Studies on Fractionation of Saline-Soluble Nucleolar Proteins on Diethylaminoethylcellulose*

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ABSTRACT: The saline-soluble proteins of Walker tumor nucleoli have been subfractionated on DEAE-cellulose using NaCl gradients. The most outstanding feature of the proteins eluted from the column was their high content of acidic amino acids. In the fractions eluted with 0.2 and 0.4 M NaCl, 30 and 40% of the total amino acids were acidic amino acids. Other characteristic features of the fractions eluted from the DEAE-cellulose column were the low arginine and tyrosine content in most of the fractions. With increasing concentrations of NaCl, there was a decrease in the isoleucine content of the proteins eluted. A high serine content was found in the fractions eluted with 0.05, 0.1, and 0.2 M NaCl. The results of these studies suggest that a variety of proteins are present in the saline extracts of nucleoli of the Walker tumor.

Increasing evidence has accumulated to show that the major part of the dry weight of nucleoli is protein (Birnstiel *et al.*, 1963, 1964; Busch, 1965; Caspersson and Schultz, 1940; Desjardins and Busch, 1964; Grogan *et al.*, 1966; Horn and Ward, 1957; Menzies, 1962; Muramatsu *et al.*, 1963; Vincent, 1955, 1957, 1963). Both basic (Busch, 1965; Caspersson and Schultz, 1940; Grogan *et al.*, 1966; Hnilica *et al.*, 1966; Liau *et al.*, 1965) and acidic proteins are represented; the majority of the protein is acidic protein (Birnstiel *et al.*, 1964; Busch, 1965; Desjardins and Busch, 1964; Grogan *et al.*, 1966; Hnilica *et al.*, 1966; Liau *et al.*, 1965). The presence of acidic protein in the nucleolus has been related to the role of the nucleolus in the biosynthesis of ribonucleoprotein (Birnstiel *et al.*, 1963, 1964; Vincent, 1955, 1963), but, as in the case of the nuclear acidic proteins, few studies have been made on their constituents or properties.

With the development of techniques for isolation of nucleoli (Desjardins *et al.*, 1965, 1966), large amounts of nucleoli have been isolated and a partial subfractionation of the nucleolar protein has been made (Birnstiel *et al.*, 1964; Grogan *et al.*, 1966; Hnilica *et al.*, 1966; Liau *et al.*, 1965). The basic proteins of the nucleoli are histones and their gross properties are similar to those of the nuclear histones (Grogan *et al.*, 1966; Hnilica *et al.*, 1966). Based on solubility, the acidic proteins can be subdivided into two classes. One class is readily soluble in dilute salt solutions. The other class of protein is a residual protein fraction which remains insoluble after acid extraction of histone. This protein can be solubilized in dilute alkali and has properties similar to a lipoprotein which has been shown to be present in whole nuclei (Carver and Thomas, 1952; Dallam and Thomas, 1953; Engbring and Laskowski, 1953; Mayer and Gulick, 1942; Wang, 1966; Wang *et al.*, 1950, 1953).

The present investigation was conducted to further study the acidic nucleolar proteins using nucleoli isolated by the large-scale isolation technique developed in this laboratory (Desjardins *et al.*, 1965, 1966). The salt-soluble proteins were chosen for study because of the ease with which they are initially solubilized. The amino acid compositions were determined on fractions obtained from nucleoli of the Walker tumor after they were fractionated on DEAE-cellulose.

Materials and Methods

Animals. The animals used in this study were male albino rats obtained from Cheek-Jones Co. (Houston, Texas), weighing 175–250 g. A saline suspension of Walker carcinosarcoma cells was injected subcutaneously into six to eight sites of the abdominal wall 6–7 days prior to sacrifice. The animals were sacrificed by decapitation and the tumors rapidly removed and transferred to a vessel containing ice-cold 0.25 M sucrose. The average yield of tumor per rat was 8–10 g. The method utilized for isolating nucleoli has been previously described (Muramatsu *et al.*, 1963; Desjardins *et al.*, 1965, 1966; Grogan *et al.*, 1966). Utilizing this method, approximately 7.3×10^{10} nucleoli/kg of tumor was obtained; the number of nucleoli/ml of packed preparation was approximately 1.6×10^{10} (Desjardins *et al.*, 1966), and 2–3 ml of packed volume was obtained for each preparation. Three separate experiments were carried out.

Extraction of Nucleoli. Isolated nucleoli were extracted with 0.14 M NaCl containing 0.01 M sodium citrate (pH 7.2) in the cold laboratory by homogeniza-

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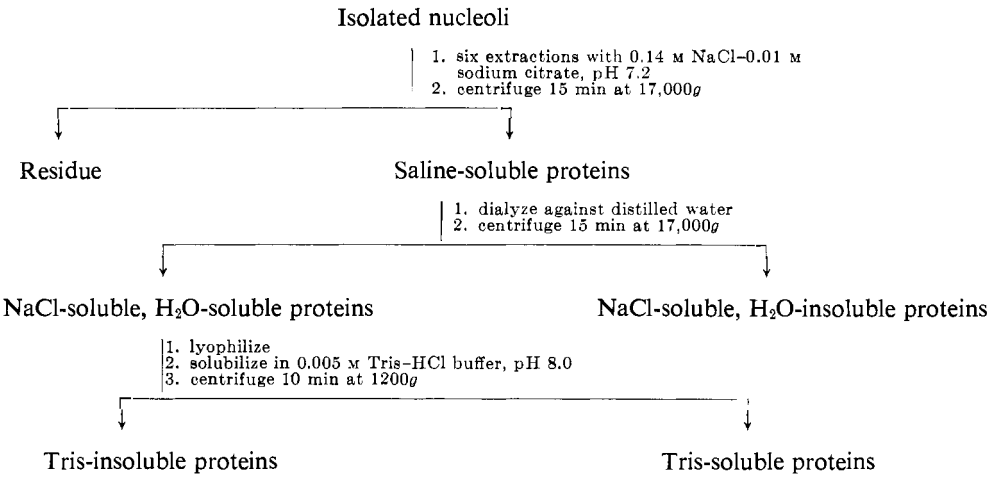
CHART I: Procedure for the Extraction of the Saline-Soluble Proteins of Walker Tumor Nucleoli.



tion of the sample in the saline solution (Hnilica and Busch, 1963; Johns *et al.*, 1961) as is shown in Chart I. The nucleoli were extracted six times with the saline solution and after each extraction the homogenates were centrifuged at 17,000*g* for 15 min. The supernatants (50–100 ml) were pooled and dialyzed 48 hr against four changes of 4 l. of distilled water. After dialysis, the pooled extracts were centrifuged 15 min at 17,000*g* to spin out the water-insoluble protein (NaCl soluble, H_2O insoluble) that precipitated out during the dialysis period. Both the supernatant (NaCl soluble, H_2O soluble) and water-insoluble material were then lyophilized to obtain the dry protein.

Column Preparation and Elution. The NaCl-soluble, H_2O-soluble proteins were fractionated on the anion exchanger DEAE-cellulose (0.98 mequiv/g of standard absorbent capacity, Bio-Rad) as described previously (Peterson and Sober, 1956; Sober and Peterson, 1958). DEAE-cellulose (3 g) was passed through a series of several washes including water, 0.5 N HCl, water, 0.5 N NaOH, and water, respectively. A slurry of the exchanger was made with distilled water and poured into a 0.9-cm i.d. column to a height of 20 cm. The column was then equilibrated with 0.005 M Tris–HCl buffer, pH 8.0. The NaCl-soluble, H_2O-soluble proteins (30–60 mg) were dissolved in 4 ml of the 0.005 M Tris–HCl buffer and centrifuged 10 min at 1200*g* to remove the Tris-insoluble protein. The above supernatant was then placed on the DEAE-cellulose column and allowed to adsorb. The column was washed with the 0.005 M Tris buffer until all of the unadsorbed material had been removed from the column. Fractions of 2 ml were collected throughout the run, and the flow rate of the column was approximately 8 ml/hr. The column effluent was monitored by reading each sample at 230-, 260-, and 280-mμ wavelengths. All chromatographic procedures were conducted at 4°.

Proteins were eluted from the column by applying stepwise increments of NaCl in buffer. Maintaining the pH of the column at pH 8.0 with 0.005 M Tris buffer, stepwise gradients of 0.025, 0.05, 0.1, 0.2, 0.4, and 0.6 M NaCl were added. The column was eluted with each salt concentration until the 230-mμ adsorption returned to base line. Concentrations higher than 0.6 M NaCl did not produce a further release of protein from the column. Therefore, once the 0.6 M NaCl elution was completed, the column was washed with 0.2 N HCl and 0.2 N NaOH, respectively. All of the pooled fractions were dialyzed 48 hr against four changes of 4 l. of distilled water. They were then lyophilized to obtain the dry protein.

Electrophoresis. Several different techniques were utilized for electrophoresis of the NaCl-soluble, H_2O-soluble proteins. Disc gel electrophoresis described by Davis (1964) was utilized at pH 8.6. Both 7.5 and 15% acrylamide were used, with 6 M urea and without urea. In addition to gels run at pH 8.6, the method that has been described for disc gel electrophoresis of histone at pH 4.5 was also used (Fambrough and Bonner, 1966). Urea was used in these gels to eliminate aggregation due to hydrogen bonding of the proteins. Starch gel electrophoresis at pH 8.6 and 2.0 was also performed on the NaCl-soluble, H_2O-soluble proteins as was described earlier (Hnilica and Busch, 1963).

Other Analytical Procedures. Amino acid analyses were made with the aid of an automatic amino acid analyzer (Spackman *et al.*, 1958). Samples run on the analyzer were hydrolyzed 20 hr at 110° in constant-boiling HCl. DNA content of the NaCl-soluble, H_2O-soluble protein was determined using Burton's modification of the diphenylamine reaction (Burton, 1956). RNA was determined by the method of Drury (1948).

Results

Properties of the Original 0.14 M NaCl-Soluble Proteins. It was found previously that the saline-soluble nucleolar proteins composed 21% of the total nucleolar protein of the Walker tumor. During dialysis of the whole saline-soluble protein fraction, a precipitate formed. The precipitate accounted for 1% of the total

TABLE I: Amino Acid Content of the Proteins Fractionated on DEAE-Cellulose.[a]

Fraction	NaCl and H_2O Soluble	Break-through	NaCl (0.05 M)	NaCl (0.1 M)	NaCl (0.2 M)	NaCl (0.4 M)	HCl (0.2 N)	NaOH (0.2 N)	Tris Insoluble	NaCl Soluble, H_2O Insoluble
Alanine	8.3	7.5	8.3	8.8	8.3	6.2	7.4	7.5	7.7	8.3
Arginine	4.5	5.2	3.2	4.0	3.4	4.8	5.8	5.1	7.1	5.9
Aspartic acid	10.7	8.3	9.5	10.3	12.4	17.8	10.1	9.7	10.5	9.5
Half-cystine	1.7	0.7	0.9	1.5	0.7	0.9	Trace	Trace	Trace	Trace
Glutamic acid	13.9	12.6	13.7	16.8	19.1	21.3	14.2	12.8	11.7	11.9
Glycine	8.5	10.4	11.5	9.9	6.2	7.5	7.0	8.8	8.5	9.1
Histidine	2.6	1.9	2.4	1.4	1.5	1.0	2.0	1.8	2.6	2.6
Isoleucine	3.5	3.0	2.3	2.4	2.6	2.0	3.5	4.6	4.1	3.8
Leucine	7.6	6.1	5.7	3.5	5.6	5.6	8.3	9.1	8.7	8.6
Lysine	9.3	9.8	8.3	8.4	10.4	8.6	8.6	7.2	8.0	9.7
Methionine	1.2	1.2	0.8	0.6	0.9	1.2	2.1	2.2	1.8	1.9
Phenylalanine	3.1	2.1	2.5	1.8	2.3	2.1	3.0	3.4	3.5	3.1
Proline	5.8	8.0	6.3	5.0	6.0	4.3	4.4	5.6	4.7	4.5
Serine	7.8	9.2	12.4	14.9	10.2	7.7	9.4	7.8	6.9	6.7
Threonine	5.4	6.6	6.2	5.2	4.9	3.1	5.8	5.6	5.2	5.4
Tyrosine	1.4	0.8	0.7	0.9	0.8	0.8	1.7	1.5	2.1	2.0
Valine	6.4	5.6	5.0	4.7	5.2	4.6	6.5	6.9	6.4	6.2
Acidic:basic	1.5	1.2	1.7	2.0	2.1	2.7	1.5	1.6	1.3	1.2

[a] The values are percentages of total moles of amino acid recovered and are averages of two to four experiments. The value for glycine was corrected for nucleic acid present. The average standard deviation was 9.8% of the value presented.

nucleolar protein and was designated as the NaCl-soluble, H_2O-insoluble protein and the supernatant is the NaCl-soluble, H_2O-soluble protein. The NaCl-soluble, H_2O-insoluble protein represented approximately 5% of the total saline-soluble proteins. Both protein groups were acidic proteins (Table I). The protein that precipitated during dialysis was very similar to the soluble protein in amino acid analysis but contained more arginine and less glutamic acid than the NaCl-soluble, H_2O-soluble proteins. The NaCl-soluble, H_2O-insoluble protein had an acidic:basic amino acid ratio of 1.2 compared to 1.5 for the soluble material. The NaCl-soluble, H_2O-soluble proteins were chromatographically analyzed.

The NaCl-soluble, H_2O-soluble protein fraction contained some nucleic acid. As indicated by the ratio of 1.3 for optical density at 260 and 280 mμ, respectively, and by the orcinol assay, RNA composed approximately 2% of the dry weight. No DNA was found in either protein fraction.

Patterns from DEAE-Cellulose. The patterns for the elution of the NaCl-soluble, H_2O-soluble proteins from the DEAE-cellulose column are shown in Figure 1. Eight major peaks in addition to the "breakthrough" peak are present. Each peak corresponds to each of the eluents applied to the column. An average of 80% of the sample applied to the column was recovered as calculated by measuring the 230-mμ absorption of the effluents. The average percentage of the protein eluted from the column represented by each is shown in Table II. The fractions eluted with 0.2 M NaCl, 0.4 M NaCl, and 0.2 N NaOH accounted for approximately 60% of the total protein eluted from the column.

Amino Acid Composition of Proteins Fractionated on DEAE-Cellulose. The amino acid composition of the proteins fractionated on DEAE-cellulose and that of the proteins insoluble in 0.005 M Tris is shown in Table I. The protein that was not soluble in 0.005 M Tris represented approximately 3% of the total NaCl-soluble, H_2O-soluble proteins and had an amino acid composition very similar to the NaCl-soluble, H_2O-insoluble proteins. The amino acid composition of protein in the "breakthrough" peak was similar to the Tris-insoluble protein. However, it did contain slightly more lysine and slightly less aspartic acid. There was an increased content of serine. In these proteins, the proline content was high.

The proteins, eluted from the column with 0.025 and 0.6 M NaCl, were obtained in very small amounts, and a reproducible amino acid analysis was not obtained. However, both protein fractions were acidic proteins. Glycine was present in high concentration in the 0.025 M NaCl fraction, and could not be accounted for by RNA contamination since the 260:280 mμ

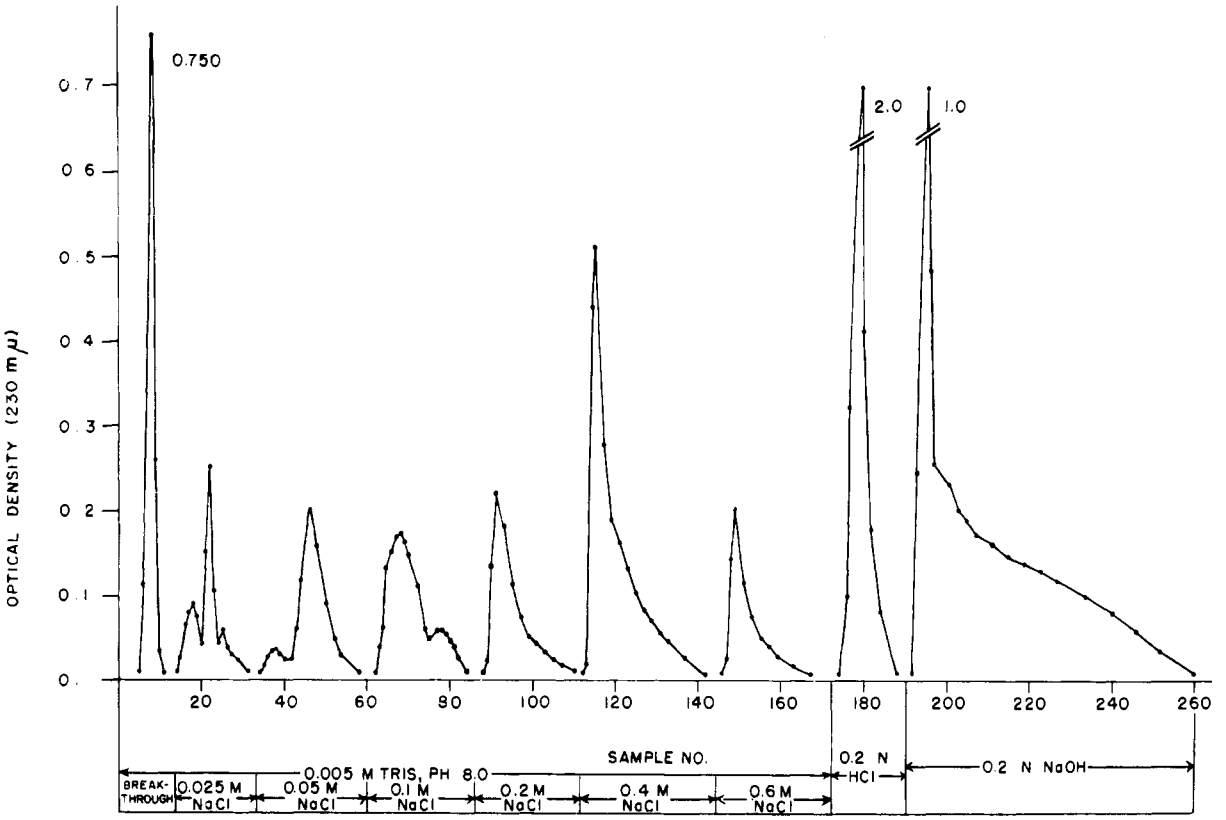


FIGURE 1: The elution pattern of saline-soluble nucleolar proteins on DEAE-cellulose.

ratio of this protein was less than 1.0. The serine content was also high in both fractions. In the fraction eluted with 0.05 M NaCl the content of serine and glycine was high. The arginine content was lower than that of the proteins in the "breakthrough" peak (Table I). The protein in this fraction has an acidic:basic amino acid ratio of 1.7. The protein eluted with 0.1 M NaCl was very similar to that eluted with 0.05 M NaCl. The high contents of glutamic acid and serine were the outstanding features of this peak.

The proteins eluted from the DEAE-cellulose column with 0.2 M NaCl were very acidic proteins. Aspartic and glutamic acid composed more than 30% of the total amino acids and glutamic acid alone accounted for approximately 19%. Serine, aspartic acid, and lysine were present in high concentrations, but serine was not as high as in the fractions eluted earlier. The proteins eluted with 0.4 M NaCl contain the largest percentage of acidic amino acids, *i.e.*, glutamic acid and aspartic acid composed approximately 40% of the total amino acids. The aspartic acid content was almost twice and the serine content almost one-half of that of the fractions eluted with 0.2 M NaCl.

The proteins eluted with 0.2 N HCl and 0.2 N NaOH have similar amino acid compositions, which are like those of the original NaCl-soluble, H_2O-soluble, and the Tris-insoluble fractions. There was a lower lysine content in the fraction eluted with 0.2 N NaOH which results in a higher ratio of acidic:basic amino acids in these proteins.

Electrophoresis. The gel electrophoresis of the NaCl-soluble, H_2O-soluble proteins was attempted. The bulk of these proteins did not migrate significantly either on starch gel or on disc gel electrophoresis. At pH 4.5 the proteins penetrated the polyacrylamide gel, but the degree of penetration was small. Four bands were identified, but the major band remained close to the origin and a very slight band moved in front of it. When urea was used in the gels to prevent aggregation by hydrogen bonding, there was no significant change in the behavior of the proteins on the gels.

Discussion

It is known that the nucleolus plays an important role in synthesis of ribonucleoprotein. Since so little is known about the properties of nucleolar proteins, this present investigation was designed to characterize the nucleolar acidic proteins. Their behavior on DEAE-cellulose, their amino acid composition, and their behavior on gel electrophoresis were studied.

Chromatography on DEAE-cellulose seems to be effective for fractionation of the nucleolar saline-soluble proteins. The most striking differences noted between the protein fractions separated on DEAE-cellulose was the high ratio of acidic:basic amino acids. The ratio

TABLE II: The Average Percentage of the Total Protein Eluted from the DEAE-Cellulose Represented by Each Eluent Peak.[a]

Eluent	% of Total Protein Eluted from the Column
Breakthrough	8.4 ± 1.0
0.025 M NaCl	5.0 ± 2.3
0.05 M NaCl	4.0 ± 0.8
0.1 M NaCl	13.9 ± 0.8
0.2 M NaCl	20.9 ± 6.6
0.4 M NaCl	16.5 ± 4.9
0.6 M NaCl	1.9 ± 1.5
0.2 N HCl	9.7 ± 11.1
0.2 N NaOH	20.5 ± 2.4

[a] The total protein represented by each peak was calculated from the number of micromoles of amino acid recovered from the amino acid analyzer and the values are averages of three experiments. The mean of standard deviation for the percentage of protein recovered in each peak was 3.5%.

gradually increased as the salt concentration increased. The proteins eluted with 0.4 M NaCl were very acidic proteins with glutamic acid and aspartic acid composing approximately 40% of the total amino acids. Like the viral proteins (Knight *et al.*, 1962) and plant proteins such as gliadin and zein (Tristram and Smith, 1963), these proteins contain the most acidic residues demonstrated in biological materials and certainly the most found in mammalian cells. These fractions were not low molecular weight peptides since none were dialyzable. Another property of these protein fractions was the high serine content in the fractions eluted with 0.05, 0.1, and 0.2 M NaCl. This finding suggests that a phosphoprotein or a lipoprotein might be present.

The failure of these proteins to migrate on gel electrophoresis may stem from a very high molecular weight, denaturation, or the presence of RNA in the protein which could prevent the protein from entering the gel. If RNA formed a complex with the protein, a product with a high molecular weight could result and the protein might be prevented from penetrating the gel. Similar behavior of acidic protein of whole nuclei on gel electrophoresis has also been noted (Steele and Busch, 1963).

The function of nucleolar proteins has not been defined although a number of nuclear enzymes have been found to be localized to the nucleolus (Siebert *et al.*, 1966), and the over-all amino acid composition of nucleolar proteins is very similar to that of the ribosomal proteins (Busch, 1965). The precise role of the ribosomal proteins is not clear, and their function has been largely unstudied. It is possible that the saline-soluble acidic proteins of the nucleolus are precursors of the lipid-bound nuclear and ribosomal proteins. Studies on this point can be made now that some fractionation of these proteins has been effected.

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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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