Neurotoxins of *Bungarus multicinctus* Venom. Purification and Partial Characterization†

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ABSTRACT: The purification to homogeneity of nine neurotoxic components of the venom of *Bungarus multicinctus* is described. The purified components include α-bungarotoxin and two other α-type synaptic toxins and β-bungarotoxin and five other β-type synaptic toxins. The purified toxins have been characterized by electrophoresis, isoelectric focusing, amino acid analysis, and N-terminal amino acid determination. The α-type synaptic neurotoxins constitute a discrete class with molecular weights of 7000–8500, isoelectric points (p*I*) of 9.0–9.2, and N-terminal isoleucine or methionine. The β-type synaptic neurotoxins constitute a second group with molecular weights of 20 000–22 000 and p*I* = 8.8–9.7. Fractions 10 through 13 exhibit a chain structure consisting of a 6000–7000 light chain and a 11 000–15 000 heavy chain apparently covalently stabilized by interchain disulfides. Fractions 9A and 14 were single chains of 11 000–14 000 which resemble the sequenced β-type synaptic neurotoxin notexin (Halpert, J., and Eaker, D. (1975), *J. Biol. Chem. 250,* 6990). All of the β-type synaptic toxins have a single tryptophan and N-terminal aspartic acid or asparagine.

The protein neurotoxins of snake venoms have received increasing attention both as systems for study of structure–function relationships (Tu, 1973; Karlsson, 1974) and as tools in the study of synapses (Changeux et al., 1970; Von Hahn and Honegger, 1974). The majority of purified toxins from the Elapidae and Hydrophidae families are highly specific postsynaptic neurotoxins targeted to the nicotinic acetylcholine receptor of the neuromuscular junction (Lee, 1972). In some Elapid venoms there occur presynaptic neurotoxins directed to the nerve terminal of the neuromuscular junction (Lee, 1972). Both toxin varieties are found in the venom of the Formosan banded krait, *Bungarus multicinctus* (Lee et al., 1972). Two predominant components of the venom, α-bungarotoxin (postsynaptic) and β-bungarotoxin (presynaptic), have been studied in some detail (Mebs et al., 1972; Kelly and Brown, 1974). There are other functional homologues of these neurotoxins in the same venom which have not been structurally characterized. Initial pharmacological studies (Lee et al., 1972; Dryden et al., 1974) have suggested that several components should be examined more closely for their possible neurochemical application. We have presented a fractionation scheme and some toxicological and spectral properties of the bungarotoxins (Eterović et al., 1975). In this paper, we present details for further purification and several aspects of structural analysis which will facilitate reproducible identification of the same toxins in different laboratories. In addition, we can now begin to identify generic features of the two pharmacological classes of bungarotoxins. In the case of postsynaptic neurotoxins, a picture of striking structural conservation has emerged (Yang, 1974). For presynaptic toxins, less is known. However, they appear to share phospholipase A_2 enzymatic activity and some sequence homology (Halpert and Eaker, 1975; Strong et al., 1976).

Experimental Section

Materials

Suppliers of the reagents and materials were: *Bungarus multicinctus* crude venom, Miami Serpentarium; guanidine hydrochloride (ultrapure), Heico; *N*-bromosuccinimide, 99% pure reagent grade, Aldrich; iodoacetic acid, K & K Laboratories; Bio-Rex 70, Bio-Gel P30, acrylamide, and bisacrylamide, Bio-Rad; Sephadex G-50 and CM-Sephadex C-50, Pharmacia; *Bungarus multicinctus* horse serum anti-venom, Temed,[1] trypsin, α-chymotrypsin, leucine aminopeptidase, and carboxypeptidases A and B, Sigma; dansyl amino acids, dansyl chloride, dimethyl suberimidate, 4 N methanesulfonic acid ampules, and constant boiling HCl ampules, Pierce Chemical; ampholines, LKB-Produkter AB; polyamide sheets (Mikropolyamid F1700), Schleicher and Schuell; Diaflo ultrafiltration membranes, Amicon; and CM-cellulose, Whatman. *N*-Bromosuccinimide was recrystallized twice from water before use; iodoacetic acid was recrystallized from petroleum ether (30–60 °C) and diethyl ether.

Methods

Fractionation of Crude Venom. Thirteen components were obtained by ion-exchange chromatography on CM-Sephadex (Eterović et al., 1975).

Purification of CM-Sephadex Fractions. For the first experiments, pure α-bungarotoxin was obtained from fraction 3 by rechromatography on CM-cellulose (Eterović et al., 1975). For later experiments, fractions were concentrated by pressure dialysis to 1–2 mL and applied to a 2.6 × 40 cm Se-

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[1] Abbreviations used: UV, ultraviolet; AChE, acetylcholinesterase; α-Bgt, α-bungarotoxin; Asx, aspartic acid/asparagine; β-Bgt, β-bungarotoxin; β-NAD, nicotinamide adenine dinucleotide; CM, carboxymethyl; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MCD, magnetic circular dichroism; NBS, *N*-bromosuccinimide; $NaDodSO_4$, sodium dodecyl sulfate; Temed, *N,N,N′,N′*-tetramethylethylenediamine; BAPNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride.

phadex G-50 (fine) column. The column was run at 4 °C with upward elution by 0.1 M ammonium acetate (pH 6.8) at 20 mL/h. Peaks of absorbance at 280 nm detected by a Chromatronix Model 220 UV monitor were pooled and then desalted and concentrated by ultrafiltration (UM-2 Diaflo). These fractions were applied to a 2.5 × 20 cm column of Bio-Rex 70 (sodium form, 200–400 mesh). The column was rinsed with 150 mL of 50 mM sodium acetate (pH 6.0) and the proteins were eluted with a linear gradient of 0–1 M NaCl (1.5 L) in the same buffer. Lethality was determined after each chromatographic step (Eterović et al., 1975). Final fractions were desalted, concentrated, and either lyophilized or stored frozen in dilute neutral buffer. Lyophilized components were stored desiccated at −20 °C.

Assay of Enzymatic Activities. Acetylcholinesterase (EC 3.1.1.7) was determined by the method of Ellman et al. (1961). The substrate was acetylthiocholine at a final concentration of 6×10^{-4} M. As a blank for each determination, identical assays were done in the presence of 10^{-5} M eserine (an anticholinesterase). Hyaluronidase (EC 4.2.99.1) was measured by the turbidimetric method of DiFerrante (1956) with hyaluronic acid at several concentrations. Phosphomonoesterase (EC 3.1.3.1) activity was measured with *p*-nitrophenyl phosphate as a substrate following the method of Lowry (1957). Nucleotide pyrophosphatase (NADase, EC 3.6.1.9) was determined by the method of Kornberg and Pricer (1950) with 8 or 27 mM β-NAD. Phospholipase A (EC 3.1.1.4) was measured by the titrimetric method of Saito and Hanahan (1962). Proteolytic activities were screened using azocasein (Fritz et al., 1974). Arginine esterase was measured by the hydrolysis of BAPNA (*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride) which produces a product absorbing at 405 nm. Proteinase inhibitor assays employed BAPNA as a synthetic substrate for purified trypsin (Sigma type XI) to which assay aliquots were added (Fritz et al., 1971).

Electrophoresis. Phenol:acetic acid:urea gels were prepared by an adaptation of the procedure of Takayama et al. (1966). Sodium dodecyl sulfate–urea gels were prepared by the method of Eipper (1974). Sodium dodecyl sulfate gels were prepared according to Laemmli (1970).

For cross-linking, 200 μg of lyophilized protein was dissolved in 20 μL of 0.2 M triethanolamine (pH 8.5) containing 100 μg of dimethyl suberimidate and incubated for 3 h at 25 °C (Davies and Stark, 1970). The cross-linked products and standards were then solubilized and fractionated by the Laemmli $NaDodSO_4$ gel procedure. For reduction and alkylation of cross-linked proteins, 100 μg of protein was incubated for 17 h in 0.2 M sodium borate buffer (pH 9.0) with 1% mercaptoethanol. Iodoacetic acid (1 mg) was added and protein was incubated for an additional hour in the dark at 25 °C.

Polyacrylamide gradient slab gels, incorporating the buffer system of Laemmli (1970), were prepared by increasing the concentration of acrylamide in the direction of migration. A linear gradient of acrylamide (10–20% or 17.5–20%), with the ratio of acrylamide to bisacrylamide (20:1 w/w), was cast as the separating gel and overlaid by a 5% stacking gel. Each gel accommodated up to 10 samples (10–50 μg of protein) which were boiled with and without mercaptoethanol (5%) for 2 min and analyzed by electrophoresis.

Gels were stained in all cases by the procedure of Fairbanks et al. (1971).

Isoelectric Focusing. Isoelectric focusing in a pH 3–10 gradient was performed according to the procedures of Vesterberg (1972), Fawcett (1968), and Trump and Singer (1970) using 7.5% and 10% polyacrylamide gels and both chemical and photopolymerization. The pH profile was determined by slicing a control gel containing no protein into 2-mm sections which were incubated in 250 μL of deionized water overnight and then the pH of each was measured. Proteins were fixed and rinsed with repeated washes of 10% Cl_3CCOOH over 4 days and then stained by either Coomassie Blue, Naphthol blue black, or the Schiff's procedure. Where elution of the focused material was desired, fixation was omitted and the gel was sliced and sections were incubated as above in water.

N-Terminal Amino Acid Determination. Protein (30 nmol) was dansylated and hydrolyzed as delineated by Gros and Labouesse (1969) with the modifications of Zanetta et al. (1970). Dansyl amino acids were chromatographed on 3 × 3 cm Schleicher and Schuell polyamide sheets with two-dimensional development in 3% formic acid followed by benzene:acetic acid (9:1 v/v). To resolve dansylaspartic and dansylglutamic acid, a subsequent development in the second direction with ethyl acetate:methanol:acetic acid (20:1:1 v/v) (Briel and Neuhoff, 1972) was used. Unknowns were identified by comparison to standard dansyl amino acids.

Amino Acid Analysis. Toxins (1 to 1.5 mg) were hydrolyzed with 1 mL of constant boiling HCl in sealed tubes maintained at 110 ± 1 °C in an oil bath (Dow Corning 550). Prior to sealing, the tubes were flushed twice with dry nitrogen and evacuated to less than 20 μm Hg. Hydrolyses were run for 20 to 72 h. Hydrolysates were analysed in a Beckman Model 120C using the two-column system of Spackman et al. (1958). Values for serine, threonine, cystine, tyrosine, and histidine were corrected to zero time and values for valine, leucine, isoleucine, alanine, glycine, and phenylalanine were extrapolated to plateau levels at long hydrolysis times. In cases where an appreciable decomposition of cystine occurred, the values for proline were also corrected to zero time since cysteine is coeluted with proline in the system used. (Its presence can be detected by the ratio of absorbances at 570 and 440 nm which is 7.56 for proline and 1.46 for cysteine.) The best integral fit of amino acid residues to the experimental data was obtained by a computer analysis (Katz, 1968). Results were weighted to give different reliabilities to different amino acids and to account for either absolute or percentage error generally following the recommendations of Katz.

Independent composition analyses, including tryptophan, were obtained by hydrolysis in 4 N methanesulfonic acid (115 °C, 22 h) after the procedure of Simpson et al. (1976). Tryptophan values were confirmed by oxidation with *N*-bromosuccinimide in both the presence and absence of urea (Spande and Witkop, 1967), and by magnetic circular dichroism in 6 M guanidine hydrochloride (Barth et al., 1972). Total sulfur was obtained from x-ray fluorescence (see below) and compared with values for cystine plus methionine. Titration with DTNB was used for detection of free sulfhydryl groups (Robyt et al., 1971).

Carboxymethylated reduced toxins were prepared by reducing 3–5 mg of protein dissolved in 1 mL of 0.1 M Tris base (pH 8.5) with a 100-fold molar excess of solid dithiothreitol over protein. Samples were maintained at 25 °C overnight under a nitrogen barrier and solid iodoacetic acid was then added. Samples were incubated for an hour in the dark, and the reaction was quenched by acidification. Carboxymethylated derivatives were isolated by gel filtration on a 1.6 × 50 cm Sephadex G-10 (superfine) column equilibrated with 10% acetic acid. The CM-toxins were lyophilized and stored. Cystine content was obtained as carboxymethylcysteine after acid hydrolysis.

Sulfur Determination by X-Ray Fluorescence. Total sulfur content of the protein samples was determined with nondis-

TABLE I: Enzymic Activities in Fractions of the Venom of *Bungarus multicinctus*.

Fraction	μmol hydrolyzed/mg of protein·min			Phosphomono-esterase (nmol/mg·min)	Hyaluronidase[b] (μg/mg·min)	Arginine esterase (μmol/min·mg of protein)
	Acetylcholin-esterase	Phospho-lipase A[a]	Nucleotide pyrophospha-tase			
2	12	12.3	0.1	6.7		
3	37	24.0	4.8	4.3		
α-Bgt[c]						
4 + 5	739	3.2	10.5	7.3		8.4
6	80		0.1	19.9		0.5
7	2		*	17.3		
8	*[d]		*	6.5		
9	*		*			
10	*		*			
11	*					
12	*				3.2	
13	*				6.0	
14	*				25.0	

[a] Activity was assayed in the medium of Saito and Hanahan (1962), giving no detectable levels in fractions 9 to 14. However, addition of sodium deoxycholate to these fractions stimulated an endogenous phospholipase activity (Strong et al., 1976). We have not included the deoxycholate-dependent results for the β-toxins as they are a misleading gauge of contamination, and will be reported in more detail elsewhere. [b] Hyalaronidase was inhibited by excess substrate. Optimum substrate concentrations varied with the concentration of enzyme and so did the ratio of enzyme to optimum substrate concentration and the specific activities. The numbers shown were obtained with 150 μg of protein per mL, and the substrate concentrations of 43, 144, and 192 μg/mL, respectively. [c] α-Bgt was purified from fraction 3 by gradient elution from CM-cellulose. [d] Trace activity. Absence of number indicates no detectable activity.

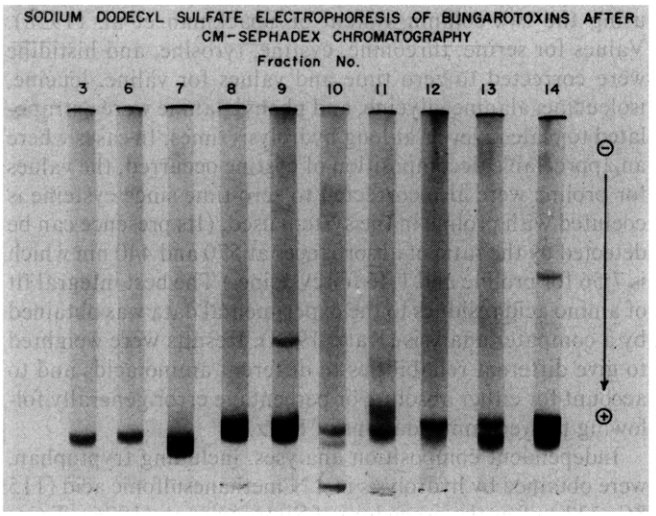

FIGURE 1: Sodium dodecyl sulfate electrophoresis of protein fractions after first chromatographic step. Protein (20 μg) was applied in 20 μL of Laemmli solution (1970) for fractions 3 and 6. In all other fractions, 40 μg was applied in 40 μL of Laemmli solution. These are 12% gels run at 4 mA/gel until the marker bromophenol blue had reached the gel end. Gels were stained and destained as in Methods.

persive vacuum soft x-ray fluorescence spectrometer (Hebert and Street, 1974). The spectrometer features six anodes which provide characteristic x-rays for sample excitation and determinations of the elements from oxygen to iron. The present experiments were primarily aimed at a sulfur determination and only a cursory examination was made to determine the presence of Na, Mg, Al, Si, P, Cl, K, and Ca at μg/mL level in some samples. Analysis used 50 μL of aqueous solution or homogeneous suspension containing 14 to 53 μg of protein. An important consideration in performing vacuum soft x-ray analyses is the amount of incident radiation which may be converted to heat in the sample. This is expecially true of biological samples which may contain easily decomposed to volatile components. Repeated analyses with several of the protein sample disks over periods of hours, and then again after several weeks, revealed no observable sample deterioration or decline in sulfur content.

Results

Purification. Initial fractionation on CM-Sephadex separated the crude venom into 13 protein peaks as indicated by positive Lowry reaction and UV absorbance (Eterović et al., 1975). Eleven of the protein fractions were lethal to mice. Fraction 3 contained the toxin designated α-Bgt and fraction 10 contained the toxin previously designated β-Bgt. Earlier work has provided a basis to classify fractions 3 and 6 thru 8 as "α-type" synaptic neurotoxins and fractions 9 thru 14 as "β type". It is believed that fractions 3 and 6 thru 8 are postsynaptic neurotoxins and that fractions 9 thru 14 are presynaptic neurotoxins (Lee et al., 1972; Dryden et al., 1974, Eterović et al., 1975). Purity was determined by assay of enzymatic activities detectable in the venom (Lee et al., 1972) and by sodium dodecyl sulfate disc gel electrophoresis (Laemmli, 1970).

Our patterns for four enzyme activities (Table I) resembled that reported by Lee et al. (1972). No proteolytic or proteolytic inhibitor activities were found in crude venom or in chromatographic fractions. A thrombin-like arginine esterase was detected in fraction 4 + 5 (so designated because of variable chromatographic resolution). After the CM-Sephadex separation, fractions 2 thru 7 were the most heavily contaminated by enzymes whereas fractions 9 thru 11 contained only traces of nucleotide pyrophosphatase and AChE. Further purification of fraction 3 from CM-cellulose yielded pure α-Bgt which showed no activity for the enzymes tested. When 10–40 μg of each fraction was subjected to $NaDodSO_4$ electrophoresis on 7.5 or 10% gels, they displayed a high degree of homogeneity, with the exception of fractions 2 and 4 + 5. These latter fractions manifested multiple high molecular weight bands probably due to the enzymes which had been detected by their activities (Table I). All other fractions were 90–100% low molecular weight material (Figure 1). Higher loading of the gels permitted detection of additional high molecular weight contaminants in some fractions, in agreement with the enzyme assays.

TABLE II: Summary of Gel Filtration Data of Bungarotoxins.

Fraction	Neurotoxic[a] act.	Earlier Names[b] Lee	Dryden	K_{av}[c] Sephadex G-50	Bio-Gel P30	App[d] mol wt
3 (α-Bgt)	α type	II_2	5c	0.28	0.21	15 000–16 000
7	α type	III_1	7	0.28	0.21	15 000–16 000
8	α type	III_2	8	0.28	0.21	15 000–16 000
9A	β type	IV_2	9	0.28	0.17	15 000–17 500
10 (β-Bgt)	β type	V	10	0.12	0.14	20 000–21 000
11	β type			0.11	0.13	20 500–21 500
12	β type	VI	11	0.10	0.12	21 000–22 000
13	β type	VII	13	0.11	0.13	20 500–21 500
14	β type	VIII	12	0.08	0.10	22 000–23 000

[a] Activities defined by toxicology (Eterović et al., 1975) and pharmacological studies (Lee et al., 1972; Dryden et al., 1974). [b] From Lee et al., 1972; Dryden et al., 1974. [c] K_{av} is defined as $(V_e - V_0)\cdot(V_t - V_0)^{-1}$, where V_e is the elution volume of the peak concentration of a solute; V is the void volume (indicated by blue dextran); V_t is the total column bed volume (indicated by potassium dichromate) (Ackers, 1975). [d] From calibration curve of K_{av} vs. (molecular weight) using chymotrysinogen, myoglobin, lysozyme, ribonuclease, cytochrome *c*, pancreatic trypsin inhibitor, and insulin as standards.

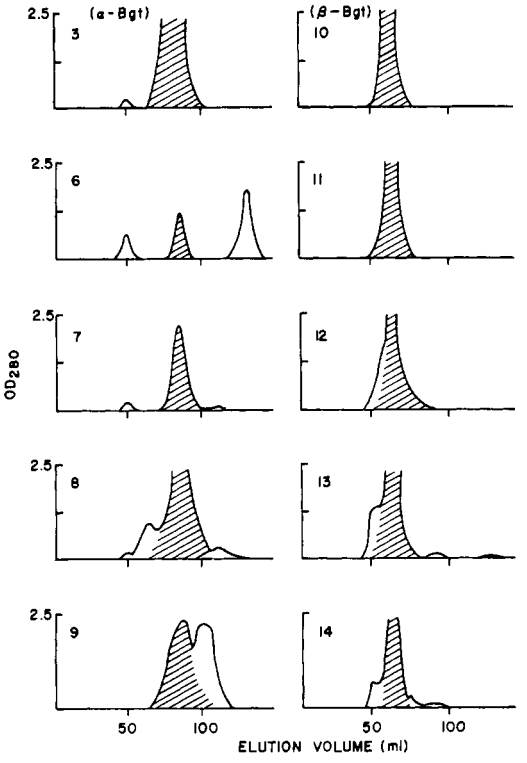

FIGURE 2: Gel filtration of fractions from first chromatographic step on Sephadex G-50. Hatched peaks indicate the toxic components.

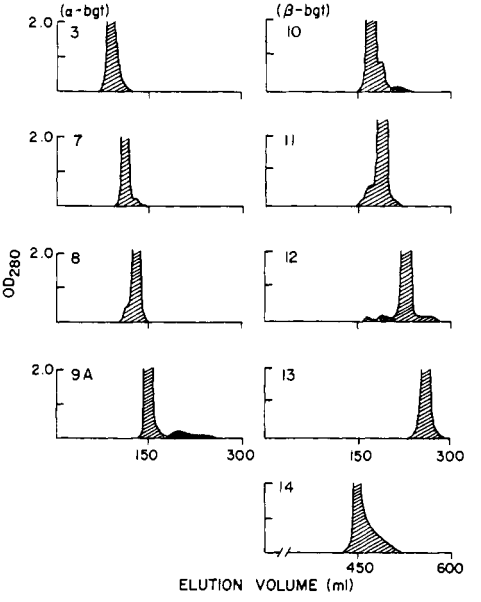

FIGURE 3: Bio-Rex 70 rechromatography of Sephadex G-50 fractions. Hatched peaks indicate components lethal to mice. Cross-hatched peaks were not lethal.

To eliminate high molecular weight contaminants, proteins were fractionated by gel filtration on Sephadex G-50 (Figure 2). Fractions 3, 6, 7, and 8 contained several components, including nontoxic material excluded from the gel at the void volume. A small tryptophan-containing peptide, whose properties are consistent with an identified snake venom tripeptide (Kato et al., 1966), was present in fraction 6. The toxic components of 3 and 6 thru 8 all eluted at identical volumes. The α-toxins eluted at a position calibrated at 15 000–16 000, twice the expected volume. To investigate whether this was attributable to dimerization under the mild conditions employed, 0.2 M NaCl was incorporated into the column eluent. The elution positions were not altered. Indeed, comparable calibrated weights were obtained by preparative scale chromatography on a 2.6 × 40 cm Bio-Gel P30 column, although there was adsorption to Bio-Gel P30 since yields were reduced to 70–80% with pronounced trailing of peaks. Fraction 9 was separated into two components, one toxic (9A) and one nontoxic (9B).[2] Fractions 12 thru 14 had varying degrees of contamination with proteins eluting both before and after the toxic components. In all cases, there was quantitative recovery of the applied protein. The toxins of fractions 10 thru 14 eluted in an identical position with an apparent molecular weight of 20 000–22 000 (Table II).

After removal of nontoxic high molecular weight materials, isoelectric focusing in polyacrylamide gel revealed that several fractions were significantly cross-contaminated. A final fractionation step and gradient elution was introduced using the cation exchanger Bio-Rex 70 (Figure 3). Final fractions gave single bands on phenol:acetic acid:urea gels, NaDodSO$_4$-urea gels, and upon isoelectric focusing (Figure 4).

Stability and Storage of Purified Toxins. Lethality was stable to extremes such as acid pH, high temperature, nonre-

[2] By the procedure previously described (Eterović et al., 1975), the LD_{50} of fraction 9A was 0.36 μg/g of mouse (0.33–0.40), and the LD_{100} was 0.55 μg/g of mouse. Dosages up to 2 μg/g of 9B were not lethal to mice. We had previously reported a LD_{50} of 0.50 μg/g for the unresolved fraction 9. Fraction 9A retained the characteristic of a slow time to death.

TABLE III: Molecular Weights of Bungarotoxins and Their Constituent Polypeptide Chains.

Fraction	$NaDodSO_4$ gradient[a] slab gel	Reduced/$NaDodSO_4$[a] gradient slab gel	DMS/$NaDodSO_4$[b] Cylindrical gels	Reduced/alkylated[b] DMS/$NaDodSO_4$ cylindrical gels
3	8000	8000	8400	8100
7	8000	8000	8700	7800
8	5000	5000	7000	6800
9A	21 000	11 500	16 000	14 500
10	21 500	8500	8500	6000
		11 500	18 000	15 000
11	20 500	7000	9000	6800
		11 500	18 500	15 000
12	21 000	7000	8400	7000
		11 500	18 000	15 000
13	21 500	7000	7600	7200
		12 500	13 500	14 000
14	21 500	11 300	11 500	11 000
		12 000		
		12 500		

[a] Measured with gradients of acrylamide from 17.5 to 20% (2 experiments); and 10 to 20% (1 experiment). [b] Measured on 12% and 15% Laemmli (1970) $NaDodSO_4$ gels. DMS, dimethyl suberimidate.

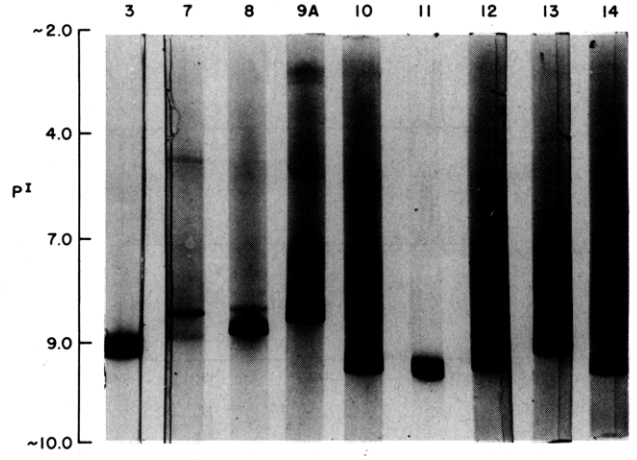

FIGURE 4: Polyacrylamide gel isoelectric focusing of fractions after second chromatographic step. Forty micrograms of each fraction was focused in 10% gels at 100 V for 1 h and then 200 V for 2 h. Gels were exhaustively fixed and rinsed in 10% Cl_3CCOOH and stained as in Methods.

ducing denaturants, and brief exposure to organic solvents. The native toxins are relatively resistant to proteolytic damage by chymotrysypsin, trypsin, pepsin, carboxypeptidases, and aminopeptidases. They can be stored frozen or lyophilized. There is no tendency for the toxins to aggregate on freeze-drying (Karlsson et al., 1972) but the toxins do aggregate to less active forms with repeated freezing and thawing. They are very soluble at all pHs.

The toxic activities are susceptible to cystine cleavage by either reducing agents or basic pH. The β-type toxins are much more sensitive to alkaline damage and will denature above pH 10. The α-toxins, on the other hand, can be stored up to pH 12 for short periods without damage. Alkaline denaturation is immediately detectable by an increase in turbidity indicative of reduced solubility. Control experiments showed that the toxins are resistant to cleavage by products of bacterial contamination, but are readily deamidated with a concomitant change in the isoelectric point.

Isoelectric Focusing. The purified toxins were all highly basic proteins (p*I* > 9) (Figure 6). This was corroborated by eluting the unfixed basic proteins and testing for toxicity. In the presence of urea, multiple bands were observed after isoelectric focusing. This may reflect either natural variation in the "isotoxins" of each purified venom component or a carbamoylation artifact (Stark et al., 1960). Storage of the β-type toxins in urea at pH 9 resulted in a rapid reduction in activity paralleled by a loss of free amino groups as determined by ninhydrin. Although fresh recrystallized urea was used in all experiments, it is possible that the minor bands with lower p*I*s may be carbamoylated derivatives.

Selective staining indicated that no purified component was a glyco- or lipoprotein.

Purity and Molecular Weight by $NaDodSO_4$–Polyacrylamide Gel Electrophoresis Technique. In $NaDodSO_4$ gel systems in the absence of a reducing agent, single bands were observed in all fractions, with the exception of fraction 14, which gave evidence of heterogeneity. With reduction, however, fractions 10 thru 13 split into two electrophoretic bands. Since fractions 10 thru 13 gave single electrophoretic bands in the strongly dissociative but nonreducing systems described above, the constituent chains may be maintained by interchain disulfides in the native molecule.

The initial results with $NaDodSO_4$ gels (Figure 1) and gel electrofocusing (Figure 4) suggested that these proteins might prove difficult to accurately analyze by conventional $NaDodSO_4$ gel procedures since, like the anomalous histones (Panyim and Chalkley, 1971), they are small and highly basic. Thus, several more elaborate approaches were used. Estimates of the unreduced weight were obtained by calibration of a linear gradient $NaDodSO_4$ slab gel. Although this procedure has limitations, we nevertheless obtained molecular weights consistent with other techniques. Fraction 7 was very similar to α-Bgt. On the other hand, fraction 8 appeared inordinately small by this procedure, approximately 5000, which may be due to unreliability from inadequate standards. All of the β-types appeared to be 20 000–22 000 in agreement with gel filtration. Running the toxins on identical gradients under reducing conditions did not alter the size of fractions 7 and 8, indicating single polypeptide chains. Smaller chains were obtained in all β-type toxin fractions. Fractions 10 thru 13 had chains of very similar weights, 7000–8000 for the lighter and 11 500–12 500 for the heavier. By comparison of results with and without reduction with β-mercaptoethanol, fractions 9A and 14 appeared to be homodimers. Fraction 14 was notably more heterogeneous, giving three separate heavier bands with reduction.

The cross-linking procedure of Davies and Stark (1970),

TABLE IV: Amino Acid Composition of Bungarotoxins (Fractions 7 through 14).[a]

	Fraction 7		Fraction 8		Fraction 9A		Fraction 10		Fraction 11		Fraction 12		Fraction 13		Fraction 14	
	Moles/Mole	Integer	Moles/Mole	Integer	Moles/Mole	Integer	Moles/Mole	Integer	Moles/Mole	Integer	Moles/Mole	Integer	Moles/Mole	Integer	Moles/Mole	Integer
Lys	5.23	5	6.42	6	7.90	8	13.18	13	14.32	14	15.77	16	18.88	19	12.92	13
His	1.15	1	1.34	1	2.77	3	4.82	5	5.79	6	6.06	6	6.25	6	3.21	3
Arg	4.77	5	5.76	6	10.82	11	14.05	14	14.11	14	15.12	15	17.26	17	8.71	9
Asp	8.50	9	8.91	9	18.13	18	23.01	23	22.27	22	22.77	23	23.86	24	12.08	12
Thr	7.11	7	5.62	6	9.27	9	10.28	10	11.30	11	11.19	11	10.17	10	6.94	7
Ser	3.44	4	3.79	4	4.40	4	3.42	4	6.93	7	6.83	7	5.97	6	5.32	5
Glu	5.10	5	5.98	6	8.39	8	12.29	12	11.82	12	12.09	12	13.21	13	7.86	8
Pro	5.00	5	1.89	2	5.28	5	6.94	7	8.18	8	7.84	8	9.82	10	6.07	6
Gly	4.55	5	2.97	3	12.63	13	17.72	18	15.97	16	16.09	16	16.29	16	8.20	8
Ala	3.26	3	2.15	2	9.24	9	11.38	11	10.87	11	10.64	11	11.35	11	5.92	6
½-Cys	9.78	10	9.94	10	14.04	14	16.29	16	20.18	22	22.13	30	20.78	26	12.57	12
Val	2.70	3	1.82	2	2.29	2	5.00	5	4.54	5	3.69	4	5.58	6	3.93	4
Met	1.00	1	0.94	1	1.61	2	1.95	2	1.97	2	2.04	2	1.61	2	0.61	1
Ile	2.61	3	2.81	3	4.92	5	8.48	9	8.47	9	8.21	8	8.53	9	5.67	6
Leu	3.19	3	3.10	3	4.70	5	7.38	7 (8)	6.32	6	5.90	6	7.61	8	4.90	5
Tyr	1.84	2	4.26	4	9.93	10	13.28	14	13.82	14-15	16.10	16	13.18	14-15	7.06	9
Phe	2.26	2	3.18	3	4.15	4	6.02	6	5.99	6	6.13	6	6.14	6	2.72	3
Trp	0.95	1	0.84	1	0.98	1	0.86	1	0.92	1	0.89	1	0.92	1	0.54	1
Total no. of aa/mole	74		72		131		177		186		198		204		118	
Mol wt	8300		8500		15100		20400		21500		23000		23700		13800	
N terminus	Met		Met		Asx		Asx		Asx		Asx		Asx		Asx	

[a] Moles of amino acid per mole of protein were calculated from the μmol of amino acid obtained from the analyses and the μmol of protein hydrolyzed. This last value was calculated from A_{280} of the solution to be hydrolyzed and the ϵ_{280} of the respective protein. Values for half-cystine which are underlined were obtained by substracting the values for methionine from total sulfur content obtained from x-ray fluorescence spectrum (Table V). Values for tyrosine which are underlined were obtained from MCD spectra (Table VI). Values are extrapolated from at least one analysis at each time point; 20–22, 44, and 68–72 h. Every fraction has more than one determination at the 20–22 h time point, including a methanesulfonic acid hydrolysis for tryptophan.

employed to give a separate measure of oligomeric structure, gave very similar results to those obtained using $NaDodSO_4$ gels with the exception that the chain values were uniformly larger. This is frequently observed with cross-linked proteins, even when calibrated against cross-linked standards (Davies and Stark, 1970). To reduce the overall charge on the cross-linked molecules (unchanged by dimethyl suberimidate cross-linkages), they were reduced and carboxymethylated and again electrophoresed on disc gels with comparably treated standards. Values for the heavy and light chains in the β type and the single chain of the α type were all lower. These results are summarized in Table III.

Amino Acid Composition. Tables IV and V summarize the data of amino acid analyses. The existence of a single tryptophan was originally indicated from the magnetic circular dichroism spectra (Hanley, Vickery, and Bennett, manuscript in preparation). Results obtained from 22-h methanesulfonic acid hydrolyses of the fractions agreed very closely with those obtained by HCl hydrolyses and gave independent evidence for the existence of a single tryptophan. Subsequent quantitative studies using NBS oxidation (Figure 5) also indicated a single tryptophan whose modification eradicated its contributed to the fluorescence spectra (Figure 5). The residual fluorescence was characteristic of tyrosine.

Except for fractions 8 and 14, molecular weights obtained from computer analysis of amino acid composition agree well with the values from other techniques. Fraction 8 appears smaller than α-Bgt by $NaDodSO_4$ gel electrophoresis, but larger by amino acid composition. From their basic isoelectric point, we predict that the bulk of the glutamic and aspartic acid residues are amidated.

Notable destruction of threonine, serine, cystine, and tyrosine was observed; therefore, values were extrapolated to zero time. Even with correction, the serine and threonine values may still be low owing to their nonlinear destruction kinetics in these proteins. The tyrosine content was compared with independent

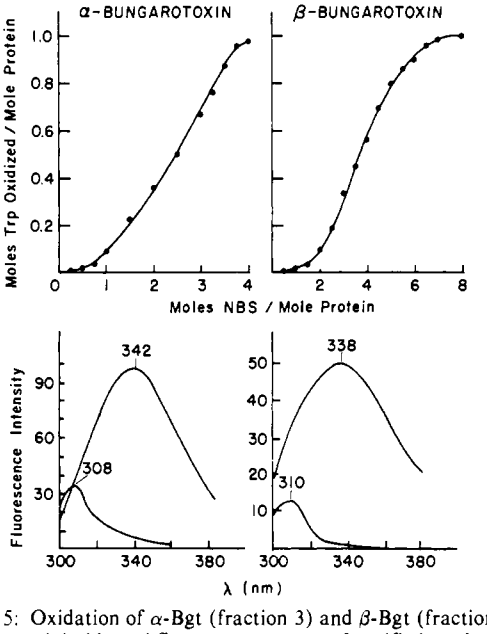

FIGURE 5: Oxidation of α-Bgt (fraction 3) and β-Bgt (fraction 10) by *N*-bromosuccinimide and fluorescent spectra of purified products of reaction. After oxidation, proteins were desalted on Sephadex G-25 and lyophilized. One hundred micrograms of protein was used in all cases to obtain the spectra on a Hitachi Perkin-Elmer MPF 2A with excitation at 286-nm and 6-nm slit widths. Oxidized samples were excited at 280 nm.

TABLE V: Determination of Sulfur Content of Bungarotoxins by X-Ray Fluorescence.

Fraction	Moles of S/mole of protein[a]	Fraction	Moles of S/mole of protein[a]
α-Bgt	10.4	10 (β-Bgt)	17.8
7	11.8	11	24.1
8	10.2	12	31.6
9A	16.3	13	27.2
		14	13.1

[a] Protein molar concentration was calculated from A_{280} and the extinction coefficient.

values obtained from magnetic circular dichroism, and we have given these values preference in this determination.

Although alanine and methionine are absent from many Elapid toxins (Karlsson, 1974), alanine (4-6 mol fraction percent) was found in all the bungarotoxins. Methionine concentrations were the lowest of all the amino acids. There were consistently higher tyrosine-to-tryptophan ratios in the β-type than in the α-type toxins. Except for the high proportion of basic residues and the high cysteine content, the compositions of the toxins were very similar to the average found for soluble, globular proteins (Jukes et al., 1975).

Complete reduction and alkylation were difficult to achieve in the β-type toxins 9A thru 14. Long incubation times (24-36 h) with strong denaturants (6 M guanidine hydrochloride or 2 M guanidine perchlorate) and a large molar excess of reducing agent were necessary. Carboxymethylcysteine values after acid hydrolysis were low in several instances which may reflect the known acid hydrolytic instability of CM-amino acids. No evidence was obtained for reaction with lysines or histidines under these alkylating conditions. α-Type toxins could be reduced and alkylated satisfactorily under standard conditions.

N-terminal amino acid analyses of α-Bgt gave the expected isoleucine (Mebs et al., 1972; Clark et al., 1972), which was recovered as a mixture of isoleucine and its alloisomer. Fractions 7 and 8 gave methionine as the N-terminal amino acid and each fraction 9A thru 14 gave Asx as the N-terminal amino acid. No attempt was made to quantitate the N-terminal amino acid liberation and dansylated arginine and ε-lysine were observed at low levels in all samples.

The x-ray fluorescence determinations served two purposes: (1) an independent determination of total protein sulfur, assumed to represent the cystine plus methionine content; and (2) the analysis of ionic contamination. The latter was of interest to judge the success of desalting and to screen for the presence of any tightly bound ionic cofactors. There were no appreciable amounts of Na, Mg, Al, Si, P, Cl, K, or Ca in the samples.

Cysteine content was very high in these proteins, as with other snake venom neurotoxins (Tu, 1973). The high proportion of disulfides accounts for the resistance of these toxins to nonreducing denaturation and susceptibility to reduction (Eterović et al., 1975). Free sulfhydryl groups were not found in any fraction. The number of half-cystines plus methionine agrees within 10% of the amino acid analyses values for fractions 7 and 8. The values for fractions 9A and 14 agree within a single sulfur per protein. We found 16 half-cystines per 20 500 daltons for fraction 10 (β-Bgt) from both the x-ray and amino acid analyses. This value contrasts with the 20 half-cystines reported by Lee et al. (1972) and Kelly and Brown (1974). The larger number is very likely caused by contamination with fraction 11, which has 22 half-cystines. Sulfur

TABLE VI: Tyrosine-Tryptophan Ratios and Extinction Coefficients of Bungarotoxins.

Fraction	Tyr/Trp[a]	Integral value	ϵ_{max}[b] $(mM\ cm)^{-1}$	0.1% A_{280}[c] (cm^{-1})
α-Bgt	2.2	2	10.5	1.32
7	2.2	2	10.9	1.31
8	4.0	4	13.5	1.59
9A	10.2	10	21.3	1.41
10 (β-Bgt)	13.7	14	29.6	1.45
11	14.5	14-15	29.8	1.39
12	16.1	16	35.4	1.54
13	14.5	14-15	34.2	1.44
14	8.9	9	20.1	1.46

[a] Method of calculation based on Barth et al. (1972). [b] Maximum was found to be 278-279 nm for the α-type fractions and 277-278 nm for the β-type fractions. [c] These values were calculated from the values for ϵ_{280} and molecular weight estimates from Table IV.

values from x-ray fluorescence for fractions 12 and 13 were considerably higher than the total sulfur from amino acid composition. We have chosen the x-ray values as correct, attributing the low cystine obtained after hydrolysis to incomplete compensation by the extrapolation to zero time. Corroboration for higher cystine content in these fractions has come from the magnitude of a near-UV circular dichroism disulfide signal which we have demonstrated to be linearly dependent on the cystine content (Hanley, Vickery, and Bennett, manuscript in preparation).

Extinction Coefficients. For the convenience of subsequent workers, we have determined the extinction coefficients of the various purified fractions using a magnetic circular dichroism technique (Hanley, Vickery, and Bennett, manuscript in preparation) and confirmed (within 3%) the results by quantitative amino acid analysis of samples with known absorption. These values are indicated in Table VI.

Discussion

The natural heterogeneity of the neurotoxins in *Bungarus multicinctus* venom makes it an ideal source for the purification of either α-type or β-type toxins. However, with partially purified toxins, subsequent pharmacological and biochemical studies may be quantitatively, or indeed, qualitatively, compromised. α-Type bungarotoxin can be obtained in high yield and purity after only two chromatographic steps (Eterović et al., 1975). On the other hand, our experience with the other fractions of bungarotoxin has established that obtaining them in a homogeneous condition required more extensive repurification. β-Bungarotoxin is a good example. Previous groups have reported amino acid compositions which differ in the amount of cystine and serine from that we reported here (Lee et al., 1972; Kelly and Brown, 1974) (Table VII). We attribute this to the cross-contamination of fraction 10 (β-Bgt) with fraction 11 since fraction 11 was not separated in the earlier fractionation schemes. Kelly and Brown (1974) declared β-Bgt "pure" after a single chromatographic resolution of crude venom into only six components, which makes it likely that the described properties are an average of several β-type fractions. More recently, Wernicke et al. (1975) reported a persistent contaminant of their β-Bgt preparation detectable on cellulose acetate electrophoresis. Donlon et al. (1975) have reported a "β-Bgt" that is the most basic fraction in the crude venom and has a molecular weight near 11 000. In both of these cases, the degree of contamination or identity of their purified component cannot be gauged without an amino acid analysis. The prop-

TABLE VII: Comparisons with Bungarotoxin Amino Acid Compositions.

	α-Bgt	Fr 7	Fr 8	Fr 10 β-Bgt	β-Bgt[a] (Kelly)	β-Bgt[b] (Lee)	Notexin[c]	Fr 9A	Fr 14
Lys	6	5	6	13	13	13	11	8	13
His	2	1	1	5	5	5	3	3	3
Arg	3	5	6	14	15	14	5	11	9
Asp	4	9	9	23	24	22	18	18	12
Thr	7	7	6	10	12	12	3	9	7
Ser	6	4	4	4	6	6	3	4	5
Glu	5	5	6	12	13	12	7	8	8
Pro	8	5	2	7	8	8	5	5	6
Gly	4	5	3	18	18	16	10	13	8
Ala	5	3	2	11	12	11	9	9	6
½-Cystine	10	10	10	16	20	20	14	14	12
Val	5	3	2	5	4	4	4	2	4
Met	1	1	1	2	2	2	2	2	1
Ile	2	3	3	9	9	8	4	5	6
Leu	2	3	3	7	7	7	4	5	5
Tyr	2	2	4	14	13	13	10	10	9
Phe	1	2	3	6	6	6	5	4	3
Trp	1	1	1	1	4	3	2	1	1
Total	74	74	72	177	191	182	119	131	118
Mol wt	7983	8350	8540	20440	22130	21110	13580	15100	13770

[a] Kelly and Brown (1974). [b] Lee et al. (1972). [c] Halpert and Eaker (1975).

erties of the toxin studied by the latter group are very close to those of our fraction 14.

The first group of toxins consisting of the α-type toxins 3 (α-Bgt), 7 and 8, has identical preparative Sephadex G-50 elution volumes, but the apparent molecular weights were twice those anticipated. The use of 0.2 M NaCl did not alter this behavior, but it should be noted that Mebs et al. (1972) used 0.5 M NaCl at pH 8.5 to determine the gel filtration weight of α-Bgt and obtained a lower value. Dimerization of toxins has been noted before (Tu, 1973) and may be a consequence of the high concentration of the toxins. Alternatively, the toxins may be sufficiently asymmetric so as to behave like a larger molecule owing to rapid rotation in solution. Consistent with this, asymmetry has been observed by x-ray diffraction studies of erabutoxin (Low et al., 1976).

Variations in the mobility of fractions 7 and 8 make it difficult to gauge their weights relative to that of α-Bgt or to judge whether any of these are behaving anomalously. For example, on $NaDodSO_4$ gradient gels (Table III and Figure 6), fraction 8 has a higher mobility than α-Bgt, but on phenol:acetic acid:urea gels and on $NaDodSO_4$–urea gels it has lower mobilities. In view of the sensitivity of $NaDodSO_4$ techniques to charge effects (Panyim and Chalkley, 1971), it is possible that this is the source of the variation.

Sequencing studies of postsynaptic neurotoxins have established that they fall into two categories: (1) type I or "short" neurotoxins (60–62 residues, 4 disulfides, 6800–7000); and (2) type II or "long" neurotoxins (71–74 residues, 5 disulfides, 7800–8100) (Tu, 1973; Yang, 1974). Fractions 7 and 8 may be long neurotoxins by these criteria, but have the N-terminal methionine found in some short neurotoxins. Their higher content of arginine and aspartic acid than in α-Bgt is more typical of the class of long neurotoxins than is the content in α-Bgt (Yang, 1974). It is possible that the final word on their similarities and differences to α-Bgt must await sequencing.

The second group consists of the β-type neurotoxins fractions 9A thru 14. Fractions 10 thru 13 are very similar, each having 1 tryptophan, the same N-terminal amino acid, similar isoelectric points, molecular weights, and comparable amino acid

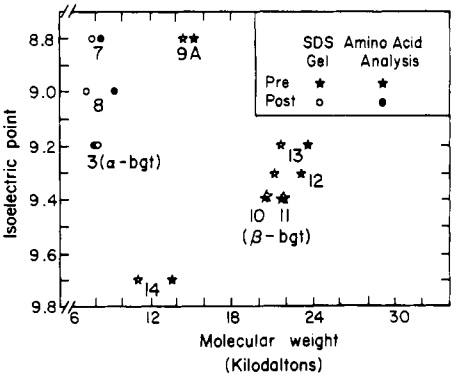

FIGURE 6: Comparison of isoelectric points and molecular weights of purified bungarotoxins. The horizontal distance between the open and solid symbols indicates the agreement of the two methods of estimation of molecular weights. It was apparent that the postsynaptic toxins (fractions 3, 7, and 8) cluster at lower isoelectric points and molecular weights than the presynaptic toxins (10, 11, 12, and 13). Two toxin fractions (9A and 14) possess different physical properties.

compositions (with the exception of increasing cystine content from fractions 10 thru 13) (Table IV and Figure 6). They can be separated into smaller chains, but only under reducing conditions and with complete loss of biological activities (Hanley and Bennett, manuscript in preparation). For this reason, we prefer to term these "chains" rather than subunits, since they cannot, so far, be isolated with a retention of any activity. When fractions 10 thru 13 are reduced and alkylated, the light chain is not detectable in $NaDodSO_4$ gel systems unless first cross-linked. Subsequent isolation of the light chain has established that it in turn may be composed of smaller chains, similar to crotoxin A (Horst et al., 1972). It is refractory to N-terminal amino acid analysis, which explains the origin of the single N-terminal Asx in β-type fractions. We would predict that the molecular weight values obtained with reduced/alkylated/cross-linked chains would most nearly approximate the true weights.

The amino acid compositions presented by Lee et al. (1972)

and Kelly and Brown (1974) for β-Bgt differ from ours in the cystine and serine content, and in the tryptophan content (Table VII). We have established by methanesulfonic acid hydrolysis, MCD, and NBS oxidation that there is a single tryptophan in each of the fractions 9 thru 14. This tryptophan is localized to the heavy chain (Hanley and Bennett, manuscript in preparation) and is partially buried, as indicated by its shift in fluorescence maximum (Eterović et al., 1975) toward 330 nm and in its relative inaccessibility to NBS (consuming 8 mol per tryptophan oxidized) (Figure 5).

The molecular weight reported by Lee et al. (1972) for β-Bgt has been recalculated by us to be about 22 000 based on the amino acid composition. The "subunit" weights reported by Kelly and Brown for β-Bgt, 8800 and 12 400, are higher and lower, respectively, than our best values for these chains. Kelly and Brown do not give an isoelectric point for their purified component, but their description suggests it is a weakly basic protein, which is clearly not the case. All of the β-type toxins are highly basic.

Fractions 9A and 14 are distinct in their toxicology (Eterović et al., 1975), structure, and antigenic properties. They resemble notexin, the sequenced presynaptic neurotoxin (Halpert and Eaker, 1975). The amino acid compositions are compared in Table VII. They are both single chains homologous to the heavy chains of the fractions 10 thru 13 (Hanley and Bennett, manuscript in preparation). The ambiguity in the pharmacological action of fraction 9 may be due to small amounts of contaminating α-type toxin. Since fraction 9 marks the transition in the chromatographic profile from α to β-type toxins, we have observed that as much as 30% of the material in fraction 9 can consist of α-type contaminants after the initial fractionation step. Fraction 14, on the other hand, has an unusual pharmacology that is currently the subject of more extensive research. Both of these fractions exhibit the myotoxic effects of notexin (Hanley, unpublished observations).

Although a great deal of research has been directed to α- and β-bungarotoxin, the other toxic components in the crude venom of *B. multicinctus* have been previously neglected. Nonetheless, the presence of structural variants of these "parent" toxins should interest workers in two respects: (1) the similarity of their chemical properties makes complete purification of a particular component difficult; and (2) the possibility of exploring functional correlates of their structural heterogeneity exists. The toxicological (Eterović et al., 1975) and pharmacological (Dryden et al., 1974) differences of particular fractions suggest such investigations should be illuminating.

Acknowledgments

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Resonance Raman Spectra of Methemoglobin Derivatives. Selective Enhancement of Axial Ligand Vibrations and Lack of an Effect of Inositol Hexaphosphate†

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ABSTRACT: Resonance Raman spectra have been obtained for the OH^-, N_3^-, and F^- derivatives of methemoglobin by excitation in the 550–650-nm region. A selective enhancement with excitation in the charge-transfer bands is observed for peaks at 413 and 497 cm^{-1} and a doublet at 471 and 443 cm^{-1} in the N_3^-, OH^-, and F^- complexes, respectively. These peaks are assigned to Fe-axial ligand stretches on the basis of: (1) a 20-cm^{-1} shift of the 497-cm^{-1} peak of the hydroxide complex to lower energy on isotopic substitution of $^{18}OH^-$ for $^{16}OH^-$; (2) the proximity of the 413-cm^{-1} Raman peak to the 421-cm^{-1} IR peak previously assigned to the Fe-N_3^- stretch in a model heme-azide complex [Ogoshi, H., Watenabe, E., Yoshida, Z., Kincaid, J., and Nakamoto, K. (1973), *J. Am. Chem. Soc. 95,* 2845]; (3) the selective appearance of the 471- and 443-cm^{-1} peaks in the Raman spectra of the F^- complex. The doublet observed at 471 and 443 cm^{-1} in the F^- derivative may reflect a heterogeneity in the heme cavity due to hydrogen bonding of H_2O to the F^- ligand in both the α and β subunits, as has been previously suggested based on x-ray diffraction results (Deatherage, J. F., Loe, R. S., and Moffat, K. (1976), *J. Mol. Biol. 104,* 723). It is suggested that the frequency of the Fe-F^- vibration reflects the out-of-plane distortion of the Fe from the heme plane. The lack of a shift in the frequency of the Fe-F^- vibration suggests that there is little or no movement of the iron with respect to the heme plane upon the addition of inositol hexaphosphate, which is thought to alter the allosteric equilibrium between the R and T forms of methemoglobin. This result is consistent with a recent x-ray crystallographic study of an IHP complex of MetHb-F^- (Fermi G., and Perutz, M. F. (1977), *J. Mol. Biol. 114,* 421). Excitation profile measurements suggest that the charge-transfer band in methemoglobin OH^- like that in methemoglobin N_3^- is z polarized, while in methemoglobin F^- the charge transfer transition is mixed with a π to π^* transition.

Resonance Raman spectroscopy can serve as a structural probe for biological molecules such as hemoglobin and myoglobin (Spiro, 1975, and references therein; Yamamoto et al., 1973; Kitagawa et al., 1975; Ozaki et al., 1976). Upon excitation within the absorption bands of the heme chromophore a selective enhancement occurs in the intensity of the Raman peaks resulting from heme vibrations (Spiro, 1975). In previous reports the dominant Raman bands which have been observed appear to result from in-plane porphyrin macrocyclic modes and occur at energies between 600 and 1700 cm^{-1}. This is because excitation occurred within π to π^* electronic transitions of the porphyrin macrocycle such as in the α, β, and Soret bands. The energies of some of these Raman peaks have been shown to be sensitive to the oxidation state, spin state, and/or planarity of the metal with respect to the porphyrin plane (Spiro, 1975; Spaulding et al., 1975). However, vibrational modes of the iron in heme complexes, such as Fe-axial ligand modes, are rarely observed (Brunner, 1974; Spiro and Burke, 1976) because the π orbitals involved in the α, β, and Soret bands are poorly conjugated with the metal orbitals (Asher and Sauer, 1976). Unfortunately, these vibrational modes are precisely the ones that contain the greatest information on ligand binding in heme proteins. In addition, these modes would be expected to be sensitive to constraints imposed by the protein such as the proposed tension on the heme iron by the proximal histidine during the transition between the R and T allosteric forms (Perutz, 1972).

Recently, Asher and Sauer (1976) demonstrated the specific enhancement of vibrational modes involving the metal when excitation occurred within the charge-transfer bands of manganese(III) etioporphyrin, suggesting that a similar enhancement of vibrational modes which involve the metal should occur upon excitation into charge-transfer bands of heme. The heme in methemoglobin, like manganese(III) porphyrins, has electronic transitions between 600 and 640 nm which have been assigned to charge-transfer bands (see Smith and Williams, 1970). Excitation within these bands should enhance vibrational modes such as axial ligand stretches. We have utilized a tunable dye laser which can excite within these charge-transfer bands and report here a study of ligation properties of methemoglobin (Asher et al., 1977; Asher, 1976).

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