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BIOCHEMICAL STUDIES ON THE TOXIC NATURE OF SNAKE VENOM

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ABSTRACT

I. The Disulfide Bonds of Cobrotoxin and their Relationship to Lethality.

Clarification of the status of sulfur bonds in the biologically-active protein is very important not only for the establishment of structure but also for the elucidation of their relation to the biological activity. The total content of half cystine plus cysteine of cobrotoxin was measured to be 12.0 by disulfide interchange reactions. No sulfhydryl groups were detected by spectrophotometric titration with p-chloromercuribenzoate and N-ethylmaleimide even after cobrotoxin was treated with 8 M urea. Since no methionine was found on amino acid analysis, the sulfurs in cobrotoxin were proved to be exclusively in the form of disulfide bonds. On reduction with β-mercaptoethanol, cobrotoxin display 11.6 to 12.0 sulfhydryl groups and loses its lethality concurrently. However, the inert, fully reduced cobrotoxin yields biologically active product with complete lethality and antigenicity, specific rotation close to the native value, and an infrared spectrum identical to native cobrotoxin on reoxidation. The results clearly indicate that the integrity of the disulfide bonds in cobrotoxin is essential for lethality.

II. Optical Rotatory Dispersion Study of Cobrotoxin

The present communication deals with the optical rotatory dispersion (ORD) study of the native, reduced and reoxidized cobrotoxin preparations in the wavelength range 220-300 mμ, and the discovery of a positive Cotton effect at 233 mμ for the native cobrotoxin. The mean residue rotation corrected for the refractive index of the solvent at 233 mμ was found to be +1,250°, suggests that cobrotoxin contains about 22 % of the left-handed α-helix in the molecule. This is a very distinct feature for a natural protein consisting of L-amino acids to have a left-handed helical structure. In contrast, the ORD curve of the reduced cobrotoxin manifests simple dispersion curve with negative value of $[m']_{233}$ (-2,100°), indicates that cobrotoxin loses left-handed helix and becomes random structure after the reductive cleavage of its disulfide bonds. However, the reoxidized cobrotoxin gave essentially the same ORD curve as the native cobrotoxin, shows that correct reformation of left-handed α-helix occurred by air-oxidation of the reduced cobrotoxin.

III. Studies on Fluorescent Cobrotoxin

The present study was initiated to investigate the properties of fluorescent cobrotoxin after fluorescein thiocarbamylation, and applied the immunofluorescent procedure to define and determine quantitatively any residual antigen that might be adherent to the supposedly purified antibody. Three to four moles of fluorescein were incorporated into each molecule of cobrotoxin when the toxin was allowed to react with more than 4-fold molar excess of fluorescein isothiocyanate. In this system none of the cobrotoxin remained unlabeled. The UV maximum of the fluorescent cobrotoxin is shifted to the shorter wavelength and the lethality decreased to 1/7.5, while the cross-reaction with anti-cobrotoxin sera was not altered. It suggests that in cobrotoxin the antigenic sites are different from the active site(s) of toxicity which was blocked by fluorescein thiocarbamylation. Fluorescent cobrotoxin was used to define and determine the degree of separation of the antigen from its antibody. The immune precipitates were completely dissolved and dissociated in 0.53 M formic acid-0.15 M NaCl: complete separation of fluorescent antigen from the antibody was achieved on Sephadex G-100 column in the same solvent. The purified antibody was proved to be free from faint traces of antigen and 100 % precipitable with cobrotoxin as measured by quantitative precipitin reaction.

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I. The Disulfide Bonds of Cobrotoxin and their Relationship to Lethality.

1. Introduction

Since the high sulfur content in the toxic ingredients of snake venom and its possible relationship to the venom toxicity were discovered, many studies on the nature of the sulfur in snake venoms have been carried out (1-5). However, the nature of the sulfur bonds and their association with the toxicity of snake venoms remain unknown.

Clarification of the status of sulfur bonds in the biologically active proteins is very important not only for the establishment of structure but also for the elucidation of their relation to the biological activity. In this study the sulfur distribution of the crystalline toxin, cobrotoxin, was established and the integrity of the disulfide bonds for its lethality was proved.

2. Materials and Methods

Cobrotoxin was prepared from Formosan cobra (Naja naja atra) venom as previously described (7). NN'-Di-DNP-L-cystine and N-ethylmaleimide were obtained from Sigma Chemical Company. Urea was a Mallinckrodt reagent, and was recrystallized from aqueous ethanol. EDTA was a Fischer certified reagent.

A commercial preparation of PCMB was purified according to the procedure of Boyer (8). A portion (20 mg) of the well-dried preparation was completely dissolved in 1 ml. of 1 N NaOH and diluted with 0.05 M sodium pyrophosphate buffer (pH 8.1). Stock solutions thus prepared were standardized by measurement of the absorbance at 232 mμ at pH 7.0 after appropriate dilution. Bio-Gel P-2 was purchased from Bio-Rad Laboratories, and β-mercaptoethanol was a product of Eastman Organic Chemicals.

a. Determination of the half-cystine plus cysteine content of cobrotoxin by disulfide interchange reaction

The equilibrium concentration of the mixed disulfide was determined by the method of Glazer and Smith (9), in the protein concentration range of 0.033 to 0.2 μmole/ml of reaction mixture. The rate of interchange was followed until the rate of change of absorbance of the aqueous phase at 357 mμ became constant. At the end of 21 days the equilibrium was reached, and the amount of mono-DNP-cystine presented was determined. The ratios of μmoles of mono-DNP-cystine/μmoles of protein were plotted against the protein concentration, and the total content of half-cystine plus cysteine was obtained by extrapolation to zero protein concentration.

b. Determination of sulfhydryl content

(1) N-Ethylmaleimide: Determinations involving N-ethylmaleimide were carried out by the method of Alexander (10). The reaction was carried out in 0.1 M phosphate buffer (pH 6.8) with 1.2×10^{-3} M N-ethylmaleimide and 0.83×10^{-4} M cobrotoxin which had been pretreated with 8 M urea for 20 h at room temperature. The reaction mixture devoid of N-ethylmaleimide served as a blank, and the spectrophotometric measurements were made in 1-cm matched silica cells in the Beckman Model DK$_2$ recording spectrophotometer. The difference in absorbance at 300 mμ between the reacted and unreacted N-ethylmaleimide solutions was divided by the molar extinction coefficient of 620 for the calculation of sulfhydryl contents.

(2) PCMB: The spectrophotometric method of Boyer (8) was employed for these determinations.

(a) Titration of the protein with PCMB. For this purpose increments of PCMB were added to the cells containing the protein in 0.025 M Tris-HCl-8 M urea (pH 8.0). Spectrophotometric measurements were made in the conventional manner with the Model DU Beckman spectrophotometer at 250 mμ.

(b) Titration of PCMB with the protein. Identical increments of the protein solution were added to a cell containing 3 ml of buffered PCMB and to a blank cell containing the same volume of 0.05 M phosphate buffer (pH 7.0) alone. The contents were mixed by inversion of each cell covered with a piece of Parafilm, and the absorbance at 250 mμ was read after each addition. The observed absorbance was corrected for dilution, and plotted against the volume of protein added. The endpoint was obtained from the intersection of the two lines, and the sulfhydryl content was calculated.

c. Reduction of cobrotoxin with β-mercaptoethanol

Reduction was carried out essentially according to the procedure of Anfinsen and Haber (11). In a representative experiment, 100 mg of cobrotoxin was dissolved in 5 ml of a freshly-prepared 8 M solution of recrystallized urea, and 0.1 ml of β-mercaptoethanol (1 μl/mg of protein) was added. The glass-stoppered tube was flushed with N_2 and left at room temperature for 4 h. The pH was then adjusted to 3.5 with glacial acetic acid, and the entire solution was applied to a column (2 cm x 52 cm) of Bio-Gel P-2 which had previously been equilibrated with 0.1 M acetic acid containing 1 mM EDTA. The column was developed with the same solvent, fractions of 7 ml were collected, and the absorbance at 280 mμ was measured.

The reduced cobrotoxin emerged in the void volume as a narrow peak in fractions 9-11, but the odor of β-mercaptoethanol was first detected in fraction 13; the remaining reagents were eluted in

fractions 18-22. The reduced cobretoxin was extremely stable at pH 3 in 0.1 M acetic acid containing 1 mM EDTA: it could be stored in a refrigerator for several days without decrease of the sulfhydryl content.

d. Reoxidation of reduced cobrotoxin

An aliquot of reduced cobretoxin in 0.1 M acetic acid was applied to a Bio-Gel P-2 column (2 cm x 52 cm), pre-equilibrated with 0.05 M phosphate buffer (pH 7.1) containing 0.1 M NaCl, and eluted with the same buffer. Reduced cobrotoxin, now in phosphate buffer pH 7.1, was allowed to stand, without shaking or bubbling, in a tube open to the air at room temperature (25-27°). After suitable intervals of time, aliquots were taken for determination of lethality and free sulfhydryl groups.

e. Ring test

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

f. Infrared absorption spectra

Infrared spectra of cobrotoxin preparations were recorded with the Perkin-Elmer Model 137 infrared spectrophotometer with a spectral slit width of 25 mm. The infrared pellets were prepared from 1 mg of cobrotoxin mixed with 300 mg of KBr.

g. Lethality

Venom lethality was measured by intraperitoneal injection of the venom solution into mice, N.I.H. strain, as previously described (12). In the mice weighing 18 ± 1 g, the L.D.50 of the cobrotoxin was approximately 0.065 mg/kg body weight.

3. Results

a. Sulfur distribution of cobrotoxin

The total content of half-cystine plus cysteine of cobrotoxin was measured by the disulfide interchange reaction (9), with the results shown in Fig. 1. The number of groups capable of undergoing interchange with di-DNP-cystine in conc. KCl was 12.0.

No free sulfhydryl groups were detected by spectrophotometric titration with PCMB even after cobrotoxin was treated with 8 M urea. The results of spectrophotometric assays using N-ethylmaleimide (10) also showed that sulfhydryl groups were absent from cobrotoxin. The half-cystine content of 12 moles therefore

indicates the presence of 6 disulfide bonds in each molecule of cobrotoxin.

b. Reduction of cobrotoxin

In order to examine the possible relation of the disulfide bonds in cobrotoxin to its lethality, cobrotoxin was dissolved in 8 M urea solution, and a reducing agent, β-mercaptoethanol, was added to split the disulfide bonds. Cobrotoxin in 8 M urea solution retains full lethality even after exposure for 24 h. However, on addition of β-mercaptoethanol, the lethality decreased immediately, and after 1 h almost all the lethality was lost. The results reveal the essentiality of the intact disulfide bonds for the venom toxicity.

In the experiment described in METHODS, reduced cobrotoxin was separated from urea and reducing agent by passing it through a column of Bio-Gel P-2, equilibrated with 0.1 M acetic acid containing 1 mM EDTA (pH 3). The sulfhydryl content of the reduced cobrotoxin was then titrated spectrophotometrically with PCMB and N-ethylmaleimide, and was found to contain 11.6 and 11.8-12.0 residues per molecule respectively. The values show that reduction with β-mercaptoethanol was essentially complete under the conditions used.

c. Oxidation of reduced cobrotoxin

The lethality of cobrotoxin was lost almost completely after reductive cleavage of disulfide bonds in concentrated urea solution. However, the possibility of some accompanying side reaction was not excluded. If we could reform the disulfide linkages and reestablish the active conformation of cobrotoxin, i.e. restore the toxicity, by gentle oxidation, it would be certain that the loss of lethality of cobrotoxin by reduction is not due to any side-effects but to the specific cleavage of the disulfide bonds into sulfhydryl groups. Recently, reactivation of the reduced enzymes by oxidation with air has been successful for pancreatic ribonuclease (13,14), taka-amylase A (15), lysozyme (16-18), trypsin (19), and several other enzymes. Therefore, it is of interest to determine if the inert, fully-reduced cobrotoxin could regain lethality by oxidation with air.

Reduced cobrotoxin in 0.05 M phosphate buffer (pH 7.1) was allowed to stand in air, without shaking or bubbling. The results illustrated in Fig. 2 show a gradual increase in lethality during a 3-day period, which was accompanied by a decrease in sulfhydryl content. Conversion of sulfhydryl groups in reduced cobrotoxin to disulfide bonds proceeds rather slowly and linearly at pH 6.39 as shown in Fig. 3, and the rate becomes faster at high pH values.

The effect of protein concentration on the reactivation of reduced cobrotoxin was studied over a protein concentration

range of 0.11 to 2.5 mg/ml at pH 7.1. As seen from Table I, the reactivation proceeded gradually at almost the same rate between protein concentrations of 1.2 and 2.5 mg/ml, and the lethality was almost recovered after 3 day's exposure to air. However, the recovery of lethality was found much lower with decreases in protein concentration. This phenomenon was also observed with reduced ribonuclease T1 by Kasai (20). This may be due to the adsorption of the protein on the surface of the container.

As shown in Table II, a large increase in levorotation was observed on reduction of cobrotoxin. A similar change was also observed on oxidation of cobrotoxin with performic acid. These observations apparently reflect the destruction of the secondary structure. However, the specific rotation returned closely to the native value on oxidation.

The decreased cross-reaction with antisera to cobrotoxin of the reduced cobrotoxin also reverted to the native after reoxidation. Reduced and performic acid-oxidized cobrotoxins showed infrared spectra distinct from that of native cobrotoxin, while reoxidized cobrotoxin not only regained the complete lethality but also revealed a spectrum practically identical with that of native cobrotoxin.

4. Discussion

In the present studies, the disulfide bonds of cobrotoxin were cleaved completely with β-mercaptoethanol in the presence of 8 M urea. The sulfhydryl groups resulting were estimated to be 11.6-12.0 moles/molecule of cobrotoxin in accord with the value obtained from disulfide interchange reactions for the total half-cystine content of 12.0. However, no sulfhydryl group could be detected in the native cobrotoxin, even after cobrotoxin had been treated with 3 M urea. These facts indicate that cobrotoxin contains six disulfide bonds and is devoid of sulfhydryl groups. Since no methionine was found on amino acid analysis, it appears that the sulfur in cobrotoxin is present exclusively in the form of disulfide bonds.

The presence of disulfide bonds maintains the specific secondary structure of the toxin, and may also stabilize the structure against the effects of 8 M urea; it is significant that no loss of lethality was detected when the cobrotoxin was treated with this reagent. However, when the disulfide bonds are broken with a reducing agent, the characteristic secondary structure is lost, with a concurrent increase in levorotation, and the cobrotoxin is completely inactivated. Nevertheless, by gentle reoxidation in air, complete reactivation occurs with resumption of full lethality, indicating that correct reformation of disulfide bonds occurs.

During the course of the oxidation of reduced cobrotoxin, the lethality returned gradually while the sulfhydryl groups disapperred. However, the rate of restoration of the lethality did not proceed in parallel with the decrease in sulfhydryl groups: only 23 % returned after 72 % of sulfhydryl groups had disappeared (Fig. 4), but it returned more quickly thereafter. Complete lethality was regained when the protein concentrations were in an appropriate range. The results suggest that reformation of all the original disulfide bonds is necessary for the recovery of full lethality of cobrotoxin.

Micheel and Slotta studied the nature of sulfur in the active principle of snake venom, and found that the sulfur in the toxic proteins played an important role in the toxicity of venom. Slotta and coworkers (3,4) during their crotoxin studies suggested that the sulfur in all snake venoms occurs in simple disulfide bonds, and that the integrity of the disulfide bonds is essential for their toxic activities. However, Micheel and Schmitz detected no disulfide bonds in their neurotoxin obtained from cobra (Naia flava) venom.

The high sulfur content and the presence of cystine-form sulfur in Formosan cobra venom were also observed by Sato, Hirano and Takino (5) and Sasaki (6). However, no conclusive evidence for the nature of the sulfur in toxic protein has been provided so far, and their association with venom toxicity remains to be established. In this study, the direct evidence for the nature of the sulfur, presented exclusively in the form of disulfide bonds, is provided. The results also give firm support to the suggestion of Slotta that the integrity of the disulfide bonds is essential for the venom toxicity.

5. Conclusions

Cobrotoxin contains six disulfide bonds, and is devoid of sulfhydryl groups. On reduction with β-mercaptoethanol, cobrotoxin displays 11.6 to 12.0 sulfhydryl groups and loses its lethality concurrently. The inert, fully reduced cobrotoxin yields biologically active toxin on oxidation. The rate of oxidation and restoration of lethality is dependent upon the pH and the protein concentration. The cross-reaction with antisera to cobrotoxin, infrared spectrum, and levorotation are restored to their original values upon reoxidation of the reduced cobrotoxin. The results of the present study clearly indicate that the integrity of the disulfide bonds in cobrotoxin is essential for lethality.

II. Optical Rotatory Dispersion Study of Cobrotoxin

This section deals with the optical rotatory dispersion (ORD) study of the native, reduced and reoxidized cobrotoxin preparations in the wavelength range 220-300 mμ, and the discovery of a positive Cotton effect at 233 mμ for native and reoxidized cobrotoxin. The ORD of the cobrotoxin preparations and the circular dichroism (CD) measurements were performed with a Jasco spectropolarimeter model ORD/UV-5 with a circular dichroism attachment.

As shown in Fig. 5, the native cobrotoxin has a positive Cotton effect with peak at 233 mμ and the mean residue rotation corrected for the refractive index (21) of the solvent, 0.95 M sodium acetate buffer, pH 5.9, at 233 mμ ($[m']_{233}$) was found to be +1,250°. In general, the ORD curves of protein and polypeptide having right-handed α-helix have a negative trough at 233 mμ, and the left-handed α-helix of poly-D-glutamic acid shows a positive peak at 233 mμ. Therefore, it suggests that cobrotoxin contains left-handed α-helix in the molecule. In contrast, both the ORD curves of the reduced cobrotoxin and of the reduced-carboxymethylated (CM) cobrotoxin manifest simple dispersion curves with negative value of $[m']$ and do not show a negative trough at 233 mμ. The values of $[m']_{233}$ for these proteins are found to be -2,100° which is close to the value of -1,700° for randomly-coiled polypeptide chain consisting of L-amino acids (22). This indicates that cobrotoxin loses left-handed helix and become random structure after the reductive cleavage of its disulfide bonds. However, the reoxidized cobrotoxin gave essentially the same ORD curve as the native cobrotoxin. It indicates that correct reformation of left-handed α-helix occured by air-oxidation of the reduced protein. If we assume a value of $[m']_{233}$ = +15,000° for the left-handed α-helix (23) and -1,700° for the random coil of L-amino acid polypeptide, the value of +1,250° for cobrotoxin would mean that cobrotoxin contains about 22 % of the left-handed α-helix.

Native cobrotoxin reveals a negative CD spectrum with a minimum at 285 mμ, but not in reduced and CM cobrotoxin. Therefore, the CD band of native cobrotoxin in this region reflects asymmetry in the environment of aromatic amino acids in the native protein.

As seen in Fig. 5, the ORD curve of cobrotoxin in the presence of 7.5 M urea (pH 6.0) also reveals a positive but somewhat lower peak at 233 mμ. However, the CD spectrum at 285 mμ remained unchanged. The difference spectrum of cobrotoxin in 0.1 M sodium acetate buffer, pH 5.4, containing 8 M urea referred to the protein in the same buffer but without urea shows a red shift and have positive peaks at 285 and 292 mμ.

Fig. 6 shows the effect of 2-chloroethanol on the ORD curve of cobrotoxin. In the presence of 5 % 2-chloroethanol, the peak at 233 mμ is decreased and by 10 % 2-chloroethanol, the value of $[m']$ becomes negative, however, a maximum is still observed at 233 mμ. In the presence of more than 20 % of the reagent, the ORD becomes simple dispersion curve and the value of $[m']_{233}$ was found to be -1,900°. This indicates that the helical structure in the cobrotoxin molecule destroyed in the presence of more than 20 % of 2-chloroethanol. In this case, the negative CD spectrum due to the aromatic amino acid residues at 285 mμ was not abolished but only diminished slightly. This is the difference between the effect of 2-chloroethanol to cobrotoxin and the reductive cleavage of the disulfide bonds in cobrotoxin with β-mercaptoethanol. When 2-chloroethanol was added to CM-cobrotoxin to 50 % or 75 %, a negative trough appeared at 233 mμ. . . . This shows that CM-cobrotoxin, which is devoid of disulfide bonds, forms right-handed helix in the presence of 2-chloroethanol. The same results are usually obtained for the most of proteins.

On the basis of these facts, it is strongly suggested that the cobrotoxin has a left-handed α-helical portions in the molecule. It is a very distinct feature for a natural protein consisting of L-amino acids to have a left-handed helical structure.

III. Studies on Fluorescent Cobrotoxin

1. Introduction

The crystalline toxin, cobrotoxin (7), was obtained from Formosan cobra venom and was proved to be the main toxic protein in the venom. The anti-cobrotoxin sera were also prepared by injecting increasing doses of cobrotoxin with Freund's adjuvant into rabbits. The antibody was isolated and purified by ammonium sulfate precipitation, gel filtration and subsequent specific precipitation.

The present study was initiated to investigate the properties of fluorescent cobrotoxin after fluorescein thiocarbamylation, and applied the immunofluorescent procedure to define and determine quantitatively any residual antigen that might be adherent to the supposedly purified antibody.

2. Materials and Methods

a. Labelling of cobrotoxin. Two μmoles of

cobrotoxin were allowed to react with a varying molar ratio of fluorescein isothiocyanate (FITC, Nutritional Biochemical Corporation). The latter was initially dissolved in acetone and allowed to react with the toxin in 0.5 M carbonate buffer (pH 9.0) at 4°, under continuous stirring for 4 h. Complete separation of fluorescent cobrotoxin from unreacted fluorescein was achieved on a column of Bio-Gel P-2 (2 x 49 cm) in 0.005 M phosphate-0.1 M NaCl buffer, pH 7.2.

b. Immunization. Rabbits, weighing 2 to 2.2 kg, were immunized with cobrotoxin in complete Freund's adjuvant. The increasing doses, from 6 µg to 1.6 mg/kg body weight, were injected subcutaneously at the dorsal region over 6 day intervals for 3 months. Ten days after the last injection, the animals were bled. r-Globulin was prepared from pooled immune sera by two precipitations with decreasing concentration, 40 % and 33 % saturation, of $(NH_4)_2SO_4$, followed by gel filtration through a column of Sephadex G-200 in 0.02 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. The product showed only one band, characteristic of r-globulin, when tested by paper electrophoresis. 6.48 % of the protein was precipitable by an optimal amount of cobrotoxin.

c. Separation of fluorescent antigen from anti-cobrotoxin antibody. r-Globulin of anti-cobrotoxin was allowed to react with fluorescent cobrotoxin at the predetermined equivalence point. Precipitates were washed 3 times with cold 0.15 M NaCl, dissolved in minimum amount of 0.53 M formic acid-0.15 M NaCl, and applied to a Sephadex G-100 column (2 x 47 cm) equilibrated with 0.53 M formic acid-0.15 N NaCl. The column was developed with the same solvent and each 5 ml fraction was collected at a rate of 18 ml/h. Protein concentration was determined by Folin method (24) and fluorescent intensity was measured in an Aminco-Bowman spectrophotofluorometer, employing an exciting wave length of 488 mµ and an emission wave length of 520 mµ, after neutralization of the effluent fractions. The fluorescent cobrotoxin preparation used in this experiment (which was allowed to react with 4-fold molar excess of FITC) could be detected at levels of 0.01 µg/ml.

d. Precipitin reaction. The quantitative precipitin reactions were performed essentially according to the procedure of Heidelberger and Kendall as described by Kabat and Mayer (25). Increasing amounts of antigen were added to a constant amount of antibody in a total volume of 1.0 ml 0.02 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. The tubes were incubated for 30 min at 37° and overnight in the refrigerator. The precipitates were washed 3 times with 0.5 ml of cold 0.15 M NaCl, after which they were dissolved in 2.0 ml of 0.02 N NaOH and a suitable aliquot was taken for the determination of protein by Folin method.

Paper electrophoresis was carried out according to the procedure for serum protein with Spinco Model R Paper Electrophoresis Cell. After fixation and color development, electropherogram was taken by Spinco Model RB Analytel with filter 500 mμ. Fluorescent band on paper strip was observed under Long wave ultraviolet detector, Model SL 3660, Ultraviolet Products Inc., South Pasadena, Calif.

The spectrophotometric measurements were made either with Beckman Model DU or Model DK2 recording spectrophotometer. Lethality measurements and ring tests were performed as previously described (12,26).

3. Results and Discussion

Cobrotoxin was fluoresceinated under the condition described in METHOD with varying molar ratio, from 0.5 to 16 of FITC. Spectrophotometric analysis at 495 mμ and protein content measurement indicated that as high as 3 to 4 moles of fluorescein reacted/molecule of cobrotoxin (Table III). Paper electrophoretic separation of fluorescent cobrotoxin from unlabelled cobrotoxin was obtained with veronal buffer, pH 8.6. Cobrotoxin migrated towards cathode while the fluorescent cobrotoxin did not migrate either to cathode or anode and remained on the point of application. As seen from Fig. 7, no free cobrotoxin left when it had been allowed to react with 4-fold molar excess of FITC. In all fluoresceinated preparations none of the free FITC were detected on paper electrophoresis, proved that complete separation of unreacted FITC was achieved by gel filtration on Bio-Gel P-2.

The UV absorption maximum of the fluorescent cobrotoxin is shifted to shorter wavelength from 277-278 mμ to 273-275 mμ. As shown in Table III, although the pronounced decrease in lethality was observed, the cross-reaction with antisera to cobrotoxin remains intact. It suggests that antigenic sites are different from the active site(s) of toxicity and the fluorescein thiocarbamylation of the toxic protein through free amino groups did not alter the integrity of antigenic specificity, by which the major toxic site(s) would be blocked with retaining only the residual lethality. Therefore, the fluorescent cobrotoxin might not only be used to detect the trace amounts of antigen which might remaining in combination with supposedly purified antibody, but also a very valuable tool for the production of antibody because of its low toxicity.

Fig. 8 shows the separation of fluorescent cobrotoxin from anti-cobrotoxin on Sephadex G-100 in 0.53 M formic acid. Complete separation of fluorescent antigen from anti-cobrotoxin was achieved under the conditions described. 97.0 % of the fluorescence was recovered in the antigen peak, while, fluorescence was barely detectable in the antibody peak.

The effluent fractions on antibody peak were pooled and dialyzed against cold 0.02 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl for 2 days. After removal of the small amount of precipitates which appeared, no fluorescence was detectable in the purified antibody preparation. The yield of antibody from γ-globulin fraction was 5.96 %.

As illustrated in Fig. 9, the purified antibody was 100 % precipitable with cobrotoxin as measured by quantitative precipitin reaction. Therefore, 0.53 M formic acid not only dissolved the immune precipitates at room temperature but also dissociated antigen-antibody complexes and elution from Sephadex G-100 with 0.53 M formic acid resulted in a complete separation of antigen from antibody.

Using the fluorescent tagging technique for quantitative determinations of the degree of separation of fluorescent antigen from its antibody, Bennett and Haber (27) found that great difficulty was encountered in the preparation of the antigen-free antibody when dealing with protein antigens. From the cases studied, they concluded that only when a structural change in antigen is produced, can complete dissociation be observed. Givol *et al.* (28) have separated egg white lysozyme from its antibody on Sephadex G-75 at pH 1.8. In this system 15 % of the antigen remained with the antibody and was nondissociable. Approximately 95 % of the contaminating antigen was then precipitated by adjusting the pH to 7.0 and antibody containing only 0.06 % antigen was recovered in 60 % yield. It is fortuitous, however, in the anti-cobrotoxin purification, total separation was achieved at room temperature in 0.53 M formic acid-0.15 M NaCl without alteration of native configuration of cobrotoxin. 100 % precipitable antibody was recovered in 92 % yield from γ-globulin fraction, which contained 6.48 % antibody, as shown in Fig. 9.

The importance of conformation in the reactivity of RNase with anti-RNase has been emphasized by Mills and Haber (29), who demonstrated that disulfide interchange, without other covalent alteration, would completely alter the antigenic identity. Oxidation of RNase with resultant molecular disruption has also been shown to abolish reactivity with anti-RNase (30). It was also demonstrated in our previous studies (26,31) that when the disulfide bonds in cobrotoxin are broken with a reducing agent, the characteristic secondary structure is lost with concurrent inactivation. However, by gentle reoxidation in air, complete reactivation occurs with resumption of full lethality and antigenicity, indicating that the integrity of the secondary structure is essential for the antigenic specificity.

4. Conclusions

Three to four moles of fluorescein were incorporated into each molecule of cobrotoxin when the toxin was allowed to react with more than 4-fold molar excess of fluorescein isothiocyanate. In this system none of the cobrotoxin remained unlabelled. The UV maximum of the fluorescent cobrotoxin is shifted to the shorter wavelength and the lethality decreased to 1/7.5, while the cross-reaction with anti-cobrotoxin sera was not altered. It suggests that in cobrotoxin the antigenic sites are different from the active site(s) of toxicity which was blocked by fluorescein thiocarbamylation.

Fluorescent cobrotoxin was used to define and determine the degree of separation of antigen from its antibody. The immune precipitates were completely dissolved and dissociated in 0.53 M formic acid-0.15 M NaCl; complete separation of fluorescent antigen from the antibody was achieved on Sephadex G-100 column in the same solvent. The purified antibody was proved to be free from faint traces of antigen and 100 % precipitable with cobrotoxin as measured by quantitative precipitin reaction.

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APPENDIX A-1

Table I

Effect of the concentration of reduced cobrotoxin on the rate and extent of reactivation

Reduced cobrotoxin (mg/ml)	% Regain of lethality after reoxidation				
	2 h	6 h	24 h	48 h	72 h
2.5	8.3	11.7	66.3	71.1	
1.8	7.1	12.8	47.6	80.1	95.3
1.2	7.7	14.0	66.3	78.9	
0.6	7.7	9.9		66.3	
0.4	5.1	8.4		53.4	67.4
0.16	7.0	8.9	33.2	35.6	
0.11	5.1	7.1		28.3	40.0

APPENDIX A-2

Table II

Relation of SH content, lethality, and optical rotation of cobrotoxin in various states

	SH Content moles/mole	Lethality %	$[\alpha]_D^{27}$
Native cobrotoxin	0	100	-3°
Reduced cobrotoxin	11.8	2.6	-82°
Reoxidized cobrotoxin	0	100	-7°
Performic acid oxidized cobrotoxin	0	0	-73°

APPENDIX A-3

Table III

Properties of fluorescent cobrotoxin

Molar ratio of FITC to cobrotoxin	0	0.5	1	2	4	8	16
Moles of fluorescein reacted/ molecule of cobrotoxin	0	0.75	1.85	2.59	3.13	3.74	3.73
Fluorescent cobrotoxin after electrophoresis (%)	0	52.9	76.4	89.3	100	100	100
UV absorption maximum (mμ)	277–278	275–278	273–276	273–275	273–275	273–275	273–275
Lethality (%)	100	100	53.6	42.0	13.4	13.4	13.4
Antigenicity (%)	100	100	100	100	100	100	100

APPENDIX B-1

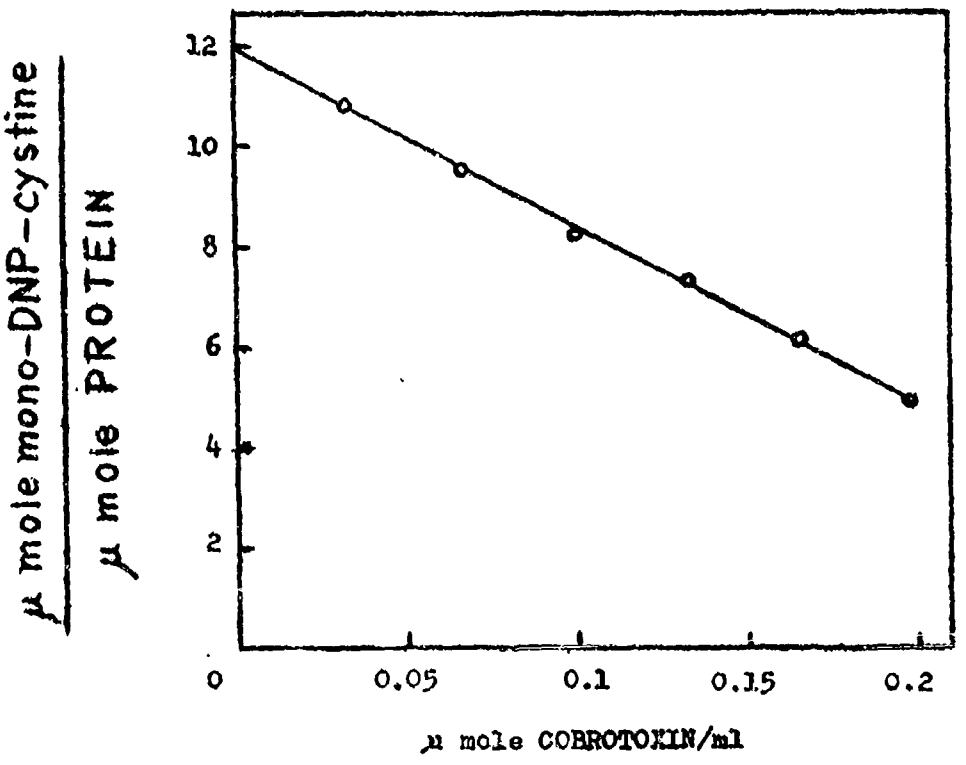

Fig. 1. Equilibrium values for the formation of mono-DNP-cystine in 9.6 N HCl at 39° as a function of cobrotoxin concentration.

APPENDIX B-2

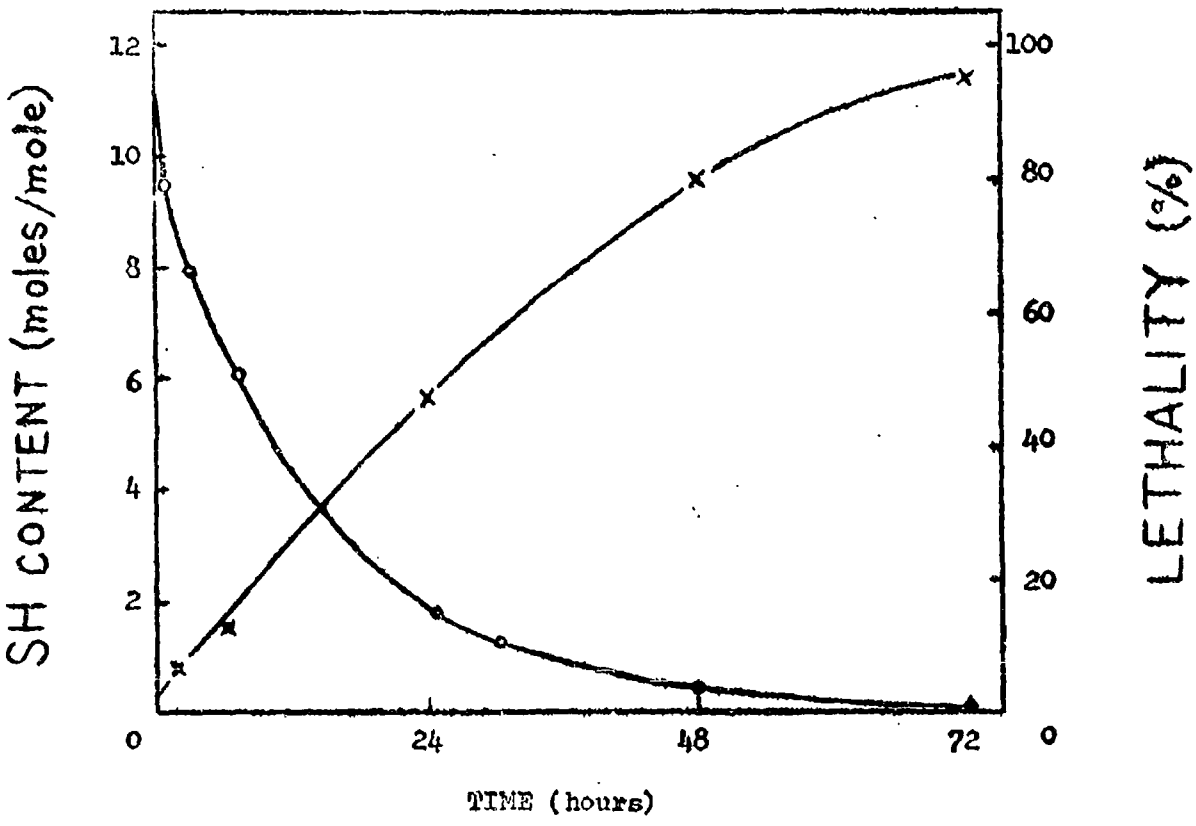

Fig. 2. Restoration of the lethality and disappearance of free sulfhydryl groups on oxidation of reduced cobrotoxin in air. The solution of reduced cobrotoxin (1.8 mg/ml) in 0.05 M phosphate buffer (pH 7.1) containing 0.1 M NaCl was stood at room temperature (25-27°). —•— SH groups; —X— lethality.

APPENDIX B-3

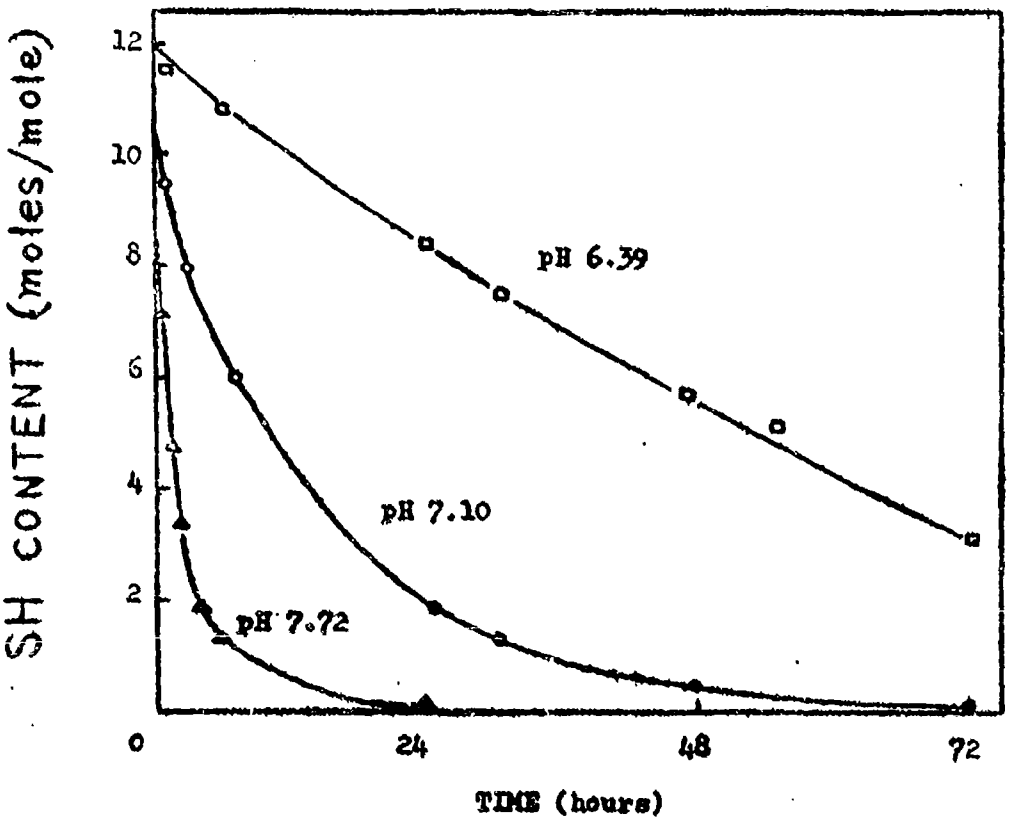

Fig. 3. Effect of pH on the reoxidation of reduced cobrotoxin. Reduced cobrotoxin was allowed to stand at room temperature in 0.05 M phosphate buffer containing 0.1 M NaCl at a protein concentration range of 1.2 to 2.5 mg/ml.

APPENDIX B-4

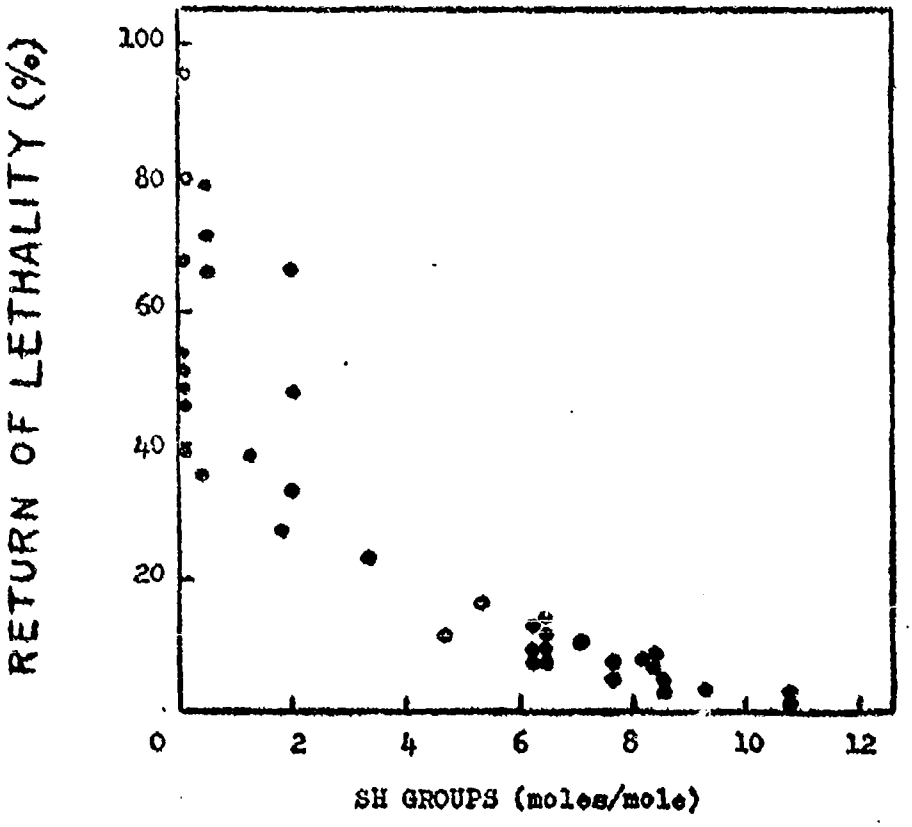

Fig. 4. The lethality of cobrotoxin at various stages of reoxidation.

APPENDIX B-5

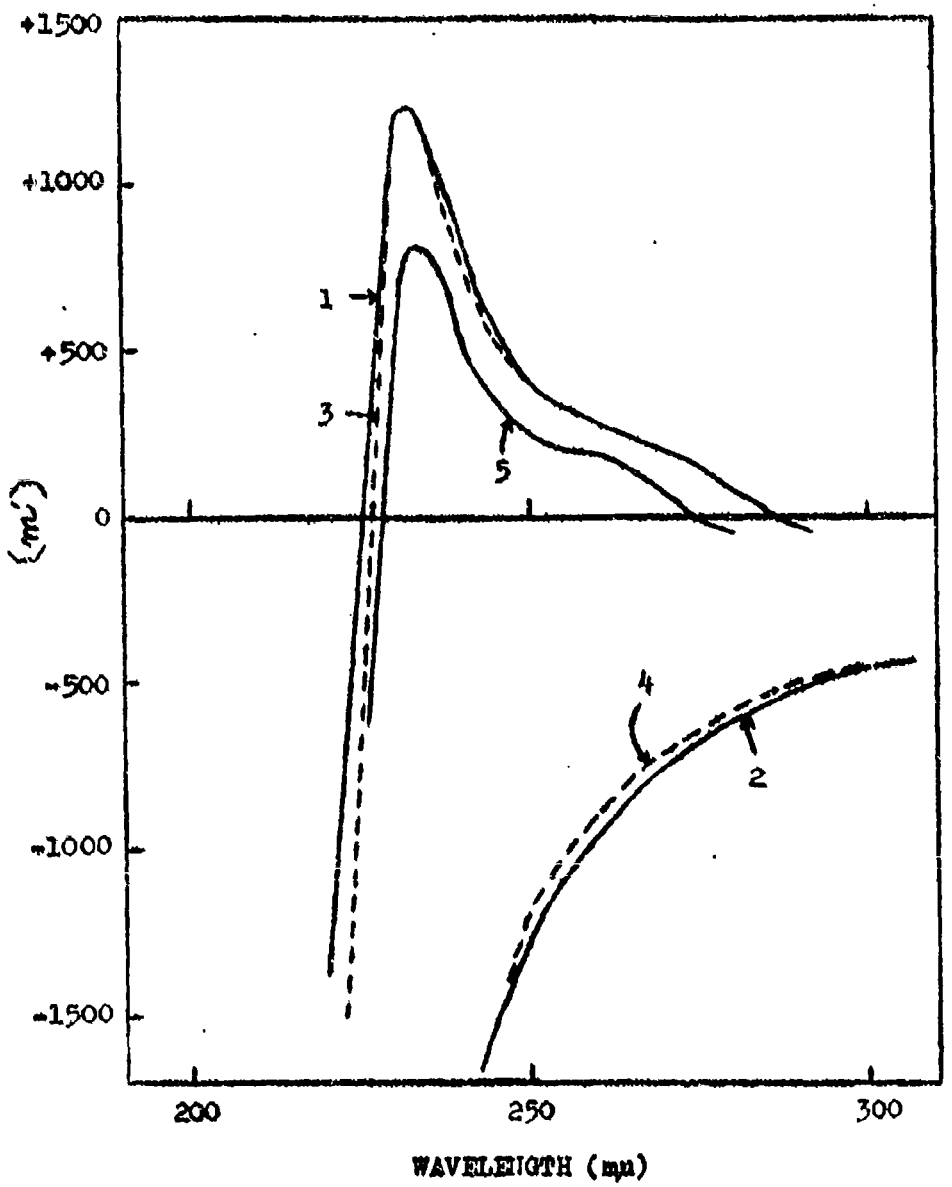

Fig. 5. Optical rotatory dispersion curves of native, reduced and reoxidized cobrotoxin preparations. Curve 1, native cobrotoxin; curve 2, reduced-carboxymethylated cobrotoxin and curve 3, reoxidized cobrotoxin in 0.05 M sodium acetate buffer, pH 5.9. Curve 4, reduced cobrotoxin in an aqueous solution and curve 5, cobrotoxin in the 0.05 M sodium acetate buffer containing 7.5 M urea.

APPENDIX B-6

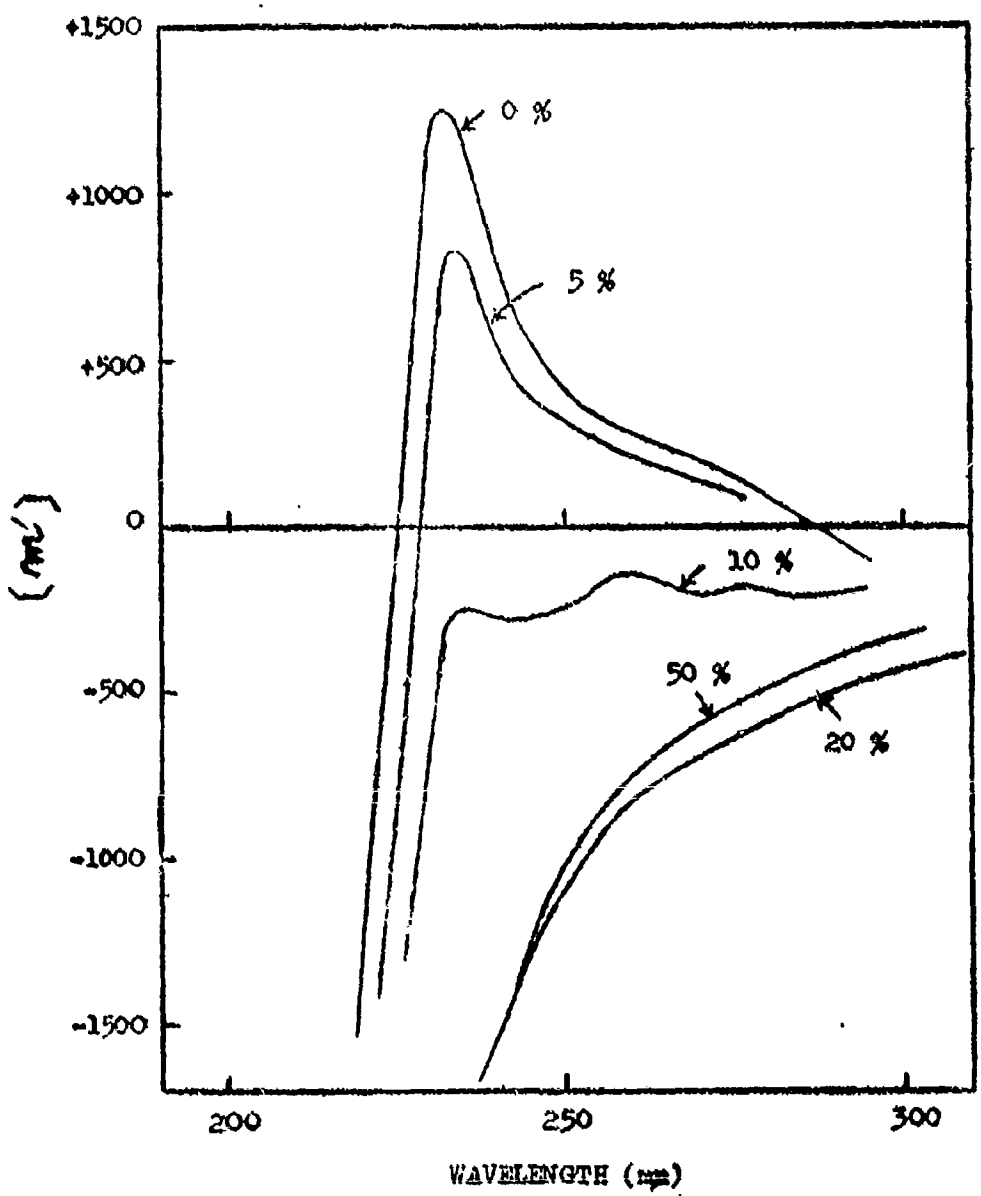

Fig. 6. Effect of 2-chloroethanol on the optical rotatory dispersion of cobrotoxin.

APPENDIX E-7

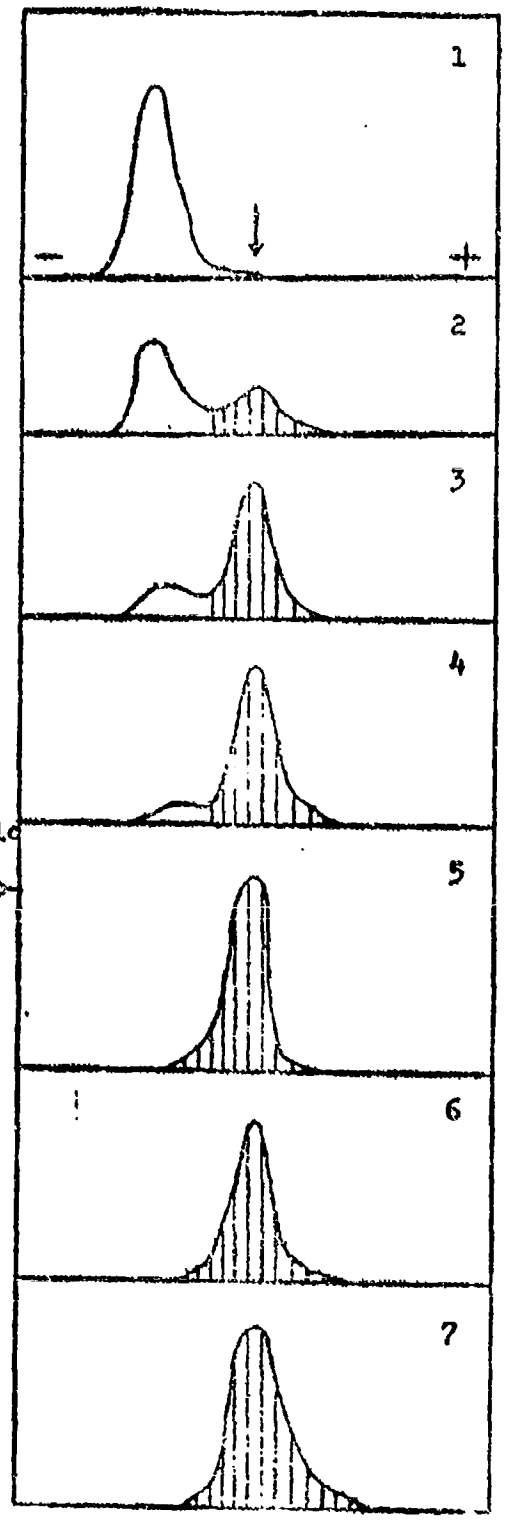

Fig. 7. Paper electrophoretic separation of fluorescent cobrotoxin from unlabelled cobrotoxin. Electrophoresis took place at room temperature in veronal buffer, pH 8.6, μ=0.075 for 16 h at 2.5 mA. The molar ratio of FITC to cobrotoxin in 1, 2, 3, 4, 5, 6 and 7 are 0, 0.5, 1, 2, 4, 8 and 16 respectively as indicated in Table III. The arrow represents the place of application and the hatched area denotes the place of fluorescence.

APPENDIX B-8

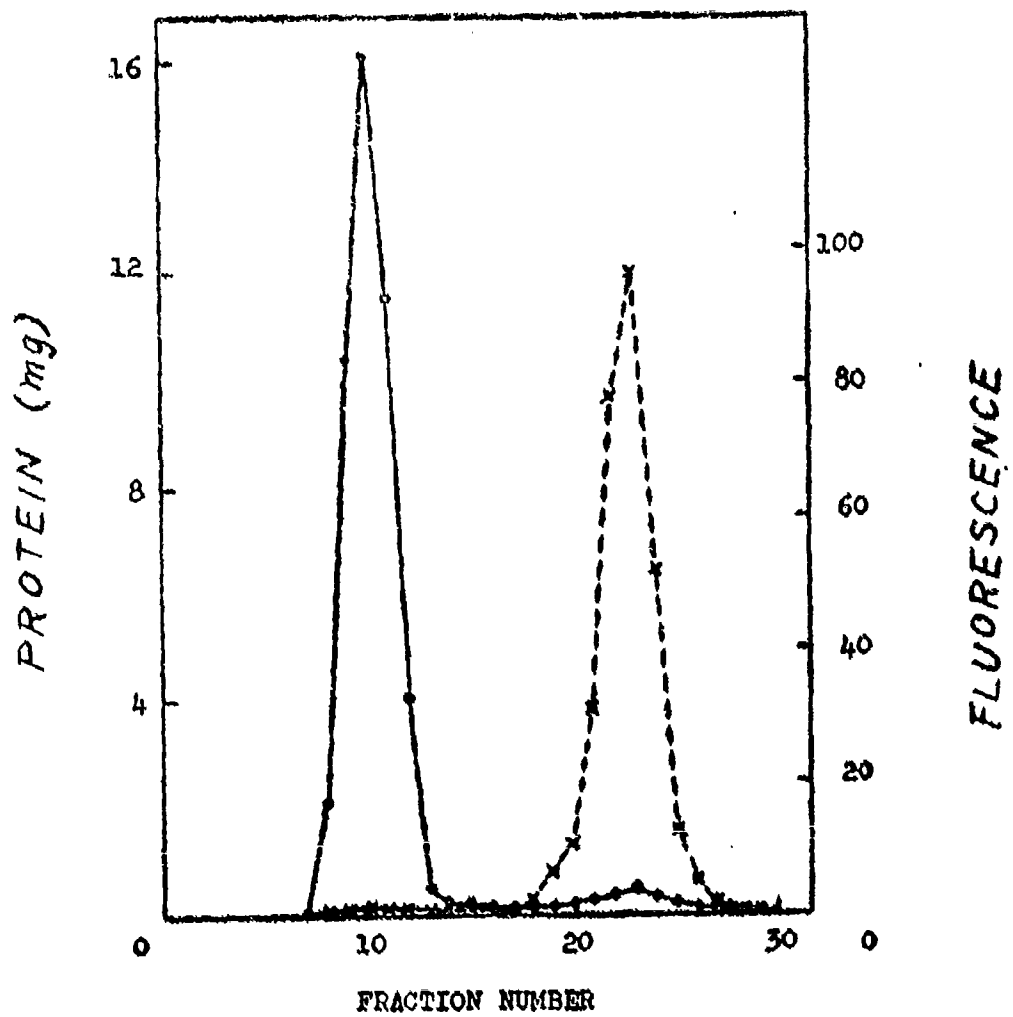

Fig. 8. Separation of fluorescent cobrotoxin from anti-cobrotoxin on Sephadex G-100 in 0.53 M formic acid. The antigen-antibody precipitates were dissolved in 0.6 ml of 0.53 M formic acid-0.15 M NaCl and eluted with the same solvent.

•——•, protein content
x--x, fluorescence in arbitrary units.

APPENDIX B-9

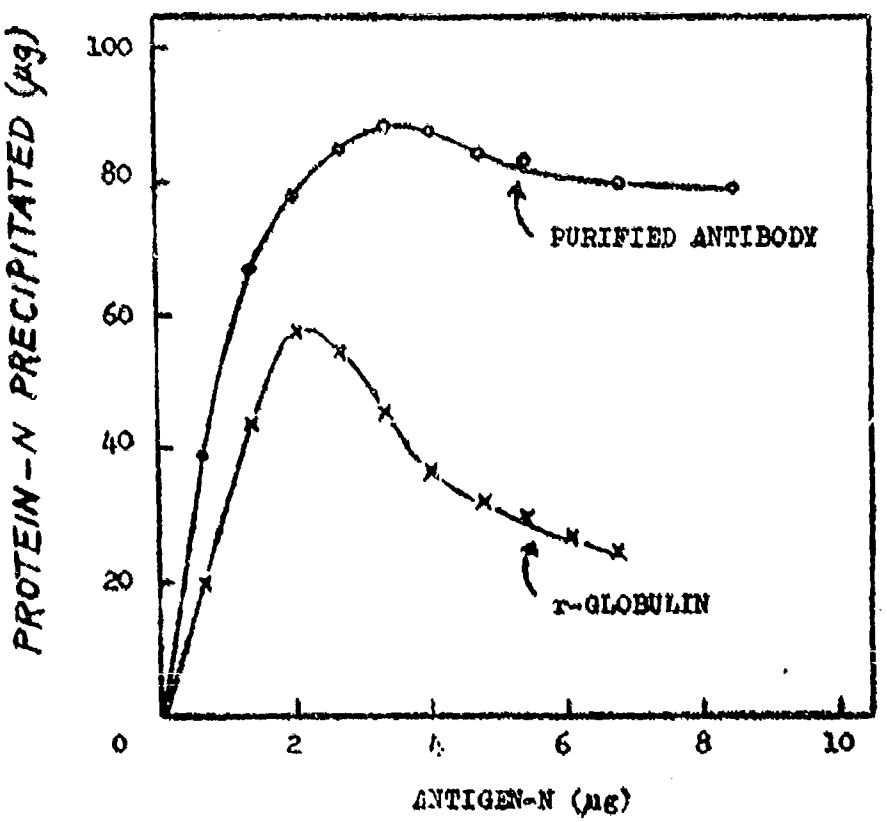

Fig. 9. Precipitin reactions of fluorescent cobrotoxin with r-globulin fraction from rabbit anti-cobrotoxin sera and of cobrotoxin with the purified antibody. r-Globulin-N (848 µg) and the purified antibody-N (84.5 µg) were used. As indicated in the text, the r-globulin fraction contained 6.48 % antibody and the purified antibody was 100 % precipitable.

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13. ABSTRACT

I. The Disulfide Bonds of Cobrotoxin and their Relationship to Lethality.

Clarification of the status of sulfur bonds in the biologically-active protein is very important not only for the establishment of structure but also for the elucidation of their relation to the biological activity. The total content of half cystine plus cysteine of cobrotoxin was measured to be 12.0 by disulfide interchange reactions. No sulfhydryl groups were detected by spectrophotometric titration with p-chloromercuribenzoate and N-ethylmaleimide even after cobrotoxin was treated with 8 M urea. Since no methionine was found on amino acid analysis, the sulfurs in cobrotoxin were proved to be exclusively in the form of disulfide bonds. On reduction with -mercaptoethanol, cobrotoxin displays 11.6 to 12.0 sulfhydryl groups and loses its lethality concurrently. However, the inert, fully reduced cobrotoxin yields biologically active product with complete lethality and antigenicity, specific rotation close to the native value, and an infrared spectrum identical to native cobrotoxin on reoxidation. The results clearly indicate that the integrity of the disulfide bonds in cobrotoxin is essential for lethality.

II. Optical Rotatory Dispersion study of Cobrotoxin

The present communication deals with the optical rotatory dispersion (ORD) study of the native, reduced and reoxidized cobrotoxin preparations in the wavelength range 220-300 mμ and the discovery of a positive Cotton effect at 233

(Cont'd)

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for the native cobrotoxin. The mean residue rotation corrected for the refractive index of the solvent at 233 mμ was found to be +1,250°, suggests that cobrotoxin contains about 22 % of the left-handed α-helix in the molecule. This is a very distinct feature for a natural protein consisting of L-amino acids to have a left-handed helical structure. In contrast, the ORD curve of the reduced cobrotoxin manifests simple dispersion curve with negative value of ($[m']$) 233 (-2,100°), indicates that cobrotoxin loses left-handed helix and becomes random structure after the reductive cleavage of its disulfide bonds. However, the reoxidised cobrotoxin gave essentially the same ORD curve as the native cobrotoxin, shows that correct reformation of left-handed α-helix occurred by air-oxidation of the reduced cobrotoxin. (Author)

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14. KEY WORDS	LINK A		LINK B		LINK C	
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