were carried out at 25 °C at an AcChE concentration of $2 \times 10^{-7}$ M active sites, with 2% dimethylformamide in the reaction mixture.

increase their activity (Blumberg & Vallee, 1975; Holmquist et al., 1976). These esters, as well as several newly synthesized *N*-methyloxycarbonylamino acid active esters, have now been employed in the modification of AcChE and have been found to inactivate this enzyme. Table I shows the residual activity of the enzyme after incubation with various such reagents at pH 7.9, 25 °C, for 30 min, at both moderate (0.18 M) and high ionic strength (1.62 M). Since the active esters hydrolyze rapidly in aqueous media, reaction with the enzyme occurs only within the first 20–30 min and no further inactivation was observed after longer periods of incubation. The extent of inactivation was always greater at the higher ionic strength value. The aliphatic derivatives Ac-Ala-ONSu and Ac-Val-ONSu are very poor inactivators even at a concentration of 1 mM reagent. The aromatic derivatives, Ac-Phe-ONSu and Ac-Trp-ONSu, as well as Moc-Phe-ONSu, are also weak inactivators, although somewhat more efficient than the aliphatic derivatives. Additional aromatic and bulky substitutions in the para position of the phenyl ring in Ac-Phe-ONSu or Moc-Phe-ONSu, however, bring about a remarkable increase in the effectiveness of the inhibitors. Thus, 0.05 mM concentrations of Moc-Phe(4DapA)-ONSu, Moc-Phe(4DnsNH)-ONSu, and Moc-Phe(4DnpNH)-ONSu cause 56%, 71%, and 90% inactivation, respectively. Of particular interest are the 2,4-dinitrophenyl derivatives, Ac-Tyr(Dnp)-ONSu and Ac-Phe(4DnpNH)-ONSu. Although the two compounds are structurally very similar, the latter is a much more efficient reagent than the former. Such a difference between these two reagents indicates that subtle structural features are involved in their interaction with the AcChE molecule.

*Effect of Ionic Strength and pH on Inactivation of AcChE by Ac-Phe(4DnpNH)-ONSu.* Since the active ester, Ac-Phe(4DnpNH)-ONSu, inactivates AcChE more efficiently than the other esters tested (Table I), and in addition exhibits a characteristic absorption maximum at 370 nm (Blumberg & Vallee, 1975), further studies on the inactivation of AcChE were performed using this particular reagent. Figure 1A shows that raising the concentration of NaCl from 0.02 M to 0.18 M and to 1.62 M markedly increases the rate and the extent of inactivation by 25 $\mu$M reagent at pH 7.9, 25 °C. The leveling-off tendency of the activity–time profiles is characteristic of modification with reagents that hydrolyze rapidly during the inactivation process. From the degree of inactivation after 80 s of incubation, it can be estimated that the reaction in 1.62 M NaCl is ca. 10-fold faster than in 0.02 M NaCl. A ca. 12-fold increase in the rate of inactivation, as a result of increasing the ionic strength from 0.02 M to 1.62 M, was also seen when the enzyme was modified with the organophosphorus reagent, DEFP. Since both the active ester and DEFP are uncharged molecules, the observed increase in the rate of inactivation may be due to an effect of ionic strength on the conformation of the enzyme (Changeux, 1966; Mooser & Sigman, 1974), or alternatively, to an ionic-strength-dependent change in the apparent $pK_a$ of a functional group in the active site of the enzyme (Rosenberry, 1975).

Raising the pH from 7.0 to 7.9 and to 8.9 also markedly increases the rate of inactivation of the enzyme by 25 $\mu$M Ac-Phe(4DnpNH)-ONSu in 0.18 M NaCl, 25 °C (Figure 1B). The increase in the extent of inactivation is somewhat less than expected from the increase in the initial rates, due to the more rapid rate of hydrolysis of the reagent at the higher pH. From the degree of inactivation after 80 s, the reaction at pH 8.9 is more than 20-fold faster than at pH 7.0. In contrast, the response of the enzyme to reaction with DEFP changed only slightly in the same pH range. Thus, the rate of inactivation by DEFP at pH 8.9 is only 30% higher than the rate at pH 7.0. This difference in pH-dependence makes it unlikely that a change in the apparent $pK_a$ of a single functional group is responsible for the similar ionic strength dependence of inactivation by the active ester and the organophosphorus reagent discussed above; moreover, it provides a first indication that the active ester and the organophosphorus reagent react with the enzyme at different sites or by different mechanisms.

*Effect of Reagent Concentration on Inactivation of AcChE by Ac-Phe(4DnpNH)-ONSu.* The time course of inactivation of AcChE by varying concentrations of Ac-Phe(4DnpNH)-ONSu was followed at constant pH and ionic strength. Again, the activity decreased within the first 20–30 min, and there was no further decrease in activity after longer periods of incubation. Both the rate and the extent of inactivation increase with increasing concentration of the reagent (Figure 1C).

The increase in the rate and extent of inactivation in response to increased concentration of the reagent (Figure 1C) is, however, less than expected from simple models for inactivation by a reagent that reacts with an enzyme in a second-order reaction and in parallel undergoes spontaneous hydrolysis (Melchior & Fahrney, 1970). Thus, from the residual activity

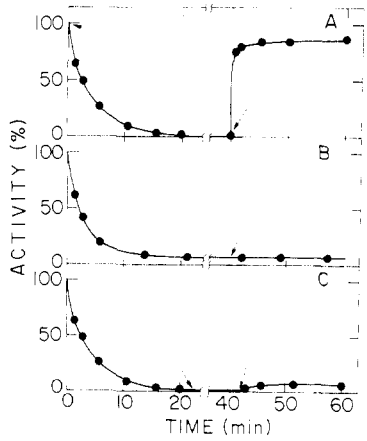


FIGURE 2: Modifications of AcChE with DEFP and Ac-Phe(4DnpNH)-ONSu, and the effect of 2-PAM on the modified enzyme. Modification was carried out on AcChE, $2 \times 10^{-7}$ M active sites, at 25 °C in 0.18 M NaCl-0.016 M Tris, pH 7.9. Treatment with 2-PAM was performed on the enzyme after it had been diluted 200-fold in 0.1 M NaCl-0.01 M Tris-0.1% gelatin, pH 8.0. (A) Modification with DEFP, $5 \times 10^{-7}$ M, for 40 min, followed by treatment with 2-PAM, $5 \times 10^{-4}$ M (dotted arrow). (B) Modification with Ac-Phe(4DnpNH)-ONSu, $1 \times 10^{-4}$ M, for 40 min and treatment with 2-PAM, $5 \times 10^{-4}$ M (dotted arrow). (C) Sequential modification, first with DEFP, $5 \times 10^{-7}$ M, for 20 min, and then with Ac-Phe(4DnpNH)-ONSu, $1 \times 10^{-4}$ M, for another 20 min (full arrow), followed by treatment with 2-PAM, $5 \times 10^{-4}$ M (dotted arrow). Activity was assayed as described in the text.

after treatment with 12 $\mu$M Ac-Phe(4DnpNH)-ONSu (55%), it would be predicted that 100 $\mu$M reagent would produce a residual activity of less than 1%, whereas in fact a value of 6% is obtained. This raises the possibility that the reaction is not simply second order, and that binding of reagent to the enzyme may precede the covalent reaction. Since the active ester hydrolyzes rapidly, it is not possible to measure its affinity for the enzyme by ordinary binding procedures. However, the affinity of Ac-Phe(4DnpNH), the product of hydrolysis of the reagent, was measured by inhibition. It was found that in 0.1 M NaCl-0.01 M Tris-0.1% gelatin, pH 8.0, 25 °C, Ac-Phe(4DnpNH) inhibits the AcChE-catalyzed hydrolysis of acetylthiocholine noncompetitively with an inhibition constant $K_i \sim 1.5 \times 10^{-4}$ M. This suggests that at the concentrations employed for inactivation the active ester may also bind to the enzyme prior to reaction.

*Sequential Modifications of AChE with DEFP and Ac-Phe(4DnpNH)-ONSu.* Since certain active esters inactivate AcChE rather efficiently (Table I), it was interesting to examine whether or not they react with the catalytic site serine similarly to the other acylating agents which serve as inactivators of the enzyme (Aldridge & Reiner, 1972). Figure 2A recapitulates the known finding (Wilson & Ginsburg, 1955) that inactivation of AcChE by DEFP is readily reversed by low concentrations of 2-PAM. In contrast, inactivation by the active ester Ac-Phe(4DnpNH)-ONSu is not reversed by 2-PAM under the same conditions (Figure 2B). This difference in response to treatment by 2-PAM does not, in itself, imply that the two reagents react with different residues, since the acyl moieties of the two reagents are very different chemical entities.

Figure 2C shows the effect of sequential modifications, first with DEFP, resulting in inactivation of the enzyme, and then with the active ester. It can be seen that the product of this sequential treatment is not reactivated by 2-PAM. If the two reagents exerted their inhibitory effects by modification of the same residue, then once that residue was covalently blocked by the organophosphorus reagent there could be no reaction

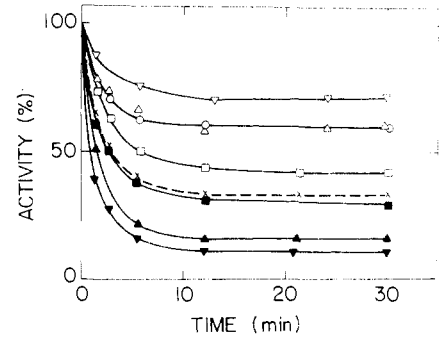


FIGURE 3: Effect of quaternary ligands on modification of AcChE by Ac-Phe(4DnpNH)-ONSu. AcChE, $2 \times 10^{-7}$ active sites, was modified with 25 $\mu$M reagent in 0.082 M sodium phosphate, pH 7.9, 25 °C, in the presence of the appropriate quaternary ligand. (X) No ligand; (▼) 1 mM PMI; (▲) 1 mM PTMA; (■) 0.1 mM Tensilon; (□) 0.1 mM MAC; (△) 0.1 mM propidium; (○) 1 mM gallamine; (▽) 1 mM decamethonium.

with the active ester, and 2-PAM would be capable of reactivating the inhibited enzyme. Since 2-PAM does not reactivate the enzyme, the two reagents must interact at different sites and, furthermore, blocking of the active site serine by the organophosphorus reagent does not prevent reaction of the active ester with the residue which it modifies.

*Effects of Quaternary Ligands on the Rate of Inactivation.* Since the active ester, Ac-Phe(4DnpNH)-ONSu, inactivates AcChE by reaction at a site distinct from the catalytic site serine, the question arises whether it reacts at the anionic site or elsewhere. This question was examined by testing the effect of quaternary ligands on the rate of inactivation. The monoquaternary derivatives Tensilon, PTMA and PMI, which are all known to bind preferentially at the anionic site of the enzyme (Wilson & Alexander, 1962; Taylor & Lappi, 1975), were found either to have no effect or to accelerate the inactivation process (Figure 3). Another monoquaternary derivative, MAC, also known to bind at the anionic site (Mooser et al., 1972; Shinitzky et al., 1973) differs from the above ligands in its larger size and bulkiness. This ligand retards the inactivation process to some extent (Figure 3). These findings imply that modification occurs outside the anionic site and suggest that it may occur near it. The other ligands tested, gallamine and propidium, which are believed to bind preferentially at the peripheral site (Belleau et al., 1970; Changeux, 1966; Taylor & Lappi, 1975) and decamethonium, which is believed to bind simultaneously at the anionic site and the peripheral site, with its decamethylene chain bridging these two sites (Bergmann et al., 1950; Mooser & Sigman, 1974), all effectively retard the inactivation process.These results indicate that modification by the active ester occurs in a region extending away from the anionic site toward the peripheral site of the enzyme.

*Reactivation of the Modified AcChE by Hydroxylamine.* Although 2-PAM fails to reactivate AcChE modified by Ac-Phe(4DnpNH)-ONSu under the conditions employed for reactivation of DEFP-inactivated enzyme, it was found that high concentrations of $NH_2OH$ (0.5 M, pH 8.0) reactivate the modified enzyme rather effectively. In different experiments, employing a variety of ionic strength values and reagent concentrations for inactivation, reversal of activity by $NH_2OH$ yielded 70–90% of the initial activity. In one particular experiment a relatively high concentration of enzyme was employed. The enzyme, $1.1 \times 10^{-5}$ M active sites, in 1 M NaCl-16 mM Tris, pH 8.0, was first inactivated with $2.5 \times 10^{-5}$ M reagent and after 10 min treated with an additional portion of $2 \times 10^{-5}$ M reagent (Figure 4). The residual activity after 30 min was 11%. When the modified enzyme was di-

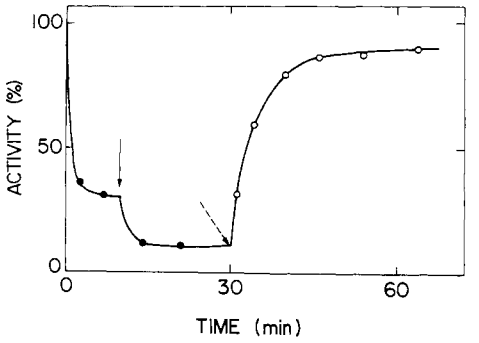


FIGURE 4: Inactivation of AcChE by Ac-Phe(4DnpNH)-ONSu (closed symbols) and reactivation by hydroxylamine (open symbols). AcChE, 2.5 mL, $1.1 \times 10^{-5}$ active sites, in 1 M NaCl-0.016 M Tris, pH 8.0, was inactivated in two steps, first by reaction with $2.5 \times 10^{-5}$ M reagent and, after 10 min, by reaction with additional freshly added $2.0 \times 10^{-5}$ M reagent (full arrow). After 30 min, 1.2 mL of the mixture was taken and treated with 0.3 mL of 2 M $NH_2OH$-0.3 M Tris, pH 8.0 (dotted arrow), to a final concentration of 0.5 M $NH_2OH$-0.075 M Tris, pH 8.0. Activity was assayed on acetylthiocholine as described in the text.

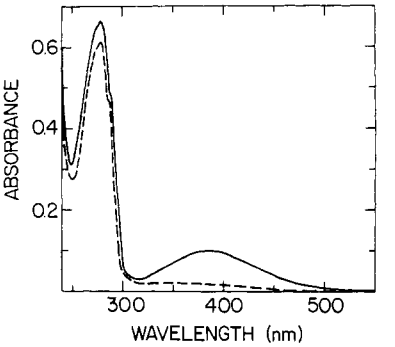


FIGURE 5: Absorption spectra of AcChE modified with Ac-Phe(4DnpNH)-ONSu before (—) and after (---) deacylation with hydroxylamine. Modification with Ac-Phe(4DnpNH)-ONSu and deacylation of the modified enzyme were carried out as described in Figure 4. The enzyme modified for 30 min and the modified enzyme subsequently deacylated by $NH_2OH$ for 50 min were both dialyzed for 24 h at 4 °C against four changes of 0.1 M NaCl-0.01 M $Na_2HPO_4$, pH 7.0. Measurements of spectra were performed on the dialyzed enzyme preparations, diluted to $\sim 0.43 \times 10^{-5}$ M in catalytic subunits based on $\epsilon_{280} = 144\,000$ per catalytic subunit.

alyzed for 24 h against 0.1 M NaCl-0.01 M $Na_2HPO_4$, pH 7.0, no increase in the residual activity was observed. Treatment of the modified enzyme with 0.5 M $NH_2OH$, pH 8.0, however, regenerates 90% of the initial activity, with a half-life of ca. 3 min (Figure 4). Comparison of this rate of reactivation with the rate of deacylation of different acyl model compounds (Holmquist et al., 1976) shows the closest similarity to the rate of deacylation observed with $O^{4'}$-acyltyrosine model compounds, although the rate of reactivation of the modified AcChE is ca. fivefold slower. This suggests that inactivation by the active ester is due to modification of hydroxyl group(s) of tyrosine(s) but that the protein environment affects the reactivity of the acyl-enzyme linkage.

*Stoichiometry of the Modification by Ac-Phe(4DnpNH)-ONSu.* Using sufficiently high concentrations of AcChE during modification by Ac-Phe(4DnpNH)-ONSu allows quantitation of the reaction and evaluation of the spectral properties of the modified enzyme, since the Ac-Phe(4DnpNH) chromophore in aqueous solution absorbs maximally at 370 nm with $\epsilon_{370} = 18\,500$ and $\epsilon_{280} = 7000$ (Blumberg & Vallee, 1975). Figure 5 (full curve) shows the spectrum of the enzyme modified as described above, after removal of excess reagents and products by dialysis for 24 h against four 100-mL changes of 0.1 M NaCl-0.01 M $Na_2HPO_4$, pH 7.0. The enzyme contains 1.2 groups of covalently bound reagent per catalytic subunit of enzyme and exhibits 11% of the activity of the native enzyme. Treatment of the modified enzyme with 0.5 M $NH_2OH$, pH 8.0, for 50 min, followed by extensive dialysis, resulted in deacylation of 0.9 residues of covalently bound reagent per catalytic subunit of AcChE (Figure 5, dotted curve) concomitantly with recovery of 90% of the activity of the native enzyme. Thus, inactivation and its reversal are due respectively to modification and regeneration of one residue per subunit, presumably of one particular tyrosine.

It is noteworthy that the spectrum of the modified enzyme exhibits a maximum at ~385 nm rather than at 370 nm, indicating interaction of the covalently attached chromophore with the surface of the enzyme molecule.

## Discussion

The results described above show that *N*-hydroxysuccinimide active esters of amino acid derivatives can serve as inactivators of electric eel acetylcholinesterase (Figures 1 and 4; Table I), and that esters containing certain aromatic substituents are particularly effective. Unlike other acylating agents such as organophosphates, carbamates, and sulfonyl halides, which inactivate AcChE by reacting with the active-site serine (Aldridge & Reiner, 1972), we have provided evidence that one such ester, Ac-Phe(4DnpNH)-ONSu, inactivates AcChE by reacting with another amino acid residue. The principal observation supporting this contention is the experiment demonstrating that the active ester can react with AcChE subsequent to blocking of the active-site serine with DEFP (Figure 2). The observed pH dependence of acylation by Ac-Phe(4DnpNH)-ONSu (Figure 1B), and the rate of deacylation of the modified AcChE by hydroxylamine (Figure 4) suggest that a tyrosine phenol group is the most likely candidate for the site of acylation. This assignment is supported by earlier kinetic (Bergmann et al., 1956) and chemical (Fuchs et al., 1974) evidence implicating tyrosine as important for catalytic activity of AcChE, as well as by the fact that Ac-Phe(4DnpNH)-ONSu has been shown to attack primarily tyrosine residues in thermolysin and other neutral proteases (Blumberg & Vallee, 1957; Holmquist et al., 1976).

The greatly increased potency achieved by use of active esters of various substituted aromatic derivatives of amino acids (Table I) may result from binding by hydrophobic forces or by charge transfer to the surface of the AcChE molecule. Existence of hydrophobic areas extending beyond the anionic site, or distinct from it, has been previously inferred from studies on the affinities and rates of reaction of homologous series of organophosphate inhibitors (Kabachnik et al., 1970) and on the affinities of various acridine derivatives (Steinberg et al., 1975), as well as from chemical modification experiments indicating separate binding sites for acetylcholine and indophenyl acetate (Purdie & McIvor, 1966; O'Brien, 1969; Meunier & Changeux, 1969; Fuchs et al., 1974). However, Hetnarski & O'Brien (1972, 1973) have provided indirect evidence suggesting that charge transfer, as well as hydrophobic forces, may be involved in interaction of aromatic carbamates with AcChE, and Shinitzky et al. (1973) have demonstrated formation of a charge-transfer complex between the quaternary inhibitor, *N*-methylacridinium, and a tryptophan residue in the active site of electric eel AcChE. The large red shift which we observe on reaction of Ac-Phe(4DnpNH)-ONSu with AcChE is similar to that observed by Eisen and

co-workers (Eisen & Siskind, 1964; Little & Eisen, 1967) on binding of Dnp derivatives to anti-Dnp antibodies, which they attributed to a charge-transfer complex between the Dnp ligand and a tryptophan indole ring. Detailed spectral and fluorometric studies on the AcPhe(4DnpNH)-modified AcChE should clarify the possible presence of a tryptophan residue in the site of interaction.

The effects of quaternary ligands on the rate of modification of AcChE by Ac-Phe(4DnpNH)-ONSu (Figure 3) support the view that the reagent is reacting with a region outside the anionic site, as suggested by the observation that Ac-Phe(4DnpNH) serves as a noncompetitive inhibitor. Our finding that ligands such as gallamine and propidium, which are believed to bind to peripheral anionic sites (Changeux, 1966; Belleau et al., 1970; Taylor & Lappi, 1975), partially inhibit modification, while decamethonium, which appears to span the two anionic sites (Bergmann et al., 1950; Mooser & Sigman, 1974), can almost completely block modification, suggests that the region in question is located between the two sites.

Several of the substituted amino acid *N*-hydroxysuccinimide active esters which inactivate AcChE also inactivate horse serum cholinesterase (acylcholine hydrolase; EC 3.1.1.8), active esters with bulky aromatic substituents being especially effective (Blumberg & Silman, unpublished results). These preliminary results suggest that the acylamino acid active ester derivatives will also prove useful in studies on cholinesterase.

Our results demonstrate that, by appropriate choice of conditions, it is possible to almost totally inactivate AcChE by covalent insertion of a single acyl group per catalytic subunit (Figures 4 and 5). The fact that some of the most potent active esters tested have a high absorption or intrinsic fluorescence suggests that they can be used as spectral probes of the reactive region, and that their interaction can also be studied with appropriate covalent or reversible probes of the catalytic (Mooser et al., 1972; Shinitzky et al., 1973; Berman & Taylor, 1977) or peripheral (Taylor & Lappi, 1975) anionic sites. *N*-Hydroxysuccinimide active esters of amino acid derivatives thus provide a new tool for probing the topography of the surface of the AcChE molecule.

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