Proc. 17, 1116.
Spackman, D. H., Stein, W. J., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Steele, W. J., and Busch, H. (1963), *Cancer Res. 23*, 1153.
Tristram, G. R., and Smith, R. H. (1963), *Advan. Protein Chem. 18*, 308.
Vincent, W. S. (1955), *Intern. Rev. Cytol. 4*, 269.
Vincent, W. S. (1957), *Anat. Record 133*, 346.
Vincent, W. S. (1963), *Proc. Intern. Congr. Genet., 11th, The Haque*, 343.
Wang, T. Y. (1966), *J. Biol. Chem. 241*, 2913.
Wang, T. Y., Kirkham, W. R., Dallam, R. D., Mayer, D. T., and Thomas, L. E. (1950), *Nature 165*, 974.
Wang, T. Y., Mayer, D. T., and Thomas, L. E. (1953), *Exptl. Cell Res. 4*, 102.

Purification and Some Properties of the Neurotoxins of *Androctonus australis* Hector*

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ABSTRACT: The venom of *Androctonus australis* contains two neurotoxins (I and II) the purification of which has been carried out by extraction with water, Sephadex G-50 filtration, and equilibrium chromatography on Amberlite CG-50 and DEAE-Sephadex in ammonium acetate buffers. The neurotoxins were homogeneous by polyacrylamide gel electrophoresis, equilibrium chromatography on Amberlite CG-50, and end-group determination. The molecular weight is 6822 for toxin I and 7249 for toxin II as determined by amino acid composition and confirmed by sedimentation equilibrium. The amino acid composition of the toxins is different but similarities are found: 63 and 64 amino acids for toxins I and II, lack of methionine in both and of glutamic acid in toxin I, and presence of four disulfide bridges in both toxins. End-group analysis and alkylation studies show that both toxins consist of a single peptide chain ended by lysine at its N terminal and by threonine (toxin I) and glycine (toxin II) at its C terminal.

Starch gel and disc electrophoresis mobilities indicate the basic character of both toxins. The molar extinction coefficients of toxins I and II in 0.5 M acetic acid are 10.71 and 18.08 $\times$ 10^3 at 275 and 276 mμ, respectively. The LD_{50} of the toxins dissolved in saline containing serum albumin and determined by intravenous injection into 20-g mice is 19 μg/kg for toxin I and 10 μg/kg of toxin II. These values indicate that the neurotoxins of *A. australis* are among the more toxic of the hitherto known animal neurotoxins.

Previous investigations (Miranda and Lissitzky, 1961; Miranda *et al.*, 1964a,b) have shown that the venom of two North African scorpions each contained two neurotoxins, the purification of which has been carried out by reversible retention on Sephadex G-25 in water and ion-exchange chromatography on Amberlite IRC-50. The toxic proteins purified by this method were homogeneous in the ultracentrifuge, by zone electrophoresis in starch gel, and by equilibrium chromatography on Amberlite IRC-50. The complete absence of methionine in both toxins was an additional criterion of purity. Molecular weights of 11,000 and 16,000 were obtained by ultracentrifugation for each toxin.

Further studies (unpublished experiments) have shown that the treatment of the toxins at pH values removed from neutrality led to their dissociation into subunits. This paper describes a new method of purification giving the pure monomeric neurotoxins of *Androctonus australis*. Evidence is given that each toxin is composed of a single polypeptide chain.

Material

The venom was obtained by electrical stimulation of the postabdomen of animals collected in the area of *Tozeur* (Tunisia). It was obtained from F. G. Celo (Zweibrücken, Germany) and stored in the dried form.

2-Mercaptoethanol was purchased from Gallard-Schloesinger (Garden City, N. Y.), iodoacetic acid and iodoacetamide from Nutritional Biochemicals Corp. (Cleveland, Ohio), human serum albumin (five times crystallized) from Immunology Inc. (Lombard, Ill.), and 2-bromoethylamine from Eastman Kodak (New York). Amberlite CG-50 was obtained from Rohm and Haas (Philadelphia, Pa.), Sephadex and DEAE-

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Sephadex A-50 from Pharmacia (Uppsala, Sweden), and Bio-Gel P_2 and P_6 from Bio-Rad (Richmond, Calif.).

All other chemicals were of analytical grade. All solutions were made with deionized quartz-redistilled water.

Methods

All chromatography was performed at room temperature using calibrated glass columns fitted with sintered glass supports. Ammonium acetate buffers were prepared by adjusting the pH of solutions of ammonium acetate with concentrated ammonium hydroxide or acetic acid as required. These solutions will be referred to further as ammonium acetate buffers. The pH measurements (±0.005) were made with a Beckman 1019 pH meter and the concentration of NH_4^+ ion was determined by the Kjeldahl method. Solutes in the column effluents were concentrated and ammonium acetate removed by *two* consecutive lyophilizations.

Toxic lyophilisates were dissolved in the buffer chosen for equilibrium chromatography. Flow rates higher than 50 ml/hr were controlled by a mP1 pump (Bühler, Tubingen, Germany) and flow rates lower than 50 ml/hr with a Miniflow 4501 pump (LKB, Stockholm, Sweden). Protein concentrations were analyzed by spectrophotometry at 280 mμ.

Determination of Toxicity. LD_{50} were calculated according to Behrens and Karber (1935). Toxic samples dissolved in 0.3 ml of physiological saline were injected subcutaneously or into the tail vein of 20-g Swiss mice. The relative toxicity of diluted solutions has been found to decrease as the purity of the toxins increased. This effect might be explained by the denaturation of the toxins at very low concentrations and the effect is, in fact, reversed by the addition of proteins (unpublished results). Because of this observation, toxic solutions used for *in vivo* tests were supplemented with human serum albumin (final concentration, 2 mg/ml). The purification of neurotoxins was followed by the determination of the specific toxicity, arbitrarily defined as the number of LD_{50} per absorbance unit at 280 mμ.

Water Extraction and Dialysis. The crude venom (2–12 g) was extracted with water in five consecutive operations (total volume, 100 ml/g of venom). The extract was centrifuged (4000*g* for 10 min) and the supernatant was dialyzed against water for 48 hr in 23/32 Visking cellulose tubing. Insoluble nontoxic material formed during dialysis was eliminated by centrifugation and the toxic solution was freeze dried and stored at −10°.

Dextran Gel Filtration. An amount of the extract corresponding to 0.5 g of crude venom was dissolved in 0.1 M ammonium acetate, pH 6.9 (10 ml), and filtered at a flow rate of 250 ml/hr through a column (5 × 100 cm) of Sephadex G-50 beads fine grade, equilibrated in the same buffer.

Chromatography on Amberlite IRC-50. Amberlite IRC-50 sieved to obtain the 200–400 mesh fraction (Amberlite CG-50) was washed and cycled according to Hirs (1955) and used with the minor adjustments for toxin purification as previously described (Miranda, 1964). The required amount of resin in the hydrogen form was equilibrated with the desired ammonium acetate buffer. Column dimensions were 4 × 150 cm and flow rate was to 48 ml/hr. Two steps of equilibrium chromatography on Amberlite were performed. The first one in 0.2 M ammonium acetate at pH 6.70 was used to fractionate the toxic fractions isolated from two Sephadex G-50 filtrations (corresponding to 1 g of crude venom and dissolved in 10 ml of buffer). Two toxic peaks (I and II) were obtained and lyophilized separately.

After the DEAE-Sephadex step described below, both toxic fractions were submitted to a second equilibrium chromatography on Amberlite CG-50 in 0.2 M ammonium acetate at pH 6.30 for toxin I and at pH 6.70 for toxin II. The amount of toxic material chromatographed on a 4 × 150 cm column corresponded to 2–6 g of crude venom for the toxin I fraction and 1–3 g for the toxin II fraction.

Chromatography on DEAE-Sephadex. The anion-exchange gel DEAE-Sephadex A-50 beads 40–120 μ was cycled through the Cl^- and OH^- forms using HCl and NaOH (0.5 M) as indicated by the supplier. The gel in the basic form was equilibrated with 0.1 M ammonium acetate, pH 8.50, and the required amount of gel was poured into a column to obtain a 2 × 200 cm bed.

Lyophilized toxic fractions obtained from the first Amberlite fractionation corresponding to 1 g of crude venom for toxin I fraction and 0.5 g for toxin II fraction were dissolved in 5 ml of equilibrating buffer for chromatography. Toxic fractions coming from several DEAE-Sephadex runs were submitted to the final Amberlite fractionation described above.

Molecular Weight Determination. A Spinco Model E analytical ultracentrifuge equipped with interference optics was used. In sedimentation velocity experiments, the pure neurotoxins were dissolved in 0.5 M acetic acid to obtain concentrations from 0.8 to 1%, introduced in a synthetic boundary cell, and spun at 59,780 rpm (20 ± 0.1°). Photographs were taken each 8 min for 50 min.

The molecular weight was calculated from sedimentation equilibrium experiments done at 13,410 rpm for 88.5 hr (toxin I) and 15,220 rpm for 116 hr (toxin II) at 20 ± 0.1°. The neurotoxins were dissolved in 0.2 M ammonium acetate, pH 6.9, for the latter determinations. Initial concentration was evaluated by refractometry in a Brice Phoenix differential refractometer at 430, 546, and 589 mμ.

Ultraviolet Absorption Spectra. The absorption spectra were obtained with a recording Beckman DK 2 spectrophotometer. The absorbance values at characteristic wavelengths were also measured in a Beckman DU. The neurotoxins desiccated over P_2O_5 to constant weight were dissolved in 0.5 M acetic acid at a concentration of 0.03%.

Zone Electrophoresis. Starch gel zone electrophoresis was carried out in borate buffer at pH 8.6 (Smithies,

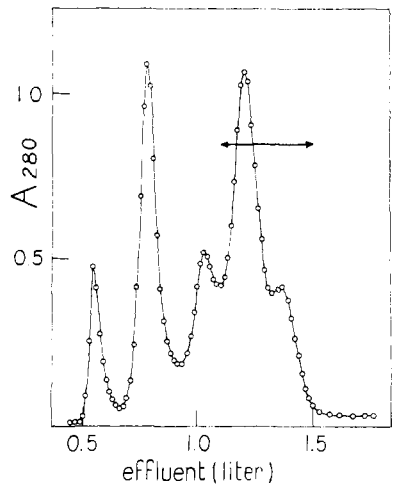

FIGURE 1: Gel filtration of the water extract of crude venom (0.537 g) on a Sephadex G-50 column (5 × 100 cm) in 0.1 M ammonium acetate, pH 6.9. Flow rate: 200 ml/hr; fractions of 15 ml. The horizontal arrow indicates the toxic fractions.

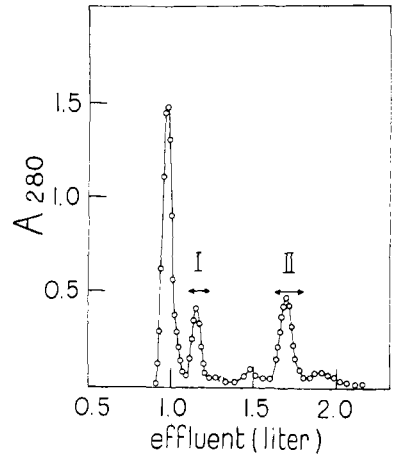

FIGURE 2: Amberlite CG-50 equilibrium chromatography of the toxic fractions from two Sephadex G-50 filtrations (see Figure 1). Equilibration and elution with 0.2 M ammonium acetate, pH 6.70. Column: 4 × 150 cm; flow rate: 48 ml/hr; 12-ml fractions. The horizontal arrows indicate the position of the toxic fractions corresponding to toxins I and II.

1955) and disc electrophoresis in a formate–formic acid buffer, pH 3.6, containing 8 M urea (Davis, 1964). Protein bands were stained with Amido Black. The stained toxin bands faded rather rapidly, and photographs of the electrophoregrams were taken as soon as possible.

Amino Acid Composition. Samples (1 mg) of the native neurotoxins or their reduced and alkylated derivatives were digested in 6.0 N HCl for 20, 70, 120, and 200 hr in sealed evacuated tubes (Moore and Stein, 1963). The hydrolysates were analyzed (Piez and Morris, 1960) in an amino acid AutoAnalyzer (Technicon). Corrections were made for losses during hydrolysis of serine, threonine, tyrosine, and half-cystine (Piez *et al.*, 1961).

The precision of analysis calculated from 20 determinations with standard runs and performic acid oxidized ribonuclease was 1–1.5%.

The total cystine content was determined directly on the native and the reduced and S-carboxymethylated toxins. Tryptophan was estimated spectrophotometrically (Beaven and Holiday, 1952).

Search for Sulfhydryl Groups. Titration with *p*-mercuribenzoate (Boyer, 1954) was carried out on 0.5 mg of each toxin. In the same conditions with human serum albumin (five times crystallized), we have found 0.54 SH group instead of 0.6. The possible presence of cysteine was also investigated by alkylation of the toxins without prior reduction. Toxin (2 mg) was treated with iodoacetamide (10 mg) as described below in Tris-acetate, pH 8.6, containing 8 M urea or 7.4 M guanidine. The alkylation was carried out for 15 min at room temperature and in the dark. The toxin was recovered by filtration of Bio-Gel P_2 in 1 M acetic acid and hydrolyzed for 20 and 70 hr in 6.0 N HCl.

Preparation of the Reduced and Alkylated Toxins. The method will be described for toxin II. Pure toxin II (16 mg) was dissolved in 3 ml of 0.25 M Tris-acetate, pH 8.6, containing 8 M urea and 16 mg of EDTA (Crestfield *et al.*, 1963). After 14 hr at 50°, nitrogen was bubbled through the solution and the toxin was reduced with 0.160 ml of 2-mercaptoethanol for 1 hr at 25°. Alkylation was performed by adding 0.416 mg of iodoacetic acid dissolved in 1.2 ml of the Tris–urea–EDTA buffer (pH readjusted to 8.6 with 10 N NaOH). Nitrogen was bubbled through the solution and the mixture was allowed to stand 15 min in the dark at room temperature. Reagents were eliminated by filtration on Bio-Gel P_2 in 30% acetic acid. The reduced and S-carboxymethylated toxin was excluded from the gel and could be recovered quantitatively by lyophilization. The reduction and alkylation of toxin I was performed in similar conditions on a 2-mg sample.

In another series of experiments, 2 mg of toxin II has been reduced as described above and alkylated with 200 mg of 2-bromomethylamine hydrobromide dissolved in 1 ml of the Tris–urea–EDTA solution. After 5 hr at room temperature in the dark, the modified toxin was recovered by filtration on Bio-Gel P_2 in 1 M acetic acid.

Amino- and Carboxy-Terminal Amino Acids. The N-terminal residue of each toxin was determined by the dinitrofluorobenzene method (Sanger, 1945). The dinitrophenylation was carried out on 4 mg of the native toxins. The DNP proteins were hydrolyzed with redistilled 6.0 N HCl in sealed evacuated tubes for 18 hr at 110°. The DNP-protein hydrolysate was diluted sixfold and extracted with peroxide-free ether. The ether extract, as well as the aqueous phase, was analyzed by paper chromatography (Levy, 1954;

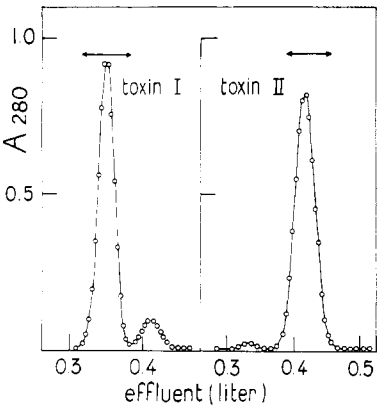

FIGURE 3: DEAE-Sephadex A-50 equilibrium chromatography of toxic fractions I and II obtained from CG-50 separation (Figure 2). Equilibration and elution with 0.1 M ammonium acetate, pH 8.50. Column: 2 × 200 cm; flow rate: 24 ml/hr; 4.5-ml fractions. The amount of fractions I and II obtained, respectively, from 1.074 and 0.537 g of crude venom was applied separately on two identical columns. Horizontal arrows indicate the position of toxicity.

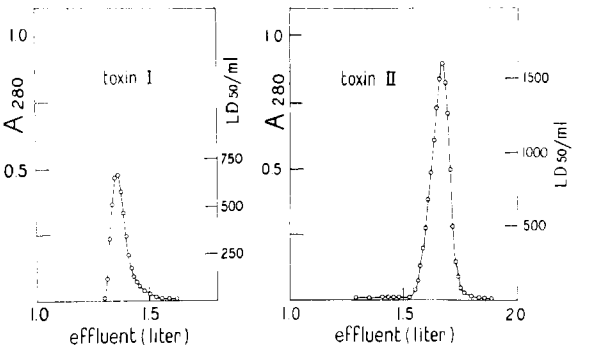

FIGURE 4: Amberlite CG-50 equilibrium rechromatography of the toxic fractions I or II eluted from DEAE-Sephadex (Figure 3). Columns of 4 × 150 cm equilibrated with 0.2 M ammonium acetate, pH 6.30 (toxin I) or pH 6.70 (toxin II). Flow rate: 48 ml/hr; 12-ml fractions. The amount of material applied to columns corresponded to 3.224 g of crude venom for toxins I and II.

Biserte and Osteux, 1955).

The C-terminal residue was determined by hydrazinolysis (Niu and Fraenkel-Conrat, 1955). The experiments were carried out on 3 mg of each toxin for 15 hr at 100°. Hydrazine in excess was eliminated over concentrated H_2SO_4 in a vacuum desiccator. After benzaldehyde treatment, the aqueous phases were washed with peroxide-free ether, lyophilized, and analyzed by column chromatography in the Technicon AutoAnalyzer.

Results

Isolation of the Neurotoxins. The procedure is described for a 3.224-g venom sample. The crude venom was extracted with water and dialyzed, and the dialysate was divided into six equal fractions which were lyophilized separately. Each lyophilisate was dissolved in 10 ml of 0.1 M ammonium acetate, pH 6.90, and filtered on Sephadex G-50 in the same buffer. The toxic fraction (Figure 1) was freeze dried. The lyophilisates obtained from two filtrations were dissolved in 10 ml of 0.2 M ammonium acetate, pH 6.70, and applied to a column of Amberlite CG-50 equilibrated with the same buffer. For all further chromatographic runs elution was made with the equilibration buffer. Two neurotoxic peaks were observed and have been arbitrarily designated as I and II according to their order of elution from the column (Figure 2). The toxic fractions I and II were pooled separately. The amount corresponding in toxicity to 1.074 (toxin I) or 0.537 g of crude venom (toxin II) was dissolved in 5 ml of 0.1 M ammonium acetate, pH 8.50, and submitted to equilibrium chromatography on DEAE-Sephadex in the same buffer (Figure 3). The last step of purification was equilibrium chromatography on Amberlite at pH 6.30 for toxin I and pH 6.70 for toxin II. The exchange capacity of the resin bed used (4 × 150 cm) allowed the treatment of the two toxic fractions obtained from the whole starting batch in a single run. The elution profiles (Figure 4) for each toxin showed a single absorbance peak, each point of which had a constant specific toxicity.

Table I summarizes the results obtained by the purification procedure; 53 mg of toxin I and 86 mg of toxin II corresponding to 1.7 and 2.7% of the crude venom were obtained. The total toxicity recovered in pure toxins was 65% of that contained in the venom. Reproducible results were obtained when several 10-g batches of venom were submitted to the same purification procedure.

Sedimentation and Molecular Weight. For each toxin the ultracentrifuge runs were done at three concentrations (0.39, 0.53, and 0.79%). The partial specific volume calculated from amino acid composition (Cohn and Edsall, 1943) was 0.708 ml/g for toxin I and 0.704 ml/g for toxin II. Extrapolation of the molecular weight obtained at the three concentrations to zero concentration gave a molecular weight of 7300 for toxin I and 7700 for toxin II (Figure 5). These values are close to the minimum molecular weight calculated from amino acid analysis (6822 and 7249, respectively) and in agreement with the results of gel filtration (retardation on Sephadex G-50 and exclusion from Sephadex G-25). In 0.5 M acetic acid, the values obtained for the sedimentation constants extrapolated to zero concentration were 0.87 (toxin I) and 0.98 (toxin II).

Ultraviolet Spectrum. In 0.5 M acetic acid toxins I and II present an absorption maximum at 275 and 276 mμ, respectively (Figure 6). On the basis of nitrogen

TABLE I: Purification of the Neurotoxins of *A. australis* Venom.

Steps		LD_{50}[a] (no.)		Specific Toxicity[b]	Yield in Toxicity (%)[c]
Crude venom (3.224 g)			844,383	283	100.0
Extraction by water and dialysis (48 hr)			837,628	310	99.2
Filtration on Sephadex G-50			775,988	622	91.9
Equilibrium chromatography on Amberlite CG-50 at pH 6.70 (0.2 M NH_4Ac buffer)	toxic fraction I	157,900	599,512	1,240	18.7 71.3
	toxic fraction II	441,612		1,260	52.6
Equilibrium chromatography on DEAE-Sephadex at pH 8.50 (0.1 M NH_4Ac buffer)	toxic fraction I	146,923	569,959	1,602	17.4 67.5
	toxic fraction II	423,036		1,900	50.1
Equilibrium chromatography on Amberlite CG-50 (0.2 M NH_4Ac buffer)	at pH 6.30 (toxin I)	137,634	553,915	1,680	16.3 65.6
	at pH 6.70 (toxin II)	416,281		2,100	49.3

[a] Toxic solutions supplemented with serum albumin (final concentration, 2 mg/ml) were injected intravenously to 20-g Swiss mice. [b] LD_{50} per absorbance unit at 280 mμ. [c] With reference to initial step.

determinations (16.14% for toxin I and 16.95% for toxin II), quantitative recovery of amino acids after hydrolysis, and chromatography, it was calculated that the toxins desiccated over P_2O_5 to constant weight contained a residual humidity value of 5.5 (toxin I) and 9.5% (toxin II). Taking into account these values, the molecular extinction coefficients at the wavelengths noted above in 0.5 M acetic acid are 10.71×10^3 for toxin I and 18.08×10^3 for toxin II.

Amino Acid Composition. Table II shows the amino acid composition of the two toxins. The results represent the mean of three determinations after 20- and 70-hr hydrolysis. The values indicated for valine, isoleucine, and leucine in toxin I are those obtained after 200-hr hydrolysis. However, the amount of isoleucine recovered was only 2.46 residues/mole, which likely corresponds to three residues. For all the other amino acids, the values are close to integral.

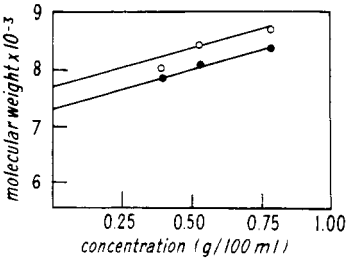

FIGURE 5: Relationship between molecular weight and concentration of toxins I and II. Molecular weight determined by sedimentation equilibrium according to Svedberg. Solution column length, 4 mm. Filled circles, toxin I; open circles, toxin II.

The total cystine content has been determined on the native toxins (Table II). After reduction and alkylation with iodoacetic acid or iodoacetamide and hydrolysis, 7.33 and 7.60 residues of *S*-carboxymethylcysteine were recovered for toxins I and II. In the case of toxin II,

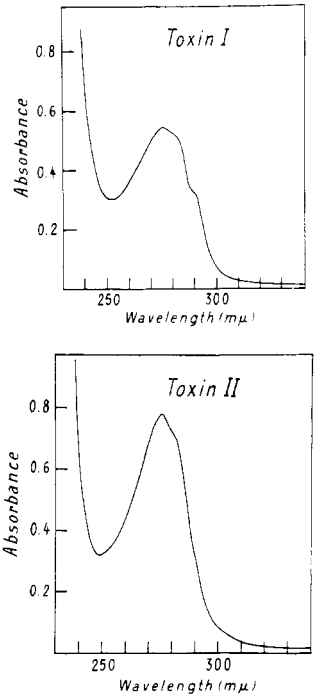

FIGURE 6: Ultraviolet absorption spectra of toxins I and II in 0.5 N acetic acid. Concentration: toxin I, 0.034 g/100 ml; toxin II, 0.031 g/100 ml.

TABLE II: Amino Acid Composition of *A. australis* Neurotoxins.

Amino Acid	Molar Ratio[a] Toxin I	Toxin II
Aspartic acid	9.04 (9)	8.13 (8)
Threonine	2.00 (2)	3.07 (3)
Serine	5.76 (6)	2.12 (2)
Glutamic acid	0.0 (0)	4.13 (4)
Proline	5.96 (6)	2.86 (3)
Glycine	6.04 (6)	7.02 (7)
Alanine	1.06 (1)	3.12 (3)
Half-cystine	7.55 (8)	7.92 (8)
Valine	4.29[b] (4)	4.08 (4)
Methionine	0.0 (0)	0.0 (0)
Isoleucine	2.46[b] (3)	0.98 (1)
Leucine	4.01[b] (4)	1.75 (2)
Tyrosine	2.83 (3)	7.04 (7)
Phenylalanine	1.01 (1)	0.99 (1)
Lysine	5.87 (6)	5.00 (5)
Histidine	0.99 (1)	1.96 (2)
Arginine	2.03 (2)	2.99 (3)
Amide NH_3	(2)	(6)
Tryptophan[c]	1.3 (1)	1.3 (1)
Total	63	64
Minimum molecular weight	6822	7249

[a] Taking phenylalanine = 1.0. Numbers in parentheses represent the closest integer. [b] After 200-hr hydrolysis. [c] Spectrophotometric determination.

one lysine residue was lost probably by alkylation into ε-carboxymethyllysine which was characterized qualitatively on the chromatograms. With the exception of cystine and lysine, the amino acid composition of reduced and alkylated toxins was identical with that of the native proteins. Histidine, which is known to react also with alkylating reagents (Gundlach *et al.*, 1959), was unaffected. The absence of cysteine in both toxins has been shown by titration with *p*-mercuribenzoate and by the absence of *S*-carboxymethylcysteine after alkylation and hydrolysis.

Tryptophan has been evaluated spectrophotometrically. In 0.5 M acetic acid molecular extinction coefficients are 10.71×10^3 for toxin I at 275 mμ and 18.08×10^3 for toxin II at 276 mμ. Those of tryptophan, tyrosine, and cystine at 275 mμ are 5.45, 1.34, and 0.16×10^3, respectively. Toxin I was shown by amino acid analysis to contain three residues of tyrosine and four of cystine; toxin II, seven residues of tyrosine and four of cystine. The calculation of the molecular extinction coefficient, if one residue of tryptophan is supposed to be present in each toxin, gives 10.11×10^3 for toxin I and 15.47×10^3 for toxin II. The ratio for the measured to the calculated value is 1.06 for toxin I and 1.17 for toxin II, in agreement with the values determined on other proteins (Wetlaufer, 1962). The absence of carbohydrates in both toxins has been shown by paper chromatography of the toxin hydrolysates (1.0 N HCl for 1 hr at 100°).

N- and C-Terminal Amino Acids. The analysis of N-terminal residues by dinitrophenylation has been performed on 0.53 μmole of toxin I and 0.45 μmole of toxin II. In both cases a single spot was observed on the chromatograms besides dinitroaniline and dinitrophenol. The spot had the R_F values of di-DNP-lysine in all the solvent systems tested. After elution from the paper, the solution exhibited the characteristic absorbance spectrum of di-DNP-lysine; 0.29 and 0.10 μmole (uncorrected for losses during chromatography and hydrolysis) has been recovered from toxins I and II, respectively.

The hydrazinolysis method performed on 0.45 μmole of toxin I and 0.39 μmole of toxin II gave 0.29 μmole of threonine for toxin I and 0.05 μmole of glycine for toxin II. No other amino acid in amount above the blank level has been found. The poor yield in glycine is in agreement with the poor recovery of this amino acid after hydrazinolysis.

Properties of Reduced and Alkylated Toxins. Both toxins, after reduction and alkylation, are excluded from Bio-Gel P_6 in 1 M acetic acid containing 8 M urea. This gel excludes proteins of molecular weight equal to or higher than 4600. The reduced and alkylated toxins dialyze less rapidly than the native across the same dialysis membrane.

Criteria of Homogeneity. Evidence for the homogeneity of the purified neurotoxins is the following. (1) Starch gel electrophoresis at pH 8.6 shows the migration of each toxin as a single band toward the cathode. In acrylamide gel electrophoresis in formate buffer, pH 3.6, containing 8 M urea each toxin also migrates as a single band, toxin II moving faster than toxin I. (2) Each toxin sediments in the analytical ultracentrifuge as a single symmetrical peak. (3) Fractions of the peak eluted from equilibrium chromatography on Amberlite CG-50 have a constant specific toxicity (Figure 4). (4) With the exception of isoleucine in toxin I all the amino acid values are close to integer (Table II); methionine is absent from both toxins and toxin I does not contain glutamic acid. (5) Only one N- or C-terminal amino acid was found in each toxin.

Discussion

The new purification procedure of the toxins of *A. australis* described in this paper differs from the previous one (Miranda and Lissitzky, 1961) by several points: (1) extensive dialysis of the aqueous venom extracts eliminates nontoxic components which interfere with subsequent steps in the purification; (2) the substitution of reversible adsorption on Sephadex G-25 by filtration on Sephadex G-50 which is better adapted to large-scale purifications; and (3) the introduction of a step of DEAE-Sephadex ion-exchange chromatog-

raphy at alkaline pH, after the chromatography on Amberlite, which is necessary to dissociate ionic complexes of the toxins with glycopeptides (unpublished experiments).

The toxicity (65% of it) contained in the crude venom is recovered in the pure neurotoxins which represent 4.4% in weight of the venom. Toxins I and II are, respectively, 9.7 and 18.5 times more active than the crude venom. The toxicity of the pure neurotoxins is very high. Tested in 20-g white mice, the LD_{50} is 19 μg/kg for toxin I and 10 μg/kg for toxin II. These values represent the highest toxic activities hitherto known for animal neurotoxins. The symptoms of poisoning[1] produced by the injection of the pure neurotoxins into the mouse or the rat are identical with those produced by the crude venom whatever the injection route. In view of the absence of enzymatic activities in the venom of *Androctonus australis* (Balozet, 1952), we may conclude that the symptomatology of the poisoning is directly and only related to the presence of the neurotoxins in the venom. According to the findings described above (Table II and text), some similarities exist between the two toxins: (1) the total number of amino acids (63 and 64); (2) the absence of methionine and cysteine, and the presence of four disulfide bridges; (3) the high amount of aromatic amino acids (five and nine residues for toxins I and II, respectively); and (4) the same amino-terminal residue of lysine. The absence of glutamic acid in toxin I is also noteworthy. Similar characteristics have been found previously for toxin I of *Buthus occitanus* (Miranda *et al.*, 1964a).

Some arguments favor the idea that both toxins are composed of a single polypeptide chain cross-linked by four disulfide bridges. (1) Only one N-terminal amino acid residue has been found in each toxin. Although the presence of N-acylated terminal amino acids has not been investigated, their existence is unlikely for only one C-terminal amino acid has been found in the two toxins in a proportion not exceeding one residue per mole. (2) After complete reduction of the disulfide bridges and alkylation, one should expect, if the toxins were made of two or several polypeptide chains, that at least one of them be retarded on Bio-Gel P_6 in a highly dispersing medium (1.0 M acetic acid–8 M urea). This has not been observed. However, it is not absolutely excluded that, if the toxins consist of more than one chain, the S-carboxymethylated chains could reassociate non-specifically.

The stability of the neurotoxins of *A. australis* toward denaturating agents such as temperature and variations of pH has been already noticed (Miranda, 1964). After solution of 1 or 2 mg of toxin in 1 ml of Tris-acetate (pH 8.6)–8 M urea and standing for 5 hr at 20°, no loss of toxicity was observed; after 18 hr at 50° in the same solvent 18% of the initial toxicity was still present. It is tempting to correlate this great stability with the low molecular weight of the toxins and their probable compact secondary structure.

The behavior of the toxins on cation or anion exchangers and their reversible adsorption on Sephadex in water (Miranda *et al.*, 1962) indicate their basic nature. Zone electrophoresis experiments suggest a pH_i near 9, that of toxin II being higher than that of toxin I. Additional support is given by the nitrogen content of the toxins determined by the Kjeldahl procedure. Toxin I was found to contain 16.14% nitrogen and toxin II, 16.95%. Taking into account the amino acid composition given in Table II, one may calculate that toxin I should contain two amide groups and toxin II six, showing an excess of two positive charges in toxin II over toxin I.

In spite of the similarities mentioned above, the over-all amino acid composition of the two toxins ap-

TABLE III: Amino Acid Composition of the Neurotoxins Isolated from Snake Venoms.

	Origin of Neurotoxins			
Amino Acid	*Naja naja atra*[a]	*Laticauda semifasciata*[b] (erabutoxin a)	*Naja nigricollis*[c] (toxine α)	*Crotalus terrificus*[d] (crotamin)
Aspartic acid	7	5	7	3
Threonine	7	5	8	0
Serine	2	7	2	3
Glutamic acid	7	8	6	2
Proline	3	4	5	4
Glycine	6	5	5	5
Alanine	0	0	0	0
Half-cystine	6	8	8	4
Valine	1	2	2	0
Methionine	0	0	0	1
Isoleucine	0	4	3	1
Leucine	2	1	2	1
Tyrosine	1	1	1	1
Phenylalanine	0	2	0	2
Lysine	3	4	6	11
Histidine	2	1	2	3
Arginine	5	3	3	2
Tryptophan	1	1 or 2	1	3
Total	53	61	61	46
Minimum molecular weight	5952	6750	6787	5450

[a] Sasaki (1957). [b] Tamiya and Arai (1966). [c] Karlsson *et al.* (1966). [d] Moura-Goncalves and Giglio (1964).

[1] Convulsive behavior, emprosthotonic and opisthotonic spasms, excessive salivation, paralysis of the hind legs, polypnea, and respiratory paralysis.

pears to be rather different. As both toxins exhibit the same pharmacological characteristics, comparative structural investigations should provide information on the part of the molecule responsible for the toxicity.

The same similarities and differences have been found in the structure of neurotoxins of different snake venoms. Table III compares the amino acid composition of the neurotoxins of *Crotalus terrificus* (Moura-Goncalves and Giglio, 1964), *Naja naja atra* (Sasaki, 1957), *Laticauda semifasciata* (Tamiya and Arai, 1966), and *Naja nigricollis* (Karlsson *et al.*, 1966). All have molecular weights ranging between 5450 and 6787, are strongly basic, and contain two to four cystine residues. Moreover, two to four amino acids, according to the considered neurotoxin, are completely absent. The curare-like mode of action of snake and especially of cobra neurotoxins is rather different from that of scorpion neurotoxins which disclose a pattern suggesting an action on the peripheral nerves as well as on the central nervous system. How these differences could be explained by the structure of the toxins secreted by these two groups of animals is at present a matter of speculation. Nevertheless, comparative structural studies between toxins of several species of scorpions and snakes should provide invaluable information on the molecular configurations involved in neurotoxicity and on the mode of action of animal neurotoxins.

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Added in Proof

The increase of the apparent molecular weight with decreasing concentration of proteins (Figure 5) could indicate that association and dissociation are occurring in the range of concentrations used. This could also reflect the existence of an electrostatic effect resulting from limitations of the buffer system.

References

Balozet, L. (1952), *Arch. Inst. Pasteur Algerie 30*, 1.
Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.
Behrens, B., and Karber, C. (1935), *Arch. Exptl. Pathol. Pharmakol. 177*, 379.
Biserte, G., and Osteux, R. (1955), *Bull. Soc. Chim. Biol. 33*, 50.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, New York, N. Y., Reinhold, p 370.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754.
Hirs, C. H. W. (1955), *Methods Enzymol. 1*, 113.
Karlsson, E., Eaker, D. L., and Porath, J. (1966), *Biochim. Biophys. Acta 127*, 505.
Levy, A. L. (1954), *Nature 174*, 126.
Miranda, F. (1964), Ph.D. Thesis, Marseille, France.
Miranda, F., and Lissitzky, S. (1961), *Nature 190*, 443.
Miranda, F., Rochat, H., and Lissitzky, S. (1962), *J. Chromatog. 7*, 142.
Miranda, F., Rochat, H., and Lissitzky, S. (1964a), *Toxicon 2*, 51.
Miranda, F., Rochat, H., and Lissitzky, S. (1964b), *Toxicon 2*, 123.
Moore, S., and Stein, W. (1963), *Methods Enzymol. 6*, 819.
Moura-Goncalves, J., and Giglio, J. R. (1964), *Intern. Congr. Biochem., 6th, New York, 2*, 170.
Niu, C. I., and Fraenkel-Conrat, H. (1955), *J. Am. Chem. Soc. 77*, 5882.
Piez, K. A., Davis, E. W., Folk, J. E., and Gladner, J. A. (1961), *J. Biol. Chem. 236*, 2912.
Piez, K. A., and Morris, L. (1960), *Anal. Biochem. 1*, 187.
Sanger, F. (1945), *Biochem. J. 39*, 507.
Sasaki, T. (1957), *J. Pharm. Soc. Japan 77*, 848.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Tamiya, N., and Arai, M. (1966), *Biochem. J. 99*, 624.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.