Amino Acid Sequence in the Region of the Reactive Serine Residue of Eel Acetylcholinesterase†

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ABSTRACT: The amino acid sequence in the region of the reactive serine residue of electric eel (*Electrophorus electricus*) acetylcholinesterase has been determined. The enzyme was inactivated with tritium-labeled pinacolyl methylphosphonofluoridate, degraded with pepsin, papain, and Pronase, and the tritium-labeled peptides were isolated with ion exchange resins. Twelve peptides were isolated from two preparations. Their partial characterization led to the following sequence by the method of peptide overlap: Gly-Gly-Glu-MePSer-Ser-Glu-Gly-Ala-Ala-Gly.

Organophosphorus anticholinesterases are known (Schaffer *et al.*, 1954) to bind irreversibly the reactive serine residue of electric eel (*Electrophorus electricus*) acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7). In unpublished work of Shaw, it has been reported (Sanger, 1963) that the amino acid sequence in the region of the reactive serine residue in eel acetylcholinesterase is Glu-Ser-Ala. In the present work, the amino acid sequence in the region of the reactive serine residue in eel acetylcholinesterase was studied more extensively. The enzyme was inactivated with tritium-labeled Soman[1] and degraded successively with pepsin, papain, and Pronase. The tritium-labeled active-site peptides were isolated chromatographically with Dowex 50-X8 and Dowex AG1-X2 and partially characterized.

Materials and Methods

Eel acetylcholinesterase was prepared in this laboratory. It was purified by ammonium sulfate fractionation, benzyl DEAE-cellulose chromatography (Kremzner and Wilson, 1964), and partially by affinity chromatography as used to purify brain acetylcholinesterase (Yamamura *et al.*, 1973). The specific activity was 100–130 mmol of acetylcholine hydrolyzed/mg of protein per hr at pH 7.4, 25°, in 0.02 M KCl, 0.001 M Veronal, 0.02 M $MgCl_2$, 0.02% azide, 0.05% bovine serum albumin, and 0.0037 M acetylcholine. Preparations with lower specific activities have been found to contain no pseudocholinesterase on the basis of their reaction with diisopropylphosphorofluoridate. Our preparations had an activity toward acetylcholine that was 200 times their activity toward butyrylcholine. Since butyrylcholinesterase has an activity toward butyrylcholine of about 2.5 times its activity toward acetylcholine (Siakotos *et al.*, 1969), it may be concluded that at least 99.5% of the enzyme activity of our preparations is due to acetylcholinesterase.

Tritium-labeled Soman was synthesized by mixing a solution of 20 mg of [^{3}H]methylphosphonodifluoridate in 1.5 ml of benzene–toluene (3:1) with 25 μl of pinacolyl alcohol (3,3-dimethyl-2-butanol) and 28 μl of triethylamine. The reaction was carried out in a glove box under dry nitrogen at room temperature. Small aliquots were analyzed for anticholinesterase potency at intervals. After 2 hr 73% of the theoretical amount of Soman had formed. This did not increase after 3 hr. The solution of Soman was washed three times with 1.4 ml of water each time, placed over Linde type 4A molecular sieve, and stored in a freezer. The specific radioactivity of the Soman was 860 mCi/mmol. A small sample of tritium-labeled Soman was mixed with authentic, unlabeled Soman and analyzed by countercurrent distribution. The radioactivity and anticholinesterase activities were distributed essentially identically indicating a radioactive purity of at least 99%.

[^{3}H]Methylphosphonodifluoridate of specific activity 1 Ci/mmol was obtained from New England Nuclear Corporation. Unlabeled Soman was obtained from the Edgewood Arsenal Chemical Research Laboratory. Pinacolyl alcohol was obtained from Aldrich Chemical Co. and dried over type 4A molecular seive. Triethylamine was purified by reaction with unlabeled methylphosphonodifluoridate and distilled.

Soman Activity. Aliquots of Soman were diluted so as to have concentrations in the range of 5×10^{-9} M when mixed with eel acetylcholinesterase having an enzymatic activity of 0.052 mmol of acetylcholine hydrolyzed/hr per ml at pH 7.4 and 25° in 0.33 M KCl, 0.01% gleatin, and 0.007 M acetylcholine chloride. Soman and enzyme were allowed to react 1–2 min, acetylcholine was added, and the residual activity was measured with a Radiometer autotitrator.

Countercurrent Distribution. Unlabeled and labeled Soman (130:1) were distributed in a 30 tube Post countercurrent apparatus which contained equal volumes of two phases formed by shaking 3 vol of cyclohexane, 2 vol of methanol, and 1 vol of 0.01 M sodium acetate buffer, pH 5.5.

Tritium-Labeled Methylphosphonylacetylcholinesterase. Approximately 1.5 μmol of acetylcholinesterase in 1700–4000 ml of 0.005 M Veronal buffer, pH 7.5, was used in a preparation. A 2.5-fold excess of tritium-labeled Soman was added with stirring. Enzyme activity was lost rapidly, the reaction being essentially complete when checked within 10 min. The solution was dialyzed to remove excess Soman and then lyophilized. The pinacolyl group is lost subsequent to reaction of the Soman with the enzyme, due to the process of "aging" (Michel *et al.*, 1967).

Proteolytic Degradation of Tritium-Labeled Methylphosphonylacetylcholinesterase. The dialyzed and lyophilized enzyme was dissolved in 60–65 ml of water and adjusted to pH 2.0 with 2 N HCl. Twenty-five milligrams of pepsin (2 × crys-

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[1] Abbreviations used are: MePSer, methylphosphonylserine; PMePSer, pinacolylmethylphosphonylserine; Soman, pinacolylmethylphosphonofluoridate.

tallized, Worthington) and a crystal of thymol were added. The digestion was carried out at room temperature. Two or three additions of 15 mg of pepsin were made on successive days after readjusting to pH 2.0. The peptic digest was transferred to an erlenmeyer flask, the pH adjusted to 5.7 with 2 N NaOH, and the following were added: 0.35 ml of 2,3-dimercapto-1-propanol, 1 ml of papain (2 × crystallized, Worthington) suspension (30 mg), and thymol. The solution was then flushed with nitrogen and kept at room temperature. The reaction was continued for 1 week with readjustment of pH to 5.7 and two further additions of papain (0.3–0.5 ml) with 0.1 ml of 2,3-dimercapto-1-propanol each time. After lyophilization the digest was chromatographed on Dowex 50-X8 with water as the eluent. The main peak material was lyophilized and extracted with 0.2 M ammonium acetate, pH 7.54. Pronase (B grade, Calbiochem) digestion was carried out by the addition of 1.3 mg of the enzyme initially and 1 mg on the second day. Thymol was added as a preservative and the digestion was continued at 40° for several days.

Column Chromatography. Before the Pronase digestion the peptides were separated with Dowex 50-X8 (hydrogen form, 200–400 mesh, 2.2 × 200 cm) with water as the eluent. After the Pronase digestion, the peptides were rechromatographed on Dowex 50-X8 (hydrogen form, 200–400 mesh, 0.9 × 150 cm) with 0.01 N HCl as the eluent. The isolated fractions were rechromatographed finally on Dowex AG1-X2 (formate form, 200–400 mesh, 0.7 × 96 cm) with concentration gradients of pyridine formate, pH 4.5. The gradients were produced in a nine-chamber Technicon gradient apparatus with 100 ml/chamber. The molar concentrations in the first gradient were 0.0125, 0.0125, 0.025, 0.025, 0.05, 0.05, 0.1, 0.1, and 0.2. In the second gradient they were 0.1, 0.1, 0.25, 0.25, 0.25, 0.5, 0.5, 1.0, and 1.0.

Edman Degradation. Several manual Edman degradation methods were used, but the one used most is the following.[2] The dried peptide (20 nmol) in a glass-stoppered centrifuge tube was dissolved in 0.2 ml of 75% pyridine (Pierce, sequanal grade), and 10 μl of phenyl isothiocyanate (Pierce, sequanal grade) was added. The solution was flushed with nitrogen, stoppered, and kept at 50° for 1 hr, then evaporated with nitrogen, and dried *in vacuo* at 60°. Cleavage was carried out by the addition of 0.2 ml of trifluoroacetic acid (Eastman, redistilled), flushing with nitrogen and standing at room temperature for 1 hr. Trifluoroacetic acid was removed by evaporating with nitrogen and drying *in vacuo* at 60°. The peptide was dissolved in 0.2 ml of water and was extracted three times with 2 ml of butyl acetate (Eastman, redistilled) each time. The thiazolinones were usually hydrolyzed with HI (Smithies *et al.*, 1971), HCl (Van Orden and Carpenter, 1964), or NaOH (Africa and Carpenter, 1966) and the amino acids determined with the amino acid analyzer. The HI hydrolysis was carried out with 0.5 ml of 57% HI (Fisher) in an O-ring joint tube (Schaffer *et al.*, 1966). The tube was evacuated, flushed three times with nitrogen, evacuated, and heated at 127° for 20 hr. In one experiment the thiazolinone was hydrolyzed to the phenylthiohydantoin by heating in 1 N HCl at 80° for 10 min (Ilse and Edman, 1963). The resulting phenylthiohydantoin was identified by paper chromatography (Sjöquist, 1960).

C-Terminal Residue. C-Terminal residues were determined, in most cases, by hydrazinolysis (Fraenkel-Conrat and Tsung, 1967). A minimum of 15 nmol of peptide was taken for each analysis. The reaction was carried out in an O-ring joint tube with 0.5 ml of redistilled 95% hydrazine (Eastman) for 24 hr at 80°. After the reaction, the hydrazine was removed by distillation *in vacuo* directly from the O-ring tube and the residue was analyzed on the amino acid analyzer.

A modification (method C) (Holcomb *et al.*, 1968) of the selective tritiation method of Matsuo was also employed. From 5 to 20 nmol of peptide was used for each analysis. After reaction and removal of the excess ^3H_2O by repeated rotary evaporation, 50 nmol of the standard amino acid mixture was added to the tritiated amino acid. This was chromatographed on the neutral plus acidic column of the amino acid analyzer without ninhydrin. The column effluent was collected in a fraction collector at a rate of 1 min/tube. The tritiated amino acid was located by selecting arbitrary fractions in the regions where amino acids were expected to be found, and counting 90% of the volume collected. This gave an indication of which amino acid was tritiated. To confirm this indication, the remaining 10% of the tritiated fraction was added to 5 nmol of a standard amino acid mixture and the resulting mixture analyzed with the amino acid analyzer, using ninhydrin. The tritiated amino acid was that one which was present in the amount of 10 nmol, while all others were present in 5-nmol quantities.

Amino acid analysis was done with a Spinco amino acid analyzer after hydrolysis of the peptide with constant boiling HCl at 110° for 22 hr. The amounts of serine and MePSer[1] were corrected by factors of 7.2 and 22.3%, respectively, to account for their destruction during acid hydrolysis. The latter factor was actually determined for phosphorylserine (Schaffer and Balakir, 1966). Tryptophan was determined by measuring fluorescence at 342 nm, with excitation at 290 nm (Teale, 1960). Glutamine was determined by analysis with the amino acid analyzer following complete hydrolysis of the peptide with aminopeptidase M (Röhm and Haas, Henley and Co., New York, N. Y.). Glutamine normally cannot be separated from serine, but when run with the pH 3.25 sodium citrate buffer alone at 30°, it is separated sufficiently to permit detection.[3] The flow rates of the buffer alone and buffer with ninhydrin were 50 and 75 ml/hr, respectively. The aminopeptidase M digestion was carried out by adding 0.2 ml of 0.1 M Tris-HCl buffer, pH 8.18, to the lyophilized peptide. It was preserved with toluene and incubated for 30 hr at room temperature.

Tritium was determined using 10 ml of scintillation solution containing 5.5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 200 ml of Triton X-100 (Packard), and 800 ml of toluene. Measurements were made with a Packard Tri-Carb liquid scintillation counter, Model 314 EX. A correction factor for quenching was obtained by the addition of standard tritiated toluene (Packard) to the unknown. The tritium content was calculated from the specific activity of the Soman.

Results

Chromatography on Dowex 50-X8 of the pepsin–papain digest of methylphosphonylacetylcholinesterase gave one main tritium-containing fraction in a 75% yield. A small fraction preceding this accounted for another 10% of the tritium. Dowex 50-X8 chromatography of the Pronase digest of the main fraction gave three radioactive fractions (Figure 1). These three fractions accounted for 91% of the largest

[2] Cebra, J. J., personal communication.

[3] Padden, C., personal communication.

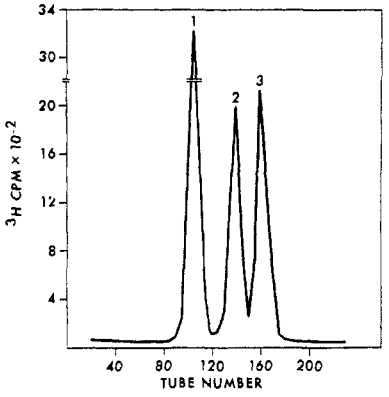

FIGURE 1: Dowex 50-X8 chromatogram of proteolytic digest of methylphosphonylacetylcholinesterase. Digest had preliminary separation with Dowex 50-X8 and a water eluent: column 0.9 × 150 cm; 0.01 N HCl eluent; flow rate 18 ml/hr; fraction size 3.6 ml.

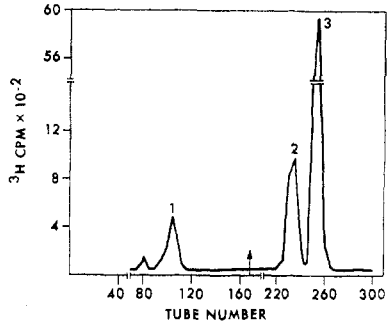

FIGURE 2: Dowex AG1-X2 chromatogram of second fraction in Figure 1: column 0.7 × 96 cm; pyridine formate; first gradient 0.0125–0.2 M, flow rate 29 ml/hr, fraction size 3.6 ml; second gradient at vertical arrow 0.1–1 M, flow rate 20 ml/hr, fraction size 2.5 ml.

pepsin–papain fraction. Each of these fractions was rechromatographed, in turn, on the anion exchanger, Dowex AG1-X2. Fraction 1 (Figure 1) yielded five subfractions accounting for 72% of its tritium. Fraction 2 (Figure 1) gave three subfractions accounting for 90% of its tritium (Figure 2). Fraction 3 (Figure 1) gave two subfractions accounting for 84% of its tritium. The amino acid compositions of the final subfractions that were partially characterized are given in Table I.

A pool of large sized subfractions was hydrolyzed with 12 N HCl at 37° to obtain smaller peptides. Two of the peptides in Table I, 41-1 and 50-4, were isolated in this way. Peptide 41-1 was obtained from a 5-day HCl hydrolysis. After removal of the HCl with a rotary evaporator, the hydrolysate was chromatographed on Dowex 50-X8 with 0.01 N HCl as the eluent. The largest fraction was rechromatographed on Dowex AG1-X2 to yield 41-1. Peptide 50-4 was obtained from a 14-day HCl hydrolysis of pooled subfractions. This hydrolysate was chromatographed on Dowex 50-X8 with water as the eluent to yield four fractions. The fourth fraction was 50-4.

Two of the peptides shown in Table I, 33-2 and 35-2, were isolated from peptides that had undergone two Edman degradations. Peptide 33-2 is from a Dowex 50-X8–0.01 N HCl chromatography of a peptide not shown in Table I. Peptide 35-2 was the residual peptide after two Edman degradations of peptide 22-4. It was purified by chromatography, successively on Dowex 50-X8 with 0.01 N HCl, Dowex AG1-X2 with pyridine formate, and again on Dowex 50-X8 with 0.01 N HCl.

TABLE I: Amino Acid Composition of Serine Active-Site Peptides from Eel Acetylcholinesterase.

	Molar Ratios of Amino Acids					
Peptide	Asp[a]	Ser	Glu	Gly	Ala	Ile[a]
33-2	0.39	1.1	1.0	1.7		
41-1	0.27	2.0	2.0	0.32	0.12	
47-3		1.7	2.0			
23-1	0.31	1.8	2.2	2.0	0.23	0.37
45-2		1.8	2.3	2.0		
47-2	0.26	1.9	2.2	2.0	1.8	
50-4	0.30	2.0	0.56	0.75	2.2	
35-2	0.50	1.9	0.83	2.2	2.0	
45-1		1.9	2.2	2.7	1.0[b]	
26-2	0.23	1.9	2.2	3.1	2.0	
22-4		1.2[b]	2.0	3.2	1.9	
44-5		1.8	1.8	4.0	2.1	

[a] Impurities (see text). [b] Low value (see text).

The peptides listed in Table I represent those that were most completely characterized. The methylphosphonyl content of these peptides from tritium analysis was in a molar ratio close to one. Two peptides, 47-3 and 45-1, are believed to be mixtures, but with one main component. Most of the peptides contained small amounts of impurities, particularly aspartic acid. Since such impurities only occurred in trace quantities in peptides 22-4 and 44-5, which practically encompass the entire sequence, it is felt that their presence in the other peptides does not detract from the validity of the results. Tryptophan and basic amino acids are absent in peptide 22-4. Basic amino acids were also absent in peptide 44-5 which was not further characterized. Glutamic acid occurs as such and not as glutamine in peptides 22-4 and 47-3, as determined by amino acid analysis after hydrolysis with aminopeptidase M. The serine analysis of peptide 22-4 is erroneously low. This is evident since peptide 35-2, which is the residual peptide after two Edman degradations of peptide 22-4, had a molar ratio for serine of 1.9 in two analyses. The alanine value of peptide 45-1 is also low. This molar ratio was 1.3 in the analysis of its first Edman residue using twice the amount for analysis.

A summary of the Edman degradations, hydrazinolyses, and Matsuo C-terminal findings on these peptides is given in Table II. The entire sequence of ten amino acids is determined by the method of peptide overlap. The first two Edman degradations of peptide 33-2 fix the first two residues as glycine. However, no other peptide was isolated with glycine in position 1. The glycine in position 2 was confirmed by Edman degradations on six other peptides. Position 3 was shown to be glutamic acid by Edman degradations on four different peptides and is N terminal in peptide 47-3. Position 4 is MePSer, the tritium-labeled residue. This is evident from peptide 33-2 which contained only one serine and, therefore, had to be present as MePSer. Its position would have to be C terminal to be consistent with the structures of the other peptides. Also, the residual peptide after the third Edman degradation of peptide 45-2 contained no tritium. Therefore, the third residue must have been MePSer.

It has been found (Sanger, 1963) that phosphate splits off when serine phosphate undergoes an Edman degradation, and our evidence indicates that methylphosphonic acid is likewise liberated from MePSer and remains with the residual peptide

TABLE II: Amino Acid Sequence of Serine Active-Site Peptides from Eel Acetylcholinesterase.

Peptide	Sequence
33-2	Gly[a]-Gly-(Glu,MePSer)
41-1	(Glu,MePSer,Ser)- Glu[b]
47-3A	Glu-MePSer-Ser[c]
47-3B[d]	Glu-MePSer-(Ser, Glu)
23-1	Gly-Glu (MePSer, Ser, Glu, Gly)
45-2	Gly- Glu- MePSer,(Ser, Glu)-Gly[b,c]
47-2	Gly-(Glu, MePSer, Ser, Glu, Gly,Ala)-Ala[c]
50-4	MePSer- Ser- (Glu,Gly,Ala)-Ala[c]
35-2	MePSer- Ser- (Glu,Gly,Ala,Ala, Gly)
45-1A	Gly-(Glu, MePSer, Ser, Glu, Gly)
45-1B[d]	Gly-(Glu, MePSer, Ser, Glu, Gly, Ala,Ala)-Gly[c]
26-2	Gly-(Glu, MePSer, Ser, Glu, Gly, Ala,Ala)-Gly[b]
22-4	Gly- Glu-(MePSer, Ser, Glu, Gly,Ala,Ala)-Gly[c]
44-5	(Gly,Gly, Glu,MePSer, Ser, Glu, Gly,Ala,Ala, Gly)
	Composite Sequence
	Gly-Gly- Glu-MePSer- Ser- Glu- Gly-Ala-Ala- Gly
	1 2 3 4 5 6 7 8 9 10

[a] →, Edman degradation. [b] Matsuo C terminal. [c] Hydrazinolysis. [d] Main component of assumed mixture.

TABLE III: Partial Amino Acid Sequences Near Reactive Serine Residue in Serine Enzymes.

Enzyme	Sequence[m]	Ref
Eel acetylcholinesterase	Gly- Gly-Glu-*Ser*- Ser- Glu-Gly-Ala- Ala-Gly	*a*
Pseudocholinesterase	Phe- Gly-Glu-*Ser*- Ala- Gly	*b*
Liver aliesterase	Gly-Glu-*Ser*- Ala- Gly-Gly	*c*
Chymotrypsin	Gly-Asp-*Ser*- Gly- Gly-Pro-Leu	*d*
Trypsin	Gly- Gly-Asp-*Ser*- Gly- Pro-Val- Cys- Ser-Gly	*e*
Alkaline phosphatase (*E. coli*)	Val- Thr-Asp-*Ser*- Ala- Ala- Ser- Ala	*f*
Thrombin	Gly-Asp-*Ser*- Gly	*g*
Elastase	Gly-Asp-*Ser*- Gly	*h*
Subtilisin	Thr-*Ser*- Met-Ala	*i*
Phosphoglucomutase	Ala-*Ser*-His-Asp- Gly-Glu-*Ser*- Ala- Gly-Leu-Asp-Leu	*j*
Phosphorylase *a*	Lys-GluNH$_2$- Ile-*Ser*- Val- Arg	*k*
Arthrobacter protease	Ser-*Ser*- Gly	*l*

[a] This paper. [b] Jansz *et al.*, 1959b. [c] Jansz *et al.*, 1959a. [d] Schaffer *et al.*, 1957; Oosterbaan *et al.*, 1958. [e] Schaffer *et al.*, 1958; Dixon *et al.*, 1958. [f] Schwartz *et al.*, 1963. [g] Gladner and Laki, 1958. [h] Hartley *et al.*, 1959. [i] Sanger and Shaw, 1960. [j] Harshman and Najjar, 1965. [k] Fischer *et al.*, 1959. [l] Wählby, 1968. [m] Reactive serine residue is in italics.

during extraction of the thiazolinone. In the second Edman degradation of peptide 47-3 serine phenylthiohydantoin was detected weakly by paper chromatography. However, the conversion of serine thiazolinone to serine phenylthiohydantoin is known to be only about 20% (Ilse and Edman, 1963). The serine thiazolinones from methylphosphonylserine of peptides 50-4 and 35-2 were hydrolyzed with HI and HCl, respectively. With peptide 50-4 the finding of a large amount of alanine indicated that it was mostly derived from serine thiazolinone (Smithies *et al.*, 1971). With peptide 35-2, no serine was found, the result that would be expected from the known instability of serine thiazolinone to HCl.

Position 5 is serine. Both in peptides 50-4 and 35-2 the HI hydrolysis of the second Edman thioazolinones gave mainly alanine, the known product of HI hydrolysis of serine thiazolinone. A more direct piece of evidence for the MePSer-Ser sequence was obtained due to the observation of an anomalous result with aminopeptidase M. It was noticed that the serine was completely absent in the aminopeptidase M digest of peptides 47-3 and 22-4. Since these peptides contained both MePSer and serine, it was expected that serine should appear in the analysis. An acidic component appeared at the front in the amino acid analyses of the aminopeptidase digest of these peptides. This component was isolated by chromatography on Dowex AG1-X2 where it appeared in the first gradient. On amino acid analysis it contained only serine in a molar ratio of 1.8:1 to the methylphosphonyl group (from tritium).

Position 6 was inferred to be glutamic acid from its C-terminal position in peptide 41-1 by tritium incorporation. Glutamic acid is not detected by hydrazinolysis and no C-

terminal group is found by hydrazinolysis of peptide 47-3. The presence of a small amount of serine after hydrazinolysis of 47-3 is the basis for considering it a mixture. Position 7 is clearly glycine from the results with peptides 23-1 and 45-2, particularly the latter. Position 8 was inferred to be alanine by default. In both peptides 47-2 and 50-4 alanine was determined to be C terminal by hydrazinolysis. Both peptides contain two alanines. There is no position for the other alanine which is consistent with the data besides position 8. Position 9 was found to be alanine from evidence with peptides 47-2 and 50-4. Position 10 was determined to be glycine due to its C-terminal position in peptides 45-1, 26-2, and 22-4.

Discussion

Since the discovery of the unique reactivity of a serine residue in the active site of chymotrypsin (Schaffer *et al.*, 1953), a considerable number of other serine enzymes have been shown to possess a reactive serine. The partial amino acid sequences in the region of the reactive serine residue of some of these enzymes are given in Table III. In most of these enzymes, either glutamic acid or aspartic acid precedes the reactive serine residue and either alanine or glycine follows it. In the *Arthrobacter* protease, a serine residue is also adjacent to the reactive serine residue, but in contrast to eel acetylcholinesterase it precedes, rather than follows, the reactive serine residue.

The preliminary report of Shaw (Sanger, 1963) of the sequence Glu-*Ser*-Ala around the reactive serine residue in eel acetylcholinesterase is in error.

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