AD-779 433

STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN

Chen-Chung Yang

Kaohsiung Medical College

AD 779 433

Prepared for:

Army Research and Development Group (Far East)

5 March 1974

DISTRIBUTED BY:

NTIS
National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS
BEFORE COMPLETING FORM

1. REPORT NUMBER
FE-428-8

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER
AD 779433

4. TITLE (and Subtitle)
STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN (U)

5. TYPE OF REPORT & PERIOD COVERED
Final Report
18 Dec 72 - 17 Dec 73

6. PERFORMING ORG. REPORT NUMBER
FE-428-8

7. AUTHOR(s)
Chen-chung Yang

8. CONTRACT OR GRANT NUMBER(s)
DA-RDRF-S92-544-73-G194

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Department of Biochemistry
Kaohsiung Medical College
Kaohsiung, Taiwan, Republic of China

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
2N061102B71D 00 128FE

11. CONTROLLING OFFICE NAME AND ADDRESS
U.S. Army R&D Group (Far East)
APO San Francisco 96343

12. REPORT DATE
5 March 1974

13. NUMBER OF PAGES
39

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)
Same as Block 11

15. SECURITY CLASS. (of this report)
Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Reproduced from
best available copy.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Snake venom
Venom purification
Amino acids
Toxicology
Cobrotoxin
Biochemistry
Immunology
Identification
Measurements
Analysis
Antigenic activity
Taiwan

Reproduced by
NATIONAL TECHNICAL
INFORMATION SERVICE
U S Department of Commerce
Springfield VA 22151

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Studies on the Status of Arginine Residues in Cobrotoxin

Cobrotoxin, a neurotoxic crystalline protein, was isolated from the venom of Taiwan cobra (Naja naja atra) and was proved to be the main toxic protein in cobra venom. The two-dimensional structure of the toxin has recently been established, permits a study of structure-function relationships. Preceding studies on the chemical modification of the single tryptophan, tyrosyl and histidyl residues, free amino and carboxyl groups in cobrotoxin suggested that
(Cont)

either the intact Trp-29, Tyr-25, His-32, ε-amino group of Lys-47 or γ-carboxyl group of Glu-21 is essential for full activity of the toxin.

Cobrotoxin is a basic protein having six arginine residues at the positions 28, 30, 33, 36, 39 and 59 in the sequence. In this study, selective and stepwise chemical modification of arginine residues were conducted with a group specific reagent, phenylglyoxal, at varying pH and the degree of modification in relation to the lethal activity and antigenic specificity has been studied in details.

Reaction of cobrotoxin with phenylglyoxal at pH 8.0 resulted in almost complete loss of lethal activity and four of the six arginine residues were modified consequently. However, the rate of inactivation was decreased significantly when the pH of the reaction was lowered. Only one arginine residue at position 28 was modified at pH 6.0 and the product retained full biological activity. Arg-33 is the next one modified when the reaction was carried out at pH 6.7 and the lethality drops precipitously, but the antigenic activity was not altered significantly. However, the lethal activity lost almost completely and the antigenic activity decreased about 30 % when an additional arginine residue at position 30 was modified at pH 7.5.

These results indicate that Arg-30 and Arg-33 are essential for the lethal activity and Arg-30 and Arg-36 are more closely related to the antigenic specificity of the toxin. The possible mechanism of neuromuscular blocking activity of snake neurotoxin is discussed. (Author)

II

DDC DISTRIBUTION AND AVAILABILITY NOTICE

Approved for public release; distribution unlimited.

DISPOSITION INSTRUCTIONS

Destroy this report when it is no longer needed. Do not return it to the originator.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

REPORT No. FE-428-8 (Final Report)

GRANT No. DA-RDRF-S92-544-73-4194

DA Project/Task Area/Work Unit No. 2N061102B71D 00 128FE

STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN

by

Chen-chung Yang, D. Med. Sc.

Professor of Biochemistry
Kaohsiung Medical College
Kaohsiung, Taiwan
Republic of China

January 1974

U. S. ARMY RESEARCH AND DEVELOPMENT GROUP
FAR EAST
APO San Francisco 96343

Approved for public release; distribution unlimited.

STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN

Studies on the Status of Arginine Residues in Cobrotoxin

Cobrotoxin is a basic protein having six arginine residues at the positions 28, 30, 33, 36, 39 and 59 in the sequence. In this study, selective and stepwise chemical modification of arginine residues were conducted with a group specific reagent, phenylglyoxal, at varying pH and the degree of modification in relation to the lethal activity and antigenic specificity has been studied in details.

Reaction of cobrotoxin with phenylglyoxal at pH 8.0 resulted in almost complete loss of lethal activity and four of the six arginine residues were modified consequently. However, the rate of inactivation was decreased significantly when the pH of the reaction was lowered. Only one arginine residue at position 28 was modified at pH 6.0 and the product retained full biological activity. Arg-33 is the next one modified when the reaction was carried out at pH 6.7 and the lethality drops precipitously, but the antigenic activity was not altered significantly. However, the lethal activity lost almost completely and the antigenic activity decreased about 30 % when an additional arginine residue at position 30 was modified at pH 7.5.

These results indicate that Arg-30 and Arg-33 are essential for the lethal activity and Arg-30 and Arg-36 are more closely related to the antigenic specificity of the toxin. The possible mechanism of neuromuscular blocking activity of snake neurotoxin is discussed.

ABSTRACT

Studies on the Status of Arginine Residues in Cobrotoxin

Cobrotoxin, a neurotoxic crystalline protein, was isolated from the venom of Taiwan cobra (Naja naja atra) and was proved to be the main toxic protein in cobra venom. The two-dimensional structure of the toxin has recently been established, permits a study of structure-function relationships. Preceding studies on the chemical modification of the single tryptophan, tyrosyl and histidyl residues, free amino and carboxyl groups in cobrotoxin suggested that either the intact Trp-29, Tyr-25, His-32, ε-amino group of Lys-47 or γ-carboxyl group of Glu-21 is essential for full activity of the toxin.

Cobrotoxin is a basic protein having six arginine residues at the positions 28, 30, 33, 36, 39 and 59 in the sequence. In this study, selective and stepwise chemical modification of arginine residues were conducted with a group specific reagent, phenylglyoxal, at varying pH and the degree of modification in relation to the lethal activity and antigenic specificity has been studied in details.

Reaction of cobrotoxin with phenylglyoxal at pH 8.0 resulted in almost complete loss of lethal activity and four of the six arginine residues were modified consequently. However, the rate of inactivation was decreased significantly when the pH of the reaction was lowered. Only one arginine residue at position 28 was modified at pH 6.0 and the product retained full biological activity. Arg-33 is the next one modified when the reaction was carried out at pH 6.7 and the lethality drops precipitously, but the antigenic activity was not altered significantly. However, the lethal activity lost almost completely and the antigenic activity decreased about 30 % when an additional arginine residue at position 30 was modified at pH 7.5.

These results indicate that Arg-30 and Arg-33 are essential for the lethal activity and Arg-30 and Arg-36 are more closely related to the antigenic specificity of the toxin. The possible mechanism of neuromuscular blocking activity of snake neurotoxin is discussed.

Table of Contents

List of Appendixes

A-1 Table I. Amino acid composition of cobrotoxin and arginine-modified derivatives. Page 11

A-2 Table II. Amino acid sequence of chymotryptic peptides from reduced and S-carboxymethylated cobrotoxin. 12

A-3 Table III. Stepwise modification of arginine residues in cobrotoxin with phenylglyoxal at varying pH. 13

A-4 Table IV. The lethality and antigenic activity of monomeric and polymeric forms separated from Arg-28 & 33 modified derivative by gel filtration on Sephadex G-50. 14

B-1 Fig. 1. Structure of cobrotoxin. Two-dimensional schematic diagram showing the arrangement of the disulfide bonds and sequence of the amino acid residues. 15

B-2 Fig. 2. Chromatography of Arg-modified cobrotoxin (at pH 8.0) on CM-cellulose column. 16

B-3 Fig. 3. Changes of lethal activity of cobrotoxin by reaction with phenylglyoxal at varying pH values. 6 mg of cobrotoxin was dissolved in 0.5 ml of buffer solution and a 100-fold molar excess of phynylglyoxal in 1.5 ml of the same buffer was added. Reaction was allowed to proceed at 27°C. After suitable intervals of time, aliquots were taken for determination of lethal activity. Buffer solutions used were: 0.1 M acetate, pH 6.0; 0.1 M phosphate, pH 6.7 or pH 7.5 and 0.2 M N-ethylmorpholine acetate, pH 8.0. 17

B-4 Fig. 4. Peptide maps of the chymotryptic hydrolysates of RCM-cobrotoxin and RCM-Arg-modified derivatives at varying pH. RCM-cobrotoxin: A; RCM-Arg-modified toxins: at pH 6.0, B; at pH 6.7, C; at pH 7.5, D; at pH 8.0, E. 18

Studies on the Status of Arginine Residues in Cobrotoxin

I. Introduction

Cobrotoxin, a neurotoxic crystalling protein, was isolated from the venom of Taiwan cobra (*Naja naja atra*) (1) and was proved to be the main toxic protein in cobra venom (2). The two-dimensional structure of the toxin has recently been established (3,4), permits a study of structure-function relationships. Preceding studies (5-9) on the chemical modification of the single tryptophan, tyrosyl and histidyl residues, free amino and carboxyl groups in cobrotoxin suggested that either the intact Trp-29, Tyr-25, His-32, ε-amino group of Lys-47 or γ-carboxyl group of Glu-21 is essential for full activity of the toxin.

Cobrotoxin is a basic protein having six arginine residues at the positions 28, 30, 33, 36, 39 and 59 in the sequence (Fig. 1). Modification with 1,2-cyclohexanedione (10,11) in strong alkaline solution (0.2 N NaOH), all arginine residues were modified and lethal activity disappeared completely. If the reaction was conducted in 0.1 M triethylamine buffer (pH 10), four residues were modified and lethality decreased to 1.6 % (unpublished observations). The reaction conditions required, however, are not mild enough for the biologically active protein and the reagent appears to react with ε-amino group of lysine residue easily.

Recently Takahashi (12) has reported that a group specific reagent, phenylglyoxal, can react highly specifically with guanidino group of arginine residue in protein under mild conditions. In this study, selective and stepwise chemical modification of arginine residues were conducted with phenylglyoxal and the degree of modification in relation to the lethal activity and antigenic specificity has been studied in details. From the results of this investigation, the arginine residues which are essential for the biological functions of cobrotoxin have been differentiated, and the positions of these residues in the amino acid sequence have also been established.

II. Materials and Methods

Cobrotoxin used in this study was prepared from Taiwan cobra (*Naja naja atra*) venom as previously described (1).

phenylglyoxal hydrate was purchased from Seikagaku Kogyo Co., Ltd. and N-ehtylmorpholine from Nakarai Chemicals, Ltd. Trypsin and chymotrypsin were the products of the Worthington Biochemical Corp. Reagent grade β-mercaptoethanol and iodoacetic acid obtained from Matheson and Coleman Co. were used. Sephadex G-25 and CM-cellulose were purchased from the Sigma Chemical Co. and urea was a Mallinckrodt reagent. All other reagents were of analytical grade.

1. Reaction of phenylglyoxal with cobrotoxin

Modification of arginine residues in cobrotoxin with phenylglyoxal was performed essentially according to the method described by Takahashi (12). To a solution of cobrotoxin (4 µmoles) in 0.5 ml of 0.2 M N-ethylmorpholine acetate buffer (pH 8.0), a 100-fold molar excess of phenylglyoxal in 3 ml of the same buffer was added, and the reaction was allowed to proceed at room temperature (27°C) for 1 h. Reactions at pH values other than 8.0 were carried out in the same way, but with other buffers. The mixture was passed through a column of Sephadex G-25 (2.5 cm x 40 cm), followed by ion exchange chromatography on CM-cellulose with a gradient of increasing salt concentration from 0.005 M to 0.5 M ammonium acetate, pH 5.8 to 6.8 (Fig. 2). The fractions of the main protein peak were lyophilized and desalted by passage through a column of Sephadex G-25 (2.5 cm x 50 cm) equilibrated with 1 % acetic acid. The protein fractions were then pooled and lyophilized.

2. Identification of arginine residues modified by phenylglyoxal

In order to differentiate the "essential" arginine residues in the amino acid sequence of cobrotoxin, the toxin was reacted with phenylglyoxal at varying pH and the modified derivatives were reduced and S-carboxymethylated (RCM) by the procedure described by Crestfield <u>et al</u>. (13), followed by chymotryptic digestion. The RCM-derivatives were dissolved in 0.1 M NH_4HCO_3 buffer (pH 8.2) to give a 1 % solution, and chymotrypsin was added (50 : 1). Digestion was carried out at 27°C for 5 h and the digest was dried over P_2O_5 in a desiccator under vacuum.

Arginine-containing peptides from chymotryptic digests were separated by a combination of high voltage paper electrophoresis at pH 5.4 with pyridine-acetic acid-water (20 : 7 : 973, by vol.) and descending paper chromatography with n-butanol-acetic acid-water-pyridine (15 : 3 : 12 : 10, by vol.), as previously described (6). Peptides on the map were developed initially with 0.2 % ninhydrin in acetone and thereafter with

Sakaguchi reagent to detect arginine-containing peptides. In this procedure, the arginine-containing peptides from chymotryptic digests can be completely separated each other and obtained in a good yield.

3. Amino acid analysis

About 0.2 μmole of protein sample was hydrolyzed in 1 ml of constant-boiling HCl (5.7 M) at 110°C for 24 h in evacuated sealed tubes. Amino acids were determined on a Technicon amino acid autoanalyzer, using norleucine as an internal standard.

4. Measurements of lethal activity

Lethality was measured by intraperitoneal injection of a progressively diluted toxin solution into mice (16-18 g), as previously described (14). Four mice of both sexes were used for each dilution, and the LD_{50} was calculated according to the 50 % end-point method f Reed and Mueuch (15).

5. Immunological procedures

Double diffusion in agar gel was performed by Ouchterlony's technique (16) as previously described (2). The quantitative precipitation reactions were carried out as described by Kabat and Mayer (17). Increasing amounts of antigen in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl were added to a constant amount of antisera in a total volume of 1 ml. The tubes were incubated for 30 min at 37°C and then left overnight at 4°C. The precipitates were washed 3 times with cold 0.15 M NaCl, after which they were dissolved in 3 ml of 0.02 M NaOH, and the absorbances were measured at 280 nm.

III. Results

1. Chemical modification of arginine residues in cobrotoxin

Changes of lethal activity of cobrotoxin by reaction with phenylglyoxal at varying pH values are shown in Fig. 3. The lethal activity decreased rapidly when the reactions were performed at pH 7.5 or higher pH and the lethality lost almost completely after 60 min. However, the rate of inactivation was decreased significantly when the pH of the reaction was lowered. The lethal activity remained unchanged when the reaction was proceeded at pH 6.0 even for 80 min.

2. Identification of arginine residues modified by phenylglyoxal

The results of amino acid analysis of the modified derivatives (Table I) showed that four of the six Arg-residues in cobrotoxin were modified when the reaction was carried out at pH 8.0. However, only one Arg-residue was modified when the reaction was carried out at pH 6.0 and essentially two and three Arg-residues were modified by the reactions at pH 6.7 and pH 7.5, respectively. All other amino acids remained essentially unchanged.

In order to determine the position of Arg-residues modified, the modified derivatives were digested with chymotrypsin after reduction and S-carboxymethylation. Peptide maps prepared from chymotryptic digests by a combination of high voltage paper electrophoresis and paper chromatography were compared with that of native toxin (Fig. 4). The Arg-containing peptides were detected by Sakaguchi reagent and the position of Arg-residue which was selectively modified with phenylglyoxal at pH 6.0 was identified as Arg-28 (Fig. 4-B). Two of the six Arg-residues which were modified at pH 6.7 were identified as Arg-28 and Arg-33 (Fig. 4-C). It can be seen in Fig. 4-D, an additional Arg-residue modified at pH 7.5 was Arg-30.

As shown in the peptide map (Fig. 4-E) of RCM-Arg-modified toxin (at pH 8.0), three Arg-59 containing peptides (C-1, C-3 and C-11, ref. Table II) and one Arg-36 & 39 containing peptide (C-13, ref. Table II) are positive for Sakaguchi test. The result of amino acid analysis (Table I) shows that four Arg-residues were modified. This suggests that either Arg-36 or Arg-39 was also modified besides Arg-28, 30 and 33. Therefore, the Arg-36 & 39 containing peptide, C-13, was cut out, eluted with 1 M acetic acid and dried. The dried material was dissolved in 0.2 ml of 0.1 M NH_4HCO_3 (pH 8.2) and 0.05 ml of trypsin solution (5 mg/ml) was added. After the mixture was incubated at 27°C for 2 h, the pH of the solution was lowered. The solution was taken to dryness for paper electrophoresis at pH 5.4 along with authentic arginine, asparagine and glutamic acid. The paper electrophoretogram shown in Fig. 5 gave three spots; two at cathodic side, of which one spot just corresponds to asparagine, and one at anodic side. No free Arg-residue was appeared. Since the peptide C-13 has the following amino acid sequence (ref. Table II),

$$\overset{36}{\mathrm{Arg}}\text{-Thr-Glu-}\overset{39}{\mathrm{Arg}}\underset{\underset{T}{\uparrow}}{-}\text{Gly-Cys-Gly-Cys-Pro-Ser-Val-Lys}\underset{\underset{T}{\uparrow}}{-}\overset{48}{\mathrm{Asn}}$$

and if only Arg-30 was modified, the following two peptides and a free asparagine will be given off after tryptic digestion,

$\overset{36}{\text{Arg}}\text{-Thr-Glu-}\overset{39}{\text{Arg}}$

Gly-Cys-Gly-Cys-Pro-Ser-Val-Lys

Asn

of which Arg-39 containing peptide gives a positive color reaction with Sakaguchi reagent. Indeed, it happened and the results indicate accordingly that Arg-36 was also modified besides Arg-28, 30 and 33 at pH 8.0.

3. Characterization of the modified derivatives

As shown in Fig. 6, the ultraviolet absorption spectrum of cobrotoxin changed extensively by reaction with phenylglyoxal. However, only Arg-28 modified derivative showed a similar spectrum around 280 nm as that of native toxin.

Almost complete loss of lethal activity (Table III) and pronounced decrease of antigenic activity (Fig. 7 and 8) were observed when four Arg-residues, Arg-28, 30, 33 & 36, in cobrotoxin were modified. However, Arg-28 modified derivative retained essentially full lethality and antigenic activity, suggests that the Arg-28 which is most accessible to modification is not essential for biological functions of cobrotoxin. The lethal activity of Arg-28 & 33 modified toxin drops precipitously, but the antigenic activity was not altered significantly. However, the lethality lost almost completely and the antigenic activity decreased 30 % when an additional Arg-residue at position 30 was modified. These results indicate that Arg-30 and Arg-33 are essential for the lethal activity and Arg-30 and Arg-36 are more closely related to the antigenic specificity of the toxin.

In order to determine whether the Arg-modified derivative forms a polymer, gel filtration on Sephadex G-50 was performed (Fig. 9). In comparison with cobrotoxin (mol. wt., 6,949) and α-chymotrypsin (mol. wt., 22,000), both the Arg-28 & 33 modified toxin and Arg-28, 30, 33 & 36 modified derivative revealed significant amount of polymerized forms and the amount of polymers increased in parallel with the degree of Arg-modification. After gel filtration of Arg-28 & 33 modified toxin, the fractions emerged in the same volumes of eluate as those of cobrotoxin and α-chymotrypsin, corresponding to the monomeric and polymerized forms were pooled for the determination of biological activities. The lethality and antigenic activity were measured after the fractions were desalted and lyophilized. As shown in Table IV, the lethal activity and antigenic activity of the monomeric form is almost the same as those of the modified derivative. Although no pronounced decrease of antigenic activity is noted, the polymerized form is completely non-toxic.

IV. Discussion

Arg-28 was found to be the most accessible to the reaction with phenylglyoxal, and it is the only one modified at pH 6.0 without alteration in biological activity of cobrotoxin. Arg-33 is the next one modified when the reaction was carried out at pH 6.7. An additional residue modified at pH 7.5 was found to be the Arg-residue at the position 30. Arg-30 and Arg-33 are essential for the lethal activity and Arg-30 and Arg-36 are more closely related to the antigenic specificity of the toxin.

There are three moles of arginine in toxin a and two in toxin b of *Laticauda semifasciata*. Only one Arg-residue was modified in both toxins after reaction with 1,2-cyclohexanedione (18). The modification did not alter the lethality of the toxins, confirms that at least one of the Arg-residues is not essential for the lethal activity of snake neurotoxins. *Siamensis* 3 toxin of *Naja naja siamensis* (19) contains five arginine residues at the positions 2, 33, 37, 68 and 70. Digestion of the toxin with protease from *Arthrobacter* removed the C-terminal tetrapeptide, ^{68}Arg-Lys-^{70}Arg-Pro, and the resulting molecule is half as toxic as the intact toxin. The result indicates that neither Arg-68 nor Arg-70 is essential for lethality. However, the toxin was inactivated by reaction with phenylglyoxal and the inactive monomeric derivative, having only one arginine residue modified, was separated.

Pharmacological properties of postsynaptic neurotoxins are the same as those of d-tubocurarine, except for the slowness of onset and the lower reversibility of the paralysis (21, 22). The latter difference is probably due to the larger molecular size of basic polypeptides in comparison to d-tubocurarine. Both short and long neurotoxins (23) show a great affinity to the acetylcholine receptor on the motor endplate (24-28). Although one cannot at present, exclude the possibility that the apparently irreversible toxin-receptor interaction might involve the formation of a covalent bond, for example, by disulfide interchange, it still seems likely that the cationic groups present in snake neurotoxins might be directly involved in the interaction with the receptors. Probably, as in the case of d-tubocurarine (Fig. 10), at least two basic groups with positive charge held at a certain distance in the molecule may be responsible for their neuromuscular blocking activity.

Chemical modification of Lys-47 with group specific reagents resulted in complete loss of lethal activity without change of the conformational properties (9,29), suggest that the positive charge contributed by the ε-amino group of Lys-47 (or guanidino group of arginine residue) which present in all sequence of snake neurotoxins (Fig. 11) is functionally essential for the biological activity of neurotoxins. The results of selective and stepwise arginine modification with group specific reagent, phenylglyoxal, at varying pH suggest that at least the Arg-residue at position 28 in cobrotoxin is not essential. However, when an additional Arg-residue at position 33 was modified the lethal activity drops precipitously, and the lethality even lost almost completely when a third Arg-residue at the same region was modified. This indicates that the cationic groups contributed by guanidino groups of Arg-residue are also functionally essential. This functionally essential cationic group located in the loop containing the sole Trp-residue; namely, the region between positions 25 and 40 (Fig. 1) which contains most of the basic residues and aromatic functional residues, and this uncross-linked loop possibly protrude outward from the molecule because of its hydrophilic properties. All postsynaptic neurotoxins sequenced so far have a sequence $\overset{33}{\text{Arg}}$-$\overset{34}{\text{Gly}}$ occurs at the same or homologous positions (Fig. 11). If there should exists an additional essential cationic group, this may be a guanidino group of Arg-33.

Therefore, it seems likely that the positive charges of the ε-amino group of Lys-47 and of the guanidino group of Arg-33 probably form salt bridges with the anionic sites of the receptors which recognize the quaternary ammonium ion of acetylcholine. The definite answer will have to await the isolation of the toxin receptors and the identification of the essential basic residues which are masked by selective chemical modification in the neurotoxin-receptor complex, and the structure analysis by X-ray crystallography may provide a basis for consideration of structure-function relationship for cobrotoxin and the studies are now being undertaken.

V. Conclusion

Cobrotoxin is a basic protein having six arginine residues at the positions 28, 30, 33, 36, 39 and 59 in the sequence. In this study, selective and stepwise chemical modification of arginine residues were conducted with a group specific reagent, phenylglyoxal, at varying pH and the degree of modification in relation to the lethal activity and antigenic specificity has been studied in details.

Reaction of cobrotoxin with phenylglyoxal at pH 8.0 resulted in almost complete loss of lethal activity and four of the six arginine residues were modified consequently. However, the rate of inactivation was decreased significantly when the pH of the reaction was lowered. Only one arginine residue at position 28 was modified at pH 6.0 and the product retained full biological activity. Arg-33 is the next one modified when the reaction was carried out at pH 6.7 and the lethality drops precipitously, but the antigenic activity was not altered significantly. However, the lethal activity lost almost completely and the antigenic activity decreased about 30 % when an additional arginine residue at position 30 was modified at pH 7.5.

These results indicate that Arg-30 and Arg-33 are essential for the lethal activity and Arg-30 and Arg-36 are more closely related to the antigenic specificity of the toxin. The possible mechanism of neuromuscular blocking activity of snake neurotoxin is discussed.

Literature Cited

1. Yang, C. C. (1965) J. Biol. Chem. 240, 1616-1618

2. Chang, C. C. and Yang, C. C. (1969) J. Immunol. 102, 1437-1444

3. Yang, C. C., Yang, H. J. and Huang, J. S. (1969) Biochem. Biophys. Acta 188, 65-77

4. Yang, C. C., Yang, H. J. and Chiu, R. H. C. (1970) Biochim. Biophys. Acta 214, 355-363

5. Chang, C. C. and Hayashi, K. (1969) Biochem. Biophys. Res. Commun. 37, 841-846

6. Chang, C. C., Yang, C. C., Hamaguchi, K., Nakai, K. and Hayashi, K. (1971) Biochim. Biophys. Acta 236, 164-173

7. Huang, J. S., Liu, S. S., Ling, K. H., Chang, C. C. and Yang, C. C. (1972) J. Formosan Med. Assoc. 71, 383-388

8. Chang, C. C., Yang, C. C., Kurobe, M., Nakai, K. and Hayashi, K. (1971) Biochem. Biophys. Res. Commun. 43, 429-434

9. Chang, C. C., Yang, C. C., Nakai, K. and Hayashi, K. (1971) Biochim. Biophys. Acta 251, 334-344

10. Itano, H. A. and Gottlieb, A. J. (1963) Biochem. Biophys. Res. Commun. 12, 405

11. Toi, K., Bynum, E., Norris, E. and Itano, H. A. (1965) J. Biol. Chem. 240, PC 3455-3457; (1967) J. Biol. Chem. 242, 1036-1043

12. Takahashi, K. (1968) J. Biol. Chem. 243, 6171-6179

13. Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622-627

14. Yang, C. C. (1964) J. Formosan Med. Assoc. 63, 325-331

15. Reed, L. J. and Muench, H. (1938) Am. J. Hyg. 27, 493-497

16. Ouchterlony Ö. (1958) Progr. Allergy 5, 1-78

17. Kabat, E. A. and Mayer, M. M. (1961) *Experimental Immunochemistry* 2nd edn, p. 22, Charles C. Thomas, Springfield, Ill.

18. Tu, A. T., Hong, B. S. and Solie, T. N. (1971) *Biochemistry* 10, 1295-1304

19. Arnberg, H., Eaker, D. and Karlsson, E. (1973) in press

20. Karlsson, E. and Eaker, D. (1972) *J. Formosan Med. Assoc.* 71, 358-371

21. Peng, M. T. (1960) *J. Formosan Med. Assoc.* 59, 1073-1082

22. Su, C., Chang, C. C. and Lee, C. Y. (1967) in *Animal Toxins* (Russell, F. E. and Saunders, P. R., eds), pp. 259-267, Pergamon Press, New York

23. Yang, C. C. (1973) *Toxicon* 11, in press

24. Lee, C. Y. and Chang, C. C. (1966) *Mem. Inst. Butantan, Simp. Intern.* São Paulo, 33, 555-572

25. Lester, H. (1970) *Nature* 227, 727-728

26. Eaker, D., Harris, J. B. and Thesleff, S. (1971) *Eur. J. Pharmacol.* 15, 254

27. Miledi, R., Molinoff, P. and Potter, L. T. (1971) *Nature* 229, 554-557

28. Changeux, J. P., Kasai, M. and Lee, C. Y. (1970) *Proc. Nat. Acad. Sci. U.S.A.* 67, 1241-1247

29. Chicheportiche, R., Rochat, C., Sampieri, F. and Lazdunski, M. (1972) *Biochemistry* 11, 1681-1690

30. Lowry, O. H., Rosebrough, N. G., Farr, A. L. and Randall, R. G. (1951) *J. Biol. Chem.* 193, 265-275

APPENDIX A-1

Table I

Amino acid composition of cobrotoxin and arginine-modified derivatives

	Residues per mole of protein				
Amino acid	Cobrotoxin	Modified derivatives			
		pH 6.0	pH 6.7	pH 7.5	pH 8.0
Aspartic acid	8	8.1	8.0	8.1	8.0
Threonine	8	7.8	7.9	7.9	7.9
Serine	4	3.9	3.8	3.9	4.0
Glutamic acid	7	7.0	7.1	7.1	7.0
Proline	2	1.8	1.8	1.9	1.7
Glycine	7	7.1	7.2	7.0	7.1
Alanine	-	-	-	-	-
Half-cystine	8	8.1	8.0	7.9	7.9
Valine	1	0.9	1.0	0.9	1.0
Methionine	-	-	-	-	-
Isoleucine	2	1.9	2.0	2.0	1.9
Leucine	1	1.0*	1.0	1.0	1.0
Tyrosine	2	2.0	1.9	2.0	1.9
Phenylalanine	-	-	-	-	-
Lysine	3	3.0	2.9	3.0	2.9
Histidine	2	2.0	1.9	1.9	1.9
Arginine	6	5.1	4.1	2.9	2.1
Tryptophan	1	1.0	1.0	1.0	1.0

* All values are expressed as molar ratios based on leucine = 1.0.

APPENDIX A-2

Table II

Amino acid sequence of chymotryptic peptides from reduced and S-carboxymethylated cobrotoxin

Peptides	Amino acid sequence	Arg-residues
C-1	H-Cys-Cys-Thr-Thr-Asp-Arg-Cys-Asn-Asn-OH	Arg-59
C-2	H-Gly-Cys-Ser-Gly-Gly-Glu-Thr-Asn-Cys-Tyr-OH	
C-3	H-Gly-Ile-Glu-Ile-Asn-Cys-Cys-Thr-Thr-Asp-Arg-Cys-Asn-OH	Arg-59
C-4	H-Thr-Gly-Cys-Ser-Gly-Gly-Glu-Thr-Asn-Cys-Tyr-OH	
C-5	H-Cys-Tyr-OH	
C-6	H-Asp-OH	
C-7	H-Leu-Glu-Cys-His-OH	
C-8	H-Gly-Ile-Glu-Ile-Asn-OH	
C-9	H-Leu-Glu-Cys-His-Asn-Gln-Gln-OH	
C-11	H-Arg-Cys-Asn-Asn-OH	Arg-59
C-12	H-Ser-Ser-Gln-Thr-Pro-Thr-OH	
C-13	H-Arg-Thr-Glu-Arg-Gly-Cys-Gly-Cys-Pro-Ser-Val-Lys-Asn-OH	Arg-36 & 39
C-14	H-Arg-Gly-Tyr-OH	Arg-33
C-15	H-Arg-Asp-His-Arg-Gly-Tyr-OH	Arg-30 & 33
C-16	H-Lys-Lys-Arg-Trp-Arg-Asp-OH	Arg-28 & 30
C-17	H-Lys-Arg-Trp-OH	Arg-28
C-18	H-Lys-Lys-Arg-Trp-OH	Arg-28

APPENDIX A-3

Table III

Stepwise modification of arginine residues in cobrotoxin with phenylglyoxal at varying pH

	Arg-residues modified	Lethality	Antigenic activity
		(%)	(%)
Cobrotoxin	None	100	100
At **pH** 6.0	Arg-28	100	98
pH 6.7	Arg-28 & 33	22.6	94
pH 7.5	Arg-28, 30 & 33	3.1	70
pH 8.0	Arg-28, 30, 33 & 36	1.6	34

APPENDIX A-4

Table IV

The lethality and antigenic activity of monomeric and polymeric forms separated from Arg-28 & 33 modified derivative by gel filtration on Sephadex G-50

	Lethality (%)	Antigenic activity (%)
Cobrotoxin	100	100
Arg-28 & 33 modified derivative	22.6	94
Monomeric form	25	95
Polymerized form	0	78

APPENDIX B-1

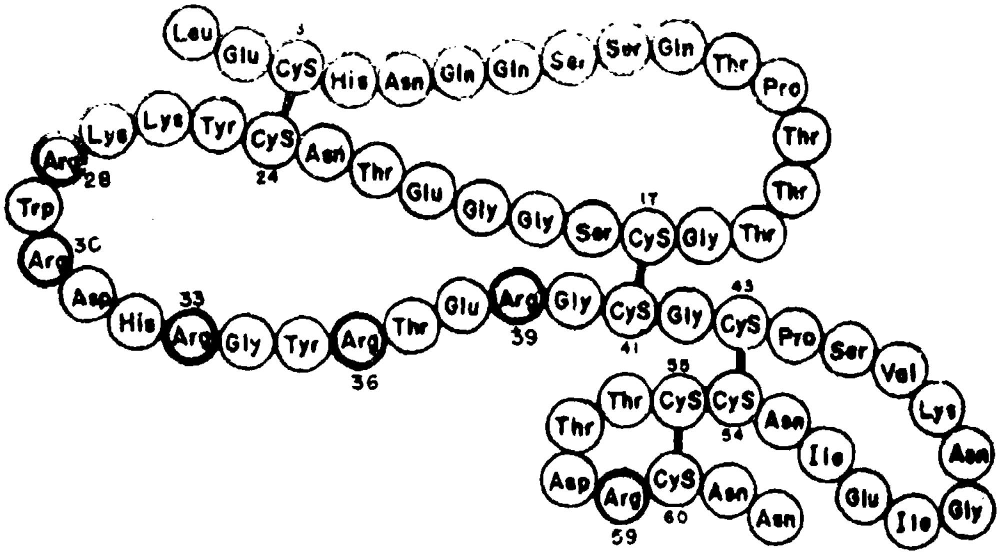


Fig. 1. Structure of cobrotoxin. Two-dimensional schematic diagram showing the arrangement of the disulfide bonds and the sequence of the amino acid residues.

APPENDIX B-2

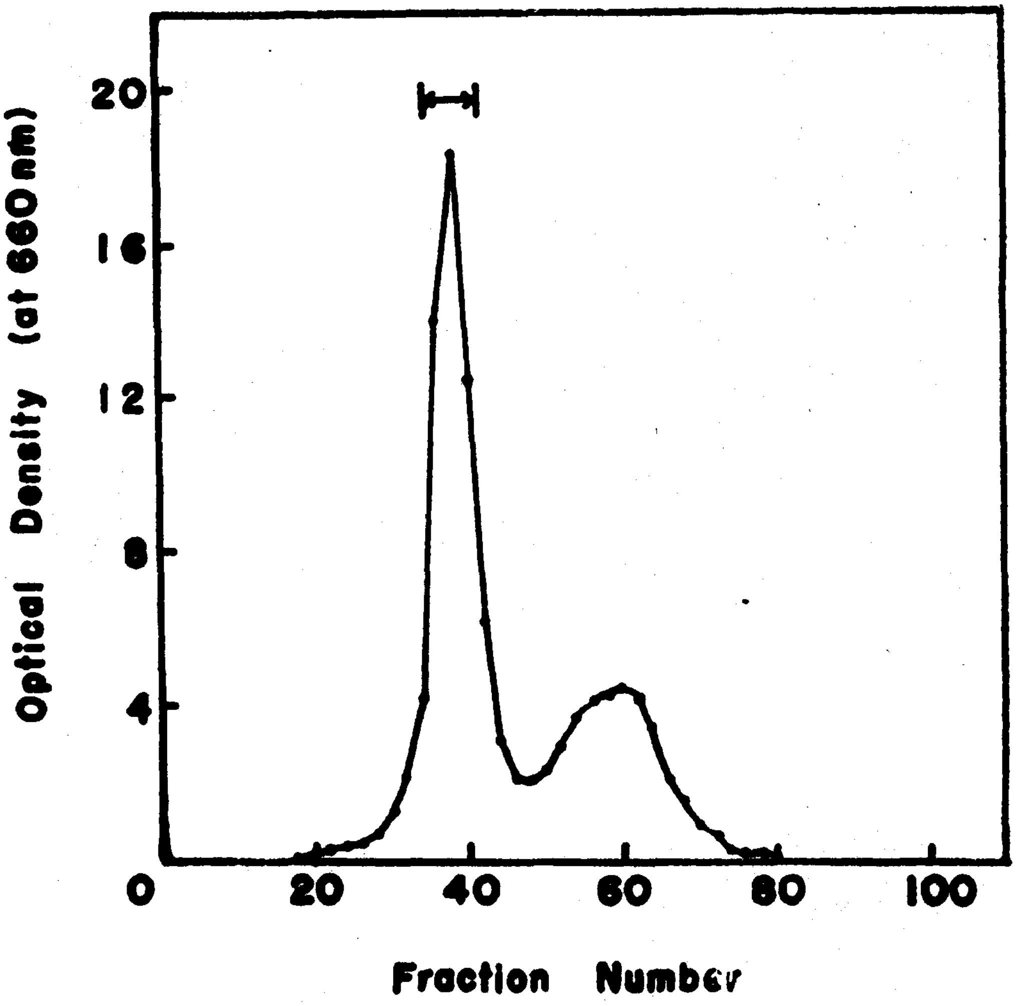


Fig. 2. Chromatography of Arg-modified cobrotoxin (at pH 8.0) on CM-cellulose column.

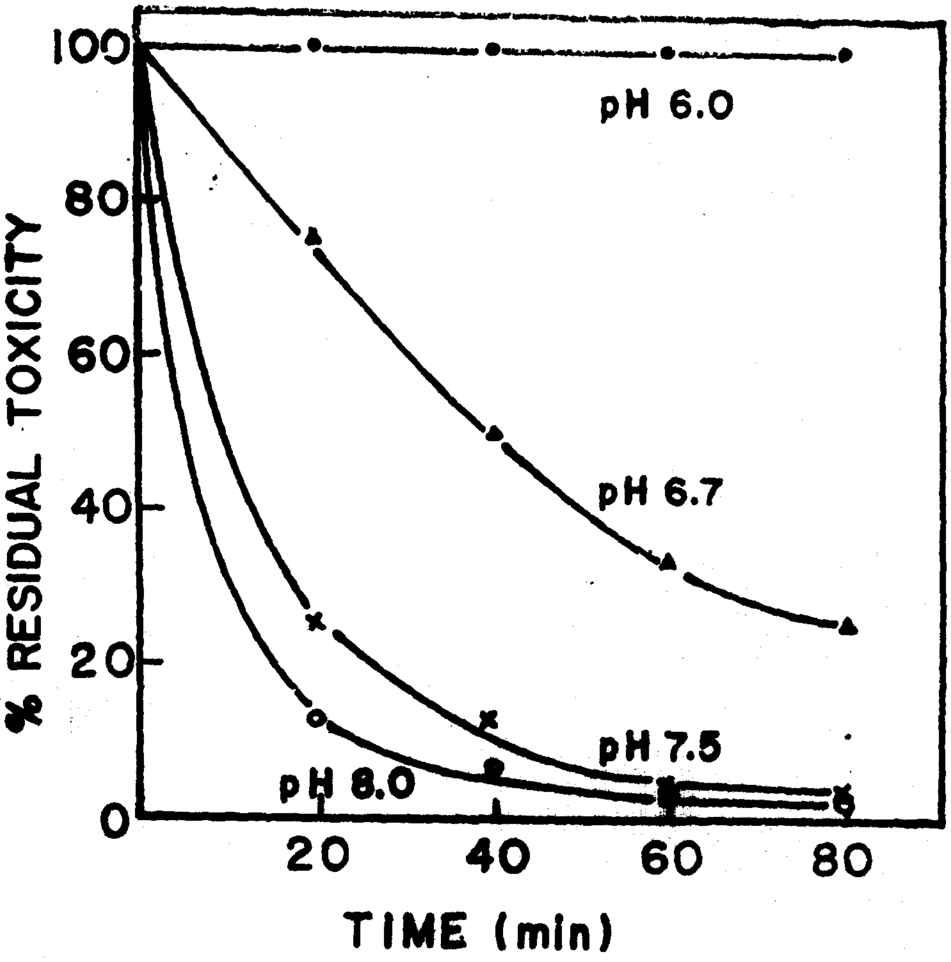


Fig. 3. Changes of lethal toxicity of cobrotoxin by reaction with phenylglyoxal at varying pH values.

6 mg of cobrotoxin was dissolved in 0.5 ml of buffer solution and a 100-fold molar excess of phenylglyoxal in 1.5 ml of the same buffer was added. Reaction was allowed to proceed at 27°C. After suitable intervals of time, aliquots were taken for determination of lethal toxicity. Buffer solutions used were: 0.1 M acetate, pH 6.0; 0.1 M phosphate, pH 6.7 or pH 7.5 and 0.2 M N-ethylmorpholine acetate, pH 8.0.

APPENDIX B-4A

Fig. 4-A

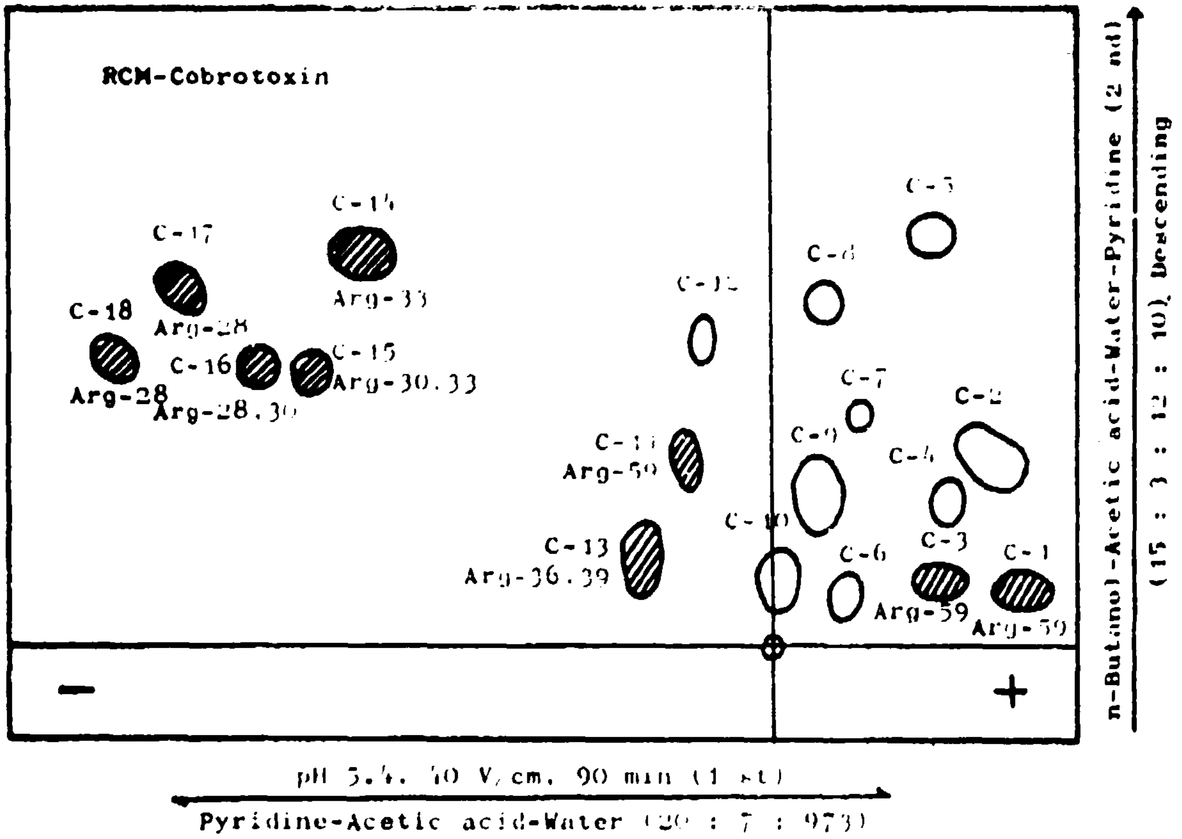


Fig. 4. Peptide maps of the chymotryptic hydrolysates of RCM-cobrotoxin and RCM-Arg-modified derivatives at varying pH. RCM-cobrotoxin: A; RCM-Arg-modified toxins: at pH 6.0, B; at pH 6.7, C; at pH 7.5, D; at pH 8.0, E.

Fig. 4-B

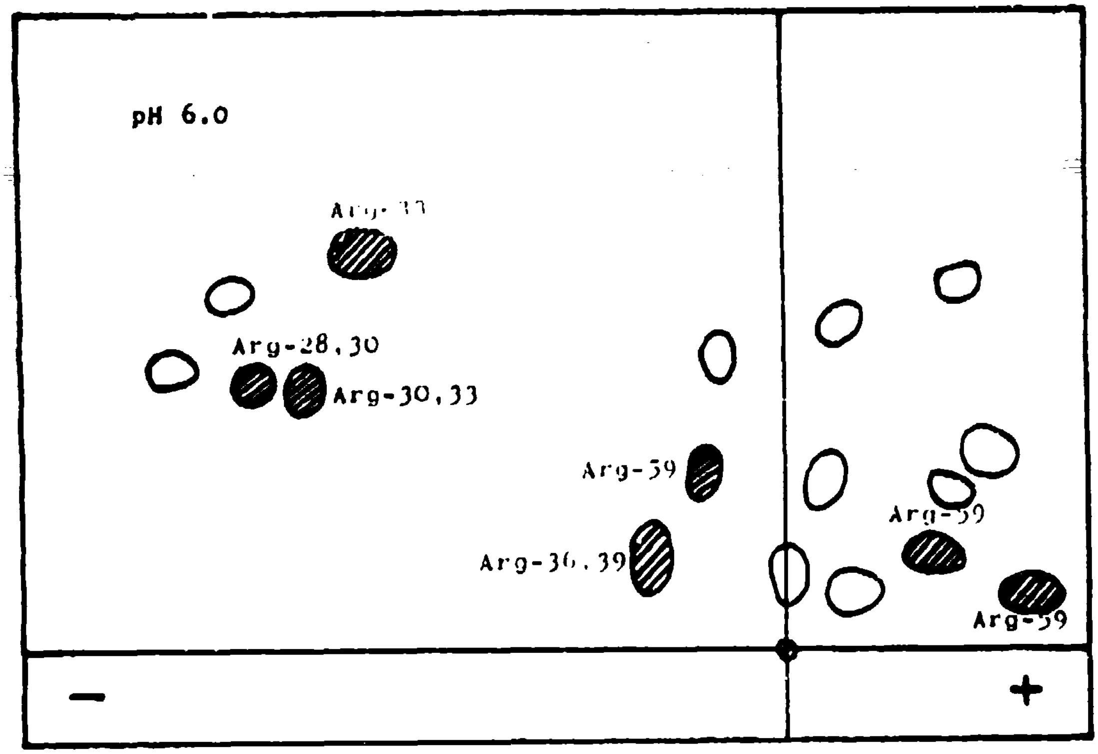

pH 6.0
Arg-33
Arg-28,30
Arg-30,33
Arg-59
Arg-36,39
Arg-59
Arg-59
−
+

Fig. 4-C

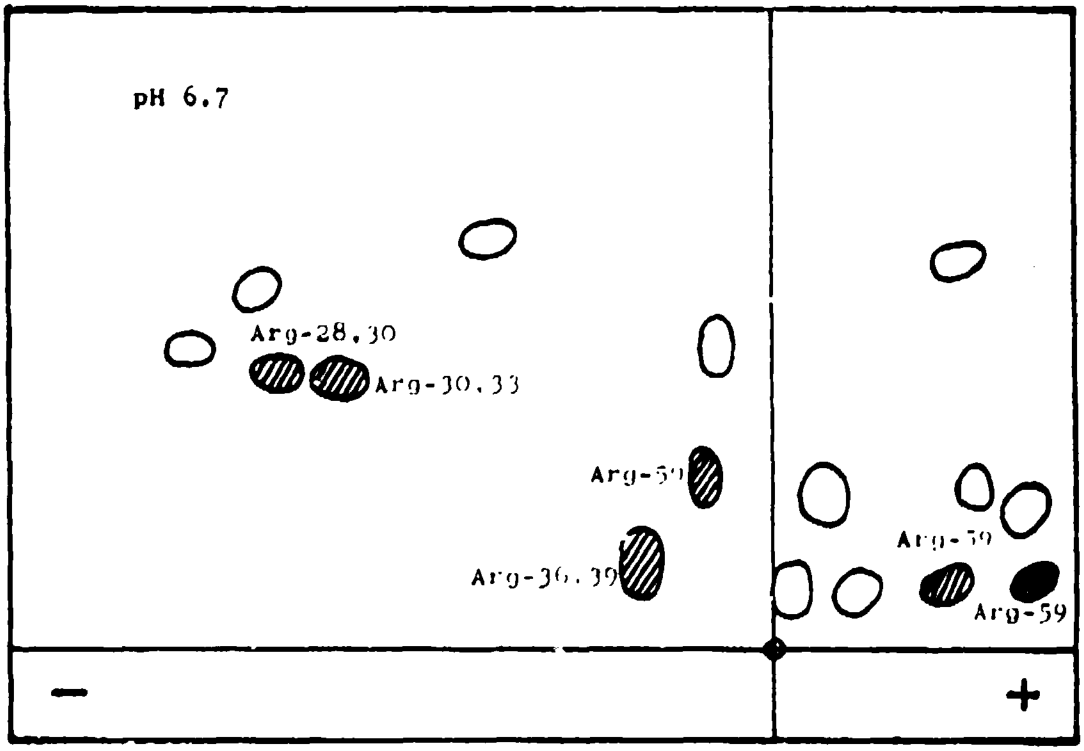

pH 6.7
Arg-28,30
Arg-30,33
Arg-59
Arg-36,39
Arg-59
Arg-59
−
+

Fig. 4-D

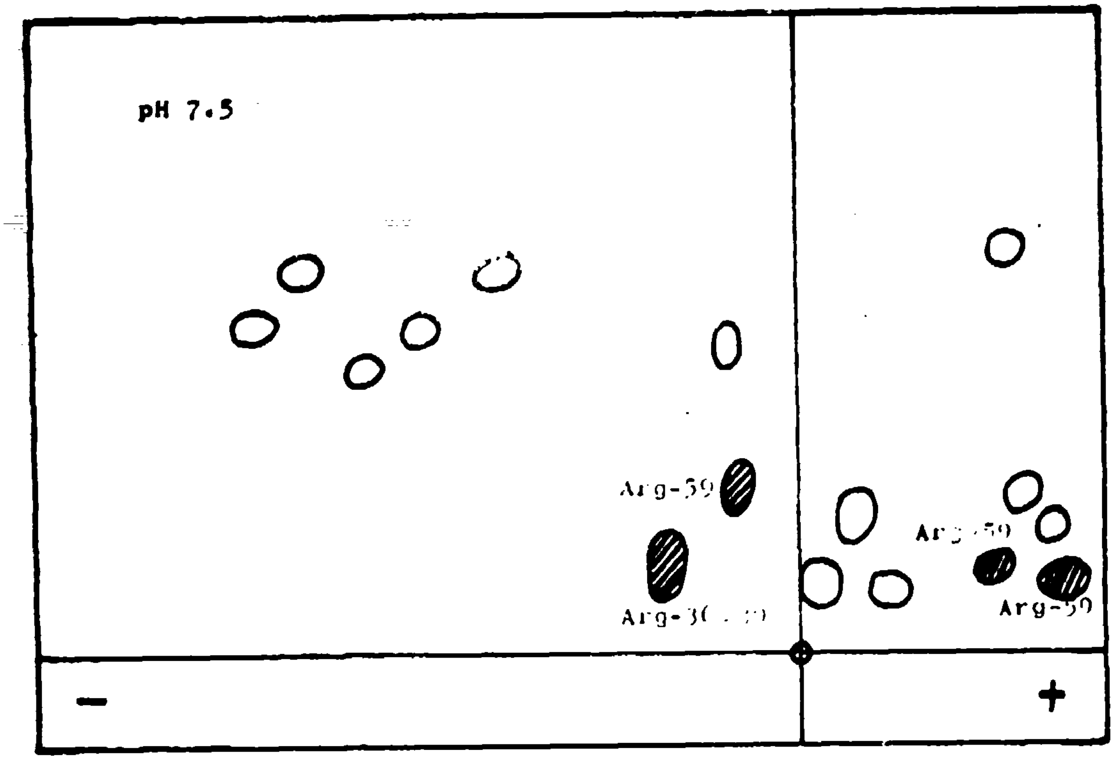
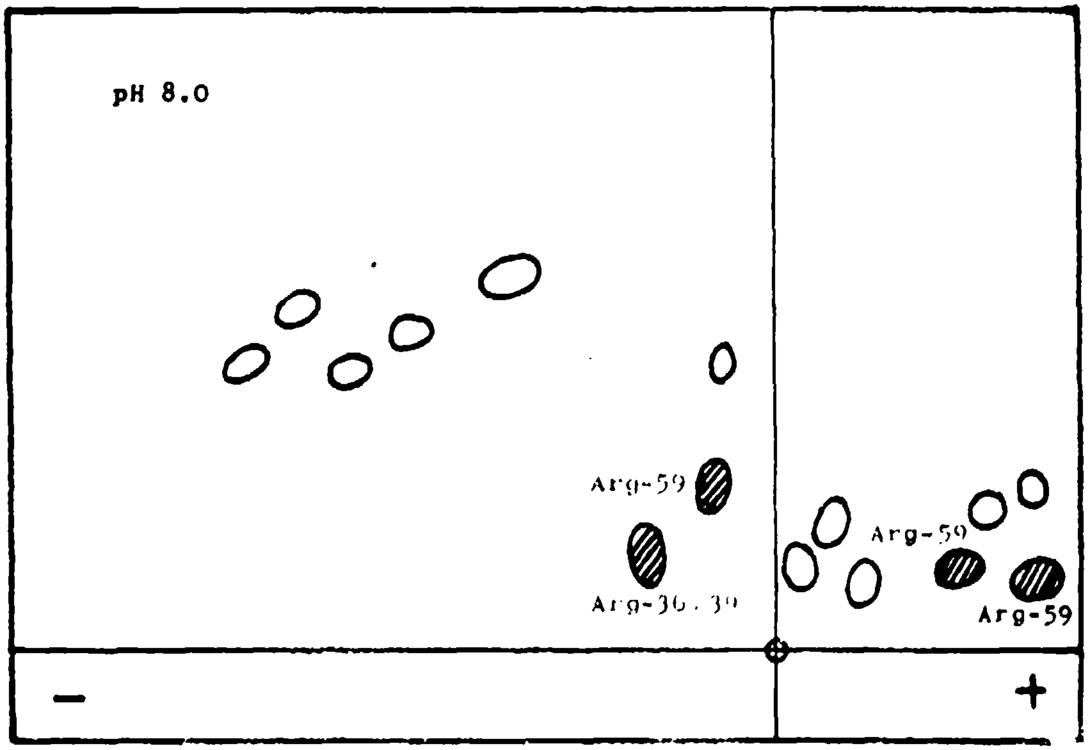

pH 7.5
Arg-59
Arg-36,39
Arg-59
Arg-59
−
+

Fig. 4-E


pH 8.0
Arg-59
Arg-36,39
Arg-59
Arg-59
−
+

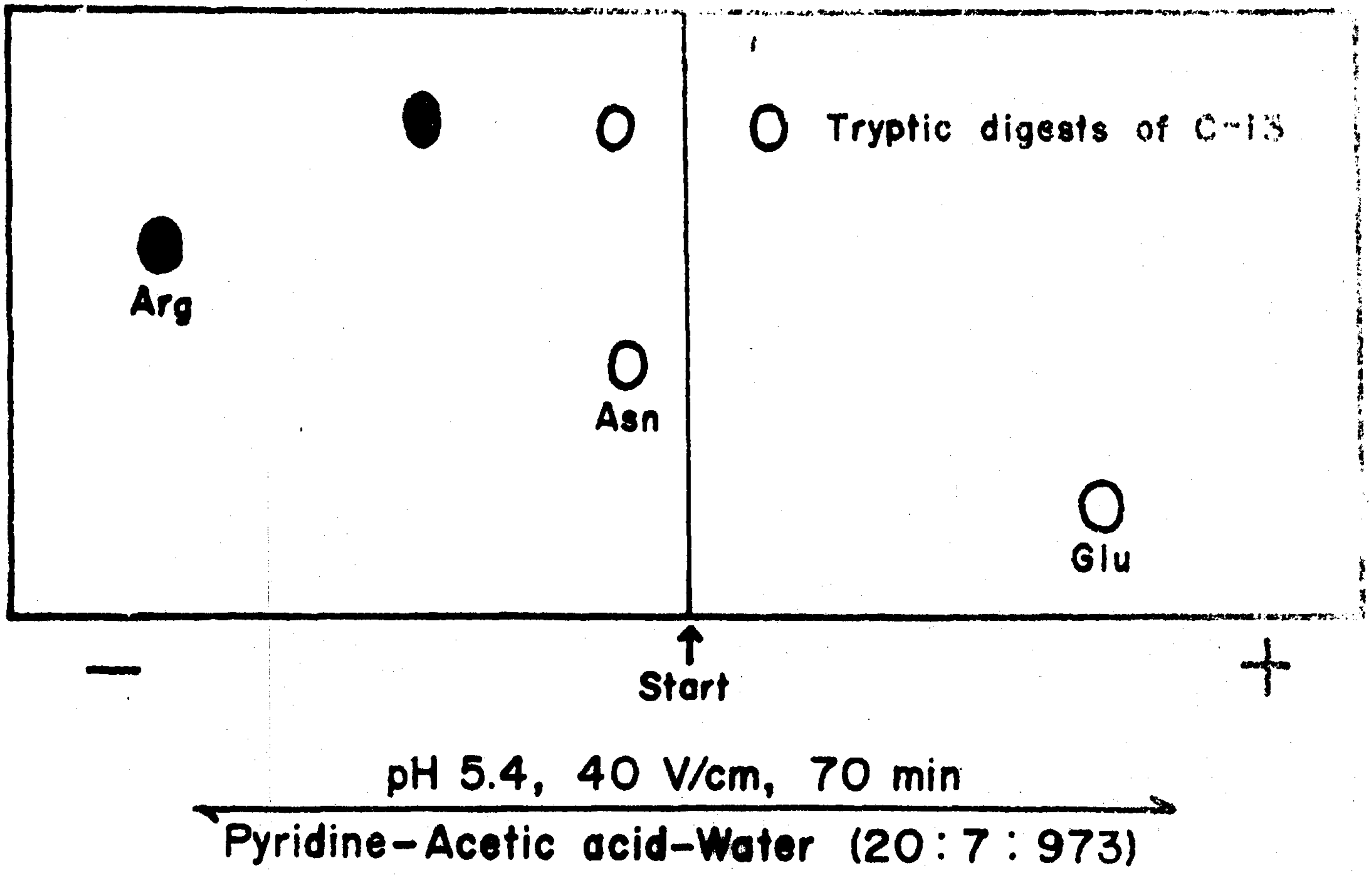


Fig. 5. Paper electrophoretogram of the tryptic digests of C-13 (Arg-36 & 39 containing peptide) from chymotryptic hydrolysates of RCM-Arg-modified cobrotoxin (at pH 8.0).

APPENDIX B-6

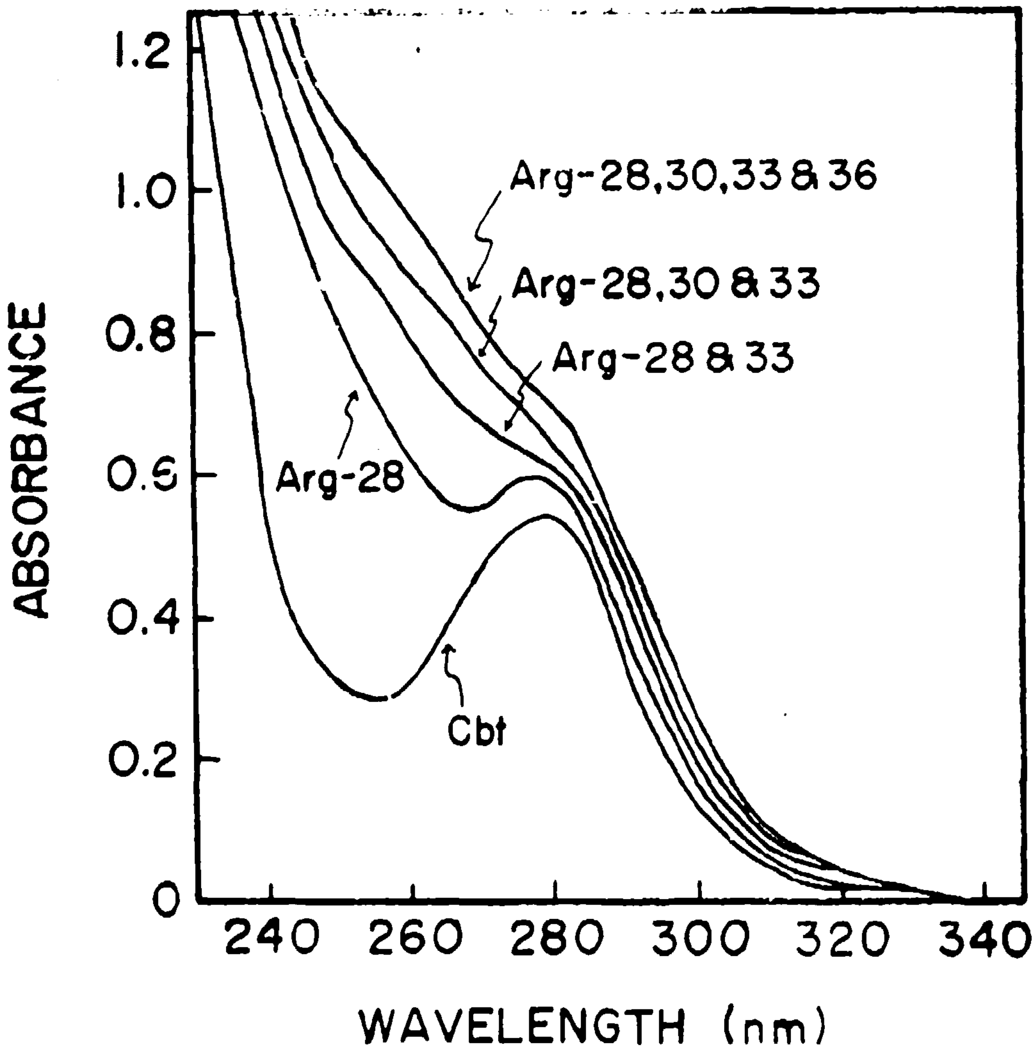


Fig. 6. Absorption spectra of cobrotoxin (Cbt) and Arg-modified derivatives. Each sample (2 mg) was dissolved in 4 ml of 0.01 M ammonium bicarbonate. Arg-28, represents the Arg-28 modified toxin; Arg-28 & 33, Arg-28 & 33 modified toxin; Arg-28, 30 & 33, Arg-28, 30 & 33 modified toxin; Arg-28, 30, 33 & 36, Arg-28, 30, 33 & 36 modified toxin.

APPENDIX B-7

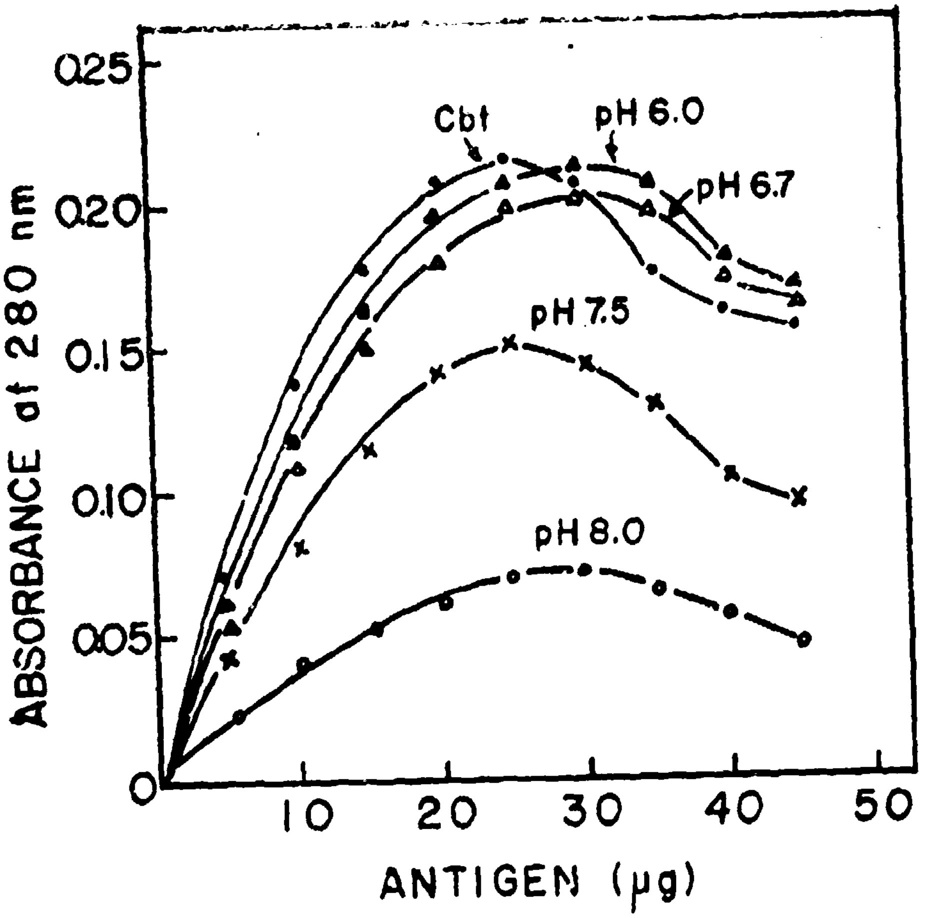


Fig. 7. Quantitative precipitin reactions of cobrotoxin and its Arg-modified derivatives with anti-cobrotoxin serum.

0.4 ml of antiserum was used in each case. ●——●, cobrotoxin; ▲——▲, Arg-28 modified toxin (at pH 6.0); ▲——△, Arg-28 & 33 modified toxin (at pH 6.7); x——x, Arg-28, 30 & 33 modified toxin (at pH 7.5); o——o, Arg-28, 30, 33 & 36 modified toxin (at pH 8.0).

APPENDIX B-8

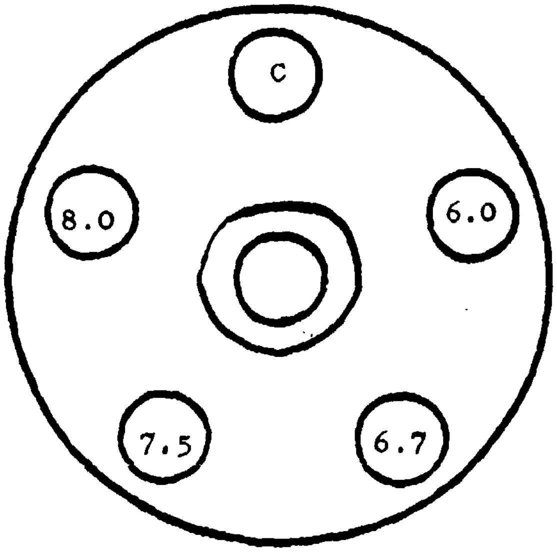


Fig. 8. Immunodiffusion in agar gel. Central well: anti-cobrotoxin serum. Surrounding wells: C, cobrotoxin; 6.0, Arg-28 modified toxin (at pH 6.0); 6.7, Arg-28 & 33 modified toxin (at pH 6.7); 7.5, Arg-28, 30 & 33 modified toxin (at pH 7.5); 8.0, Arg-28, 30, 33 & 36 modified toxin (at pH 8.0).

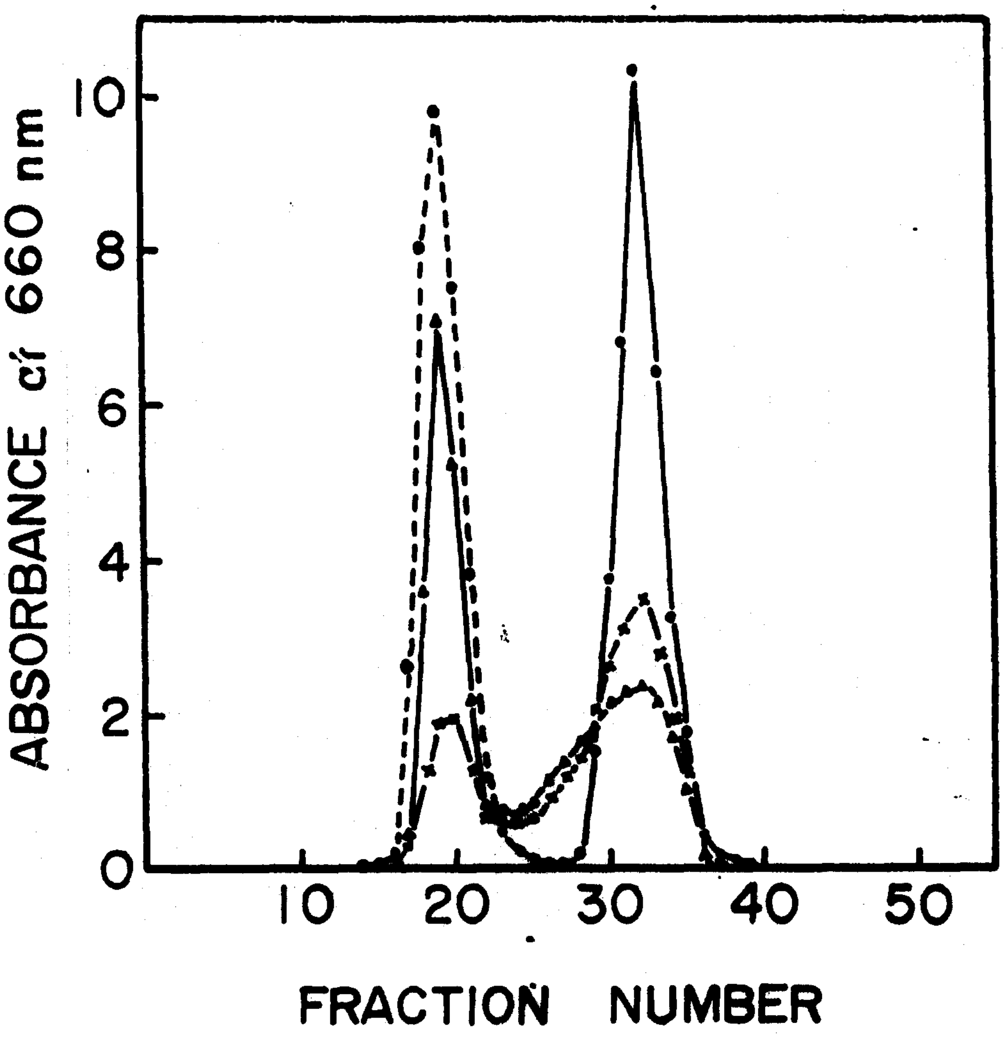
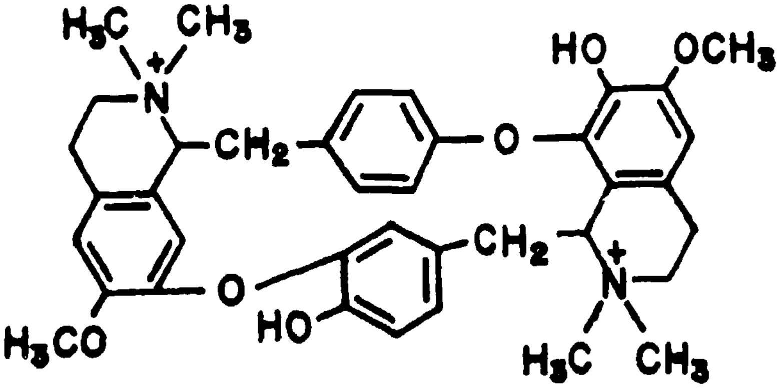


Fig. 9. Gel filtration patterns of cobrotoxin, Arg-modified derivatives and α-chymotrypsin on Sephadex G-50. The column was equilibrated with 0.067 M phosphate buffer (pH 7.4) to a constant height (2 cm x 83 cm). Each sample (10 mg) dissolved in the same buffer, was applied onto the column and eluted with the same buffer. 5 ml fractions were collected at a rate of 25 ml per h and the protein concentration was determined by the method of Lowry <u>et al</u>. (30). ●—●, cobrotoxin; ×—×, Arg-28 & 33 modified toxin; ▲—▲, Arg-28, 30 33 & 36, modified toxin; ●--● α-chymotrypsin.

APPENDIX B-10



d-Tubocurarine

Fig. 10. d-Tubocurarine

APPENDIX B-11

					28		30			3
N. naja atra	(cobrotoxin)	Tyr	Lys	Lys	Arg	Trp	Arg	Asp	His	Ar
N. haje haje α, N. nivea δ		Tyr	Lys	Lys	Arg	Trp	Arg	Asp	His	Ar
N. nigricollis	(toxin α)	Tyr	Lys	Lys	Val	Trp	Arg	Asp	His	Ar
N. melanoleuca	(toxin d)	Tyr	Lys	Lys	Gln	Trp	Ser	Asp	His	Ar
H. haemachatus	(toxin II)	Tyr	Asn	Lys	Arg	Trp	Arg	Asp	His	Ar
H. haemachatus	(toxin IV)	Tyr	Lys	Lys	Gln	Trp	Ser	Asp	His	Ar
N. nivea	(toxin β)	Tyr	Lys	Lys	Arg	Trp	Arg	Asp	His	Ar
D. polylepis	(toxin α)	Tyr	Lys	Lys	Tyr	Trp	Arg	Asp	His	Ar
E. schistosa	(toxin 4)	Tyr	Lys	Lys	Thr	Trp	Ser	Asp	His	Ar
E. schistosa	(toxin 5)	Tyr	Lys	Lys	Thr	Trp	Ser	Asp	His	Ar
L. semifasciata	(erabutoxin a)	Tyr	Asn	Lys	Gln	Trp	Ser	Asp	Phe	Ar
L. semifasciata	(erabutoxin b)	Tyr	His	Lys	Gln	Trp	Ser	Asp	Phe	Ar
L. semifasciata	(erabutoxin c)	Tyr	His	Lys	Gln	Trp	Ser	Asp	Phe	Ar
N. naja siamensis	(toxin 3)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
N. naja naja	(black cobra toxin)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
N. naja naja	(toxin 3)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
N. naja naja	(toxin 4)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
N. naja	(toxin A)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
N. melanoleuca	(toxin b)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
N. nivea	(toxin α)	Tyr	Thr	Lys	Met	Trp	CyS	Asp	-	Ar
O. hannah	(toxin a)	Tyr	Thr	Glu	Thr	Trp	CyS	Asp	-	Ar
O. hannah	(toxin b)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
D. jamesonii	(toxin II)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
D. polylepis	(toxin γ)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
D. polylepis	(toxin δ)	Tyr	Thr	Lys	Thr	Trp	CyS	Asp	-	Ar
B. multicinctus	(α-bungarotoxin)	Tyr	Arg	Lys	Met	Trp	CyS	Asp	-	Ar

Fig. 11. Amino acid seque

(Showing the region betwee
most of the basic residue

①

```
 28       30           33               36            39                                          47
Arg+Trp+Arg+Asp-His+Arg+Gly-Tyr+Arg+Thr-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Ser-Val+Lys+Asn-Gly-
Arg+Trp+Arg+Asp-His+Arg+Gly-Ser-Ile-Thr-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Ser-Val+Lys+Lys+Gly-
Val-Trp+Arg+Asp-His+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Thr-Val+Lys+Pro-Gly-
Gln-Trp-Ser-Asp-His+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Ser-Val+Lys+Lys+Gly-
Arg+Trp+Arg+Asp-His+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Thr-Val+Lys+Pro-Gly-
Gln-Trp-Ser-Asp-His+Arg+Gly-Ser+Arg+Thr-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Thr-Val+Lys+Pro-Gly-
Arg+Trp+Arg+Asp-His+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Ser-Val+Lys+Lys+Gly-
Tyr-Trp+Arg+Asp-His+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro+Lys+Val+Lys+Pro-Gly-
Thr-Trp-Ser-Asp-His+Arg+Gly-Thr+Arg+Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Gln-Val+Lys+Pro-Gly-
Thr-Trp-Ser-Asp-His+Arg+Gly-Thr+Arg+Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Gln-Val+Lys+Ser-Gly-
Gln-Trp-Ser-Asp-Phe+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Thr-Val+Lys+Pro-Gly-
Gln-Trp-Ser-Asp-Phe+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Thr-Val+Lys+Pro-Gly-
Glr-Trp-Ser-Asp-Phe+Arg+Gly-Thr-Ile-Ile-Glu+Arg+Gly-CyS-Gly-CyS-Pro-Thr-Val+Lys+Pro-Gly-

Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro-Thr-Val+Lys+Thr-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro-Thr-Val+Lys+Thr-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro-Thr-Val+Arg+Thr-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro-Thr-Val+Arg+Thr-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro-Thr-Val+Arg+Thr-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro-Thr-Val+Lys+Pro-Gly-
Met-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro+Lys+Val+Lys+Pro-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Asp-Leu-Gly-CyS-  -  -CyS-Pro-Ile-Val+Lys+Pro-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Ile-Asp-Leu-Gly-CyS-  -  -CyS-Pro+Lys+Val+Lys+Pro-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Glu-Leu-Gly-CyS-  -  -CyS-Pro+Lys+Val+Lys+Thr-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Arg+Val-Glu-Leu-Gly-CyS-  -  -CyS-Pro+Lys+Val+Lys+Ala-Gly-
Thr-Trp-CyS-Asp-  - +Arg+Gly+Lys+Ile-Val-Glu-Leu-Gly-CyS-  -  -CyS-Pro+Lys+Val+Lys+Ala-Gly-
Met-Trp-CyS-Asp-  - +Arg+Gly+Lys+Val-Val-Glu-Leu-Gly-CyS-  -  -CyS-Pro-Ser+Lys+Lys+Pro-Tyr-
```

11. Amino acid sequence of snake neurotoxins

wing the region between positions 25 and 49 which contains
t of the basic residues).

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. Conger. Biochem. 7th, Tokyo Col. VIII, 1 (1967)
4. Studies on fluorescent cobrotoxin. Biochim. Biophys. Acta 147 (1967) 600.
5. Study on ^{131}I labeled cobrotoxin. Toxicon 5 (1968) 295
6. Optical rotatory dispersion and circular dichroism of cobrotoxin. Biochim. Biophys. Acta 168 (1968) 373
7. Amino acid composition and end group analysis of cobrotoxin. Toxicon 7 (1969) 43
8. Immunochemical studies on cobrotoxin. J. Immun. 102 (1969) 1437
9. The amino acid sequence of cobrotoxin. Biochim. Biophys. Acta 188 (1969) 65
10. Biochemical and immunochemical studies on cobrotoxin. The Snake 2 (1970) 1
11. Structure-activity relationships and immunochemical studies on cobrotoxin. Radiation Sensitivity of Toxins and Animal Poisons IAEA-PL-334/6 (1970) 63
12. The position of disulfide bonds in cobrotoxin. Biochim. Biophys. Acta 214 (1970) 355
13. Biochemical studies on the toxic nature of snake venom: Cobrotoxin from Formosan cobra venom. Toxins of Animal and Plant Origin DeVries/Kochva, Gordon and Breach, London, 1 (1971) 205

14. Studies on the status of tyrosyl residues in cobrotoxin.
Biochim. Biophys. Acta 236 (1971) 164

15. Studies on the status of free amino and carboxyl groups in cobrotoxin. Biochim. Biophys. Acta 251 (1971) 334

16. Photooxidation of cobrotoxin.
J. Formosan Med. Assoc. 71 (1972) 383

17. Iodination of cobrotoxin.
Toxicon 11 (1973) 39

18. Immunochemical studies on the tryptophan-modified cobrotoxin. Biochim. Biophys. Acta 295 (1973) 595

19. Chemistry and evolution of snake venom toxins.
Toxicon in press (1973)

20. Studies on the status of arginine residues in cobrotoxin.
Biochim. Biophys. Acta in press (1974)

List of Graduate Students

Chun-chang Chang, D. Sc.

Yea-shiang Su, B. S.

Lung-hsiung Hsu, B. S.

Che-an Yang, B. S.

Hung-chin Chiu, B. S.

Ing-fong Liou, B. S.

Hsing-ming Chen, B. S.

Shu-mei Yao, B. S.