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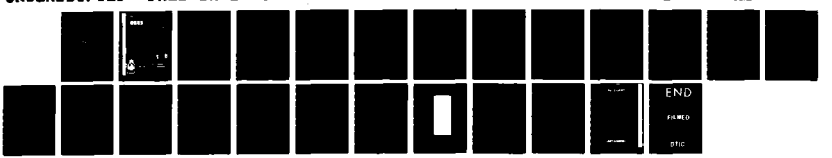

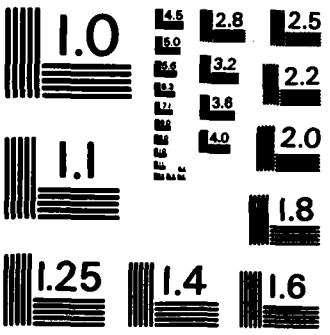

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PARTIAL PURIFICATION AND PROPERTIES OF BOVINE AND OVINE SPINAL CORD ACETYLCHOLINESTERASES (U)

by

R.G. Smyth and A.R. Bhatti

Project No. 13E20

August 1985

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ABSTRACT

Methods were developed for the solubilization of spinal cord acetylcholinesterases from cows and sheep. Several characteristics of this preparation are reported, including the results of attempts at purification. A method for bovine enzyme achieved an 80-fold purification from the spinal cord homogenate. Comparison studies with similar enzymes from other species and tissues may help elucidate the mechanism of organophosphate poisoning.

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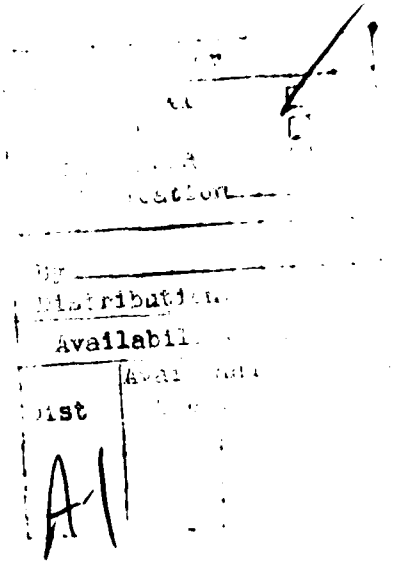

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INTRODUCTION

The biochemical mechanism of toxicity of the organophosphorus insecticides and nerve agents has been attributed to the inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) in central (1,2) and peripheral (3-5) systems. However, the inhibition of the enzyme in most tissues examined does not correlate well with the severity of symptoms. AChE from other sources may have different properties. A close relationship between the degree of inhibition and the toxic effects may implicate the tissue of origin as an important target of the organophosphates.

The properties of the spinal cord enzyme have not been examined in any detail, although significant levels are present (6). In this communication we describe the partial purification and characterization of AChE from spinal cords of cows and sheep.

MATERIALS AND METHODS

Bovine and ovine spinal cords were donated by Lakeside Packers, Brooks, Alta; bovine brain was a gift from Sherwood Packers, Medicine Hat, Alta. The tissues were frozen in liquid N_2 within 10 min after the death of the animal, and kept frozen until homogenization. In the frozen state, no loss of enzyme activity or change in other physical characteristics was observed after storage for up to two years.

N-methylnicotinic acid iodide was provided from synthesis by Dr. P.A. Lockwood, Chemistry Section, DRES. Affi-Gel 102 and EDAC* are products of Bio-Rad Laboratories, Richmond, CA. Coomassie Brilliant Blue G-250 was obtained from Fluka, Switzerland. Acetylthiocholine chloride, DTNB**, tetracaine, Triton X-100, acetylcholinesterase (type V-S, electric eel), acrylamide, and N,N'-methylenebisacrylamide were all purchased from Sigma Chemical Co., St. Louis, Mo. The disodium salt of ethylenediamine tetraacetic acid (EDTA) was obtained from J.T. Baker Chemical Co., Phillipsburg, N.J.

Tissue Preparation

Tissues were homogenized at 10% w/v in a Teflon-glass homogenizer with 10 mM Tris buffer containing 5 mM EDTA, 0.1 mM tetracaine, and 1% Triton X-100, adjusted to pH 7.0 at 22°C with HCl. The homogenates were left overnight at 4°C and centrifuged at 30000xg for 60 min with a JA-21 rotor in a Beckman J2-21M centrifuge. The supernatants were dialyzed overnight against 125 volumes of 1 mM K-phosphate buffer pH 8.0. Aliquots were taken for analysis of protein (7) and AChE activity (8), and the remaining samples

*EDAC: N-ethyl-N'-dimethylaminopropylcarbodiimide
**DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)

were centrifuged at 200,000xg for 30 min with a SW-41 Ti rotor. Aliquots of the supernatants were tested for recovery and purity of enzyme by electrophoresis. The rest of each supernatant was lyophilized for further purification by affinity chromatography.

Electrophoresis

Samples (50 µl) were run on gels containing 7.0% acrylamide and 0.2% bisacrylamide, polymerized with 1.2 mg/ml ammonium persulfate and 140 µg/ml TEMED*, in 89 mM Tris-borate-EDTA buffer pH 8.28. The gels were run for 1 hr (2 mA/gel) in a buffer of 18 mM Tris-borate-EDTA pH 8.28. Protein was detected with Coomassie blue. AChE activity was visualized on paired gels with a modification of the reagents used in the spectrophotometric procedure of Ellman (8). Immediately after running, the gels were soaked for 10 min in a mixture of 6 ml 0.1 M K-phosphate buffer pH 8.0, 200 µl 10 mM DTNB, and 100 µl 75 mM acetylthiocholine chloride. The yellow bands which developed were preserved by photography within 1 hr due to subsequent fading of the colour.

Affinity Chromatography

The general coupling procedure outlined by Merrill and McCormick (9) was followed. Affi-Gel 102 (33 ml) was mixed with 1.39 g of N-methylnicotinic acid iodide in 20 ml H_2O, and the pH was adjusted to 5.0 with 1N HCl. EDAC (2.40 g) was added and the pH was maintained at 5.0 for 1 hr. The mixture was incubated at 22°C overnight with little further change in pH. The coupled gel was washed successively with H_2O, saturated NaCl, H_2O, 0.1 N HCl, H_2O, 1 M K-phosphate buffer pH 7.0, H_2O, 20% EtOH, 50% EtOH, 80% EtOH, absolute EtOH: benzyl alcohol 1:1 w/v, 80% EtOH, 50% EtOH, 20% EtOH, H_2O, and

*TEMED: N,N,N',N'-tetramethylethylenediamine

20 mM K-phosphate buffer pH 8.0. The resulting gel (~30 ml) was used to form a 17 x 1.5 cm column.

Lyophilized samples from the ultracentrifuged extracts were dissolved in 1/20 of their original volume of H_2O. 0.5-1.0 ml was placed on the column and eluted with 50 ml of 20 mM K-phosphate buffer pH 8.0, followed by 2 M KCl in the same buffer. Fractions (2.0 ml) were collected and assayed for protein (7) and AChE activity (8). The column could be regenerated after use by washing with saturated KCl in buffer, then the phosphate buffer alone to remove salt. Multiple runs did not appreciably degrade the characteristics of the column.

RESULTS

Several homogenization media based on a Tris-HCl buffer system, variously containing NaCl, EDTA, and Triton X-100, have been suggested (6, 10-12) for extraction of AChE from the central nervous system. We examined the relative merits of these constituents for the solubilization of spinal cord AChE. Table I illustrates that a detergent such as Triton X-100 is essential for extraction of appreciable enzyme and EDTA likewise improves the AChE solubility. However, the inclusion of NaCl solubilizes other proteins from this tissue: running NaCl extracts through electrophoresis revealed a large number of protein bands. The standard medium therefore included EDTA and Triton, but omitted NaCl.

A sucrose medium, originally recommended for both brain and peripheral tissues (13-15), was less effective in extracting the enzyme from spinal cord (20-50% of the activity obtained with Tris-based media). However, at 0.1 mM the tetracaine used in these systems approximately doubled AChE recovery from the Tris medium. It was therefore included in the Tris-EDTA-Triton mixture as a component of the standard homogenization medium.

An attempt was made to recover further enzyme activity from the precipitate of centrifuged samples. As seen from Table II, a resuspension of the pellet permits the extraction of another 62% of the activity in the original homogenate. Further resuspensions lead to a decline in specific activity of the extracts. It was found that most of the activity extractable by this method could be obtained from the original homogenate by preserving the sample in the refrigerator (4°C) overnight before centrifugation. This method was preferred over the manipulations involved in sequential pellet resuspension.

The thermolability of this crude extract was examined by exposure to different temperatures for 2 min. Results for the bovine enzyme are illustrated in Figure 1; similar behavior is observed from ovine AChE. No temperature effect was observed up to 37°C in this experiment. Progressive loss of enzyme activity was noted above 37°C with complete inactivation at 60°C. Solubilized enzyme samples were stored at 4°C for several days with no significant change in activity (<10%).

Partial purification of the enzyme extract was achieved by passing the crude sample through an affinity column. The results are presented in Figure 2. Approximately 20% of the total AChE activity appeared in the effluent, but the remainder adhered to the column until released by an increase in ionic strength (2 M KCl). A 9-fold increase in specific activity was thereby obtained from the bovine enzyme, resulting in a cumulative purification factor of over 80-fold from the spinal cord homogenate. However, recovery of activity from the ovine extract was less successful under the same conditions.

Figure 3 illustrates the sensitivity of the newly developed AChE visualization procedure for electrophoresis gels, based on 32 mU (50 µl) AChE per gel. The gel stained rapidly (5-10 min) and reproducibly: AChE is seen

to have a R_f ~0.1 in this system. In some gels it appeared that two closely-spaced bands of AChE activity were present; a separation of these species may be possible with further purification.

DISCUSSION

Apart from an analysis of molecular forms in the superior cervical ganglion (6), spinal cord AChE has received little attention. The 80-fold purification achieved for bovine spinal cord AChE from homogenate provides a suitable sample for the examination of the effects of nerve agents and other compounds.

It may be possible to achieve substantially improved purification by using a linear ionic strength gradient with the affinity column. Adamson (15) obtained a purification factor of 35x as a result of passing mouse brain extract through a similar affinity column with gradient elution. However, the methods and product would be species-specific, as evidenced by the poor recovery of ovine-derived enzyme with the basic procedure used here. The purification of ovine AChE was generally less successful than that of the bovine enzyme upon which the procedures were developed (Table III). Different species may require individual modification of the standard conditions. If maximum specific activity is desired, bovine spinal cord would be the preferred source using the purification sequence developed here.

The development of a new method for detection of AChE activity in acrylamide gels is rapid and suitable for the identification of protein bands containing AChE. Under the conditions described here, 10 mU of AChE activity can be detected. Gels run in parallel and treated with a protein stain gave three protein bands, suggesting that enzyme purification on the affinity

column was still incomplete.

Future plans include further purification of these spinal cord enzymes and their testing against specific acetylcholinesterase inhibitors and nerve agents. Inhibition of spinal cord AChE will be correlated with the toxicity of these compounds in the living animal.

CONCLUSIONS

Triton X-100 and EDTA aid the extraction of acetylcholinesterase from bovine spinal cord. The use of salt is contraindicated in purification procedures, since large quantities of other proteins are also solubilized.

Most of the enzyme activity in Triton-containing homogenates is soluble, but affinity chromatography of these extracts gives species-specific results.

The crude enzyme is fairly resistant to heat up to 50°C, but activity is quickly destroyed at 60°C.

Quick visualization of acetylcholinesterase in electrophoresis gels is possible with the reagents used in spectrophotometric determination, although fading of the stain precludes storage.

REFERENCES

1. E. Meeter and O.L. Wolthuis. The spontaneous recovery of respiration and neuromuscular transmission in the rat after anticholinesterase poisoning. Eur. J. Pharmacol., 2, 377-386 (1968).

2. G.K. Adams, H.I. Yamamura, and J.F. O'Leary. Recovery of central respiratory function following acetylcholinesterase intoxication. Eur. J. Pharmacol., 38, 101-112 (1976).

3. J. Clement. Toxicology and pharmacology of bispyridinium oximes - insight into the mechanism of action vs soman poisoning in vivo. Fund. Appl. Toxicol., 1, 193-202 (1981).

4. J. Clement. HI-6: Reactivation of central and peripheral acetylcholinesterase following inhibition by soman, sarin and tabun in vivo in the rat. Biochem. Pharmacol., 31, 1283-1287 (1982).

5. J.G. Clement and P.A. Lockwood. HI-6: an oxime which is an effective antidote of soman poisoning: a structure-activity study. Toxicol. Appl. Pharmacol., 64, 140-146 (1982).

6. J. Grassi, M. Vigny, and J. Massoulié. Molecular forms of acetylcholinesterase in bovine caudate nucleus and superior cervical ganglion: solubility properties and hydrophobic character. J. Neurochem., 38, 457-469 (1982).

7. M.M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254 (1976).

8. G.L. Ellman, K.D. Courtney, V. Andres Jr., and R.M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol., 7, 88-95 (1961).

9. A. H. Merrill and D.B. McCormick. Flavin affinity chromatography: General methods for purification of proteins that bind riboflavin. Anal. Biochem., 89, 87-102 (1978).

10. F. Rieger and M. Vigny. Solubilization and physiochemical characterization of rat brain acetylcholinesterase: development and maturation of its molecular forms. J. Neurochem., 27, 121-129 (1976).

11. M. Vigny, S. Bon, J. Massoulié, and V. Gisiger. The subunit structure of mammalian acetylcholinesterase: catalytic subunits, dissociating effect of proteolysis and disulfide reduction of the polymeric forms. J. Neurochem., 33, 559-565 (1979).

12. L.H. Silver and D.J. Prescott. Aggregation properties of the acetylcholinesterase from the central nervous system of Manduca sexta. J. Neurochem., 38, 1709-1718 (1982).

13. E.G. Hollunger and B.H. Niklasson. The release and molecular state of mammalian brain acetylcholinesterase. J. Neurochem., 20, 821-826 (1973).

14. E.D. Adamson, S.E. Ayers, Z.A. Deussen, and C.F. Graham. Analysis of the forms of acetylcholinesterase from adult mouse brain. Biochem. J., 147, 205-214 (1975).

15. E.D. Adamson. Acetylcholinesterase in mouse brain, erythrocytes, and muscle. J. Neurochem., 28, 605-615 (1977).

TABLE I

EXTRACTION OF PROTEIN AND ACETYLCHOLINESTERASE FROM BOVINE SPINAL CORD AS A FUNCTION OF THE HOMOGENIZATION MEDIUM

Media Constituents			Tissue Extracts		
Triton X-100 (1%)	NaCl (1M)	EDTA-Na_2 (5mM)	Protein (mg/ml)	AChE (mU/ml)	Specific Activity (mU/mg)
+	+	+	1.18	172	146
+	+	-	1.35	147	103
+	-	+	0.42	153	364
+	-	-	0.95	125	132
-	+	+	0.30	43	143
-	+	-	0.42	34	81
-	-	-	0.23	51	227

All homogenization media were based on 10 mM Tris-HCl buffer pH 7.0. Homogenized samples (10% w/v) were centrifuged immediately at 30000xg for 1 hr. The standard procedure, adopted later, included 0.1 mM tetracaine in the homogenization medium and an incubation of the homogenate at 4°C overnight before centrifugation.

TABLE II

RECOVERY OF PROTEIN AND ACETYLCHOLINESTERASE ACTIVITY FROM SUCCESSIVE EXTRACTIONS OF BOVINE SPINAL CORD

Supernatant Fraction	Protein (mg/ml)	Acetylcholinesterase (mU/ml)	Specific Activity (mU/mg)
S_1	0.82	305	372
S_2	0.52	190	365
S_3	0.50	118	236

Spinal cord was homogenized in the standard medium (10% in 10 mM Tris-HCl buffer pH 7.0 containing 5 mM EDTA-Na_2, 0.1 mM tetracaine, and 1% Triton X-100), and centrifuged at 30000xg for 60 min. The supernatant (S_1) was assayed for protein and acetylcholinesterase activity while the precipitate was resuspended in the original volume of homogenization medium. The centrifugation and resuspension process was repeated to obtain two further supernatants (S_2, S_3) which were likewise assayed for protein and AChE.

TABLE III

ISOLATION OF SPINAL CORD ACETYLCHOLINESTERASES

	Bovine					Ovine				
	Protein (mg)	AChE (U)	Specific Activity (mU/mg)	Purification Factor	Yield (%)	Protein (mg)	AChE (U)	Specific Activity (mU/mg)	Purification Factor	Yield (%)
Homogenate	147	8.29	56	1.00	100	139	6.22	45	1.00	100
Supernatant (30000xg)	13.3	4.57	344	6.14	55	16.1	4.39	273	6.07	71
Supernatant (200000xg)	8.5	4.28	504	9.00	52	8.8	2.82	320	7.11	45
Column eluate	0.47	2.13	4532	80.93	26	0.44	0.34	772	17.17	5

Frozen spinal cords were homogenized in Tris buffer (standard medium, see Table II), centrifuged at 30000xg for 60 min, ultracentrifuged at 200000xg for 30 min, and 0.5 ml chromatographed on an N-methylnicotinate affinity column. Acetylcholinesterase activity and protein were assayed after each major step of the purification.

Legends to Figures

Figure 1: Bovine spinal cord was homogenized 10% w/v in the standard medium (Table II), preserved at 4°C overnight, and centrifuged at 30000xg for 60 min. Aliquots of the supernatant were exposed to temperatures from 37-60°C in a water bath for 2 min. The enzyme activity remaining was then assayed as described in "Methods". The control aliquot maintained at 0°C had 320 mU/ml of AChE activity.

Figure 2: Lyophilized enzyme samples from bovine spinal cord were dissolved in H_2O to a final concentration of 20 mM K-phosphate buffer pH 7.0, and 0.5 ml placed on a 17 x 1.5 cm Affi-Gel 102- N-methyl-nicotinate chromatography column. 50 ml of 20 mM K-phosphate buffer pH 8.0 was run through the column followed by 100 ml of 2 M KCl in the same buffer. 2-ml fractions were collected: KCl was first detected in the eluate in fraction #55. Protein and AChE were determined for each fraction. The fractions depicted are the only ones which contained detectable AChE.

Figure 3: 50 µl samples from fraction 59 (Fig 2), containing the highest AChE activity from bovine spinal cord, were electrophoresed on polyacrylamide gels. The Ellman reaction was modified to visualize acetylcholinesterase activity (see "Methods"). Yellow bands appeared at R_f ~0.1, but faded rapidly (<1 hr), requiring photography to preserve a record of AChE detection.

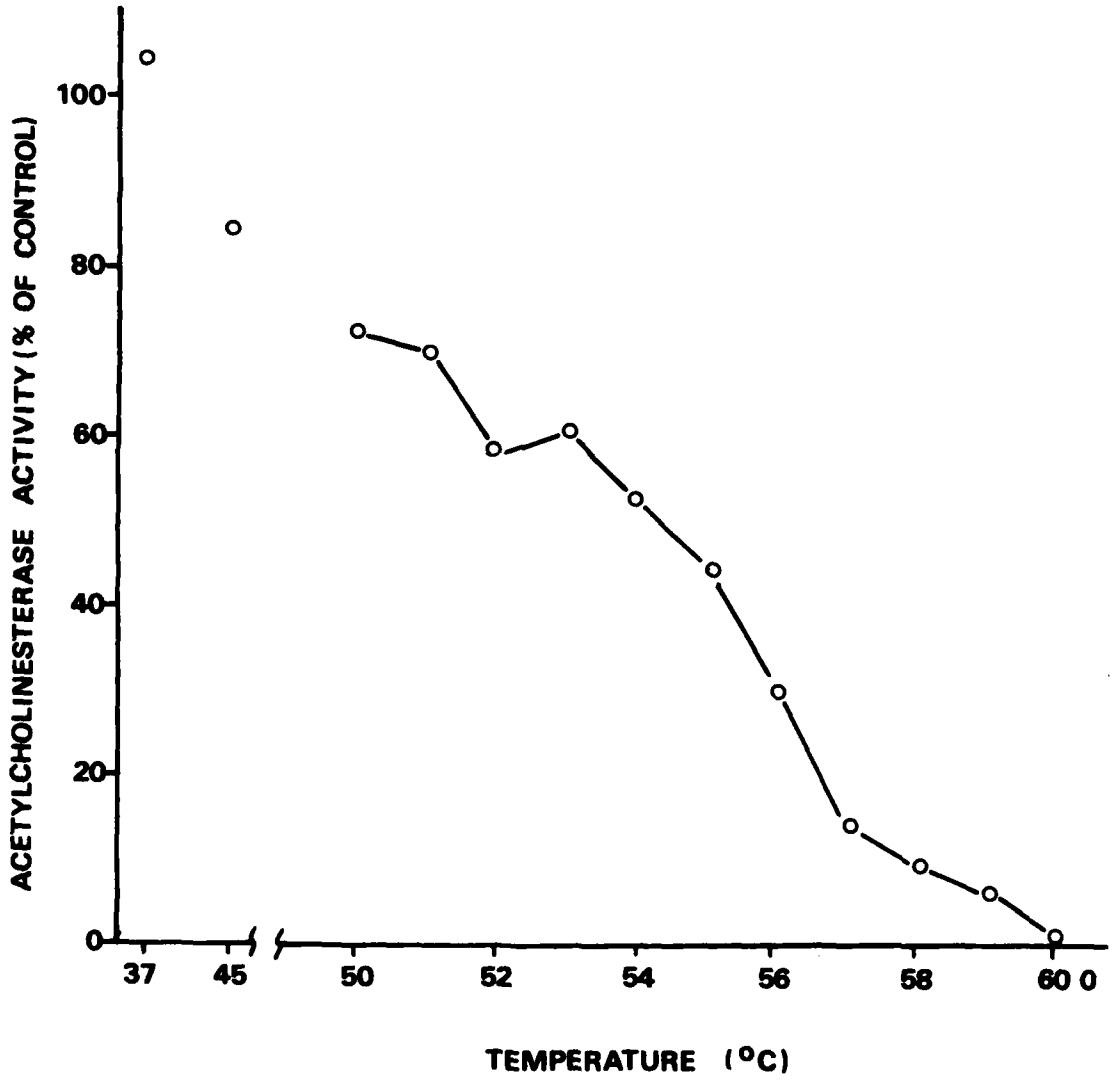

FIGURE 1: TEMPERATURE LABILITY OF BOVINE SPINAL CORD ACETYLCHOLINESTERASE

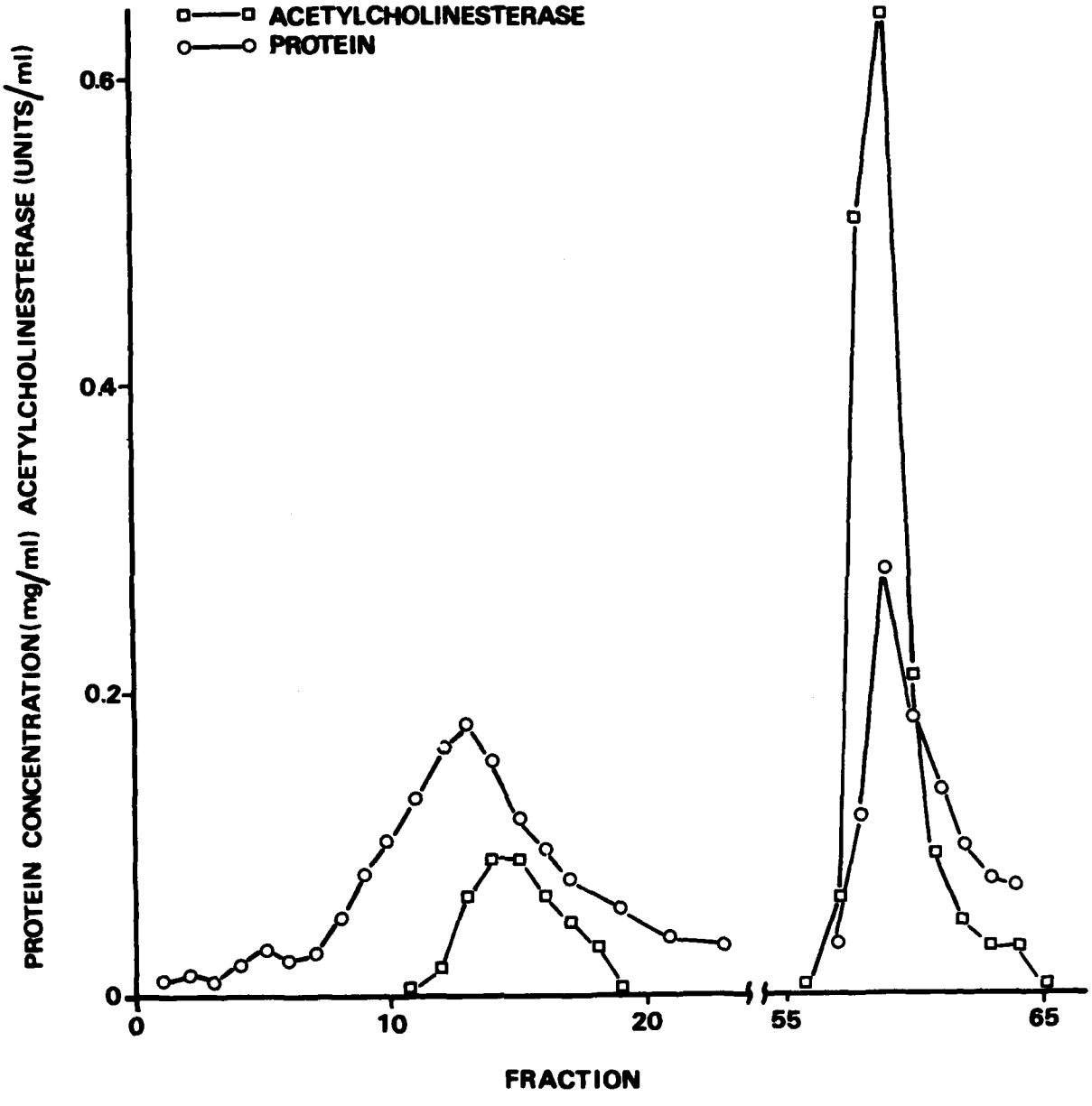

FIGURE 2: AFFINITY CHROMATOGRAPHY OF BOVINE SPINAL CORD ACETYLCHOLINESTERASE

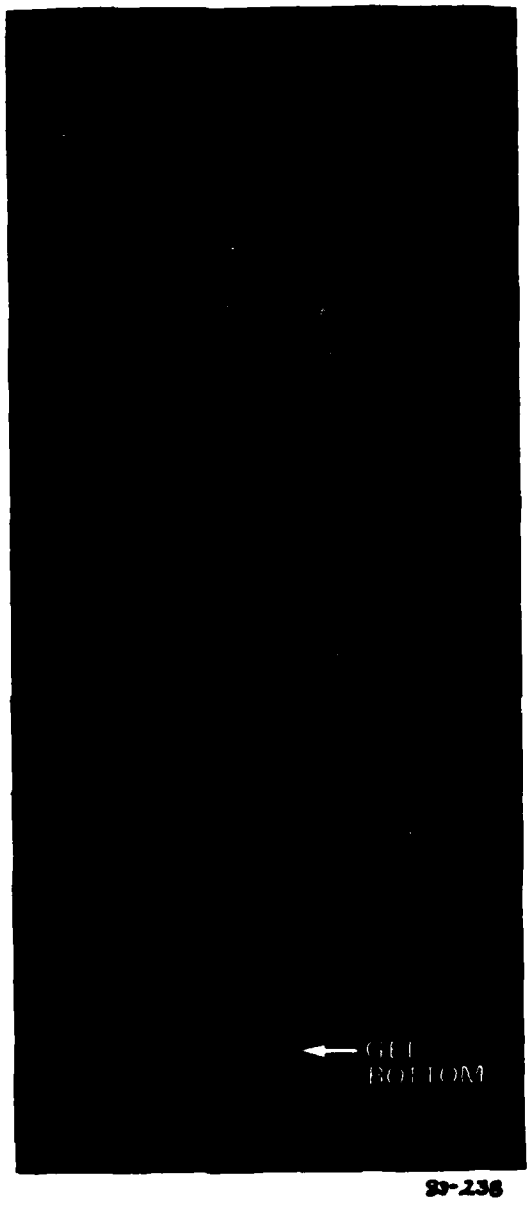

FIGURE 3: THE VISUALIZATION OF ACETYLCHOLINESTERASE ON POLYACRYLAMIDE ELECTROPHORESIS

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13. ABSTRACT

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