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(6) STRUCTURE AND FUNCTION OF COBROTOXIN.

by

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Both precipitating and non-precipitating antibodies proved to be similar with regard to molecular size and elution from cobrotoxin-Sepharose column. There was no evidence of physiochemically distinct type of immunoglobulin responsible for the non-precipitating character of antisera. Possible mechanisms for non-precipitability antibody were discussed.

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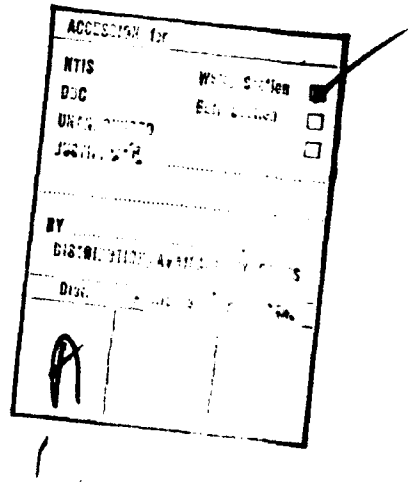

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We demonstrated that rabbits hyperimmunized with cobrotoxin in Freund's complete adjuvant do produce non-precipitating as well as precipitating antibodies. By affinity chromatography of supernatants obtained from precipitin reaction at the maximum precipitation on a column of cobrotoxin-Sepharose, the non-precipitating antibodies were separated from the antisera. Specific neutralizing capacity of the antibody preparations obtained from affinity chromatography was much higher than those isolated by precipitin reactions. The specific neutralizing capacity of the non-precipitating antibody and its papain fragment increased 23-fold and 27.6-fold over that of the antisera, respectively.

Both precipitating and non-precipitating antibodies were proved to be similar with regard to molecular size and elution appearing on cobrotoxin-Sepharose column. There was no evidence that a physicochemically distinct type of immunoglobulin was responsible for the non-precipitating character of antisera and the possible mechanisms for the non-precipitability of the non-precipitating antibody were discussed.

ABSTRACT

Purification of Anticobrotoxin Antibody by Affinity Chromatography

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. Chemical studeis show that cobrotoxin is a small, basic protein consisting of a single peptide chain of 62 amino acid residues, crosslinked intramolecularly by four disulfide bridges. The complete amino acid sequence and the positions of disulfide bonds in cobrotoxin have also been established.

Cobrotoxin was successfully immobilized on Sepharose through its free amino groups without altering the antigenic activity. Coupling reaction at pH 9.0 in 0.1 M sodium bicarbonate-0.5 M NaCl yielded the best affinity adsorbent for antibody. It was found that 0.53 M formic acid-0.15 M NaCl (pH 2.05) was very efficient for the elution of antibody from cobrotoxin-Sepharose. Modification of Arg-residues did not cause any change in the coupling capacity of cobrotoxin to Sepharose, however, the antigenic activity decreased pronouncedly when Arg-30 and Arg-36 were modified.

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Both precipitating and non-precipitating antibodies were proved to be similar with regard to molecular size and elution appearing on cobrotoxin-Sepharose column. There was no evidence that a physicochemically distinct type of immunoglobulin was responsible for the non-precipitating character of antisera and the possible mechanisms for the non-precipitability of the non-precipitating antibody were discussed.

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Purification of Anticobrotoxin Antibody by Affinity Chromatography

I. Introduction

Cobrotoxin, a neurotoxic crystalline protein, was isolated from the venom of Taiwan cobra (Naja naja atra) (Yang, 1965) and was proved to be the main toxic protein in cobra venom. Chemical studies show that cobrotoxin is a small, basic protein consisting of a single peptide chain of 62 amino acid residues, crosslinked intramolecularly by four disulfide bridges (Yang et al., 1969a). The complete amino acid sequence and the positions of disulfide bonds in cobrotoxin have also been established (Yang et al., 1969b, 1970).

It has been reported in our previous immunochemical studies on cobrotoxin (Chang and Yang, 1969) that 100 % precipitable antibody could be isolated immunospecifically by gel filtration on Sephadex G-100 after the antigen-antibody complex had been dissociated with 0.53 M formic acid-0.15 M NaCl, pH 2.05. The specific neutralizing capacity of the purified antibody was 17.5 greater than that of the antisera. Molecular weight of soluble complex formed from its papain fragments and cobrotoxin provides the evidence that cobrotoxin consists of three antibody-combining sites/molecule.

In the present study, we demonstrated that rabbits hyperimmunized with cobrotoxin in Freund's complete adjuvant do produce non-precipitating as well as precipitating antibodies. By affinity chromatography of supernatants obtained from precipitin reaction at the maximum precipitation on a column of cobrotoxin-Sepharose, the non-precipitating antibodies were isolated from the anticobrotoxin sera. The specific neutralizing capacity of the non-precipitating antibodies and its papain fragment increased 23-fold and 27.6-fold over that of the antisera, respectively. Both precipitating

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Abbreviations: CMC, 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate; EDC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl; TNBS, 2,4,6-trinitrobenzene sulfonate; HNB bromide, 2-hydroxy-5-nitrobenzyl bromide; Tris, tris-(hydroxymethyl)-aminomethane; TNP-trinitrophenyl; IgG, immunoglobulin G.

and non-precipitating antibodies were proved to be similar with regard to molecular size and elution appearing on cobrotoxin-Sepharose, and the possible mechanisms for the nonprecipitability of the non-precipitating antibody were discussed.

II. Materials and Methods

Cobrotoxin used in this study was prepared from Taiwan cobra (Naja naja atra) venom as previously described (Yang, 1965). CNBr-activated Sepharose 4B, CH-Sepharose 4B and AH-Sepharose 4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. 1-Cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMC), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl (EDC), Sephadex G-25, G-100 and G-200, and CM-cellulose were obtained from Sigma Chemical Company. 2,4,6-Trinitrobenzene sulfonate (TNBS) was obtained from Tokyo Kasei Co., O-methylisourea-HCl from Nutritional Biochemical Corp., phenylglyoxal hydrate and 2-hydroxy-5-nitrobenzyl bromide (HNB bromide) from Seikagaku Kogyo Co., Ltd. Mercuripapain was a product of Worthington Biochemical Corp., Freehold, N. J. All other reagents were of analytical grade.

1. Modification of Lys-residues in cobrotoxin with TNBS

Cobrotoxin was reacted with 1.1-fold and 2.2-fold molar excess of TNBS, respectively (Chang et al., 1971). The reaction was carried out in 0.1 M borate buffer (pH 8.6) at 25°C for 1 h. The mixture was passed through a column of Sephadex G-25 (2.5 x 40 cm), followed by ion exchange chromatography on CM-celluiose with a gradient of increasing salt concentration from 0.005 M to 0.5 M ammonium acetate, pH 5.8 to 6.8. The fractions of the main protein peak were lyophilized and desalted by passage through a column of Sephadex G-25 equilibrated with 1 % acetic acid. The protein fractions were then pooled and lyophilized.

When the toxin was reacted with 1.1-fold molar excess of TNBS, the ε-amino group of Lys-27 was selectively trinitrophenylated without altering the activity of the toxin. However, complete loss of the activity was observed when Lys-27 & 47 were modified with 2.2-fold molar excess of TNBS.

Guanidinated cobrotoxin was prepared by dissolving 3 μmoles of cobrotoxin in 2 ml of 0.5 M O-methylisourea-HCl solution which had been adjusted to pH 10.8 with 6 M NaOH. The reaction was allowed to proceed at 4°C for 72 h. The mixture was then passed through a column of Sephadex G-25, and the protein fractions were pooled and lyophilized.

TNP-guanidinated cobrotoxin was prepared by trinitrophenylation of the guanidinated cobrotoxin with 10-fold molar excess of TNBS in 0.1 M borate buffer (pH 8.6).

2. Modification of Arg-residues in cobrotoxin by phenylglyoxal

Selective and stepwise modification of Arg-residues with phenylglyoxal at varying pH was conducted essentially according to the method of Takahashi (1968) as previously described (Yang et al., 1974). Cobrotoxin (4 μmoles) was dissolved in 0.5 ml of appropriate 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 for 1 h. Buffer solutions used were: 0.1 M acetate, pH 6.0; 0.1 M phosphate, pH 6.7 or pH 7.5; 0.2 M N-ethyl morpholine acetate, pH 8.0. The mixture was passed through a column of Sephadex G-25, 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. The fractions of the main protein peak were lyophilized and desalted by passage through a column of Sephadex G-25 equilibrated with 1 % acetic acid. The protein fractions were then pooled and lyophilized.

Only Arg-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 Arg-residue at position 30 was modified at pH 7.5.

3. Modification of tryptophan residue in cobrotoxin with HNB bromide

The alkylation of cobrotoxin with HNB bromide was carried out under standard conditions (Barman and Koshland, Jr., 1967)

as previously described (Chang and Hayashi, 1969). Cobrotoxin (2 μmoles) was preincubated for 20 h at 37°C in 3 ml of 10 M urea which had been adjusted to pH 2.7 with concentrated HCl. After incubation, a 10-fold molar excess of HNB bromide in 0.3 ml of dry acetone was added with a pipette below the surface of the protein solution with vigorous agitation. After reaction for 1 h at room temperature (27°C) the modified toxin was separated from excess reagent on a column (2.5 x 40 cm) of Sephadex G-25 equilibrated with 0.18 M acetic acid (pH 2.7) and the protein fractions were pooled and lyophilized.

4. Antisera

Anticobrotoxin sera were prepared by injecting increasing doses of cobrotoxin with Freund's complete adjuvant into rabbits weighing 2.0 to 2.5 kg. From 6 μg to 1.6 mg/kg body weight were injected subcutaneously into the right and left thigh alternating at 6-day intervals during a period of 3 months and the rabbits were bled 9 days after the final injection.

Immunoglobulin G (IgG) was prepared from the pooled sera by precipitation with ammonium sulfate at a decreasing order of saturation, 40 % and 33 %, followed by gel filtration on a column of Sephadex G-200.

5. Isolation of purified precipitating antibody

IgG was allowed to react with cobrotoxin at the predetermined equivalence point. The resultant antigen-antibody precipitates were washed three times with cold 0.15 M NaCl, then dissolved in 1 ml of 0.53 M formic acid-0.15 M NaCl, pH 2.05, and applied to a column of Sephadex G-100 (2 x 47 cm) equilibrated with the same solvent. The column was developed with 0.53 M formic acid-0.15 M NaCl and 4 ml fractions were collected at a rate of 18 ml/h. The protein was determined by the procedure of Lowry et at. (1951) and the fractions containing the antibody were pooled and dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl.

6. Digestion and fragmentation of the purified antibody

Papain digestion of the purified antibody was carried out according to the procedure of Porter (1959). Purified

antibody (140 mg) in 10 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.01 M cysteine and 2 mM ethylenediaminetetraacetic acid (EDTA) was digested with 1.4 mg of mercuripapain at 37°C. After 16 h, the papain digest was dialyzed against water with constant stirring and several changes of the outer liquid over a period of 32 h, and then overnight against 0.01 M sodium acetate buffer, pH 5.5. The crystalline precipitates that formed during dialysis were removed by centrifugation and the hydrolysate was chromatographed on a column of CM-cellulose with a gradient of increasing salt concentration from 0.01 to 0.9 M sodium acetate, pH 5.5. The initial volume in the mixing chamber was 800 ml and fractions of 5 ml were collected at a rate of 22 ml/h.

7. Precipitin reaction

The quantitative precipitin reactions were carried out as described by Kabat and Mayer (1961). Increasing amounts of cobrotoxin in 0.02 M Tris-HCl buffer (pH 7.5)-0.15 M NaCl were added to a constant amount of antibody solution in a total volume of 1 ml. The reaction mixtures were incubated for 30 min at 37°C and then placed at 4°C overnight. The precipitates were washed 3 times with 0.5 ml of cold 0.15 M NaCl, dissolved in 3 ml of 0.1 M NaOH and the absorbance was measured at 280 nm. The amount of antibody precipitated was calculated at the point of maximum precipitation and the extinction coefficient, $E^{1\%}_{1cm}$, was taken as 14.

8. Measurement of inhibitory activity of antibody fragments and non-precipitating antibody to homologous precipitin reactions

Increasing amounts of non-precipitating antibody or its papain fragment were added to the constant amount of cobrotoxin in 0.1 ml of 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. After incubation at 37°C for 30 min, the reaction mixture was placed at 4°C for 5 h. The equivalent amount of purified antibody or IgG was then added and the precipitates formed after incubation at 37°C for 30 min, then at 4°C overnight, were determined by the method as described in the above paragraph.

9. Measurement of neutralizing capacity of antibodies

Neutralizing capacity of antibody preparations was measured by the method of LD_{50} neutralizing dose described by Lin (1962) with a slight modification. Thirty LD_{50} of cobrotoxin/ml in 0.15 M NaCl solution was mixed with equal volume of serial two-fold dilutions of antibody. After incubation for 1 h at 37°C and then place at 4°C for 2 h, the precipitates were removed by centrifugation and the supernatant solutions were injected intraperitoneally into mice, NIH strain weighing 18 ± 1 gm, in the amount of 0.2 ml/mouse. Four mice of both sexes were used for each dilution and the LD_{50} was calculated according to the 50 % end-point method of Reed and Muench (1938). The specific neutralizing capacity was expressed as the number of LD_{50} of cobrotoxin neutralized by 1 mg of antibody.

10. Coupling of cobrotoxin and its modified derivatives to CNBr-activated Sepharose 4B

A covalent coupling of cobrotoxin and its modified derivatives to CNBr-activated Sepharose 4B was carried out essentially according to the method described by Cuatrecasas and Anfinsen (Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971a, b).

The freeze-dried powder of CNBr-activated Sepharose 4B was washed and swollen in 1 mM HCl solution on a glass filter and was divided into several portions. Each portion was washed repeatedly with the buffer solution used for coupling. The buffers used were: sodium phosphate, pH 7.5; sodium bicarbonate, pH 8.3 or pH 9.0; sodium carbonate, pH 10.0. The buffer concentration was 0.1 M and each contains 0.5 M NaCl.

Cobrotoxin (6 mg) in 3 ml of coupling buffer was mixed to a 3 ml bed volume of the washed gel suspension. The mixture was stirred gently for 1 h at room temperature and left overnight at 4°C. After coupling is complete the traces of non-covalently adsorbed toxin was washed away with coupling buffer on a glass filter. The amount of protein coupled on CNBr-activated Sepharose 4B was estimated by determination of the protein in washes by the method of Lowry et al. (1951), using cobrotoxin as standard, except for TNP- and HNB-cobrotoxin which were measured spectrophotometrically at 345 nm and 410 nm, respectively (Chang et al., 1971; Chang and Yang, 1973).

The residual active groups on the gel were blocked by

treatment with 50 ml of 1 M ethanolamine at pH 8.0 for 5 h at 4°C. The excess blocking reagent was washed away with coupling buffer followed by 0.1 M acetate buffer (pH 4.0)-0.5 M NaCl and coupling buffer. The toxin-Sepharose conjugate is now ready for use.

11. Affinity chromatography of antibody preparations on a column of cobrotoxin-Sepharose

An aliquot of the cobrotoxin-Sepharose gel suspension (3 ml bed volume) was poured into a small column (1.0 x 15 cm) and equilibrated with 0.1 M $NaHCO_3$ buffer (pH 8.3)-0.5 M NaCl. Antibody preparation was applied on the column by passage through the column three times. The column was washed with 50 ml of 0.1 M $NaHCO_3$ buffer (pH 8.3)-0.5 M NaCl and 80 ml of 0.01 M acetate buffer-0.5 M NaCl (pH 5.5). The antibody was then eluted with 0.53 M formic acid-0.15 M NaCl (pH 2.05) and the effluents were neutralized immediately with 1.0 M glycine-NaOH buffer (pH 11.5). The protein fractions were pooled and dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl at cold and the precipitates formed were removed by centrifugation. The protein concentration was measured spectrophotometrically at 280 nm, and the extinction coefficient, $E^{1\%}_{1cm}$, was taken as 14.0 for antibody and 13.5 for papain fragment of the purified antibody. The antigenic activity of cobrotoxin and its modified derivatives after coupling to Sepharose was measured by the recovery of added antibody.

12. Separation of precipitating and non-precipitating antibodies

Small amounts of cobrotoxin (approximately 1/10 of the quantity required for maximal precipitation) were added to the antisera successively. After each addition the mixtures were incubated for 1 h at 37°C and at 4°C for 1 h before centrifugation. The precipitates were collected, pooled and used to prepare the precipitating antibody. The supernatant, obtained after the addition of cobrotoxin had produced no more precipitation, was used to purify the non-precipitating antibody. The supernatants were passed through a column of cobrotoxin-Sepharose repeatedly for three times. The antibodies bound were eluted with 0.53 M formic acid-0.15 M NaCl (pH 2.05) after the column had been washed with 80 ml of 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M

NaCl and 70 ml of 0.1 M acetate buffer-0.5 M NaCl (pH 4.0). The effluents were neutralized immediately with 1.0 M glycine-NaOH buffer (pH 11.5) and the fractions containing antibody were pooled and dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl.

13. Coupling of cobrotoxin to CH-Sepharose 4B and AH-Sepharose 4B

One g of freeze-dried CH-Sepharose 4B was swollen in 0.5 M NaCl, then washed with water to remove NaCl. Cobrotoxin (8 mg) dissolved in distilled water (2 ml) was added and the pH was adjusted to 4.5 with 0.1 M NaOH. The coupling reaction was initiated by the addition of soluble carbodiimide (CMC or EDC, 40 mg to 60 mg) dissolved in 2 ml of H_2O and the mixture was shaken overnight. The pH of the mixture was maintained at 4.5-5.0 with 0.1 M NaOH throughout the reaction. After wash away the excess ligand and reagents, the gel suspension was poured into a small column (1.0 x 15 cm) and equilibrated with 0.1 M $NaHCO_3$ buffer (pH 8.3)-0.5 M NaCl. The procedure used for a covalent coupling of cobrotoxin to AH-Sepharose 4B was the same as those for CH-Sepharose 4B.

III. Results

1. Immobilization of cobrotoxin on Sepharose for affinity chromatography of antibody

Cobrotoxin was successfully immobilized on Sepharose. The effect of pH on the covalent coupling of cobrotoxin to CNBr-activated Sepharose 4B, and the antigenic activity of cobrotoxin after coupling are shown in Table I. Almost complete coupling was achieved at pH 7.5-9.0 in the toxin concentration of 2 mg/ml of Sepharose.

In affinity chromatography of antibody preparations on the column of cobrotoxin-Sepharose, the antibody bound to cobrotoxin-Sepharose was eluted at low pH. In the preliminary experiments, 0.53 M formic acid-0.15 M NaCl (pH 2.05) and 0.2 M glycine-HCl buffer containing 0.5 M NaCl (pH 2.8 and 2.05) were used for elution of the bound antibody. It was found that 0.53 M formic acid-0.15 M NaCl (pH 2.05) was the most efficient and reproducible for the elution of

antibody from cobrotoxin-Sepharose.

The antigenic activity of cobrotoxin after coupling to Sepharose at various pH was measured from the recovery of the purified antibody added. As indicated in Table I, the antigenic activity of cobrotoxin was not altered very much after immobilization at pH 7.5-10.0 on Sepharose, however, it seems that coupling reaction at pH 9.0 in 0.1 M $NaHCO_3$-0.5 M NaCl yields the best affinity adsorbent for antibody.

The maximum amount of cobrotoxin which could be covalently coupled to each ml of the activated Sepharose gel was determined. As shown in Table II, although the amount of cobrotoxin coupled increased with increasing toxin concentration from 2 mg to 6 mg, the coupling capacity, on the other hand, decreased progressively. This indicates that the coupling was less efficient at higher toxin concentration. However, there is no great difference in the antigenic activity of the coupled cobrotoxin when various concentration of cobrotoxin was used for coupling reaction.

2. Separation of antibody by affinity chromatogrphy on cobrotoxin-Sepharose column

When antibody preparations were applied to a column of cobrotoxin-Sepharose, more than twice large the amounts of antibody than those determined by precipitin reaction were always recovered from anticobrotoxin sera and IgG (Table III). This fact indicates that both antisera and IgG preparations contain non-precipitating antibody which could not be detected by quantitative precipitin reaction besides the precipitating antibody.

The heterogeneity of anticobrotoxin antibody was demonstrated by stepwise elution of the antibodies adsorbed on a column of cobrotoxin-Sepharose with several different pH buffer solutions. As illustrated in Fig. 1A, although the major fraction of antibody in anticobrotoxin IgG was eluted at pH 2.05, 0.53 M formic acid-0.15 M NaCl, at least three more populations of antibodies could be separated by acetate buffer-0.5 M NaCl at pH 5.5, pH 4.0 and pH 3.5. The result indicates that several populations of antibody, differing in their affinity to cobrotoxin, are present in the anticobrotoxin IgG preparation. It is also true in both purified precipitating and non-precipitating antibodies, as illustrated in

Fig. 1B. Both purified antibody preparations after being bound to cobrotoxin-Sepharose could be eluted stepwise with the proportions compatible with that of anticobrotoxin IgG (Table IV).

3. Isolation of non-precipitating antibody

For the isolation of the non-precipitating antibody, the precipitating antibody in anticobrotoxin sera or IgG was first removed by precipitation with the serial addition of cobrotoxin until no more precipitate formed. Then the non-precipitating antibody in supernatant solution was separated by affinity chromatography on a column of cobrotoxin-Sepharose. As shown in Table V, the total antibody content in the hyperimmunized anticobrotoxin sera was high, 4.55 mg/ml, and non-precipitating antibody amounted to 57.9 % of the total.

The binding capacities of the non-precipitating antibody and its papain fragment to immobilized cobrotoxin were compared with those of the precipitating antibody preparations. As can be seen from Table III, the recoveries of the non-precipitating antibody and its papain fragment were almost the same order of magnitude as those of the precipitating antibody and its papain fragment, indicating that there are no differences in binding affinity to cobrotoxin-Sepharose between precipitating and non-precipitating antibodies.

Antibody activity of the non-precipitating antibody and its papain fragment was determined by their inhibitory activity on the homologous precipitin reactions of cobrotoxin with IgG or with purified precipitating antibody. As illustrated in Fig. 2, complete inhibition of precipitation was achieved by FI of non-precipitating antibody. Although non-precipitating antibody that was able to be co-precipitated to render otherwise soluble complexes insoluble did not inhibit the formation of insoluble complexes by precipitating antibody in the zone of antigen excess, almost complete inhibition was also achieved by non-precipitating antibody in the zone of antibody excess (Fig. 3). Approximately 1.0 mole of FI, or 0.91 mole of the non-precipitating antibody/molecule of cobrotoxin gave 50 % inhibition, while for FI of the purified precipitating antibody about 1.2 moles were required for the same degree of inhibition (Chang and Yang, 1969). These results provide the evidence that the antibody activity of the non-precipitating antibody and its

papain fragment seems to be more active than that of the precipitating antibody and its papain fragment.

4. Specific neutralizing capacity of antibody preparations

As can be seen from Table VI, although the specific neutralizing capacity of the purified antibody isolated by precipitin reaction increased 18.1-fold over that of the antisera, even higher capacity was found for both of the purified antibody and non-precipitating antibody separated by affinity chromatogrphy on a column of cobrotoxin-Sepharose. The specific neutralizing capacity of papain fragment (FI) of the non-precipitating antibody was also higher than that of FI of the purified antibody-1 isolated by precipitin reaction. These results indicate that the specific neutralizing capacity of the antibody preparations obtained from affinity chromatography was superior to those obtained from classical precipitin reactions.

5. Gel filtration of antibody preparations

The gel filtration patterns of non-precipitating antibody and its papain fragment on Sephadex G-200 were compared with those of precipitating antibody. As shown in Fig. 4, the non-precipitating antibody and its papain fragment emerged at the corresponding effluent volume as those of the precipitating antibody and its papain fragment, showing that the molecular size of precipitating and non-precipitating was similar.

6. Effects of chemical modification on the coupling capacity and antigenic activity of cobrotoxin

As shown in Table VII, the coupling capacity of cobrotoxin to CNBr-activated Sepharose 4B was not affected very much by chemical modification. Only about 10 to 15 % decrease in coupling capacity was noticed when the free amino groups were trinitrophenylated or guanidinated and the only one Trp-residue at position 29 was alkylated. The Arg-modified derivatives and the Tyr-nitrated toxins retained essentially the full coupling capacity to Sepharose as native toxin.

Antigenic activity of the modified toxins after coupling to Sepharose was measured by the recovery of the purified antibody applied on the column of toxin-Sepharose (Table VIII).

Although the modification of Arg-residues did not affect the coupling capacity of the toxin to Sepharose (Table VII), the antigenic activity decreased progressively as the modification proceed. Antigenic activity decreased to about 40 % when the 3rd Arg-residue at position 30, besides Arg-28 and Arg-33, was modified and even more pronounced decrease was noticed when an additional Arg-residue at position 36 was modified. These results are compatible with our previous finding on the status of Arg-residues in cobrotoxin (Yang et al., 1974) that Arg-30 and Arg-36 are more closely related to the antigenic specificity of the toxin. As can be seen from Table VIII, besides the Arg-modified toxins the only decrease in antigenic activity was observed in Lys-27 & 47 TNP-cobrotoxin and TNP-guanidinated cobrotoxin which decreased 17.9 % and 14.3 %, respectively.

7. Affinity chromatography on Sepharose derivatives

CH-Sepharose 4B permits the synthesis of specific adsorbents with the ligands separated from the matrix by a six-carbon long spacer group, 6-amino hexanoic acid. In this way it could minimize the steric hindrance to the protein-ligand binding process due to the matrix if there is any. However, as shown in Table IX, the coupling capacity of cobrotoxin to CH-Sepharose 4B was less effective and the antigenic activity after coupling was even worse as compared with CNBr-activated Sepharose 4B.

AH-Sepharose 4B with spacer group, 1,6-diaminohexane, has free primary amino groups available for the covalent coupling of ligands containing carboxyl groups. Therefore, cobrotoxin which has seven free carboxyl groups was coupled to AH-Sepharose 4B by a simple one-step carbodiimide coupling reaction. As can be seen in Table IX, the coupling capacity was only 10 % and the recovery of the added antibody was also very low. The results suggest that coupling of cobrotoxin to Sepharose through its free amino groups is much more efficient than through its free carboxyl groups.

IV. Discussion

Rabbits hyperimmunized with cobrotoxin in Freund's complete adjuvant do produce non-precipitating as well as precipitating antibodies and the non-precipitating antibody amounts to 57.9 %

of the total antibody. Antibody separated by a single step of affinity chromatography on a column of cobrotoxin-Sepharose is even more superior to that of the precipitating antibody purified by immune precipitin reaction with regard to their neutralizing capacity. Both populations of antibody were similar with regard to elution appearing on cobrotoxin-Sepharose and molecular size. There was no evidence that a physicochemically distinct type of immunoglobulin was responsible for the non-precipitating character of antisera.

The specific neutralizing capacity of the non-precipitating antibody and its papain fragment increased 23-fold and 27.6-fold over that of the antisera, respectively. This predicts a substantial improvement in the therapy of victims of snake bites. On the other hand, non-precipitating antibody and its papain fragment do not form insoluble aggregates with homologous antigen but form soluble antigen-antibody complexes. This may also provide valuable tools for an immunochemical approach to the elucidation of the toxic nature of snake venom.

The existence of non-precipitating antibodies has been demonstrated in many animal species since the first original observations of Heidelberger and Kendall (1935). They found that the repeated addition of small quantities of antigen to rabbit anti-egg albumin until no more precipitate formed yielded only 78 % of the antibody detected when an optimal amount of antigen was added in one step. The missing antibody was considered to be non-precipitating antibody and it was capable of being coprecipitated by the addition of fresh antiserum and antigen.

Different hypotheses have been proposed to explain why these antibodies fail to precipitate with the specific antigen, although they combine with it and co-precipitate in the presence of precipitating antibody and antigen. These hypotheses include univalency, limited recognition of antigenic determinants on complex antigens, low affinity for the antigen and particular physicochemical properties.

The supernatant, obtained after the addition of antigen and no more precipitation had produced, was used to purify the non-precipitating antibody. In principle, this method selects antibody according to its affinity for the antigen, since the antibody with the highest affinity would precipitate first. Therefore, it might be supposed that the non-precipitating antibody remaining after precipitation was simply due to its

low affinity. However, this assumption is not suitable for the present antibody, because the separation of the non-precipitating antibody was achieved by affinity chromatography on a column of cobrotoxin-Sepharose, a technique requiring antibody of high affinity. Secondly, the antibody activity of the non-precipitating antibody and its papain fragment as determined by their inhibitory activity on the homologous precipitin reactions indicated that the reactions with cobrotoxin were very firm. Therefore, the inability of the non-precipitating antibody to form insoluble complexes with cobrotoxin must be due to reasons other than low affinity.

It has been suggested that non-precipitating antibody is univalent (Marrack, 1961; Fiset, 1962), but this possibility seems to be excluded by physicochemical studies of Klinman et al. (1964), Klinman and Karush (1967) and Carter and Harris (1967). Although no detailed study of the valency of the non-precipitating antibody was performed here, the fact that its molecular size was identical to those of the precipitating antibody suggests that the molecular structures of both of immunoglobulins were the same type.

Klinman et al. (1964, 1966 1967) purified rG precipitating and rG(T) non-precipitating equine anti-p-azophenyl-β-lactoside (Lac) antibody and demonstrated that rG(T) anti-Lac was bivalent and of high affinity. They suggested that the Fab arms of the rG(T) anti-Lac are restricted in their movement reducing the probability of such antibody to crosslink with antigens, i.e., non-precipitating antibody "exhibit monogamous bivalency" by which it is unable to react with determinants on two different molecules of antigens. Measurements of Fab flexibility of rG(T) support their suggestion (Archer et al., 1973).

The results obtained by Margni and Binaghi (1972) are also compatible with the hypothesis that non-precipitability is due to particular configuration of the molecule that makes it impossible for one molecule of antibody to combine with two different molecules of antigens simultaneously.

Christian (1970) presented evidence suggesting that the failure of non-precipitating antibodies to precipitate appeared to result from the limited recognition of multiple antigenic determinents on complex antigens. Unlike the conclusions from the investigations of rabbit and equine non-precipitating anti-hapten antibodies (Carter and Harris, 1967; Klinman and Karush,

1967), there was no evidence that a physicochemically distinct type of immunoglobulin was responsible for the non-precipitating character of the anti-ovalbumin sera.

If the antibodies are capable of binding with only a small number of antigenic determinants, lattice formation of the antigen-antibody complexes will be incomplete and, depending on degree of its incompletion, the complexes will remain soluble in the precipitin reaction.

Nakamura et al, (1972) and Tamoto et al. (1972) from their studies on guinea pig non-precipitating 7S r_2-antibody to ovalbumin suggested that the non-precipitating antibodies belonged to an antibody population which reacted with some particular antigenic determinant on ovalbumin molecule and were produced in excess, as compared with other populations of antibodies. When the amount of these antibodies is too large as compared with other antibodies, only a portion of them will be able to cooperate with other antibodies to make latticework of antigen-antibody complexes (Kabat and Mayer, 1961) and the remainder will be left as free antibodies in supernatants. On the other hand, when the amount of ovalbumin added is increased to combine with all these antibodies, the formation of latticework will become incomplete owing to relatively small amount of other antibodies and antigen-antibody complexes will not precipitate.

This explanation appears to be compatible with the results obtained by other workers for non-precipitating antibodies (Heidelberger and Kendall, 1935; Heidelberger et al., 1940; Wigle and Maurer, 1957; Feinberg, 1958; Kabat and Mayer, 1961; Christian, 1970). Many investigators isolated non-precipitating antibodies from antisera by adding repeatedly small amounts of antigens to remove precipitating antibodies. Such treatments may make antisera unbalanced and leave antibody populations reacting with only one or few antigenic determinants. However, the results so far obtained have not elucidated the weak precipitability of non-precipitating antibodies. Further study will be required for elucidating this problem and the study on the non-precipitating antibody is in progress.

V. Conclusion

Cobrotoxin was successfully immobilized on Sepharose

through its free amino groups without altering the antigenic activity. Coupling reaction at pH 9.0 in 0.1 M sodium bicarbonate-0.5 M NaCl yielded the best affinity adsorbent for antibody. It was found that 0.53 M formic acid-0.15 M NaCl (pH 2.05) was very efficient for the elution of antibody from cobrotoxin-Sepharose. Modification of Arg-residues did not cause any change in the coupling capacity of cobrotoxin to Sepharose, however, the antigenic activity decreased pronouncedly when Arg-30 and Arg-36 were modified.

We demonstrated that rabbits hyperimmunized with cobrotoxin in Freund's complete adjuvant do produce non-precipitating as well as precipitating antibodies. By affinity chromatography of supernatants obtained from precipitin reaction at the maximum precipitation on a column of cobrotoxin-Sepharose, the non-precipitating antibodies were separated from the antisera. Specific neutralizing capacity of the antibody preparations obtained from affinity chromatography was much higher than those isolated by precipitin reactions. The specific neutralizing capacity of the non-precipitating antibody and its papain fragment increased 23-fold and 27.6-fold over that of the antisera, respectively.

Both precipitating and non-precipitating antibodies were proved to be similar with regard to molecular size and elution appearing on cobrotoxin-Sepharose column. There was no evidence that a physicochemically distinct type of immunoglobulin was responsible for the non-precipitating character of antisera and the possible mechanisms for the nonprecipitability of the non-precipitating antibody were discussed.

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APPENDIX A-1

Table I

Effect of pH on the coupling capacity of cobrotoxin to CNBr-activated Sepharose 4B and the antigenic activity of cobrotoxin after coupling

Coupling buffer*	Coupling** capacity (%)	Antibody		Yield (%)
		Added (mg)	Eluted (mg)	
Sodium phosphate, pH 7.5	99.0	6.2	5.3	85.5
Sodium bicarbonate, pH 8.3	99.1	6.2	5.3	85.5
Sodium bicarbonate, pH 9.0	99.2	6.2	5.6	90.3
Sodium carbonate, pH 10.0	85.0	6.2	5.1	82.3

* The buffer concentration used was 0.1 M and each contains 0.5 M NaCl.

** The amount of cobrotoxin coupled on Sepharose was determined by the difference between the amount of toxin (6 mg/3 ml of Sepharose) added and that of free toxin appeared in the washing after the coupling process. Protein was determined by the method of Lowry et al. (1951).

APPENDIX A-2

Table II

Effect of cobrotoxin concentration on coupling capacity to CNBr-activated Sepharose 4B and the antigenic activity of coupled cobrotoxin to antibody

Cobrotoxin added*	Coupling capacity (%)	Antibody		Yield (%)
		Added (mg)	Eluted (mg)	
2.0	99.2	6.2	5.6	90.3
4.0	84.3	6.2	5.4	87.1
6.0	79.0	6.2	5.7	91.9
8.0	44.1	6.2	6.1	98.4

* mg/ml of bed volume.

APPENDIX A-3

Table III

Antigenic activity of cobrotoxin after coupling on Sepharose to various antibody preparations

Antibody preparations	Antibody		Yield (%)
	Added* (mg)	Eluted (mg)	
Antisera**	6.5	15.2	233.8
IgG**	6.0	13.8	230.0
Purified precipitating antibody	6.2	5.6	90.3
Non-precipitating antibody	6.4	5.6	87.5
FI of precipitating antibody+	6.4	3.4	53.1
FI of non-precipitating antibody+	6.5	3.0	46.2

* The protein concentration was measured spectrophotometically at 280 nm and the extinction coefficient, $E^{1\%}_{1cm}$, was taken as 14 for antibody and 13.5 for FI.

** The antibody contents of the antisera and IgG preparation were determined by quantitative precipitin reactions.

\+ Obtained from chromatography on a CM-cellulose column of the purified precipitating and non-precipitating antibodies, respectively, after papain digestion.

APPENDIX A-4

Table IV

Stepwise elution of antibodies from a column of cobrotoxin-Sepharose

Buffer solution	Antibody preparations		
	IgG	Precipitating antibody	Non-precipitating antibody
0.01 M Acetate buffer-0.5 M NaCl, pH 5.5	1.3	8.0	4.6
0.1 M Acetate buffer-0.5 M NaCl, pH 4.0	9.4	8.8	13.2
0.1 M Acetate buffer-0.5 M NaCl, pH 3.5	27.3	14.2	27.5
0.53 M Formic acid-0.15 M NaCl, pH 2.05	62.0	69.0	54.7

APPENDIX A-5

Table V

Precipitating and non-precipitating antibodies in anticobrotoxin sera

	mg/ml	%	Ratio
Precipitating antibody	1.916	42.1	1.0
Non-precipitating antibody	2.634	57.9	1.375
Total antibody content	4.550	100	

APPENDIX A-6

Table VI

Comparison of the specific neutralizing capacity of antibody preparations

Antibody preparations	Specific neutralizing capacity (LD_{50}/mg)	Relative capacity
Antisera	2.5	1.0
IgG	12.1	4.8
Purified antibody-1*	45.3	18.1
Purified antibody-2**	67.4	27.0
Non-precipitating antibody	57.6	23.0
FI of purified antibody-1	43.5	17.4
FI of non-precipitating antibody	69.1	27.6

* Purified antibody-1 was isolated by gel filtration on Sephadex G-100 after the antigen-antibody precipitates had been dissociated with 0.53 M formic acid-0.15 M NaCl, pH 2.05. Therefore, it is the precipitating antibody.

** Purified antibody-2 was isolated by affinity chromatography on a column of cobrotoxin-Sepharose. The antibody was eluted with 0.53 M formic acid-0.15 M NaCl, pH 2.05 after washing with 0.1 M soldium acetate-0.5 M NaCl, pH 3.5. It contains both precipitating and non-precipitating antibodies.

APPENDIX A-7

Table VII

Effect of chemical modification on the coupling capacity of cobrotoxin to CNBr-activated Sepharose 4B

Cobrotoxin derivatives	Added (mg)	Coupled (mg)	Coupling capacity (%)
Native cobrotoxin	6.0	5.95	99.2
Lys-27 TNP-cobrotoxin	6.0	5.49	91.5
Lys-27 & 47 TNP-cobrotoxin	6.0	5.25	87.5
Guanidinated cobrotoxin	6.0	5.28	88.0
TNP-guanidinated cobrotoxin	6.0	5.12	85.3
Arg-28 modified cobrotoxin	6.0	5.94	99.0
Arg-28 & 33 modified cobrotoxin	6.0	5.96	99.3
Arg-28, 30 & 33 modified cobrotoxin	6.0	5.89	98.2
Arg-28, 30, 33 & 36 modified cobrotoxin	6.0	5.97	99.5
HNB-cobrotoxin	6.0	5.11	85.2
Tyr-35 nitrated cobrotoxin	6.0	5.97	99.5
Tyr-25 & 35 nitrated cobrotoxin	6.0	5.97	99.5

APPENDIX A-8

Table VIII

Antigenic activity of the modified cobrotoxin derivatives after coupling on Sepharose to the purified antibody

Cobrotoxin derivatives	Antibody		Yield (%)	Relative activity (%)
	Added (mg)	Eluted (mg)		
Native cobrotoxin	6.2	5.6	90.3	100.0
Lys-27 TNP-cobrotoxin	6.2	5.6	90.3	100.0
Lys-27 & 47 TNP-cobrotoxin	6.2	4.6	74.2	82.1
Guanidinated cobrotoxin	6.2	5.8	93.5	103.6
TNP-guanidinated cobrotoxin	6.2	4.8	77.4	85.7
Arg-28 modified cobrotoxin	6.2	5.4	87.1	96.4
Arg-28 & 33 modified cobrotoxin	6.2	5.2	83.9	92.9
Arg-28, 30 & 33 modified cobrotoxin	6.2	3.4	54.8	60.7
Arg-28, 30, 33 & 36 modified cobrotoxin	6.2	2.6	42.9	46.4
HNB-cobrotoxin	6.2	5.8	93.5	103.6
Tyr-35 nitrated cobrotoxin	6.2	6.2	100.0	110.7
Tyr-25 & 35 nitrated cobrotoxin	6.2	5.5	88.7	98.2

APPENDIX A-9

Table IX

Affinity chromatography on Sepharose derivatives

Sepharose derivatives	Carbodiimide used	Coupling capacity* (%)	Antibody		Yield (%)
			Added (mg)	Eluted (mg)	
CH-Sepharose 4B	CMC, 40 mg	86.5	6.2	4.1	66.2
	CMC, 60 mg	80.5	6.2	4.1	66.2
	EDC, 60 mg	94.1	6.2	3.8	61.3
AH-Sepharose 4B	CMC, 40 mg	10.1	6.2	1.8	29.0

* The amount of cobrotoxin used was 2 mg/ml of Sepharose.

APPENDIX B-1

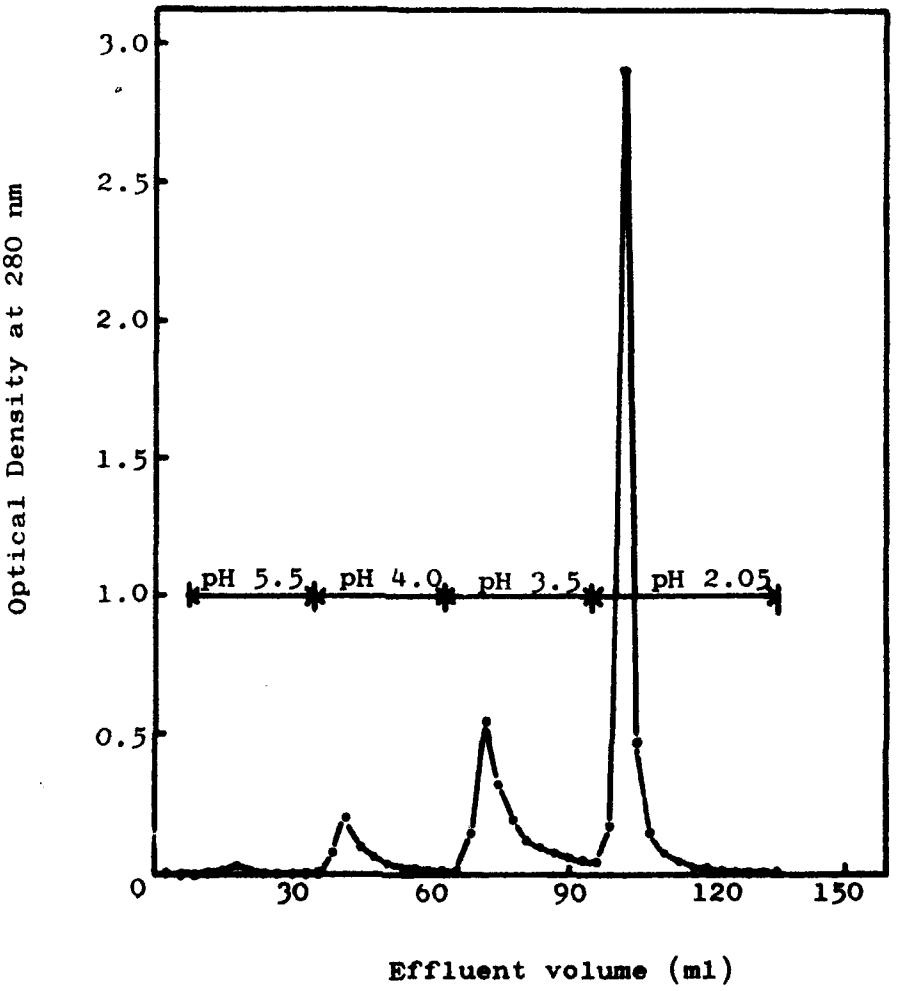

Fig. 1. Stepwise elution of antibodies from a column of cobrotoxin-Sepharose.

Antibody preparations were applied by passage through a column (1.0 x 3.5 cm) of cobrotoxin-Sepharose (cobrotoxin 2 mg/ml of Sepharose, coupled at pH 9.0) three times, and washed with 30 ml of 0.1 M $NaHCO_3$-0.5 M NaCl, pH 8.3. The buffer solutions used for stepwise elution were: 0.01 M acetate buffer-0.5 M NaCl (pH 5.5), 0.1 M acetate buffer-0.5 M NaCl (pH 4.0 and pH 3.5), and 0.53 M formic acid-0.15 M NaCl (pH 2.05).

A: Anticobrotoxin IgG 6 mg.

APPENDIX B-1

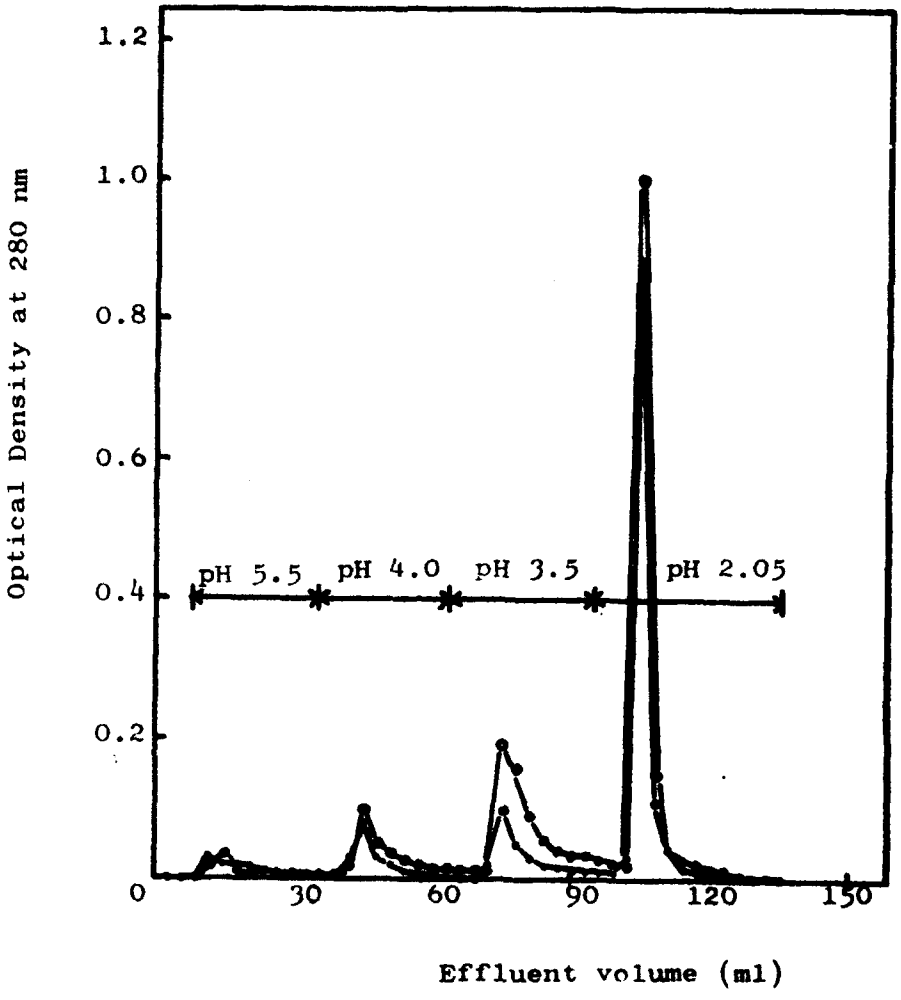

Fig. 1. Stepwise elution of antibodies from a column of cobrotoxin-Sepharose.

B: Purified precipitating antibody (•——•) 5 mg and purified non-precipitating antibody (•——•) 5.2 mg.

APPENDIX B-2

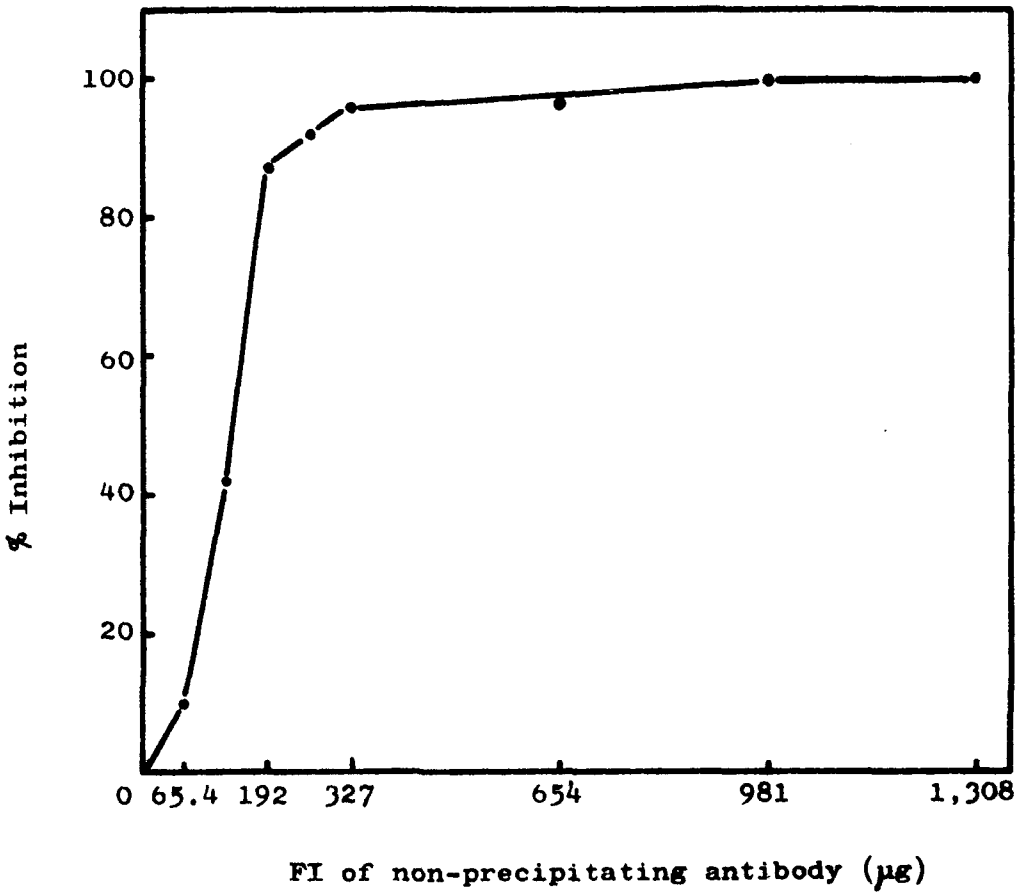

Fig. 2. Inhibition of the homologous precipitin reactions of cobrotoxin with anticobrotoxin IgG by FI of non-precipitating antibody.

Increasing amounts of FI was added to cobrotoxin (20 μg) in 0.1 ml of 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. After incubation at 37°C for 30 min the reaction mixture was placed at 4°C for 5 h. The equivalent amount of anticobrotoxin IgG was then added and the precipitates formed after incubation at 37°C for 30 min, then at 4°C overnight, were determined by the method described in the text.

APPENDIX B-3

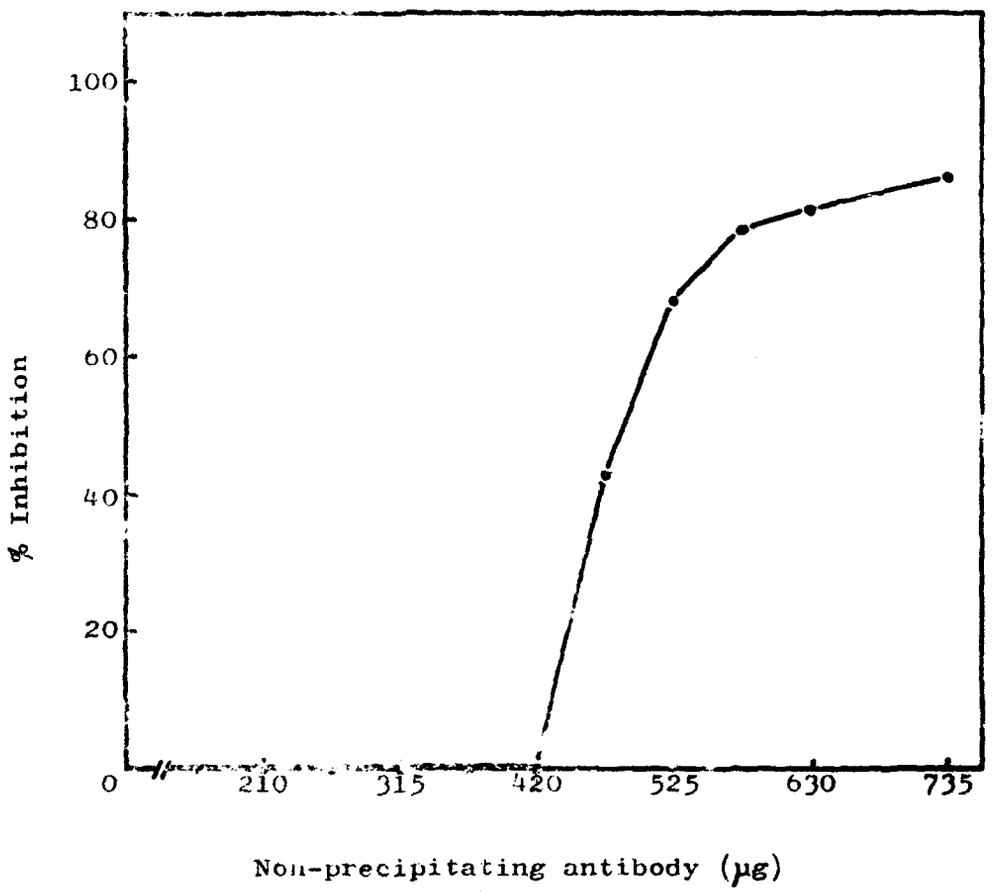

Fig. 3. Inhibition of the homologous precipitin reactions of cobrotoxin with purified precipitating antibody by non-precipitating antibody.

Increasing amounts of non-precipitating antibody was added to cobrotoxin (25 μg) in 0.1 ml of 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. After incubation at 37°C for 30 min the reaction mixture was placed at 4°C for 5 h. The equivalent amount of purified precipitating antibody was then added and the precipitates formed after incubation at 37°C for 30 min, then at 4°C overnight, were determined by the method described in the text.

APPENDIX B-4

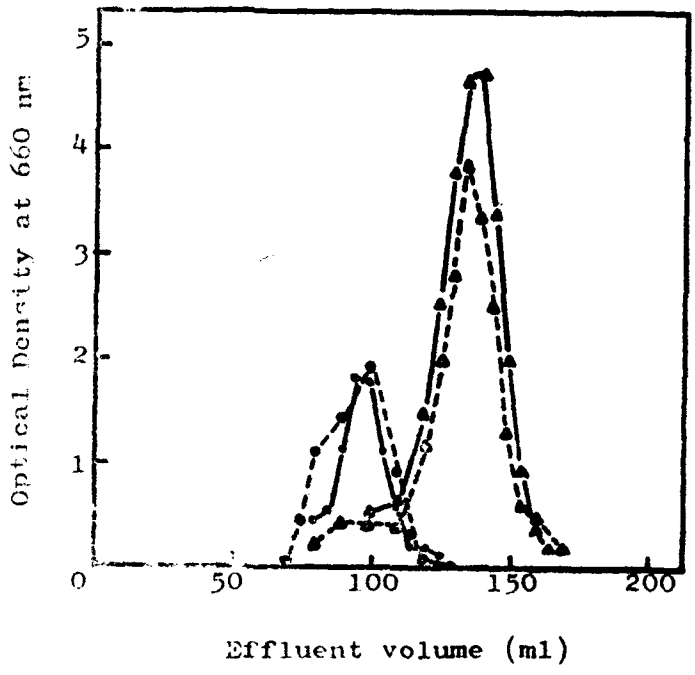

Fig. 4. Gel filtration pattern of antibody preparations on Sephadex G-200.

The column was equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl to a constant height (2 x 82 cm). The same buffer solution was used as an eluant and 5 ml fractions were collected at a rate of 25 ml/h.

- Non-precipitating antibody
- Precipitating antibody
- FI of non-precipitating antibody
- FI of precipitating antibody

List of Publications

1. The disulfide bonds of cobrotoxin and their relationship to lethality. Biochim. Biophys. Acta 133 (1967) 346-355.

2. Optical rotatory dispersion of cobrotoxin. J. Biochem. 61 (1967) 272-274.

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