Raftery, M. A., Vandlen, R. L., Reed, K. L., & Lee, T. (1975) *Cold Spring Harbor Symp. Quant. Biol. 40*, 193–202.
Raftery, M., Hunkapillar, M., Strader, C., & Hood, L. (1980) *Science (Washington, D.C.) 208*, 1454–1457.
Reiness, G., & Hall, Z. (1981) *Dev. Biol. 81*, 324–331.
Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.
Seed, B. (1982) *Nucleic Acids Res. 10*, 1799–1810.
Shorr, R. G., Dolly, O., & Barnard, E. (1978) *Nature (London) 274*, 283–284.
Shorr, R., Lyddiatt, A., Lo, M., Dolly, O., & Barnard, E. (1981) *Eur. J. Biochem. 116*, 143–153.
Sobel, A., Weber, M., & Changeux, J. P. (1977) *Biochemistry 16*, 215–224.
Stevenson, A., Harrison, R., & Lunt, G. (1981) *Eur. J. Biochem. 115*, 91–97.
Symington, J., Green, M., & Brackman, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 177–181.
Tzartos, S., & Lindstrom, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755–759.
Tzartos, S., Rand, D., Einarson, B., & Lindstrom, J. (1981) *J. Biol. Chem. 256*, 8635–8645.
Tzartos, S., Seybold, M., & Lindstrom, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 188–192.
Weill, C., McNamee, M. G., & Karlin, A. (1974) *Biochem. Biophys. Res. Commun. 61*, 997–1003.
Wennogle, L., Oswald, R., Saitoh, T., & Changeux, J. P. (1981) *Biochemistry 20*, 2492–2497.
Wolosin, J., Lyddiatt, A., Dolly, J., & Barnard, E. (1980) *Eur. J. Biochem. 109*, 495–505.
Wu, C. S. W., & Raftery, M. (1979) *Biochem. Biophys. Res. Commun. 89*, 26–35.

Neurotoxins from *Bungarus fasciatus* Venom: A Simple Fractionation and Separation of α- and β-Type Neurotoxins and Their Partial Characterization†

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ABSTRACT: The crude venom of *Bungarus fasciatus* has been fractionated by column chromatography, and the fractionation characteristics of three different resins have been compared. A minimum of 21 fractions can be identified under optimum conditions on Bio-Gel CM-30. Of the major fractions tested for neurotoxic activity, three showed postsynaptic (α) and four showed presynaptic (β) neurotoxic activity. The major protein component (an α-neurotoxin) has an isoleucyl N terminus and a calculated molecular weight of 14 200 based on amino acid composition. This main component contains 127 amino acid residues including 16 cysteine residues. A second less abundant α-neurotoxin of similar molecular weight has a methionyl N terminus. The isoelectric points of these toxins are 9.1 and 8.8, respectively. A third fraction also has postsynaptic (α) activity. Four other, very basic proteins have presynaptic (β) activity. Their apparent molecular weights are approximately 10 800 (two fractions), 13 100, and 19 100 as determined by sodium dodecyl sulfate gel electrophoresis. All α-toxin fractions showed a high tendency to aggregate in aqueous media; however, the presence of L-cysteine in molar excess prevents dimer formation. In the absence of L-cysteine, freeze/thaw cycling of aqueous solutions of α-toxins invariably leads to the formation of dimers which can be dissociated only under reducing conditions (β-mercaptoethanol). Conversely, only one out of four β-toxins examined tended to form dimers.

Low molecular weight neurotoxins have proven very useful in the study of biochemical aspects of neural impulse transmission (Changeux et al., 1970; Raftery et al., 1975; Heidmann & Changeux, 1978). In particular α-bungarotoxin (α-Bgt),[1] a protein which can be isolated from the crude venom of the elapid snake *Bungarus multicinctus*, was shown by Lee (1970, 1972) to block neural transmission by its action at the postsynaptic membrane of the neuromuscular junction where it binds selectively to the acetylcholine receptor (AChR).

α-Bgt was purified by Clark et al. (1972) and analyzed with respect to several characteristics including amino acid composition, isoelectric point, N-terminal amino acid, and electrophysiological properties. Isoleucine was identified as the N-terminal residue. More recently Hanley et al. (1977) reported the purification to homogeneity of nine neurotoxic proteins obtained from the venom of *Bungarus multicinctus*. These workers characterized the molecular weights, amino acid composition, and N-terminal residues of each protein. The purified fractions include α-Bgt, plus two other α-neurotoxins, and β-bungarotoxin, plus five other β-neurotoxins. Although α- and β-bungarotoxins have been studied extensively, much less is known about the activity or specificity of the other postsynaptic (α) and presynaptic (β) neurotoxins from *B. multicinctus*.

The availability of toxins other than α-Bgt with complementary pharmacological or biochemical activities would enhance the usefulness of toxin binding studies and has encouraged us to examine other venoms as sources of such toxins. Venom from *Bungarus fasciatus* was reported by Moore & Loy (1972) to contain an α-neurotoxin, but the toxin was not extensively characterized. *B. fasciatus* venom has, however,

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[1] Abbreviations: α-Bgt, α-bungarotoxin; AChR, acetylcholine receptor; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TEMED, *N,N,N′,N′*-tetramethylethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; HSA, human serum albumin; Tris, tris(hydroxymethyl)aminomethane.

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been fractionated by several workers, usually by CM-cellulose chromatography (Lu & Lo, 1974; Lo & Lu, 1976; Lin et al., 1972), and the fractions isolated have been partially characterized (Lu & Lo, 1978). These initial fractionations usually produced 11–13 fractions, several of which clearly contained multiple components. Further, the activity most extensively studied was identified as a cardiotoxin whose reported molecular weight differed between different reports. These uncertainties led us to reexamine the fractionation of *B. fasciatus* venom by using other chromatographic absorbents and to further characterize the neurotoxic components. The venom can be dissociated into at least 21 distinct fractions, and we report here on properties of the major neurotoxic fractions.

Materials and Methods

Crude venom from *Bungarus fasciatus*, acetylthiocholine iodide, Coomassie Brilliant Blue G-250, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *Naja naja siamensis* crude venom, and human serum albumin (HSA) were all obtained from Sigma Chemical Co. [^{125}I]-α-Bungarotoxin was obtained from New England Nuclear Corp. Acrylamide monomer (technical grade) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were obtained from Eastman Organic Chemicals. The former was recrystallized from boiling ethyl acetate, was dried under vacuum, and performs as well as "high purity" grades. High-purity $NaDodSO_4$ was obtained from BDH Chemicals, as were dansyl chloride and dansyl amino acids. *N,N′*-Methylenebis(acrylamide), Gold Label electrophoresis grade, was obtained from Aldrich Chemical Co., Inc. Protein molecular weight markers were obtained from Schwarz/Mann, Orangeburg, NY. Ion-exchange resins Bio-Rex 70 and Bio-Gel CM-30 and *N,N′*-diallyltartardiamide were obtained from Bio-Rad Laboratories. Bio-Gel CM-2 was a generous gift from Bio-Rad Laboratories, Toronto, Canada. NP-40 (Nonidet P-40) was obtained from Particle Data Laboratories Ltd., Elmhurst, IL. Ampholytes were obtained from Fisher (LKB ampholine, pH 3.5–10) and Bio-Rad Laboratories (Bio-Lyte, pH 8–10). All other chemicals were obtained from standard suppliers, and reagent grades were used throughout.

Acetylcholine Receptor (AChR) Preparation. Sarcolemmal membrane vesicle fractions were isolated from mouse hindlimb muscles by using a differential centrifugation method originally proposed by Jones et al. (1979). The final flocculent material obtained by procedure II was then resuspended in buffer (10 mM K_2HPO_4/KH_2PO_4, pH 7.4, 100 mM NaCl, 1 mg/mL HSA, 2% Triton X-100) (buffer A) with a buffer volume of 1 mL/g of starting material. The addition of Triton X-100 to the buffer solubilizes the membrane-bound receptors (Dolly & Barnard, 1977). Such preparations routinely had a final protein concentration of 0.3–0.4 mg/mL.

Fractionation of Crude Venom. Crude venom was fractionated by ion-exchange chromatography on three synthetic resins, all containing weakly acidic carboxymethyl groups: (1) Bio-Rex 70, 100–200 mesh; (2) Bio-Gel CM-30, 100–200 mesh; (3) Bio-Gel CM-2, 100–200 mesh. All columns were 2.3 × 100 cm fitted with medium porosity disks and filled in each case to a height of 80 cm. In the case of CM-30 the disk was overlaid with 0.5 cm of fine silica sand to prevent blockage by the gel. The resins were conditioned prior to loading by three washes with 0.1 M ammonium acetate (NH_4OAc), pH 6.2, followed by one wash with 1.0 M ammonium hydroxide (NH_4OH). They were finally equilibrated by stirring for 24 h in frequently changed 0.1 M NH_4OAc buffer, pH 6.2. After being loaded with venom, the columns were usually developed with an initial wash of 0.1 M NH_4OAc. Flow rates for all columns were adjusted to approximately 10 mL/h. Specific chromatographic conditions are included with the figure legends.

Toxicity Assays. (*1*) *General Toxicity.* The toxicity was estimated according to a method originally described by Boroff & Fleck (1966) for botulinus toxin and later used by Cooper & Reich (1972) to estimate the toxicity of an α-neurotoxin from *Naja naja siamensis*. Adult white mice were used. The toxicity of venom fractions was tested by injecting 25–100-μL samples of venom fractions (initial concentration approximately 10^{-5} M assuming a molecular weight of 14000) into the tail vein of adult white mice, and the time to death was recorded in each case.

(*2*) *Neurotoxic Specificity.* Toxic venom fractions were tested for presynaptic (α) or postsynaptic (β) activity in an isolated frog nerve–muscle preparation. Gastrocnemius muscle and its innervating sciatic nerve were isolated from adult frogs (*Rana pipiens*) and mounted in an isotonic muscle contraction module (no. 270) attached to a stimulator (no. 300) (both from Harvard Apparatus Co., Inc.). The preparation was bathed at regular intervals with frog Ringer's solution and under these circumstances would respond to intermittent electrical stimulation for more than an hour. The response to topically applied acetylcholine (ACh) or toxin could be accelerated by puncturing the surface membrane with a microsyringe needle in the vicinity of the entrance of the nerve into the muscle.

In each case the system was tested for sensitivity to electrical stimuli (100 mV) and topically applied ACh (10^{-3} M) prior to use and then washed extensively.

Neurotoxicity was assessed by the ability to inhibit electrically stimulated muscle contraction. Venom fraction solutions (initial concentration approximately 10^{-5} M) were applied topically or injected just under the surface membrane. The sample was allowed to penetrate for 10 min, and its effect on electrically stimulated contraction was then assessed.

Pre- or postsynaptic activity was distinguished by the response of toxin-treated muscles to the subsequent application of 10^{-3} M ACh. Toxin fractions whose effect could be mitigated by topical application of ACh are proposed as β-toxins while those whose effect cannot be overcome by ACh are thought to be α-toxins.

Acrylamide Gel Electrophoresis. Polyacrylamide gels were prepared essentially according to the method of O'Farrell (1975). Polyacrylamide gels (15% acrylamide monomer, 20:1 monomer:bisacrylamide ratio) were cast between 1.5-mm spaced slotted glass plates (17 cm high × 14 cm wide) to a height of 14 cm. After polymerization (1 h) a 4% spacer gel was overlayed, and a slot former was inserted to create 50-μL capacity sample wells.

Gradient gel electrophoresis was performed as above except that the separating gel contained a linear gradient of 10–20% acrylamide prepared with a two-chamber (15 mL each) gradient former. In this case difficulties were encountered with the mechanical properties of the slab gels (ripping due to brittleness of the high acrylamide concentration end) when *N,N′*-methylenebis(acrylamide) was used as cross-linking reagent. Mole equivalent substitution of this cross-linker by *N,N′*-diallyltartardiamide yielded gels of superior handling quality.

Isoelectric Focusing. A modified O'Farrell procedure (1975) was employed. The polymerization mixture consisted of 2.75 g of urea, 1 mL of H_2O, 1 mL of 10% NP-40 (or glycerol), 0.66 mL of an aqueous solution containing 28.4% (w/v) acrylamide, 1.6% (w/v) *N,N′*-methylenebis(acrylamide), 0.15 mL of pH 3.5–10 LKB ampholine, and 0.10 mL of pH 8–10 Bio-Rad Bio-Lyte. The solution was adjusted to ap-

proximately pH 5.7 with 0.1 mL of 1.0 M H_3PO_4 and extensively degassed. Polymerization of the mixture was initiated by the addition of 10 μL of 10% (w/v) ammonium persulfate and 10 μL of TEMED, and the solution was immediately used to fill 1.5 mm (i.d.) × 14 cm glass tubes. When polymerization was complete, samples containing 20 μg of protein were then added to the column in 50 μL of solution containing 2% ampholyte, 8 M urea, and 0.01 M β-mercaptoethanol. Electrophoresis conditions included the following: cathode, 0.02 M NaOH, and anode, 0.01 M H_3PO_4. Samples were electrophoresed for 10 h at 200 V followed by 1 h at 800 V.

After extrusion, the gels were rinsed briefly with distilled water. The pH profile of the gels was measured by direct contact of a microelectrode (Bio-Rad "pH-philer") with the gel surface. Since the ionic strength of other ions within the gel is not known, the measured pH may vary slightly from the true pH and hence must be considered as a nominal pH value. The same interpretation must be attached to the resulting p*I* values. Following this the gels were stained (see below) to locate the protein bands, and isoelectric points were estimated by interpolation within the pH profile.

Two-Dimensional Electrophoresis. Two-dimensional electrophoresis was performed by a modified O'Farrell (1975) technique. Isoelectric focusing gels were run (200 V for 10 h). These gels were then incubated for 15 min in buffer containing 0.06 M Tris-HCl, pH 6.8, 0.1% (w/v) $NaDodSO_4$, and 5% mercaptoethanol in H_2O and subsequently overlaid on appropriate slab gels (15% acrylamide running gels overlaid with 4% acrylamide stacking gels). Electrophoresis was performed (150 V for 6 h) by using the same running buffer as described above.

Molecular Weight Determination. The method of Weber & Osborn (1969) was followed by using $NaDodSO_4$–polyacrylamide slab gel electrophoresis. Molecular weight standards were the following: bovine serum albumin, 67 000; ovalbumin, 45 000; chymotrypsinogen A, 25 000; myoglobin, 17 800; cytochrome *c*, 12 400.

Staining of Proteins on Acrylamide Gels. Gels to be stained were washed initially with 5% trichloroacetic acid, rinsed with water, stained with Coomassie Blue G (400 mg in 1 L of 1.0 N HCl), and washed again with 5% trichloroacetic acid and finally water.

N-Terminal Amino Acid Determination. N-Terminal amino acid analysis was performed by the method of Percy & Buchwald (1972), based on the method of Gros & Labouesse (1969). Aliquots taken from the column effluent (containing approximately 50 μmol of protein) were lyophilized and kept under vacuum for 16 h. To the dried residue (in 15 × 150 mm Pyrex test tube) were added 0.5 mL of 8 M urea (ultrapure), 0.15 mL of 0.4 M phosphate buffer, pH 8.2, and 0.25 mL of dimethylformamide. After the solutions were throughly mixed, 5 mg of dansyl chloride dissolved in 0.1 mL of acetonitrile was added. The solution was mixed frequently by shaking and left to react at room temperature for 30 min. The dansylated protein was precipitated by the addition of 10 mL of 10% Cl_3CCOOH and collected by centrifugation. The precipitate was washed twice with acetone and dried under vacuum; 0.3 mL of 6 N HCl was added to the dried sample, and the tube was sealed and heated to 110 °C for 4 h. The resulting hydrolyzed material was dried under vacuum and the residue extracted with 20 μL of water-saturated ethyl acetate. Two-dimensional chromatography was performed on 5 × 5 cm polyamide sheets. The chromotograms were developed in the first dimension with 1.5% formic acid and in the second dimension with benzene–glacial acetic acid (9:1 v/v). Spots were visualized under UV light and identified by comparison with known standards.

Amino Acid Composition. Protein samples (~2 mg) were hydrolyzed under vacuum either in 6 N constant boiling HCl or for the determination of tryptophan in 3 N *p*-toluenesulfonic acid containing 0.02% 3-(2-aminoethyl)indole. Samples were taken and analyses performed after 22 and 48 h. In the case of labile amino acids the values were estimated by extrapolation to zero time. The hydrolysates were analyzed on automatic amino acid analyzers, Beckman Model 121 C or Durrum D 500.

Enzyme Assay. Acetylcholine esterase (AChE) activity was measured by the method of Ellman et al. (1961) with the acetylthiocholine substrate.

Acetylcholine Receptor Binding Assays. The binding of [^{125}I]-α-bungarotoxin to acetylcholine receptors from mouse muscle was measured by a technique derived from that originally proposed by Klett et al. (1973). Unless otherwise specified reaction mixtures contained 50 μL of AChR preparation, buffer A, and [^{125}I]-α-Bgt (also in buffer A) in a final reaction volume of 250 μL. The reaction mixture was maintained at room temperature for 30 min, and the reaction was then stopped by the addition of 50 μL of buffer A supplemented with 1 mg/mL crude venom from *Naja naja siamensis* (buffer B). The resulting solution was then pipetted on to DEAE filter disks which had been prewashed with 0.5 mL of buffer A (less the HSA), followed by 100 μL of buffer A supplemented with 1.0 mg/mL crude venom from *Naja naja siamensis*, and allowed to absorb for 10 min. The filter was then washed sequentially with 100 μL of buffer B, 20 mL of buffer A (less HSA), 5 mL of water, and 5 mL of methanol. The filters were dried, and retained radioactivity was counted by liquid scintillation.

Results

Venom Fractionation. Three different resins were tested for their ability to fractionate venom.

Bio-Rex 70. Fractionation with Bio-Rex 70 is less effective than with the other gels. Approximately seven peaks can be identified, two of which (peaks 5 and 6) subsequently showed postsynaptic neurotoxic activity. Although useful in the purification of these two peaks, Bio-Rex 70 offers less overall resolution than Bio-Gel CM-30 or CM-2.

Bio-Gel CM-30. Venom fractionation on Bio-Gel CM-30 is shown in Figure 1. Two different elution conditions are shown. In the first case a relatively shallow two-step elution gradient was used. In the second a steeper gradient was used, and the elution buffer was supplemented with cysteine to provide a reducing medium.

The choice of elution conditions depends on the resolution needed and desired flow rates. At high ionic strengths, the bed volume of CM-30 shrinks dramatically, and the elution rate increases under constant hydrostatic pressure.

Under both conditions CM-30 resolves the venom into 20 or 21 distinct fractions, and although there is some overlap of fractions, essentially pure components can be obtained if narrow cuts of each peak are taken. For example, in Figure 1a peaks 12 (fraction 121) and 14 (fraction 134) give single bands on $NaDodSO_4$–polyacrylamide gel electrophoresis. Under reducing conditions (Figure 1b) the overall elution profile is similar to that found initially (Figure 1a); however, fewer peaks are seen in the center section of the elution profile. We ascribe this to the elimination of protein multimers under reducing elution conditions. Thus fractions 11 and 13 in Figure 1a do not appear in Figure 1b. On the basis of several criteria we equate the leading shoulder of peak 9, peaks 9, 10, 12, and

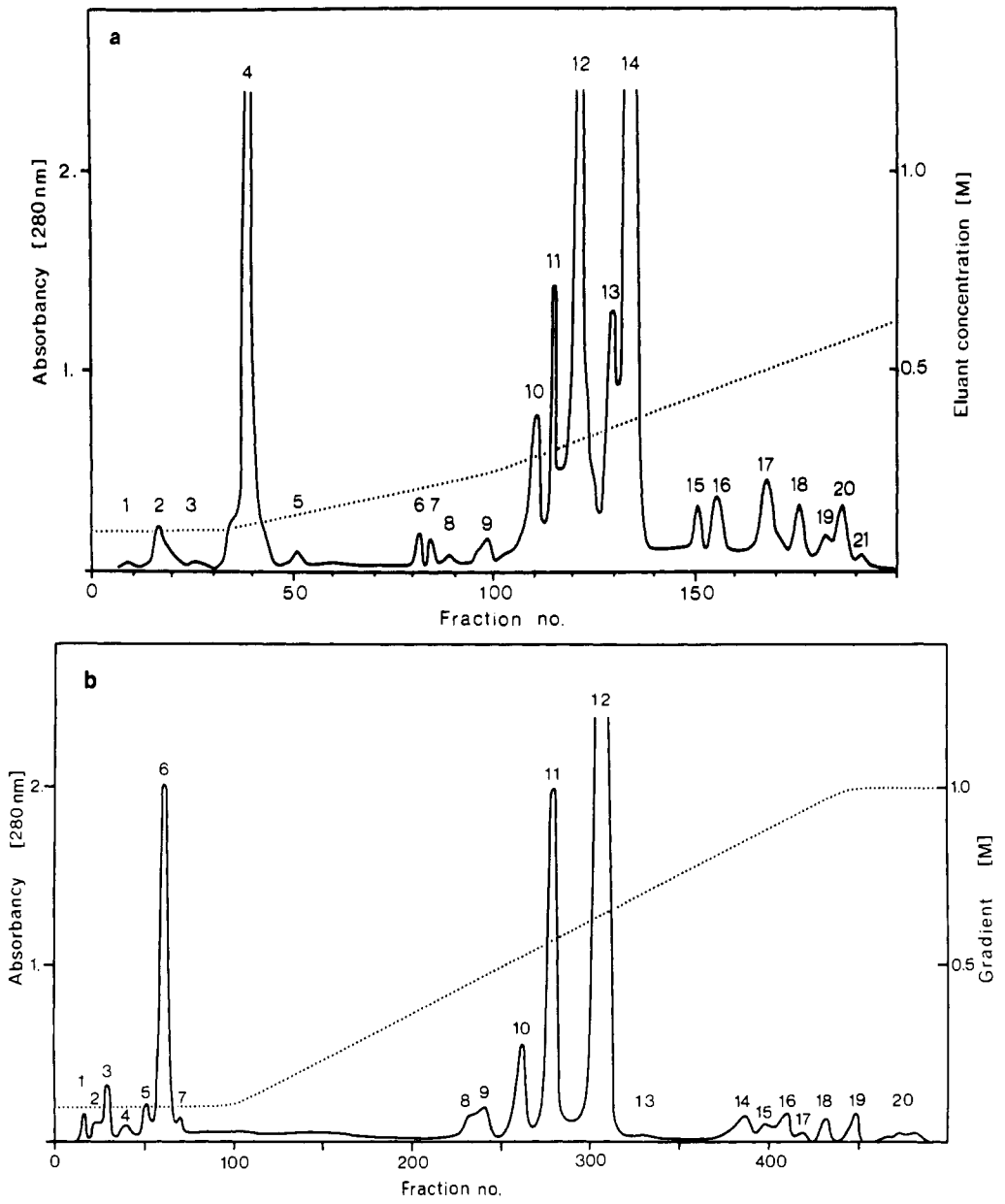

FIGURE 1: Gradient elution chromatography of crude venom on Bio-Gel CM-30. Column dimensions 2.3 × 80 cm and elution with NH_4OAc from 0.1 to 1.0 M (pH 6.2) as indicated. (a) Crude venom, 500 mg; (b) crude venom, 330 mg, plus L-cysteine, 100 mg. Absorbancy at 280 nm (—); buffer concentration (···).

14 in Figure 1a with peaks 8, 9, 10, 11, and 12, respectively, in Figure 1b. The major initial peaks in both figures, peak 4 (Figure 1a) and peak 6 (Figure 1b), are identified by their UV spectrum as guanosine. The yield of each of the major toxic components is included in Table I.

For ease of identification peaks 10, 11, and 12 obtained from CM-30 fractionation under reducing conditions are referred to as neurotoxins A, B, and C, respectively [subsequent studies reported below indicated that all three are postsynaptically acting (α) neurotoxins].

Bio-Gel CM-2. Venom fractionation on Bio-Gel CM-2 is shown in Figure 2. The CM-2 resin offers less overall resolution than does CM-30, and a maximum of only 13 or 14 fractions can be identified. However, it still provides excellent resolution into pure protein fractions of peaks 5 and 8, the two major neurotoxins (B and C) which are equivalent to peaks 11 and 12 in Figure 1b. Peak 8 (fractions 59–69) is completely free of contaminating proteins as determined by $NaDodSO_4$ gel electrophoresis, and a narrow cut of peak 5 (fraction 41) also gives a single protein when subjected to $NaDodSO_4$ electrophoresis. Peak 4 again is guanosine.

Toxicity. (*a*) *General.* The toxicity of the major protein peaks was tested in mice and/or when the protein concentration was low by the frog muscle assay. All major peaks (absorbance at 280 nm <0.5) were toxic with two exceptions: (i) the guanosine peaks and (ii) peaks 11 and 13 in Figure 1a which either were only weakly toxic on a per gram basis or showed no toxic effects. In mice the major fractions tested (toxins B and C) caused death when injected at doses of 2.5–10 μg of toxin/g of body weight. The time to death in these cases was quite variable and lay between 3 and 45 min. At an input of 0.8 μg/g of toxin no deaths occurred. The injected animals became "ill" (difficulty with controlled, coordinated voluntary muscle movement, tremors, or immobility). However, they recovered normal activity several hours postinjection.

Repeated freezing and thawing eliminate toxicity in the two major toxic peaks presumably by the formation of multimers. For example, peaks 11 and 12 (Figure 1b) were rendered nontoxic at intravenous doses of 140 μg/g after three cycles of freezing and thawing. Gel electrophoresis of each sample

Table I: Neurotoxic Specificity of Toxic Components Obtained by Chromatography of Crude Venom or Bio-Gel CM-30 under Reducing Conditions

peak no.	toxin	app M_r	neurotoxic specificity	yield[a]
8		14 000		0.7
9		12 000–14 000		1.8
10	A	14 200	α	4.6
11	B	14 200	α	23.4
12	C	15 200	α	43.0
14	B1	13 100	β	1.5
16	B2	11 200	β	1.6
18	B3	14 200	β	1.7
19	B4	11 000–14 000	β	1.9

[a] Yield is expressed as the percentage of total protein present. If the guanosine fraction is included and yield is expressed as a percentage of total material, these numbers are reduced slightly.

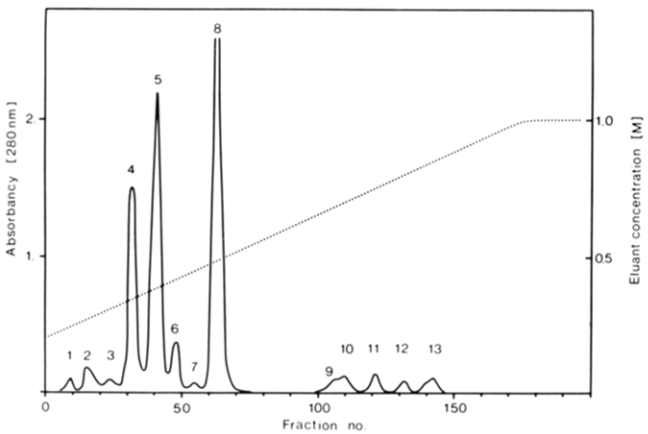

FIGURE 2: Gradient elution chromatography of crude venom (330 mg) on Bio-Gel CM-2. Column dimensions 2.3 × 80 cm and elution with NH_4OAc from 0.1 to 1.0 M (pH 6.2) as indicated. Absorbancy 280 nm (—); buffer concentration (···).

after freeze/thaw cycling showed that the monomer had disappeared and was replaced by higher molecular weight aggregates (see below).

(*b*) *Neurotoxic Specificity*. The protein fractions obtained by chromatography under reducing conditions were tested for neurotoxic specificity on frog muscle–sciatic nerve preparations. Samples from peaks 10–12 (Figure 1b) showed postsynaptic activity; i.e., no muscle contraction could be elicited by ACh application after toxin treatments. Samples from peaks 14, 16, 18, and 19 could also stop muscle contraction due to electrical stimulation within 30 min of exposure, but muscle contraction could be elicited by application of ACh (10^{-3} M). The latter proteins were therefore tentatively classed as presynaptically active (α) neurotoxins. The protein fractions tested and their neurotoxic specificity are listed in Table I.

Two-Dimensional Gel Electrophoresis of Crude Venom. Crude venom was subjected to two-dimensional polyacrylamide gel electrophoresis to obtain initial estimates of the molecular weights and isoelectric points of the venom components. An example of this separation is given in Figure 3. Approximately 19 separate spots can be identified, 13 of which are well-defined. Fractionation in the first dimension by isoelectric focusing resolves proteins with isoelectric points (p*I*s) ranging from approximately pH 3.5 to 9.4. The major proteins have p*I*s in the range of pH 8.4–9.4. Electrophoresis ($NaDodSO_4$–polyacrylamide) in the second dimension shows that the molecular weights of the protein components fall into the 12 000–19 000 range. The identity of the major proteins was

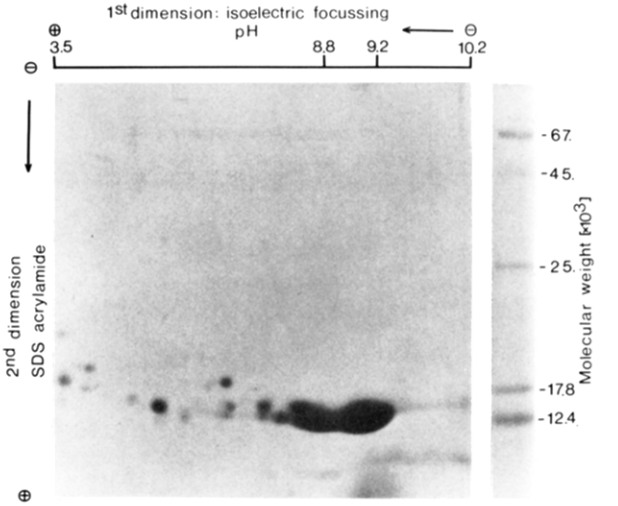

FIGURE 3: Two-dimensional gel electrophoresis of crude venom from *Bungarus fasciatus*. Protein input 50 μL of crude venom. First dimension 1.5 × 110 mm, 5% polyacrylamide disc gel containing pH 3.5–11 ampholytes (see Materials and Methods). Second dimension 1.5 × 170 × 140 nm, 15% polyacrylamide slab gel containing 0.1% $NaDodSO_4$ in Tris–glycine buffer, pH 8.8.

established by parallel electrophoresis of purified individual venom fractions. The two most heavily stained spots with coordinates p*I* = 8.8 and 9.2, M_r 14 000 (both), were identified as α-neurotoxins A and C, respectively. Neurotoxins B, $\beta_1\beta_2\beta_3$, and β_4 do not enter the isoelectric focusing gel under the nominal pH conditions and would appear to the right of the gel illustrated.

$NaDodSO_4$ Gel Electrophoresis of α-Neurotoxins. The major α-neurotoxins were then subjected to further gel analysis. Aliquots or fresh samples of A, B, and C were electrophoresed in $NaDodSO_4$ as described under Materials and Methods, and each produced a single band of M_r 12 000–16 000 (not shown). Duplicate samples were subjected to a cycle of freezing (overnight) and thawing and then electrophoresed (a) without further treatment of (b) following vigorous reduction of the proteins. The results of the experiment are shown in Figure 4. Clearly a cycle of freezing and thawing leads to the formation of higher molecular weight aggregates (bands 1–3) and the disappearance of "monomer" protein. In the case of neurotoxin C the aggregate tends to break down during electrophoresis and routinely gives a tailed distribution with the majority of the protein aggregate of lower molecular weight than those of neurotoxins A and B. Extensive reduction of all three neurotoxins produces essentially single bands of apparent M_r 13 000–15 000 (based on the R_f's of marker proteins).

In the course of electrophoresis experiments with both *B. multicinctus* and *B. fasciatus*, an unusual qualitative result was obtained. When the venoms were resolved by $NaDodSO_4$ electrophoresis and stained with Coomassie Blue G, proteins identified as neurotoxins exhibited unusual light-scattering characteristics. When stained gels were viewed with transmitted daylight, α-Bgt and toxins A, B, and C each exhibited a copper color when viewed at an angle (i.e., scattered light). None of the other neurotoxins in *B. multicinctus* venom or proteins in *B. fasciatus* venom exhibited this unusual scattering which presumably derives from unique structural aspects of these venoms.

Isoelectric Points. The isoelectric points of each of the three major α-neurotoxins were determined as outlined under Materials and Methods. α-Neurotoxins A, B, and C have p*I*s of 9.8, 8.8, and 9.2, respectively, i.e., all are basic proteins.

Table II: Comparison of Amino Acid Composition of α-Neurotoxins A, B, and C from *Bungarus fasciatus* with α-Bungarotoxin from *Bungarus multicinctus*

	α-bungarotoxin[a] integral	no. of residues (mol/mol)					
		α-neurotoxin A[b]		α-neurotoxin B[b]		α-neurotoxin C[b,c]	
		average	integral	average	integral	average	integral
Lys	6	10.5	10	7.9	8	8.4	8
His	2	2.8	3	2.6	3	3.1	3
Arg	3	4.3	4	4.4	4	4.3	4
Asx	4	19.0	19	18.5	19	16.2	16
Thr	7	9.6	10	7.1	7	10.3	10
Ser	6	2.6	3	3.9	4	2.3	3
Glx	5	9.3	9	7.2	7	7.2	7
Pro	8	5.3	5	5.4	5	5.3	5
Gly	4	11.9	12	11.8	12	12.2	12
Ala	5	12.0	12	12.0	12	12.4	12
$^1/_2$-Cys	10	16.2	16	15.1	16	16.0[d]	16
Val	5	3.1	3	4.0	4	4.0	4
Met	1	1.3	1	1.1	1	1.0	1
Ile	2	4.9	5	4.5	5	4.8	5
Leu	2	7.3	7	6.3	6	6.2	6
Tyr	2	8.2	8	7.8	8	9.0	9
Phe	1	5.1	5	4.6	5	4.6	5
Trp	1	1.4	1	0.5	1	1.0	1
	74[e]		133[e]		127[e]		127[e]
			14 800[f]		14 200[f]		14 200[f]

[a] Values taken from Lee (1972). [b] Durrum Model D500 amino acid analyzer (two determinations each for neurotoxins A and B and three determinations for neurotoxin C). [c] Beckman Model 121C amino analyzer (two determinations for neurotoxin C). [d] The half-cystine content of α-neurotoxin C was confirmed by prior reduction with β-mercaptoethanol and the synthesis and assay of the carboxymethyl derivatives. [e] Total; values are rounded off to nearest 100 since ratios of Glu to Gln and Asp to Asn are not known. [f] Calculated molecular weight.

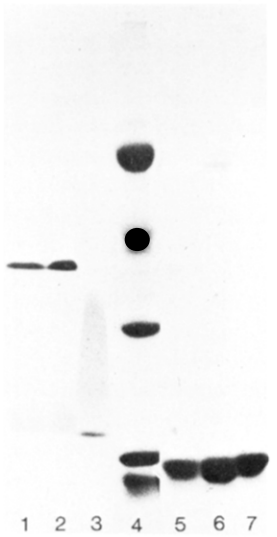

FIGURE 4: Electrophoresis of neurotoxins A, B, and C under conditions permitting oligomer formation or following vigorous reduction. Experimental conditions: polyacrylamide gradient gel (10–20%) containing 0.1% $NaDodSO_4$ in Tris–glycine buffer, pH 8.8. Electrophoresis for 12 h at 100 V. Tracks 1, 2, and 3 contain toxins A, B, and C, respectively, in Laemmli's buffer (1970). Track 4 contains marker proteins BSA, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome *c*. Tracks 5, 6, and 7 contain toxins A, B, and C, respectively, prepared as above but heated in the presence of 5% β-mercaptoethanol at 100 °C for 5 min before cooling and electrophoresis.

N-Terminal Amino Acids. The N-terminal amino acid of each α-neurotoxin was determined as outlined under Materials and Methods. α-Neurotoxins A, B, and C have N termini of aspartic acid (asparagine), methionine, and isoleucine, respectively.

Amino Acid Composition. Samples of α-neurotoxins A, B, and C were hydrolyzed for 22 or 48 h and then subjected to amino acid analysis. Multiple analyses were performed, and the averages for each amino acid were calculated. Values for labile amino acids were estimated by extrapolation of the 22- and 48-h results back to zero time. The results are presented in Table II. Free sulfhydryl groups (i.e., free half-cystine residues) in unreduced toxins were assayed according to Ellman (1959) with DTNB, and all assays were negative, i.e., all half-cystine residues are apparently present in disulfide bonds.

Enzyme Activity. Venoms have been reported to include a variety of enzyme activities (e.g., Hanley et al., 1977; Yang et al., 1960), in particular acetylcholinesterase. *B. fasciatus* venom, fractionated as outlined above (Figure 1a), was assayed for AChE (note that in this case venom fractionation was performed immediately after hydration of lyophilized venom but in the absence of cysteine which reacts in the Ellman assay). The elution profile in this case resembled Figure 1b, although the conditions represented in Figure 1a were used. AChE activity was almost completely confined to initial peak 2. When the input per assay of other column fractions was increased 50-fold, no activity was found coincident with the toxin peaks, although a small transient peak appeared ahead of the grouped α-neurotoxins.

Binding Specificity. A major rationale for the studies reported here was to find neurotoxins which would bind to acetylcholine receptors in a manner similar to that found with α-Bgt. Figure 5 shows the results of experiments that demonstrate that neurotoxin C apparently competes with α-Bgt for receptor binding. In the absence of C, α-Bgt binding is saturated with an input concentration of approximately 6×10^{-10} mol. This plateau binding level is reduced by approximately 79% when the receptor preparation is previously exposed to 4×10^{-8} M α-neurotoxin C. If the concentration of C is further increased to 1.0×10^{-7} M in the pretreatment, all subsequent α-Bgt binding is eliminated (see Figure 5, insert). In a separate experiment (data not shown), the reduction of α-Bgt binding as a function of the input concentration of C was measured. In this case the affinity of C for receptor

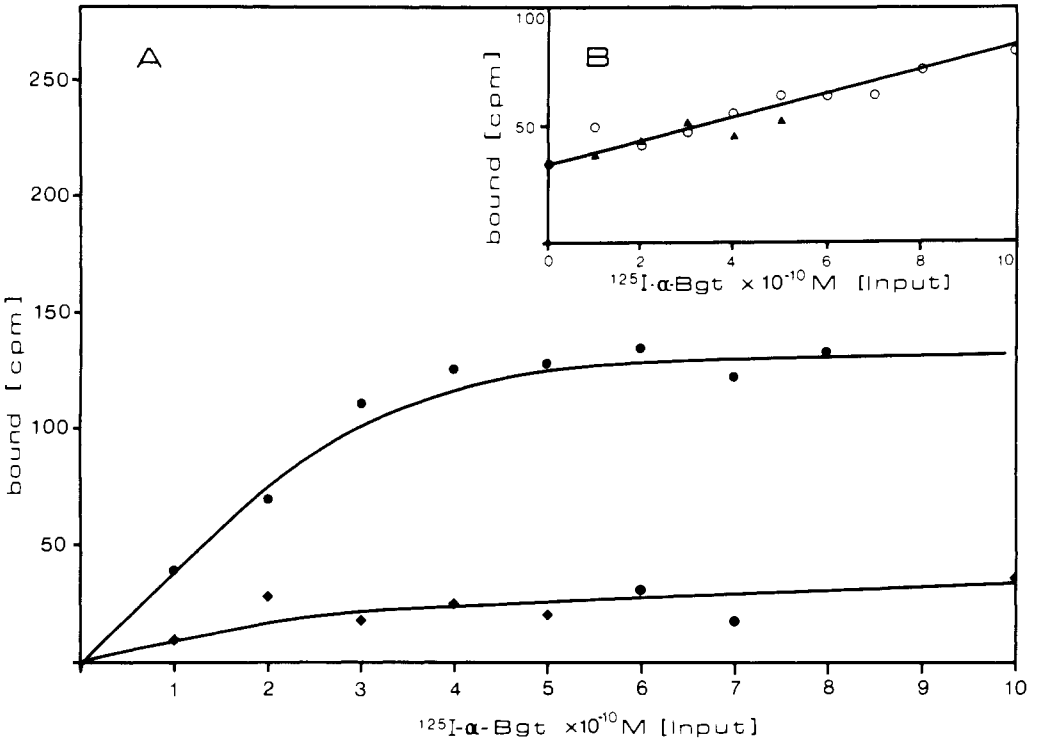

FIGURE 5: Effect of neurotoxin C on the binding of [^{125}I]-α-bungarotoxin to an acetylcholine receptor preparation from mouse muscle. [^{125}I]-α-Bungarotoxin binding assays were performed as described under Materials and Methods except when the receptors were pretreated with neurotoxin C in which cases the reaction mixtures, less α-Bgt, were incubated for 60 min at room temperature with the indicated neurotoxin C concentration, following which [^{125}I]-α-Bgt (original specific activity 16.9 μCi/μg) was added and the normal assay performed. (A) (●) [^{125}I]-α-Bgt binding in the absence of neurotoxin C; (♦)[^{125}]-α-Bgt binding to receptors pretreated with 4×10^{-8} M neurotoxin C; in both cases background (receptor-independent) binding has been subtracted. (B) (Insert) (○) Background binding of [^{125}I]-α-Bgt to filters in the absence of acetylcholine receptors; (▲) [^{125}I]α-Bgt binding to receptors pretreated with 1.0×10^{-7} M neurotoxin C.

binding sites can be calculated relative to α-Bgt binding. Using this approach we calculate $K_a = (0.1\text{–}0.2) \times 10^9$ for neurotoxin C (based upon $K_a = 1.03 \times 10^9$ for α-Bgt measured in our assay).

Discussion

Bungarotoxin (α-Bgt from *Bungarus multicinctus*) is widely used as a selective binding ligand for acetylcholine receptors (AChRs). Iodinated (^{125}I) toxin is a convenient label for AChRs, and the kinetics of dissociation have been used to discriminate between different classes of receptor (Brockes & Hall, 1975). We have looked for other selectively binding α-neurotoxins both as complements of α-Bgt and because of increasing difficulties of supply. Venom from the related elapid snake *Bungarus fasciatus* is a source of such toxin. The venom can be resolved into approximately 20 components and several have neurotoxicity similar or equivalent to that reported for *B. multicinctus* venom and in particular α-Bgt. Under conditions which minimize polymerization the venom contains at least three active α-toxins (postsynaptic). Several β-toxins (presynaptic) can also be resolved, but their characterization has not been pursued.

Comparisons of our fractionation procedure with those for *B. multicinctus* and *B. fasciatus* as reported by other workers show some similarities but also several surprising differences. Both the pattern and proportions of venom fractionation are similar to those reported for *B. multicinctus* venom (Eterovic et al., 1975; Hanley et al., 1977), i.e., an initial group of fractions which includes guanosine and several weakly toxic or nontoxic proteins, intermediate elution of three neurotoxins, one of which is present in substantially higher amounts than the other two, and finally the separation of several presynaptic neurotoxins.

The molecular weights of the toxins, however, differ substantially. Exclusion chromatography suggests a molecular weight of 15000–16000 for the *B. multicinctus* α-toxins whereas amino acid analysis indicates a weight of about 8000 (Hanley et al., 1977). In contrast *B. fasciatus* toxins all have molecular weights of approximately 14200. Toxins from both species aggregate under nonreducing conditions and lose activity under our testing regimens. Activity can be recovered by vigorous reduction which reduces or eliminates the presence of higher molecular weight oligomers.

The N-terminal amino acids and p*I*s of the α-neurotoxins are all consistent with those reported for other elapid toxins, e.g., α-Bgt [p*I* = 9.2 (Hanley et al., 1977)]. N-Terminal amino acid isoleucine (Mebs et al., 1972; Hanley et al., 1977) and neurotoxin C are identical in these respects. The only exception is neurotoxin A whose isoelectric point of 9.8 and N-terminal amino acid asparagine are more usually indicative of β-neurotoxins.

Comparison with results reported for *B. fasciatus* fractionated on CM-cellulose is more difficult. Lin et al. (1972) and Lo & Lu (1976) have both reported such fractionation and superficially their fractionations and our own are similar. When examined in detail, however, the data reported show significant differences. All separations have a sharp initial peak, two major intermediate fractions, and a group of trailing fractions. Comparison of the characteristics of each peak, however, demonstrates significant differences. The initial peak we report as guanosine whereas neither report identifies guanosine, and they disagree on the enzyme activities present in this initial peak [Lin et al. (1972), phospholipase A and cholinesterase; Lu & Lo (1978), protease and ribonuclease]. All authors identify the intermediate peaks as toxic (producing neuromuscular block) but disagree with respect to relative

proportions, toxic specificity, molecular weight, and N-terminal amino acid. For example the major peak [neurotoxin C (our report), peak VI (Lin et al., 1972), and peak VI (Lu & Lo, 1978)] has molecular weight of 14 200 (T. P. A. Kruck and D. M. Logan, this paper), 9709 and 13652 [VIa and VIb, Lin et al. (1972)], or 9351 [VI-1, Lo & Lu (1976) (note that in the latter case the toxin was initially reported to contain 85 amino acids whereas a more recent publication identifies it as containing 118 amino acids). Lin et al. identify nine fractions which with varying strength produce neuromuscular block whereas Lo & Lu identify only four. Finally the amino acid analyses of peaks that appear equivalent are similar but not the same. Overall our fractionation and the distribution of enzyme and toxic activities appear similar to those reported by Lin et al. but not those reported by Lu & Lo. While similar in these characteristics, $NaDodSO_4$ gel electrophoresis, exclusion chromatography, and amino acid analysis all indicate that the fractions we have isolated have uniformly higher molecular weights often close to double those reported elsewhere, and we are unable to identify components in the 6000–8000 molecular weight range.

We cannot be certain about the reasons for the differing results reported by the different authors. The chromatographic elution profiles are a function of the absorbent (ion-exchange resin and the ionic strength of the eluting buffers). It appears that our resin (CM-30) column has a higher number of theoretical plates than those employed by the other authors which, associated with a shallow gradient buffer system, produces much improved separation characteristics based upon the basicity of the individual components. Further, the absolute amounts of different components appear to be at variance between the published elution results of the authors cited. These differences may well be due to (i) variations between animals used as source for crude venom and/or (ii) traces of proteolytic enzymes that may be present, which will hydrolyze constituent proteins to various degrees depending upon the time lapse between harvesting the venom and freezing and lyophilizing. We observed hydrolytic activity (decreasing pH as a function of time) when crude venom dissolved in dilute buffer (0.05 M phosphate) was allowed to stand at room temperature for extended periods of time prior to loading on columns. To minimize possible proteolysis of component fractions, we now load our crude venom onto the column immediately after dissolution.

Although the molecular weights of neurotoxins A, B, and C differ substantially from that reported for α-Bgt, the amino acid composition profiles are similar in several respects. These include comparable proportions of basic amino acids lysine and arginine, large and proportionate amounts of half-cystine residues, and a single methionine and trytophan residue in each toxin. On the other hand the relative proportions of several amino acids, in particular glycine, alanine, aspartic acid (asparagine) leucine, tyrosine, and phenylalanine, are all increased substantially in the *B. fasciatus* toxins compared to toxins from *B. multicinctus*. In contrast the several α-toxins from an individual species show only slight amino acid composition differences and presumably represent variants of a single initial sequence.

Although *B. fasciatus* venom was not surveyed extensively for contaminating enzyme activities, one useful feature arises in the assay of acetylcholinesterase. With *B. multicinctus* venom, AChE is found as a contaminant of several toxin fractions including α-Bgt. *B. fasciatus* venom on the other hand can be fractionated with no AChE contamination of the major neurotoxins.

This work was undertaken in an effort to identify and characterize AChR specific toxin(s), i.e., toxins which would bind strongly and selectively as does α-Bgt. Such toxins would provide a complement to the selective binding shown by α-Bgt, allowing the possible discrimination of receptors which differ subtly but which cannot be differentiated between by α-Bgt alone. Although substantially more work needs to be done, Figure 5 illustrates data suggesting that neurotoxin C, at least, binds at or near AChR molecules and has the potential of a receptor-specific probe.

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References

Boroff, D. A., & Fleck, U. (1966) *J. Bacteriol. 92*, 1580.
Brockes, J. P., & Hall, Z. W. (1975) *Biochemistry 14*, 2092, 2100.
Changeux, J. P., Kasai, M., & Lee, C. Y. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1241.
Clark, D. G., Macmurchie, D. D., Elliot, E., Wolcott, R. G., Landel, A. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1663.
Cooper, D., & Reich, E. (1972) *J. Biol. Chem. 247*, 3008.
Dolly, J. O., & Barnard, E. A. (1977) *Biochemistry 16*, 5053.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Ellman, G. L., Courtney, K. D., Andres, V., Jr., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88.
Eterovic, V. A., Hebert, M. S., Hanley, M. R., & Bennett, E. L. (1975) *Toxicon 13*, 37.
Gros, C., & Labouesse, B. (1969) *Eur. J. Biochem. 7*, 463.
Hanley, M. R., Eterovic, V. A., Hawkes, S. P., Hebert, A. J., & Bennett, E. L. (1977) *Biochemistry 16*, 5840.
Heidmann, T., & Changeux, J. P. (1978) *Annu. Rev. Biochem. 47*, 317.
Jones, L. R., Besch, H. R., Jr., Fleming, J. W., McConnaughey, M. M., & Watanabe, A. M. (1979) *J. Biol. Chem. 254*, 530.
Klett, R. P., Fulpius, B. W., Cooper, D., Smith, M., Reich, E., & Possani, L. D. (1973) *J. Biol. Chem. 248*, 6841.
Laemmli, U. (1972) *Nature (London) 227*, 680.
Lee, C. Y. (1970) *Clin. Toxicol. 3*, 457.
Lee, C. Y. (1972) *Annu. Rev. Pharmacol. 12*, 265.
Lin, S. S., Huang, M., & Lee, C. Y. (1972) *J. Formosan Med. Assoc. 71*, 350.
Lo, T., & Lu, H. (1976) *Toxicon (Suppl. 1.)*, 161.
Lu, H., & Lo, T. (1974) *J. Chin. Biochem. Soc. 3*, 57.
Lu, H., & Lo, T. (1978) *Int. J. Pept. Protein Res. 12*, 181.
Mebs, D., Narita, K., Iwanaga, S., Samejima, Y., & Lee, C. Y. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 243.
Moore, W. J., & Loy, N. J. (1972) *Biochem. Biophys. Res. Commun. 46*, 2093.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.
Percy, M. E., & Buchwald, B. M. (1972) *Anal. Biochem. 45*, 60.
Raftery, M. A., Vandlen, R. L., Reed, K. L., & Lee, T. (1975) *Cold Spring Harbor Symp. Quant. Biol. 40*, 193.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Yang, C. C., Kao, K. C., & Chiu, W. C. (1960) *J. Biochem. (Tokyo) 48*, 714.