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BIOCHEMICAL STUDIES ON THE TOXIC NATURE OF SNAKE VENOM

by

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Kaohsiung, Taiwan
Republic of China

May 1968

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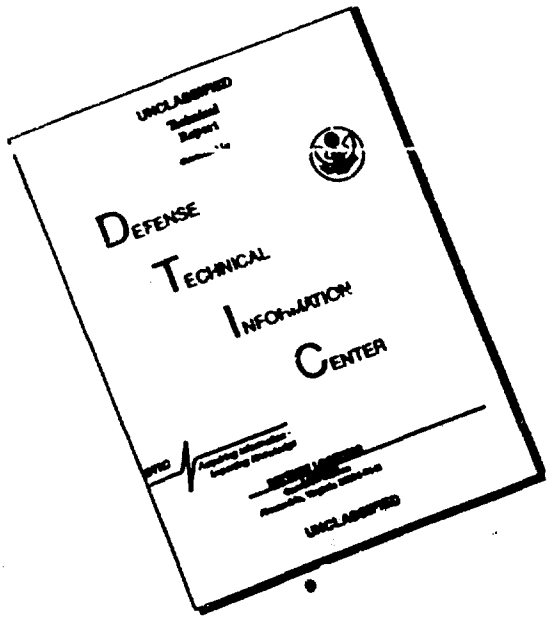

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ABSTRACT

I. Studies on I^{131} Labeled Cobrotoxin.

By taking an autoradiogram, Sumyk and co-workers found that most radioactivity concentrated in the renal cortex after injection of radioiodine labeled cobra venom into mice. Lee and Tseng also used I^{131} labeled cobra and krait venoms for their distribution and mechanism of toxicity studies. They found the highest concentration in the kidneys and the lowest in the brain, and concluded that the venom passed the brain-blood barrier with difficulty; therefore, the respiratory paralysis in mice after injection was actually peripheral in origin. Since the crystalline toxin, cobrotoxin obtained from Formosan cobra venom is thought to be the main toxic protein in the venom, the present study was initiated to investigate the properties of the I^{131} labeled cobrotoxin, its distribution and its excretion following injection.

The exhausted iodination of cobrotoxin, the crystalline toxic protein obtained from Formosan cobra venom, with I^{131} resulted in the maximum incorporation of 4 moles iodine per mole toxin without changing its lethality or antigenicity. After intravenous injection of a sublethal dose of the labeled cobrotoxin into a rabbit, about 70 % of the radioactivity was excreted in urine within 5 hours. Most of the radioactivity excreted in the urine after 20 minutes injection was shown to be in the intact cobrotoxin fraction by gel filtration, paper electrophoresis and the toxicity test. In the urine collected 4 hours after injection, half of the radioactivity appeared in the free iodine fraction.

The scanogram of rabbits in both conditions, immunized and non-immunized, showed marked different pattern in distribution of radioactivity. In the immunized rabbit the radioactivity was first shown in the region of the chest then gradually shifted in the abdominal region. Sixty-six per cent of the injected radioactivity was demonstrated in the liver of the immunized rabbit, sacrified 5 hours after injection of the labeled toxin. And most of the radioactivity was found in the soluble portion after cell fractionation. In the non-immunized rabbit the radioactivity was localized in the bladder 2 hours after injection. The excretion of the radioactivity after injection of I^{131}-cobrotoxin was much slower in the immunized rabbit than in the non-immunized rabbit.

II. Optical Rotatory Dispersion and Circular Dichroism of Cobrotoxin

In a previous paper, we have reported the optical rotatory dispersion (ORD) of a crystalline toxic protein, cobrotoxin, obtained from Formosan cobra venom, over a wavelength range of 230 to 300 mμ. It was found that the ORD curve of cobrotoxin is quite different from those of the usual proteins having right-handed α-helical structure and has a large positive peak at 233 mμ. The present paper describes the studies on the ORD and Circular dichroism (CD) of cobrotoxin over a wavelength range of 200 to 300 mμ.

The ORD curve of the native cobrotoxin had a positive peak at 207 mμ, a negative trough at 222 mμ, and a positive peak at 233 mμ with a [m'] value of + 3,400, - 1,300 and + 1,300 respectively. The very unusual ORD curve with a positive peak at 233 mμ and a negative trough at 222 mμ has been observed so far only for avidin and erabutoxin a. The CD spectrum of cobrotoxin had negative maxima at around 285 mμ and 215 mμ, and positive maxima at 228 mμ and at around 201 mμ. It is known that the CD spectrum of β-structure of poly-L-lysine shows a negative band at 217 mμ and a positive band at 195-197 mμ and that the protein having β-structure give a CD spectrum with a negative maximum at around 217 mμ. Thus it is suggested that the cobrotoxin molecule contains β-structure. The origin of the positive CD maximum at 228 mμ, which corresponds to the positive ORD Cotton effect with the peak at 233 mμ, is not clear at present. Cobrotoxin contains two tyrosyl, one tryptophanyl and eight half-cystine residues per molecular weight of 6848. It is possible that these residues contribute to the CD band at 228 mμ.

On reduction or oxidation of the disulfide bonds, the ORD and CD curves changed greatly. The ORD curve of RCM-cobrotoxin had two troughs at 208 and 226 mμ. Performic acid-oxidised cobrotoxin gave a similar ORD curve with two troughs at 210 and 230 mμ. Corresponding with this, the CD spectrum of performic acid-oxidized cobrotoxin had a negative maximum at 202 mμ and a shoulder at around 222 mμ. These ORD and CD curves are not characteristic of the completely random conformation, but closely resemble the calculated ORD curves of a mixture of a large amount of random coil and a small amount of α-helix or β-structure.

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I. Studies on I^{131} Labeled Cobrotoxin.

1. Introduction

By TAKING an autoradiogram, SUMYK and co-workers (1) found that most radioactivity concentrated in the renal cortex after injection of radioiodine labeled cobra venom into mice. Lee and Tseng (2) also used I^{131} labeled cobra and krait venoms for their distribution and mechanism of toxicity studies. They found the highest concentration in the kidneys and the lowest in the brain, and concluded that the venom passed the brain-blood barrier with difficulty; therefore, the respiratory paralysis in mice after injection was actually peripheral in origin.

Since the crystalline toxin, cobrotoxin (3) obtained from Formosan cobra venom is thought to be the main toxic protein in the venom, the present study was initiated to investigate the properties of the I^{131} labeled cobrotoxin, its distribution and its excretion following injection.

2. Materials and Methods

Cobrotoxin used in this study was prepared from Formosan cobra, Naja naja atra, venom as previously described (3). NaI^{131} was purchased from Chin-Hoa University. The standard iodine monochloride solution (4), consisting of 0.02 M of ICl, 2.0 M of NaCl, 0.02 M of KCl and 1.0 M of HCl, was prepared as follows: 21 ml of concentrated HCl was added to a solution of 0.555 g KI, 0.3517 g KIO_3 and 29.2 g NaCl. The final volume was brought up to 250 ml with water. Any iodine which had separated out was removed by repeated shaking of the solution with carbon tetrachloride. The residual carbon tetrachloride was removed by passing steam through the solution. One ml of this standard ICl solution contained 2.55 mg of iodine. The labeling solution was prepared by adding 1 mc of I^{131} to a maximum of 0.38 to 0.12 of the standard iodine monochloride solution.

a. Labeling technique

HUGLI'S method (4) of labeling serum albumin was applied with minor modifications. The procedure was as follows: 6.6 mg of the toxin in 1 ml of 1 M glycine buffer (pH 9) was injected into the labeling solution. After 10 min, the free iodine was removed by passing through a Sephadex G 25 column (1.1 x 20 cm), which was washed with water. The effluent was collected by gravity every 1.5 ml. 92-95 per cent of the iodinated I^{131} toxin was collected in fractions Nos. 5-8. The radioactivity of I^{131} was counted with a well-type scintillation counter. Protein was determined by the biuret method (5).

b. Hydrolysis of the labeled cobrotoxin (6,7)

Five mg of tyrosine, diiodotyrosine, and monoiodotyrosine, and 200 mg of NaOH were dissolved in 1 ml of iodinated toxin solution containing about 380 ug of protein, which had a maximum iodination, i.e. 4 mole iodine/mole cobrotoxin. This solution was transferred to a small pyrex test tube, which was then sealed and put into boiling water for about 20 hr. After hydrolysis, CO_2 was passed through the solution to bring the pH to about 9.0. It was kept in an ice-box. 0.01 ml of the hydrolysate, containing radioactivity of 0.1 to 0.4 μc, was applied to Whatman No. 1 filter paper for paper chromatography.

c. Paper chromatography and autoradiography

An ascending one-dimensional technique was used with Whatman No.1 filter paper. The solvent systems used were: (a) collidine $-H_2O$ (125 : 44) with a small beaker containing concentrated NH_3 placed in the bottom of the glass jar to provide an atmosphere of ammonia (8); and (b) butanol-acetic acid-H_2O (200 : 30 : 75) (8). The samples applied were protein hydrolysate and urine collected from rabbits at various periods after the injection of the labeled toxin. Development of the chromatogram was stopped 12 hr later. After drying at 25 ± 2° the paper was brought into contact with a piece of Kodak X-ray film. After 1 to 3 weeks' exposure, the film was developed. When protein hydrolysate was used as a sample, amino acid was also detected by spraying with 0.1 per cent ninhydrin alcohol solution (9).

d. Paper electrophoresis and autoradiography

A Beckman Model R electrophoresis apparatus was used. Methods employed were those commonly used for serum albumin with slight modifications. 0.01 ml of urine was applied to Whatman No. 1 filter paper. After electrophoresis, the paper was dried and brought into contact with an X-ray film. After 1 to 3 weeks' exposure, the film was developed.

e. Ring test

About 0.08 ml of rabbit anti-cobrotoxin serum was pipetted into a series of tubes (3 x 70 mm). The precipitin ring at the interface was observed at 30 min and 1 hr after an equal volume of twofold diluted antigen solution was overlaid (10).

f. Lethality

Venom lethality was measured by i.p. injection of the venom solution into mice of a N.I.H. strain (11). In the mice weighing 18 ± 1 g, the LD_{50} of the cobrotoxin was approximately 0.065 mg/kg body wt.

3. Results and Discussion

a. Iodination of cobrotoxin with I^{131}

Under the conditions described, about 80 per cent iodine was incorporated into the cobrotoxin. By chromatographic detection of free iodine, using Whatman No. 1 filter paper and methanol, the R_f values of cobrotoxin and iodine were 0 and 0.5-0.6 respectively. The labeled cobrotoxin preparation was found to contain less than 1 ~~per~~ per cent free iodine.

As shown in Fig. 1, the exhausted iodination of cobrotoxin with increasing amounts of ICl resulted in a maximum incorporation of 4 moles iodine/mole cobrotoxin. It has been reported that in iodination of some proteins, such as lactogenic hormone (6), zein (12) and insulin (13), only tyrosine is iodinated. However, histidine can also be iodinated by the method (4).

Therefore, the possibility of iodination of amino acids other than tyrosine in cobrotoxin was studied. The labeled cobrotoxin, which had a maximum iodination, i.e. 4 moles of iodine /mole of the toxin, was hydrolyzed with alkali in a sealed tube in the presence of tyrosine, monoiodotyrosine and diiodotyrosine. After 20 hr hydrolysis at 100°, paper chromatography and subsequent autoradiography were performed. The major spot on the autoradiogram was identified as diiodotyrosine, and two minor spots as monoiodotyrosine and free iodine. However, not even a trace of iodinated histidine was found. It is likely that the small amounts of the monoiodotyrosine and free iodine result from the decomposition of diiodotyrosine, which resulted from the exhausted iodination of the cobrotoxin. Tyrosine residues in cobrotoxin were, therefore, estimated to be 2 moles/mol. wt. of 6,800.

As shown in Fig. 2, the u.v. absorption curve of the labeled cobrotoxin was as changed as that of the iodinated serum albumin (14). However the lethality and antigenicity of the cobrotoxin remained unchanged even after exhausted iodination (Tables I and II), suggesting that tyrosine was not involved in the active sites of the toxicity and antigenicity. After iodination of crude venom, Lee et al. (15) found that the toxicity was reduced to 69 per cent and the curare-like activity remained unaffected. On the other hand, Yang in his early paper (16) showed that the lethality of cobrotoxin remained only 1.8 per cent after iodination using the same procedure. The results of the lethality of the cobrotoxin after iodination shown in the present paper and those of Yang's early study are contradictory; however the difference might be attributable to the different procedure of iodination used.

b. Excretion of I^{131} labeled cobrotoxin in rabbit urine

Male rabbits weighing from 2 to 2.5 kg were given 50 ml of normal saline solution by stomach tube and injected intravenously with 25 ug of the labeled cobrotoxin. Every 2 ml of urine was collected by catheterization. Radioactivity of each fraction was counted by a well-type scintillation counter. As shown in Fig. 3, about 70 per cent of the radioactivity was excreted in the urine within 5 hr. But RISA (iodinated serum albumin) was excreted in amounts less than 10 per cent.

The urine of the fraction No. 3, collected 20 min after injection, and of the fraction No. 10, collected 4 hr after injection, were passed through a Sephadex G-25 column. As shown in Fig. 4, the majority of the radioactivity in fraction No. 3 was in large molecule, while in the urine collected in fraction No. 20, the radioactivity appeared in two peaks of equal size, one large, the other a small molecule.

In order to investigate the properties of the radioactive compounds excreted in the urine, the following experiments were done. 0.01 ml urine of fraction Nos. 20 and 11, collected 4 hr and 2 hr after injection, were applied to Whatman No. 1 filter paper. After chromatography, the radioactive compounds were separated into two spots; one at the starting point as the labeled cobrotoxin; the other located as free iodine. In another experiment, 2 ml of urine, containing 4 μc of radioactivity, were collected between 13 and 20 min after injection of 800 μg labeled cobrotoxin. 0.6 ml of the same urine was injected into 2 mice, which died within 2 hr, while the injection of the same amount of urine collected before injection of the toxin did not kill mice. However, the LD_{50} of the excreted toxin was not determined. After electrophoresis of the same urine for 16 hr, the radioactive substance showed one band, slightly toward the cathode. This position was almost identical to that of the labeled cobrotoxin. It has been reported (17) that protein with a mol.wt. of 70,000 appeared in the glomerular filtrate, but some protein such as serum albumin (mol.wt. 67,500) is reabsorbed from the tubule lumen, whereas hemoglobin (mol.wt. 76,000) is excreted only when its concentration in blood exceeds a smaller amount than normal.

In the present experiment, only a small amount of the toxin (25 μg) was injected. The reason why the toxin was excreted so rapidly in urine is obscure at the present time. However, two reasons might be suggested: the toxin might freely pass through the glomerular filter without reabsorption from the tubule lumen, since the molecular weight is small (3) (18); or, the toxin might have some toxic action in the kidney and effect excretion (19).

In order to know whether cobrotoxin has any toxic effect on the renal function, radioiodinated serum albumin was

injected into rabbits with and without non-labeled cobrotoxin and urine was collected. The excretion of radioiodinated serum albumin in urine within 5 hours after injection was 5-6 %. Cobrotoxin does not affect the excretion significantly (Fig. 5). Analysis of creatinine, uric acid, sugar and total nitrogen in urine show no difference before and after injection of cobrotoxin. It seems that cobrotoxin does not affect the renal function in our experimental condition. Therefore, it is concluded that rapid excretion of the injected I^{131} labeled cobrotoxin is not likely due to the change of renal function by cobrotoxin.

c. Distribution of I^{131} labeled cobrotoxin in immunized and non-immunized rabbits

Male rabbits weighing from 2.0 to 2.5 kg were immunized by injecting increasing doses of cobrotoxin with Freund's complete adjuvant. Six to 360 µg per kg body weight were injected subcutaneously into the footpads at 6-day intervals during a period of two months. Six to 15 days after the last shot, the immunized rabbits were injected I^{131}-labeled cobrotoxin (500-750 µg) intravenously.

The scanograms of the whole immunized rabbits after injection of the labeled toxin (500 µg) were shown in Fig. 6. Radioactivity was first revealed in the region of the chest (Fig. 6a), 3 hours after injection, then gradually shifted in the abdominal region (Fig. 6b, c, d). On the contrary, in the non-immunized rabbit, the radioactivity was localized in the bladder 2 hours after injection of 40 µg of the labeled toxin. (Fig. 7).

Five hours after injection, a rabbit was sacrified and the radioactivity in each organ was measured. As seen from Table III, 66 % of the injected radioactivity was demonstrated in the liver, while, spleen has the highest I^{131}-count per gm tissue basis. It is obvious that I^{131} content in liver was observed on the chest region in the scanogram whereas I^{131} content in Kidney and bladder was observed in the abdominal region. As shown in Table IV, most of the radioactivity were found in the soluble fraction after cell fractionation. Small amounts found in microsomes, nucleus, and mitochondrial fractions may be due to the contamination of soluble fraction during fractionation.

The scanogram of rabbit 4 hours after injection (Fig. 6b), showed radioactivity in the neck region, which demonstrated that deiodination took place in the immunized rabbit after injection of I^{131} labeled cobrotoxin.

The excretion of radioactive substances after injection

of I^{131}-labeled toxin was much slower in the immunized rabbits than in non-immunized rabbit. In non-immunized rabbit, approximately 70 % of the injected radioactivity was excreted in the urine within 5 hours, while in the immunized rabbit, only 5-7 % of the injected radioactivity was excreted in 4 hours, and 60-70 % of the radioactivity was excreted within 4 days (Fig. 8).

I^{131}-labeled serum protein have been used in the study of the fate of protein after injection into the immunized and non-immunized rabbits. It was demonstrated that the only trace amount of the labeled protein remained in blood for several hours after injection into the non-immunized rabbits, but the most of the I^{131}-labeled serum protein was removed from the blood soon after the injection to the immunized rabbits. It is interpreted that I^{131} labeled serum protein-antibody complex, which formed in the immunized animal is phagocited by the liver or spleen cells. Phagocytosis of radioiodinated serum albumin-antibody complex was also found in mouse peritoneal cell. Apparent prolonged retention of the radioactivity after injection of I^{131}-labeled cobrotoxin in the immunized rabbits than in non-immunized rabbit was demonstrated in this experiment.

4. Conclusion

The exhausted iodination of cobrotoxin, the crystalline toxic protein obtained from Formosan cobra venom, with I^{131} resulted in the maximum incorporation of 4 moles iodine per mole toxin without changing its lethality or antigenicity. After intravenous injection of a sublethal dose of the labeled cobrotoxin into a rabbit, about 70 % of the radioactivity was excreted in urine within 5 hours. Most of the radioactivity excreted in the urine after 20 minutes injection was shown to be in the intact cobrotoxin fraction by gel filtration, paper electrophoresis and the toxicity test. In the urine collected 4 hours after injection, half of the radioactivity appeared in the free iodine fraction.

The scanogram of rabbits in both conditions, immunized and non-immunized, showed marked different pattern in distribution of radioactivity. In the immunized rabbit the radioactivity was first shown in the region of the chest then gradually shifted in the abdominal region. Sixty-six per cent of the injected radioactivity was demonstrated in the liver of the immunized rabbit, sacrified 5 hours after injection of the labeled toxin. And most of the radioactivity was found in the soluble portion after cell fractionation. In the non-immunized rabbit the radioactivity was localized in the bladder 2 hours after injection. The excretion of the radioactivity after injection of I^{131}-cobrotoxin was much slower in the immunized rabbit than in the non-immunized rabbit.

II. Optical Rotatory Dispersion and Circular Dichroism of Cobrotoxin

1. Introduction

In a previous paper (20), we have reported the optical rotatory dispersion (ORD) of a crystalline toxic protein, cobrotoxin, obtained from Formosan cobra venom, over a wavelength range of 230 to 300 mμ. It was found that the ORD curve of cobrotoxin is quite different from those of the usual proteins having right-handed α-helical structure and has a large positive peak at 233 mμ. The present paper describes the studies on the ORD and circular dichroism (CD) of cobrotoxin over a wavelength range of 200 to 300 mμ.

2. Materials and Methods

Cobrotoxin used in this study was prepared from Formosan cobra (Naja naja atra) venom as previously described (3).

a. Preparation of the reduced and S-carboxymethylated cobrotoxin

Reduction and alkylation of cobrotoxin was performed according to the method described by Crestfield, Moore and Stein (21). 300 mg of cobrotoxin was dissolved in 10 ml of 0.2 M Tris-HCl buffer (pH 8.2) containing 8 M urea and 0.3 ml of β-mercaptoethanol was added. The glass-stoppered tube was flushed with N_2 and left at room temperature for 4 h. For alkylation, 900 mg of iodoacetic acid in 2.3 ml of 2 N NaOH was added with constant stirring and the pH of the solution was maintained at pH 8.6 with the aid of 2 N NaOH. After 30 min the solution was placed on a column (2 x 52 cm) of Sephadex G-25-80 for desalting. The reduced and S-carboxymethylated (RCM-) cobrotoxin emerged in the void volume was pooled and lyophilized.

b. Performic acid-oxidized cobrotoxin

Performic acid-oxidized cobrotoxin was prepared as previously described (16). 30mg of cobrotoxin was dissolved in 1 ml of the mixture of 88 % formic acid and 30 % H_2O_2 (9 : 1, v/v), which was preincubated for 1 h at room temperature then at 5° for 30 min, the mixture was then incubated for 3 h at 2° to 4°. After 10 ml of ice cold water was added, the mixture was lyophilized. The dried material was redissolved in 3 ml of water and was again lyophilized.

c. ORD and CD measurements

ORD and CD measurements were made using a Jasco spectropola-

rimeter model ORD/UV-5 with a CD attachment. The ORD data were expressed in terms of the reduced mean residue rotation ($[m']$). Molecular ellipticity, $[\theta]$, was obtained by the equation, $[\theta]=3300(\varepsilon_L - \varepsilon_R)$, where $(\varepsilon_L - \varepsilon_R)$ is the difference between the molar extinction coefficients for left and right circularly polarized light. The average residue weight was used in calculation of $(\varepsilon_L - \varepsilon_R)$.

3. Results and Discussion

Fig. 9 shows the ORD and CD curves of the native cobrotoxin over the wavelength range of 200 to 310 mμ. The ORD curve had a positive peak at 207 mμ, a negative trough at 222 mμ, and a positive peak at 233 mμ with a $[m']$ value of + 3,400, - 1,350 and + 1,300 respectively. The ORD of this protein above 230 mμ has already been reported in a previous paper (20). The very unusual ORD curve with a positive peak at 233 mu and a negative trough at 222 mμ has been observed so far only for avidin (22) and erabutoxin a. The ORD and CD of the latter protein will be reported in an accompanying paper. The CD spectrum of cobrotoxin had negative maxima at around 285 mμ ($[\theta]$ = - 300), and 215 mμ ($[\theta]$ = - 1,350) and positive maxima at 228 mu ($[\theta]$ = + 4,800) and at around 201 mμ ($[\theta]$ = + 10,000). It is known that the CD spectrum of β-structure of poly-L-lysine shows a negative band at 217 mμ and a positive band at 195 - 197 mμ (23, 24) and that the protein having β-structure such as Bence-Jones proteins (25) and silk fibroin (26) give a CD spectrum with a negative maximum at around 217 mμ. Thus it is suggested that the cobrotoxin molecule contains β-structure. The origin of the positive CD maximum at 228 mu, which corresponds to the positive ORD Cotton effect with the peak at 233 mu, is not clear at present. The positive CD band at 228 mμ was also observed for Kunitz trypsin inhibitor (27), Bence-Jones proteins (25), α-Bungarotoxin (28), erabutoxin a (29), and avidin (22). In the case of Kunitz trypsin inhibitor (30) and Bence-Jones proteins (31), it is found that the CD band at 228 mμ is the most sensitive index for a conformational change among the CD bands. Cobrotoxin contains two tyrosyl, one tryptophanyl and eight half-cystine residues per molecular weight of 6848 (32). It is possible that these residues contribute to the CD band at 228 mμ (30, 33).

Fig.10 shows the ORD and CD curves of RCM-cobrotoxin and performic acid-oxidized cobrotoxin in aqueous solutions. On reduction or oxidation of the disulfide bonds, the ORD and CD curves changed greatly. The ORD curve of RCM-cobrotoxin had two troughs at 208 and 226 mμ. Performic acid-oxidized cobrotoxin gave a similar ORD curve with two troughs at 210 and 230 mμ. Corresponding with this, the CD spectrum of performic acid-oxidized cobrotoxin had a negative maximum at 202 mμ and a shoulder at around 222 mμ. These ORD and CD curves are not characteristic

of the completely random conformation. The ORD curves shown in Fig. 10 closely resemble the calculated ORD curves of a mixture of a large amount of random coil and a small amount of α-helix or β-structure (34).

4. Conclusion

The ORD curve of the native cobrotoxin had a positive peak at 207 mμ, a negative trough at 222 mμ, and a positive peak at 233 mμ with a $[m']$ value of + 3,400, - 1,350 and + 1,300 respectively. The very unusual ORD curve with a positive peak at 233 mμ and a negative trough at 222 mμ has been observed so far only for avidin and erabutoxin a.

The CD spectrum of cobrotoxin had negative maxima at around 285 mμ and 215 mμ, and positive maxima at 228 mμ and at around 201 mμ. It is suggested that the cobrotoxin molecule contains β-structure.

On reduction or oxidation of the disulfide bonds, the ORD and CD curves changed greatly. The ORD curve of RCM-cobrotoxin had two troughs at 208 and 226 mμ. Performic acid-oxidized cobrotoxin gave a similar ORD curve with two troughs at 210 and 230 mμ. Corresponding with this, the CD spectrum of performic acid-oxidized cobrotoxin had a negative maximum at 202 mμ and a shoulder at around 222 mμ. These ORD and CD curves are not characteristic of the completely random conformation, but closely resemble the calculated ORD curves of a mixture of a large amount of random coil and a small amount of α-helix or β-structure.

III. Amino Acid Composition of Cobrotoxin

Amino acid composition of cobrotoxin has been determined by the use of Technicon amino acid Autoanalyzer. As seen from Table V, cobrotoxin is composed of 15 kind of the common amino acids and is devoid of alanine, methionine and phenylalanine. Although a molecular weight of 11,000 was determined formerly by the method of Archibald for cobrotoxin, the minimal molecular weight calculated from amino acid analysis (61 residues of amino acids) is close to 6,800. It is noteworthy that with the exception of crotamin (Table V, last column) all the neurotoxins obtained from snake venoms contain 4 disulfide bridges and possess almost the same number of amino acids in the molecule.

The N-terminal amino acid was determined to be leucine by DNP- and PTC-methods. The C-terminal amino acid was identified as asparagine by the selective tritium-labelling procedure of Matsuo et al. and carboxypeptidase digestion.

The work on the amino acid sequence determination is in progress. The reduced and S-carboxymethylated toxin was digested with trypsin DCC (Diphenyl carbamyl chloride) and chymotrypsin in 0.1 N NH_4HCO_3, at pH 8.5. After 6 hours the reaction was stopped by freezing. The freeze-dried material was dissolved in a starting buffer solution and was loaded on a column (1.5 x 27 cm) of Dowex 50 x 2. The column was developed with the buffer linearly increasing the pH and the ionic strength from 0.1 M pyridine-formate of pH 3.1 to 2.5 M pyridine-acetate of pH 5 by the aid of an Autograd consisting of 9 chambers. The flow rate was adjusted to 70 ml per hour and the appropriate tubes under the chromatographic peaks were pooled and lyophilized (Fig. 11).

Homogeneity of the peptide fractions obtained by column chromatography was examined by both paper chromatography and paper electrophoresis (Fig. 12). The heterogeneous fractions were further purified with 2 % pyridine acetate buffer at pH 5.4 by high voltage paper electrophoresis. The peptides finally obtained in homogeneous state were used for structure studies. Amino acid composition (Table VI and VII) was determined on the Technicon amino acid Autoanalyzer, and the amino acid sequence of the peptides are being determined by the degradation procedure of Edman.

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APPENDIX A-1

Table I

Lethality of the I^{131} labeled cobrotoxin in mice.

Dose (µg)	Numbers of mice		Sg	Ds	Ds
	Died	Survived	(Survived at this and greater dose	(Died at this and smaller dose)	Sg-Ds (% mortality)
0.9	0	4	4	0	0
1.8	4	0	0	4	100
3.7	4	0	0	4	100

LD_{50} calculated from above data by Reed-Muench method was 0.07 mg/kg body wt.

APPENDIX A-2

Table II

Ring tests of rabbit anti-cobrotoxin sera with I^{131} labeled and unlabeled cobrotoxin.

Dilution factor	Cobrotoxin	I^{131} labeled cobrotoxin
256x	+	+
512x	+	+
768x	+	+
1024x	±	±

The concentration of antigen was 1 mg/ml and the dilution factor was 2.

APPENDIX A-3

Table III

Distribution of radioactivity in various organs of an immunized rabbits after intravenous injection of I^{131} labeled cobrotoxin

Organ	Wet weight (gm)	CRM/organ	CRM/gm	% Radioactivity distribution
Spleen	1.2	8.2×10^4	6.8×10^4	2.1
Liver	48	2.6×10^6	6.1×10^4	66.6
Bladder*	47	3.6×10^5	7.5×10^3	9.3
Kidney**	15	9.3×10^4	6.2×10^3	2.4
Heart	5	1.3×10^4	2.6×10^3	0.33
Lymph node	2	6.2×10^3	3.1×10^3	0.15
Total				80.88

* including urine

** including blood

APPENDIX A-4

Table IV

Distribution of radioactivity in subcellular fractions of liver and spleen of an immunised rabbit after intravenous injection of 720 µg of I^{131} labeled cobretoxin

Subcellular fraction	% Distribution of radioactivity	
	liver	spleen
Supernatant	73	61
Microsome	13	12
Mitochondria	8	18
Nucleus	6	9

APPENDIX A-5

Table V

Amino acid composition of the neurotoxins isolated from snake venoms

	Naja naja atra (Cobrotoxin)	Naja nigricollis (Toxine α)	Laticauda semifasciata (Erabutoxin)		L. laticaudata (Laticotoxin a)	Androctonus australis (Neurotoxin)		Crotalus terrificus (Crotamin)
			a	b		I	II	
Aspartic acid	8	7	5	4	9	9	8	3
Threonine	7	8	5	5	4	2	3	-
Serine	4	2	7	7	5	6	2	3
Glutamic acid	7	6	8	8	7	-	4	2
Proline	2	5	4	4	5	6	3	4
Glycine	7	5	5	5	5	6	7	5
Alanine	-	-	-	-	-	1	3	-
Half-cystine	8	8	8	8	8	8	8	4
Valine	1	2	2	2	1	4	4	-
Methionine	-	-	-	-	-	-	-	1
Isoleucine	2	3	4	4	2	3	1	1
Leucine	1	2	1	1	1	4	2	1
Tyrosine	2	1	1	1	1	3	7	1
Phenylalanine	-	-	2	2	1	1	1	2
Lysine	3	6	4	4	4	6	5	11
Histidine	2	2	1	2	2	1	2	3
Arginine	6	3	3	3	5	2	3	2
Tryptophan	1	1	1	1	1	1	1	3
Total	61	61	61	61	61	63	64	46
Min. mol. wt.	6848	6787	6750	6770	6880	6822	7249	5450

APPENDIX A-6

Table VI

Amino acid composition of tryptic peptides from RCM-cobrotoxin

Peptides	Amino acid composition	Total residues
T-1	$(CM\text{-}cys_{0.8}; Asp_{2.0})$	3
T-2	$(CM\text{-}cys_{1.5}; Asp_{2.9}; Thr_{2.0}; Glu_{1.0}; Gly_{1.1}; Ile_{2.0})\text{-}Arg_{1.2}$	12
T-3	$(CM\text{-}Cys_{2.5}; Asp_{1.9}; Thr_{4.7}; Ser_{2.8}; Glu_{5.0}; Pro_{1.1}; Gly_{3.0}; Leu_{1.0}; Tyr_{0.9}; His_{1.0})\text{-}Lys_{1.0}$	35
T-4	$(CM\text{-}cys_{1.9}; Ser_{0.9}; Pro_{1.1}; Gly_{2.0}; Val_{0.9})\text{-}Lys_{1.0}$	8
T-5	$(Thr_{1.0}; Glu_{1.0})\text{-}Arg_{1.0}$	3
T-6	$(Asp_{1.0}; His_{1.0})\text{-}Arg_{1.0}$	3
T-7	$(Gly_{0.9}; Tyr_{0.3})\text{-}Arg_{1.0}$	3
T-8	Trp_{+} -Arg	2
T-9	$Lys_{1.1}; Arg_{1.0}$	2
T-10	Gly; Tyr (electrophoresis)	2
T-11	Arg	1

APPENDIX A-7

Table VII

Amino acid composition of chymotryptic peptides from RCM-cobrotoxin

Peptides	Amino acid composition	Total residues
C-1		
C-2	$(CM\text{-}cys_{1.3}; Asp_{1.4}; Thr_{1.0}; Ser_{0.9}; Glu_{1.2}; Gly_{3.0})$ $-Tyr_{0.8}$	10
C-3		
C-4	$(CM\text{-}cys_{1.8}; Asp_{1.0}; Thr_{1.8}; Ser_{0.7}; Glu_{1.1}; Gly_{2.9})$ $-Tyr_{0.7}$	11
C-5		
C-6	Asp	
C-7	$CM\text{-}cys_{0.8}; Glu_{0.9}; Leu_{1.0}; His_{1.0}$	4
C-8	$Asp_{1.0}; Glu_{1.0}; Gly_{0.6}; Ile_{2.0}$	5
C-9	$CM\text{-}cys_{0.8}; Asp_{1.0}; Glu_{3.0}; Leu_{0.8}; His_{1.0}$	7
C-10		
C-11		
C-12	$Thr_{2.0}; Ser_{2.0}; Glu_{1.0}; Pro_{1.0}$	6
C-13	$CM\text{-}cys_{2.2}; Asp_{0.8}; Thr_{1.1}; Ser_{1.0}; Glu_{1.1}; Pro_{1.2};$ $Gly_{2.1}; Val_{1.0}; Lys_{1.0}; Arg_{1.9}$	13
C-14	$(Gly_{1.0}; Arg_{0.5})\text{-}Tyr_{0.8}$	3
C-15	$(Asp_{1.1}; Gly_{1.0}; His_{0.9}; Arg_{1.8})\text{-}Tyr_{0.4}$	6
C-16	$Asp_{1.0}; Lys_{2.0}; Arg_{1.9}; Trp_{+}$	6
C-17	$(Lys_{1.0}; Arg_{1.2})\text{-}Trp_{+}$	3
C-18	$(Lys_{2.0}; Arg_{1.3})\text{-}Trp_{+}$	4

APPENDIX B-1

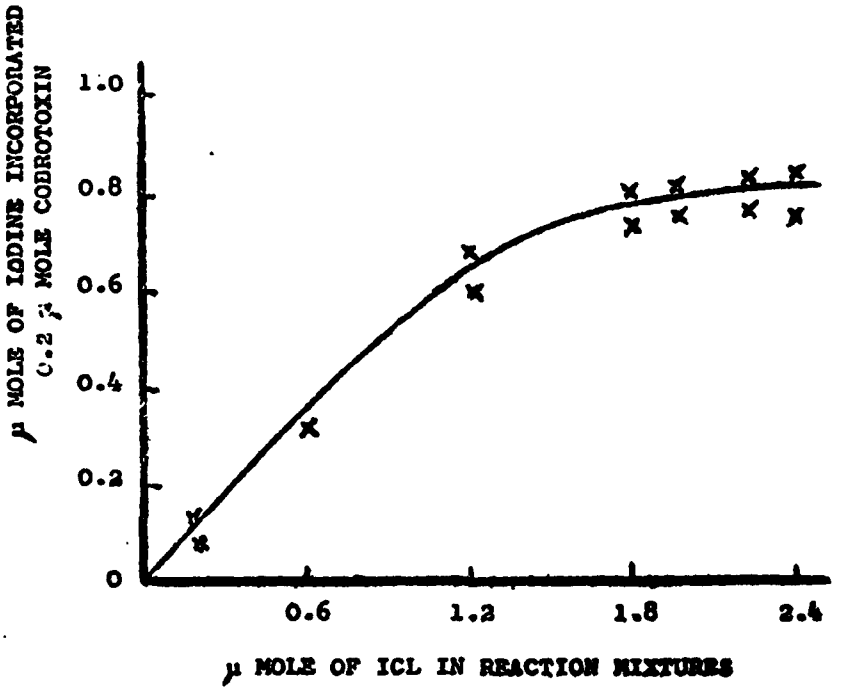

Fig. 1. Exhausted iodination of cobrotoxin with various amounts of ICl solution.
Reaction was carried out at 25 ± 2° for 20 min, in 1.5 ml glycine buffer (pH 9), containing 0.2 μ mole of cobrotoxin, 1 μc I^{131}, and various amounts of ICl solution. One ml aliquot was then passed through a Sephadex G-25 column (1.1 x 20) and eluted with water. Every 1.5 ml of the effluent was collected by gravity. About 92 to 95 per cent of protein was obtained in fractions Nos. 5-8. Percentage of incorporation was estimated by comparing the radioactivity before and after passing through the column.

APPENDIX B-2

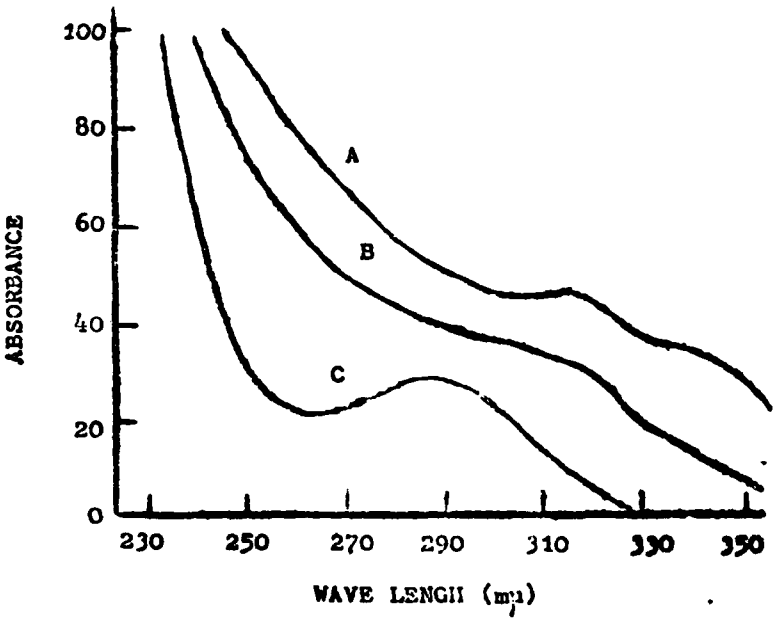

Fig. 2. Ultra-violet absorption curve of iodinated cobrotoxin and cobrotoxin plotted with a Beckman DK-2 spectrophotometer.
(a) Iodinated cobrotoxin, 4 moles iodine/mole toxin.
(b) Iodinated cobrotoxin, 2 moles iodine/mole toxin.
(c) Unlabeled cobrotoxin.

APPENDIX B-3

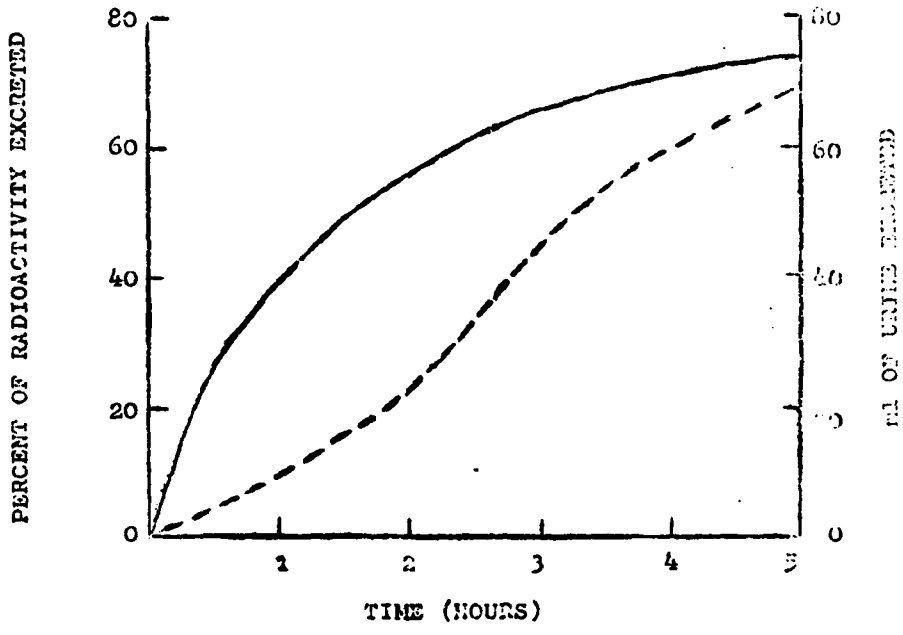

Fig. 3. Excretion of Radioactivity in urine after injection of I^{131} labeled cobrotoxin.
Urine was collected from male rabbit by catheterization immediately after injection of 25 μg toxin with 5 μc radioactivity.
———— : radioactivity excreted.
---------- : ml of urine excreted.

APPENDIX B-4

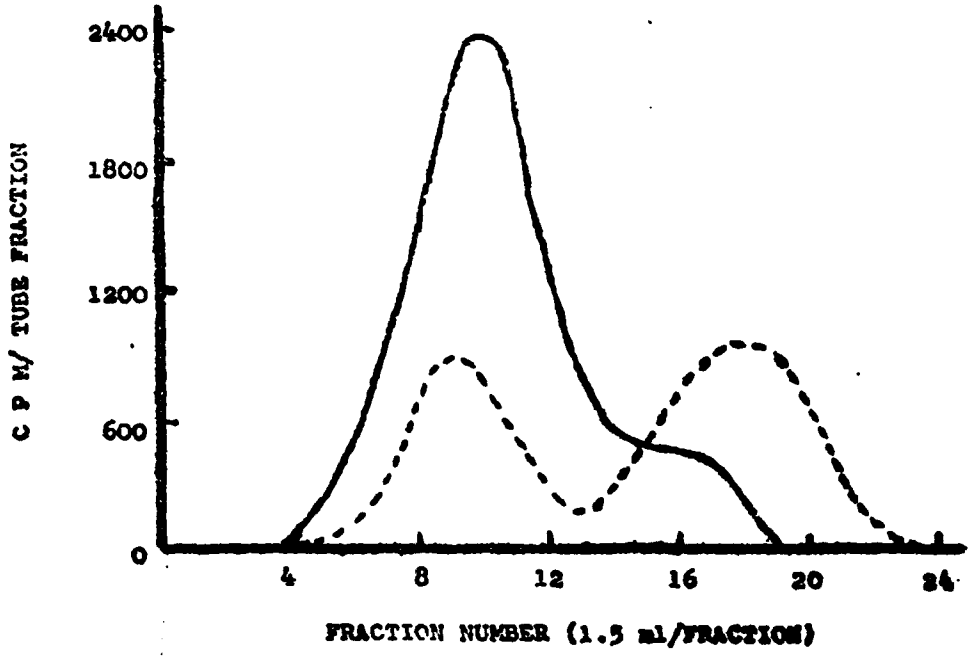

Fig. 4. Sephadex G 25 column chromatography of urine.
1 ml of the urine was applied to a Sephadex G 25 column (1.1 x 20 cm) and then eluted with water.
————— : urine of fraction No. 3.
----------: urine of fraction No. 20.

APPENDIX B-5

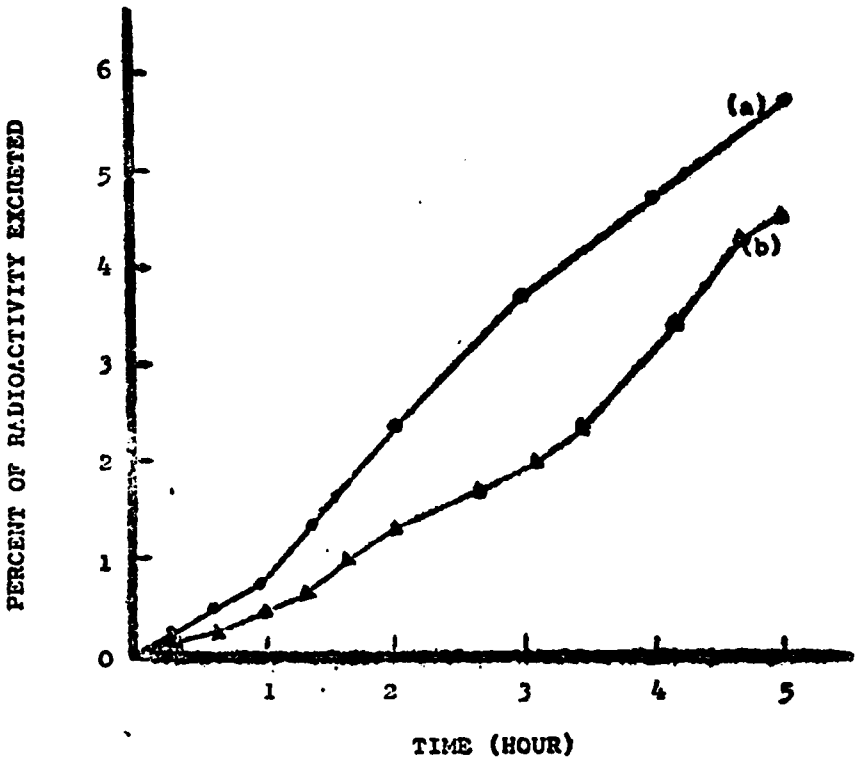

Fig. 5. Excretion of radioactivity in urine after injection of 2.6 mg radioiodinated serum albumin with (b) or without (a) cobrotoxin (30 μg).

APPENDIX B-6

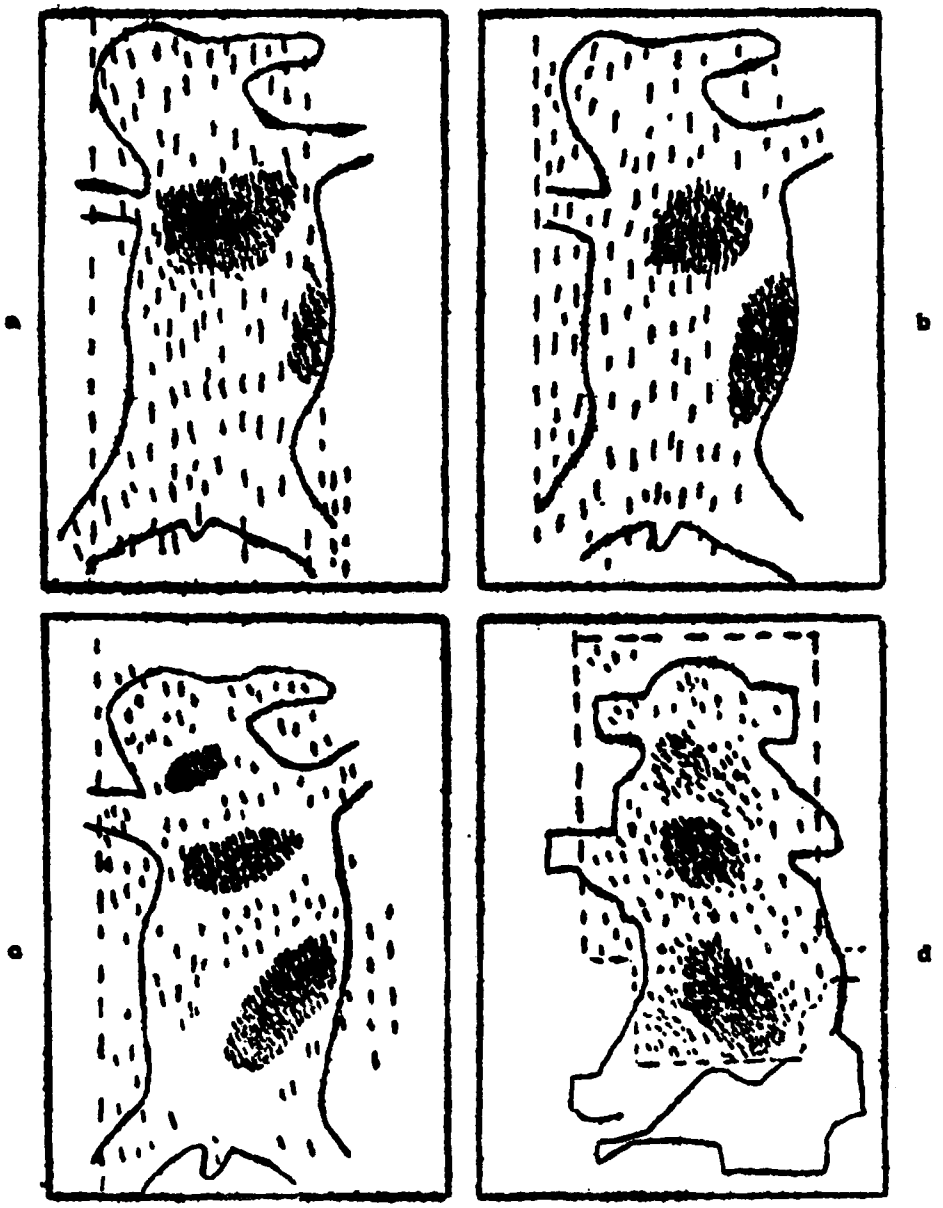

Fig. 6. Scanogram of immunized rabbit after injection of labeled toxin.
500 μg of labeled cobrotoxin was injected into 2 kg rabbit and scanograms were taken: a, 3 hours; b, 5 hours; c, 8 hours; d, 24 hours after injection.

APPENDIX B-7

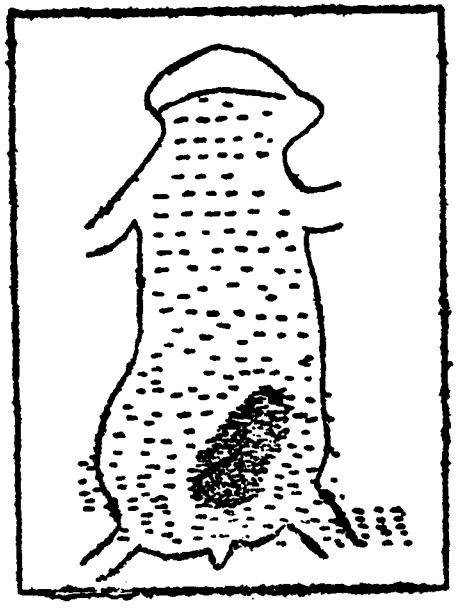

Fig. 7. Scanogram of non-immunized rabbit after injection of labeled cobrotoxin.
40 µg of labeled cobrotoxin was injected into 2.5 kg of rabbit and scanogram was taken after 2 hours.

APPENDIX B-8

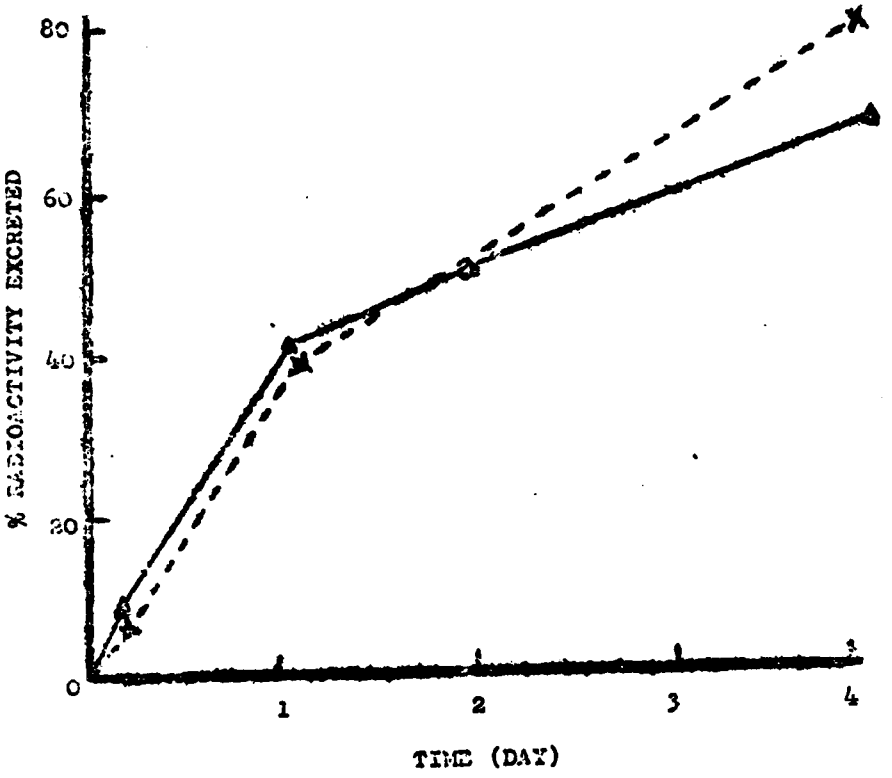

Fig. 8. Excretion of radioactivity in urine of immunized rabbits after injection of I^{131} labeled cobrotoxin.
(a) Immunized rabbit (2 kg), injected with 500 µg of I^{131} labeled cobrotoxin.
(b) Immunized rabbit (2.2 kg), injected with 700 µg of I^{131} labeled cobrotoxin.

APPENDIX B-9

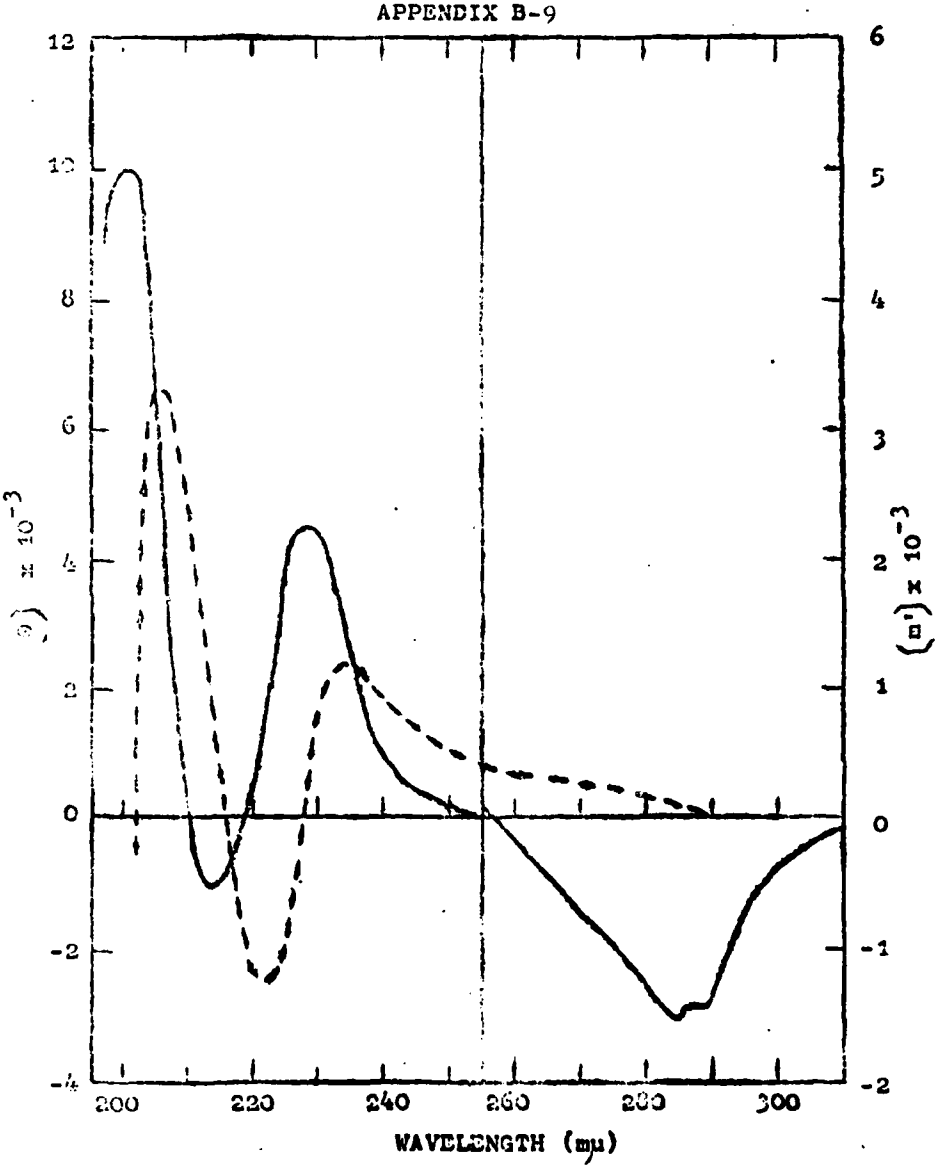

Fig. 9. ORD (----) and CD (——) of cobrotoxin in 0.1 M NaCl.
ORD measurements: Above 225 mμ, 1-cm cell, protein concentration (c) = 0.025 % or 0.5-cm, cell, c = 0.071 %; From 215 to 225 mμ, 0.1-cm cell, c = 0.071 % or 1-cm cell, c = 0.0125 %; Below 215 mμ, 0.1-cm cell, c = 0.036 or 0.014 %.
CD measurements: Above 240 mμ, 1- or 0.5-cm cell, c = 0.1 or 0.071 %; From 240 to 220 mμ, 0.2-cm cell, c = 0.1 or 0.071 %; From 220 to 210 mμ, 0.1-cm cell, c = 0.1 or 0.071 %, or 0.2-cm cell, c = 0.025 %; Below 210 mμ, 0.01-cm cell, c = 0.071 % or 0.1-cm cell, c = 0.014 %.

APPENDIX B-10

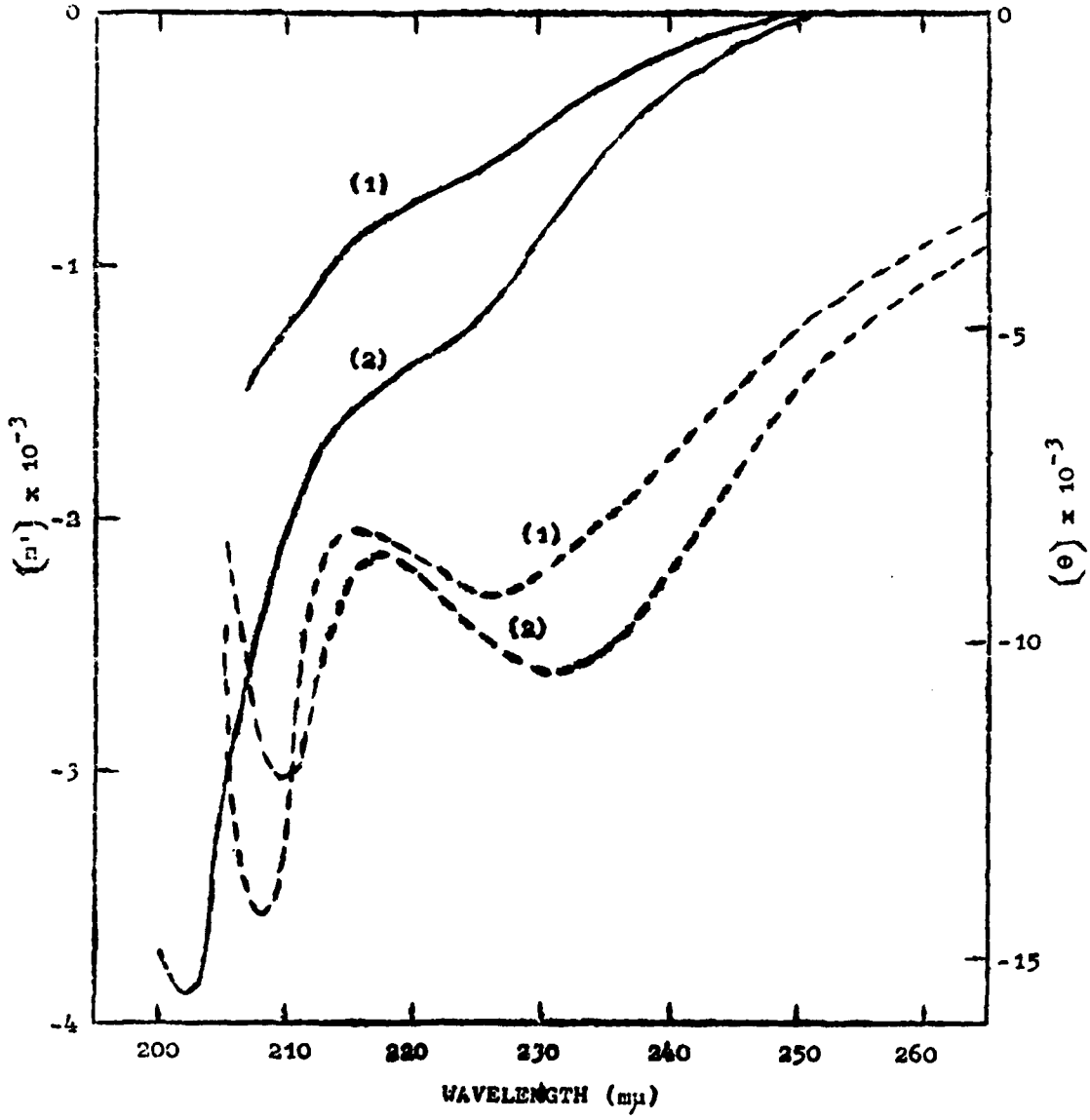

Fig. 10. ORD (----) and CD (——) of RCM- and performic acid-oxidized cobrotoxin. Curve 1, RCM-cobrotoxin in phosphate buffer at pH 5.9, ionic strength 0.1; Curve 2, performic acid-oxidized cobrotoxin in 0.1 M NaCl.

APPENDIX B-11

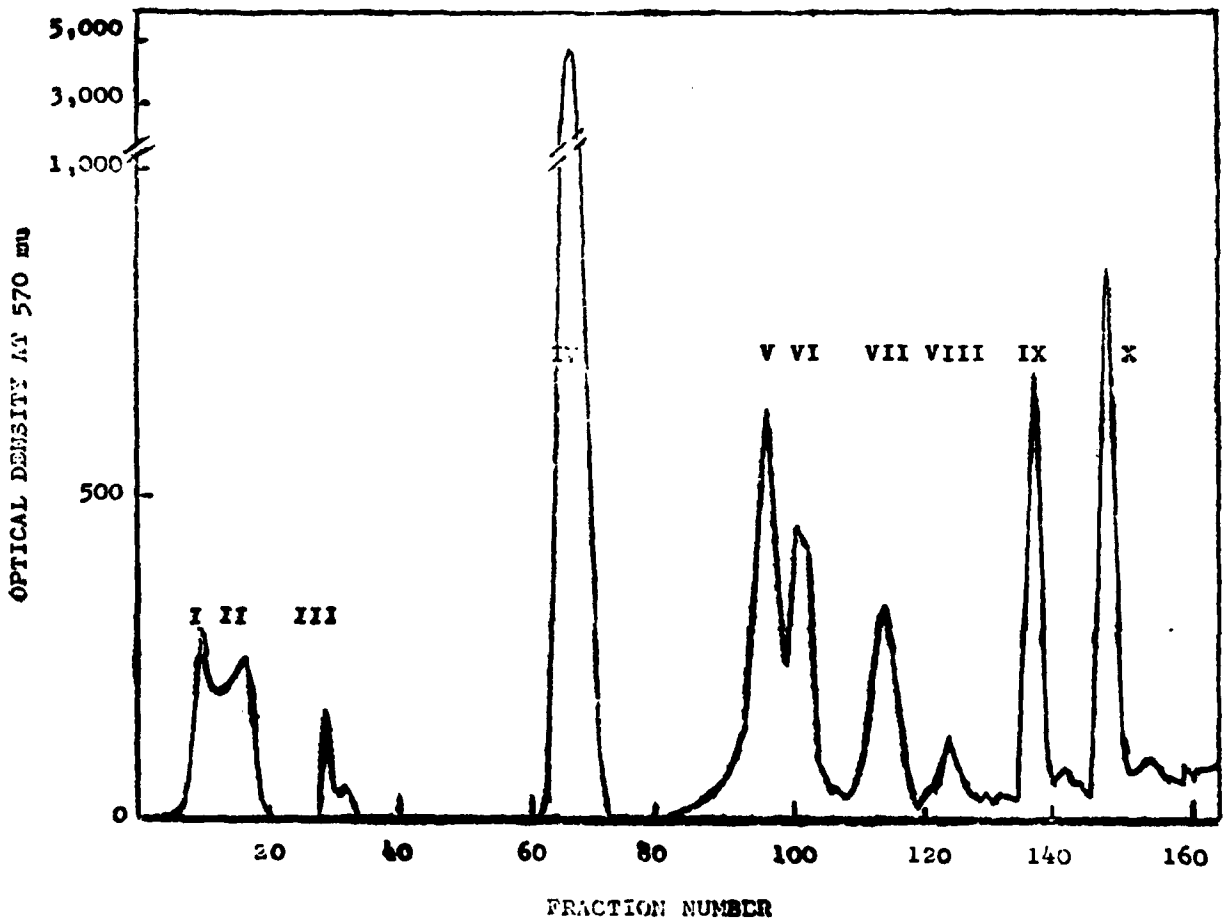

Fig. 11 ***Chromatographic fractionation of the poptides obtained by the tryptic hydrolysis of CM-cobrotoxin.***

The peptides from 120 mg of the protein were loaded on a column (1.5 x 27 cm) of Dowex 50 x 2 (200-400 mesh), which was equilibrated with 0.1 M pyridine-formate buffer, pH 3.25. The column was developed with the buffer linearly increasing the pH and the ionic strength from 0.1 M pyridine-formate of pH 3.1 to 2.5 M pyridine-acetate of pH 5 by the aid of an Autograd consisting of 9 chambers. The flow rate was adjusted to 70 ml per hour and the effluent was collected in 5.5 ml fractions. Aliquots from each tube were analyzed by the ninhydrin method.

APPENDIX B-12

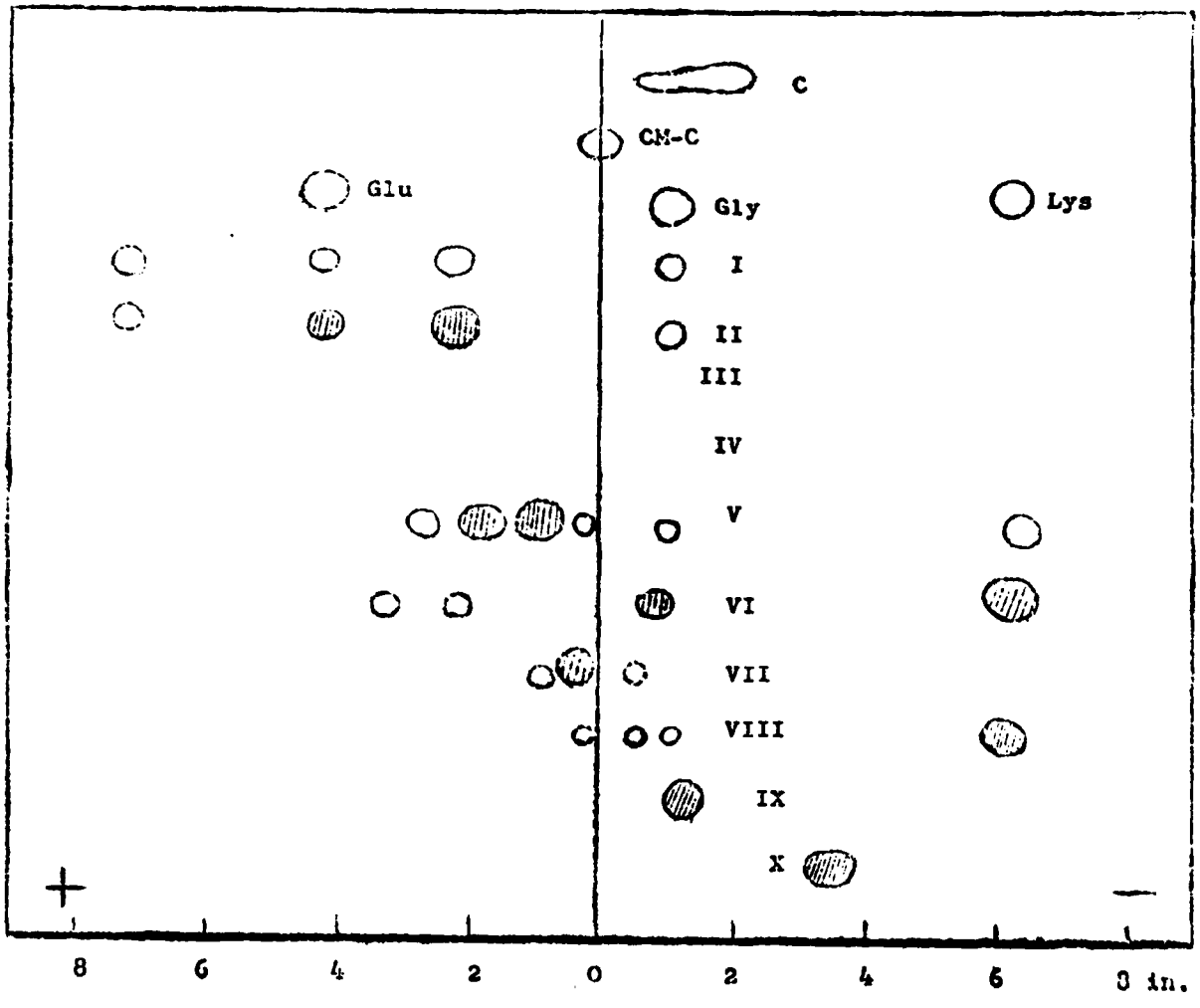

Fig. 12. Paper electrophoregram of peptide fractions obtained by the column chromatography (Fig. 11).

The electrophoresis was carried out at 2500 volts per 36 in. length of paper for 60 minutes with pyridine-acetic acid-water (7:2:490, v/v) of pH 5.4 as the electrolyte.

List of Publications

1. The disulfide bonds of cobrotoxin and their relationship to lethality. Biochim. Biophys. Acta, 133 (1967) 346.

2. Optical rotatory dispersion of cobrotoxin. J.Biochem., 61 (1967) 272.

3. Biochemical studies on the toxic nature of snake venom. Intern. Congr. Biochem., 7th, Tokyo, Col. VIII, 1 (1967).

4. Studies on fluorescent cobrotoxin. Biochim. Biophys. Acta, 147 (1967) 600.

5. Optical rotatory dispersion and circular dichroism of cobrotoxin. Biochim. Biophys. Acta, in press.

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13. ABSTRACT

I. Studies on I^{131} Labeled Cobrotoxin- The exhausted iodination of cobrotoxin, the crystalline toxin obtained from Formosan cobra venom, with I^{131} resulted in the maximum incorporation of 4 moles iodine per mole toxin without changing its lethality or antigenicity. The scanogram of rabbits in both conditions, immunized and non-immunized, showed marked different pattern in distribution of radioactivity. Sixty-six per cent of the injected radioactivity was demonstrated in the liver of the immunized rabbit, sacrified 5 h after injection of the labeled toxin. In the non-immunized rabbit the radioactivity was localized in the bladder 2 h after injection.

H. Optical Rotatory Dispersion and Circular Dichroism of Cobrotoxin- The ORD curve of the native cobrotoxin is quite different from those of the usual proteins having right-handed α helical structure and has a positive peak at 207 mμ, a negative trough at 222 mμ, and a large positive peak at 233mμ. The very unusual ORD curve with a positive peak at 233 mμ and a negative trough at 222 mμ has been observed so far only for avidin and erabutoxin a. The CD spectrum of cobrotoxin had negative maxima at around 285 mμ and 215 mμ and positive maxima at 228 mμ and at around 201 mμ. It is suggested that the cobrotoxin molecule contains β-structure. On reduction or oxidation of the disulfide bonds, the ORD and CD curves changed greatly. These ORD and CD curves are not characteristic of the completely random conformation, but closely resemble the calculated ORD curves of a mixture of a large amount of random coil and a small amount of α-helix or β-structure. (Author)

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	ROLE	WT	ROLE	WT	ROLE	WT
Snake						
Venom						
Purification						
Immunochemistry						
Chromatography						
Antibody						
Autoradiography						
Electrophoresis						
Taiwan						

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