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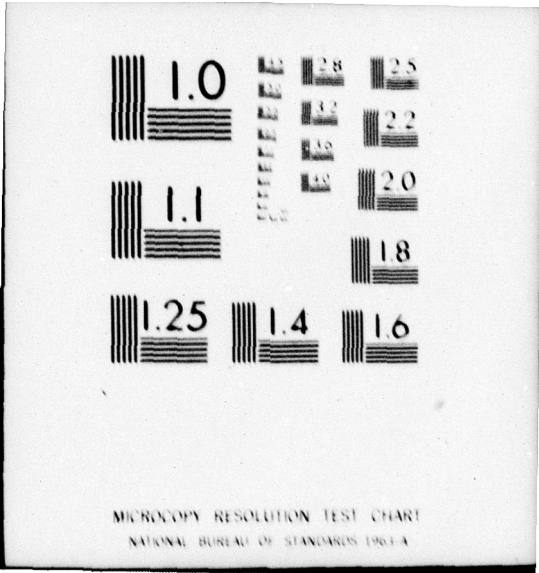
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The heterogeneity of precipitating and non-precipitating
antibodies to cobrotoxin was demonstrated by their elution
pattern on cobrotoxin-Sepharose and chromatography on DEAE-
cellulose column.Gel filtration patterns on Sepharose 6B column revealed that
the soluble complexes from non-precipitating antibody and HNB-
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cobrotoxin at a different molar ratio are emerged in the void volume, indicating that the molecular weight of the soluble complex is around 4,000,000 or larger. Unreacted free non-precipitating antibody coincided with the peak of IgG and was proved to be free from HNB-cobrotoxin. The molar ratio of antibody to antigen for the soluble complex was found to be 0.79 to 0.97 indicating that 1.58 to 1.94 molecules of non-precipitating antibody bound to HNB-cobrotoxin instead of three molecules in the case of precipitating antibody.

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STRUCTURE-FUNCTION AND IMMUNOCHEMICAL STUDIES ON
COBROTOXIN

Purification and Properties of Non-precipitating
Antibodies to Cobrotoxin*

The heterogeneity of precipitating and non-precipitating antibodies to cobrotoxin was demonstrated by their elution pattern on cobrotoxin-Sepharose and chromatography on DEAE-cellulose column.

Gel filtration patterns on Sepharose 6B column revealed that the soluble complexes formed from non-precipitating antibody and HNB-cobrotoxin at a different molar ratio are all emerged in the void volume, indicating that the molecular weight of the soluble complex is around 4,000,000 or larger. Unreacted free non-precipitating antibody coincided with the peak of IgG and was proved to be free from HNB-cobrotoxin. The molar ratio of antibody to antigen for the soluble complex was found to be 0.79 to 0.97 indicating that 1.58 to 1.94 molecules of non-precipitating antibody bound to HNB-cobrotoxin instead of three molecules in the case of precipitating antibody.

* This paper was presented at the International Symposium on Proteins at Taipei, 9 March, 1978 and will appear in International Journal of Protein and Peptide Research.

INTRODUCTION

In 1965, a neurotoxic protein, cobrotoxin, was isolated in the crystalline state from the venom of Taiwan cobra (Naja naja atra) (Yang, 1965) and was proved to be the main toxic protein in cobra venom (Chang and Yang, 1969).

In our recent study, cobrotoxin was successfully immobilized on Sepharose through its free amino groups without altering its antigenic activity (Yang et al., 1977). Rabbits hyperimmunized with cobrotoxin in Freund's complete adjuvant 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. Antibodies separated by affinity chromatography on a column of cobrotoxin-Sepharose are superior to the precipitating antibody, isolated by immune precipitin reaction, with regard to their neutralizing capacity.

The present paper deals with some properties of the non-precipitating antibody and the mechanism of non-precipitability of the non-precipitating antibody with its homologous antigen, cobrotoxin.

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MATERIALS AND METHODS

Cobrotoxin was prepared from Taiwan cobra (Naja naja atra) venom as previously described (Yang, 1965). CNBr-activated Sepharose 4B, Sepharose 6B, Sephadex G-25, G-100 and G-200, and CM-Sephadex C-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. CM-cellulose, DEAE-cellulose, β -mercaptoethanol and iodoacetic acid were obtained from Sigma Chemical Company. Freund's (complete) adjuvant Stat-Pack [®] was obtained from Calbiochem, 2-hydroxy-5-nitrobenzyl bromide (HNB bromide) from Seikagaku Kogyo Co., Ltd., and polyethylene glycol 1000 from E. Merck, Darmstadt. Mercuripapain and pepsin were products of Worthington Biochemical Corp., Freehold, N.J. All other reagents were of analytical grade.

A covalent coupling of cobrotoxin to CNBr-activated Sepharose 4B was carried out essentially according to the method described by Cuatrecasas and Anfinsen (Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971) as previously described (Yang et al., 1977).

Alkylation of cobrotoxin with HNB bromide was carried out under standard conditions of Barman and Koshland (1967) as described by Chang and Hayashi (1969).

Preparation of antisera

Anticobrotoxin serum was prepared by injecting increasing amounts of cobrotoxin with Freund's complete adjuvant into rabbits weighing 2.0 - 2.5 kg. From 6 μ g to 1.6 mg per kg body weight were injected subcutaneously into the right and left thigh alternating at weekly intervals during a period of 3 months and the animals were bled 9 days after the final injection.

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

Antisera to Fab fragments were prepared as follows: Immunization of goats with Fab fragments of rabbit precipitating and non-precipitating antibodies were performed by a series of intramuscular injections with decreasing doses of Fab fragments into male goats weighing about 10 kg. The initial dose, 20 mg of Fab fragment dissolved in 1 ml of 0.15 M NaCl and emulsified with an equal volume of Freund's complete adjuvant, was injected into each thighs. Then 10 mg and 2.5 mg each of the Fab fragment dissolved in 1 ml of 0.15 M NaCl and emulsified with an equal volume of Freund's adjuvant (complete : incomplete = 1 : 8, by volume) was injected at the second and third week, respectively. Two weeks later, an additional

immunization with the same dose used for the third week was injected and the goats were bled 9 days after the final injection.

Separation of precipitating and non-precipitating antibodies

Small amounts of cobrotoxin (approximately 1/10 of the quantity required for maximal precipitation) were successively added to IgG. After each addition the mixture was incubated for 40 min at 37°C and at 4°C for 4 hr before centrifugation. The precipitates were collected, pooled and used to prepare the precipitating antibody as previously described (Chang and Yang, 1969). The combined precipitates were washed three times with cold 0.15 M NaCl, then dissolved in a minimal amount of 0.53 M formic acid-0.15 M NaCl, pH 2.05. Complete separation of the antibody from antigen was achieved by gel filtration on a column of Sephadex G-100 using the same acid.

The supernatant, obtained after the addition of cobrotoxin produced no more precipitation, was used to purify the non-precipitating antibody. It was passed through a column of cobrotoxin-Sepharose three times. The bound antibodies 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 NaHCO₃ 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 immediately neutralized with 1.0 M glycine-NaOH buffer (pH 11.5) and the fractions containing antibody were pooled and dialyzed against 0.01 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl.

Digestion and fragmentation of antibody, precipitin reaction, measurement of inhibitory activity of non-precipitating antibody and its papain fragment to homologous precipitin reactions, and preparation of peptide maps of the peptic hydrolysates of Fab fragments were performed as previously described (Chang and Yang, 1969; Yang et al., 1974, 1977).

RESULTS AND DISCUSSION

Production of antibodies during immunization

The production of antibodies, precipitating and non-precipitating, in rabbits following a serial injection of cobrotoxin with Freund's complete adjuvant was surveyed at various intervals during immunization. The content of precipitating antibody was determined by quantitative precipitation reactions and that of non-precipitating antibody by affinity chromatography on cobrotoxin-Sepharose column. As shown in Fig. 1, the non-precipitating antibody was produced in parallel with that of precipitating antibody and the amount of non-precipitating antibody was higher than that of precipitating antibody at the same period of immunization. After completion of 3 months hyperimmunization, the total antibody content in anticobrotoxin sera was 4.55 mg per ml with the non-precipitating antibody amounting to 2.63 mg per ml or 57.8 % of the total antibody.

Isolation of non-precipitating antibody by affinity chromatography

For the isolation of the non-precipitating antibody, the precipitating antibody in antisera or IgG was first removed by

precipitation upon a serial addition of cobrotoxin (approximately 1/10 of the quantity required for maximal precipitation) until no more precipitate formed. After each addition the mixture was incubated for 40 min at 37°C and at 4°C for 4 hr before centrifugation. The precipitates were collected, pooled and used to prepare the precipitating antibody as previously described (Chang and Yang, 1969). The supernatant was used to purify the non-precipitating antibody. It was passed through a column of cobrotoxin-Sepharose three times. The bound antibodies were eluted with 0.53 M formic acid-0.15 M NaCl (pH 2.05) after the column had been washed with 0.1 M NaHCO₃ buffer (pH 8.3) containing 0.5 M NaCl and 0.1 M acetate buffer-0.5 M NaCl (pH 4.0). The effluents were immediately neutralized with 1.0 M glycine-NaOH buffer (pH 11.5) and the fractions containing antibody were pooled and dialyzed against 0.01 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl.

As illustrated in Fig. 2, although the major fraction of non-precipitating antibody was eluted at pH 3.0, 0.1 M glycine-HCl buffer-0.5 M NaCl, at least three more populations of antibodies could be separated by acetate buffer-0.5 M NaCl at pH 5.5, 4.0 and 3.5. The major fraction of precipitating antibody, however, was eluted at pH 2.5 with several more populations at

different pH values. The results suggest that several populations of antibody, differing in their affinity to cobrotoxin, are present in both antibody preparations and also indicate that there are some differences in binding affinity to cobrotoxin-Sepharose between precipitating and non-precipitating antibodies.

The heterogeneity of precipitating and non-precipitating antibodies was also demonstrated by chromatography on DEAE-cellulose. As shown in Fig. 3, although the major fractions obtained from cobrotoxin-Sepharose column at pH 2.5 and pH 3.0 for precipitating and non-precipitating antibodies, respectively, were applied, the elution profile revealed more than two peaks.

Peptide maps of the peptic hydrolysates of Fab fragments

The single fractions, eluted from DEAE-cellulose column at the 4th step by 0.033 M phosphate buffer-0.3 M NaCl (pH 7.8) for precipitating and non-precipitating antibodies (Fig. 3), were subjected to papain fragmentation. The fractions were digested with mercuripapain and chromatographed on a column of CM-cellulose. The Fab fragments obtained were digested with pepsin after reduction and carboxymethylation. The peptic hydrolysates were separated by a combination of high-voltage paper electrophoresis and descending paper chromatography. As shown in Fig. 4, striking differences in peptide maps were observed between the two preparations, indicate that the

amino acid sequences of the Fab fragments of precipitating and non-precipitating antibodies are different each other.

Antibody activity of non-precipitating antibody

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 purified precipitating antibody. Approximately 1.0 mole of papain fragment, or 0.91 mole of the non-precipitating antibody per mole of cobrotoxin gave 50 % inhibition, while for papain fragment of the purified precipitating antibody about 1.2 moles were required for the same degree of inhibition (Chang and Yang, 1969). These results show that the 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.

Effect of polyethylene glycol on precipitation of antigen-antibody complex

The effect of polyethylene glycol on the precipitation of antigen-antibody complex formed from guinea-pig non-precipitating antibody and its homologous antigen, α -amylase, has been demonstrated by immunodiffusion in agar gel as a precipitin line (Mori et al., 1976). In this study, we demonstrated the effect of polyethylene glycol on quantitative precipitin reaction of rabbit

non-precipitating antibody with its homologous antigen, cobrotoxin. As shown in Fig. 5, about 20 % of the soluble complex formed from non-precipitating antibody and cobrotoxin was precipitated in the presence of 4 % and 8 % of polyethylene glycol.

Antigenicity of precipitating and non-precipitating antibodies

In order to investigate the immunological cross-reaction between precipitating and non-precipitating antibodies, Fab fragments of both antibodies were immunized into goats, respectively. As shown in Fig. 6, both Fab fragments were capable of producing homologous as well as cross-reactive antibodies with almost the same extent in goats, suggesting that the antigenicity of the both antibodies are practically the same.

Gel filtration of soluble complex formed from non-precipitating antibody and HNB-cobrotoxin

As seen from the gel filtration patterns of soluble complex on a column of Sepharose 6B (Fig. 7), the soluble complexes formed from non-precipitating antibody and HNB-cobrotoxin at a different molar ratio are all emerged in the void volume, indicating that the molecular weight of the soluble complex is around 4,000,000 or larger.

Unreacted free non-precipitating antibody coincided with

the peak of serum IgG and was proved to be free from HNB-cobrotoxin. As illustrated in Fig. 7C, excess HNB-cobrotoxin emerged with a larger effluent volume from 425 ml to 465 ml.

The "valence" or number of antibody-combining sites per antigen molecule was obtained by doubling the molar ratio of antibody to antigen present in the precipitates formed at equivalence. The molar ratio of precipitating antibody to cobrotoxin was found to be 1.5 to 1.6 at equivalence (Chang and Yang, 1969). Therefore, the valence of cobrotoxin was 3.0 to 3.2 indicating that cobrotoxin (as well as HNB-cobrotoxin, Chang and Yang, 1973) consists of three antibody-combining sites per molecule.

The molar ratio of antibody to antigen for the soluble complex was found to be 0.79 to 0.97 (Table I) indicating that 1.58 to 1.94 molecules of non-precipitating antibody bound to HNB-cobrotoxin instead of three molecules in the case of precipitating antibody.

Christian (1970) presented evidence suggesting that the failure of non-precipitating antibodies to precipitate appeared to result from the limited recognition of multiple antigenic determinants on complex antigens. 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 incompleteness, the complexes will remain soluble in the precipitin reaction (Kabat and Mayer, 1961).

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 the latticework of antigen-antibody complexes, 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 and also with the results of present study on the molar ratio of non-precipitating antibody to HNB-cobrotoxin for the soluble complex.

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 (Yang et al., 1977). This may allow a substantial improvement in the therapy of victims of snake bites. 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 nature of antigenic determinants on cobrotoxin molecule.

Acknowledgements

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Yang, C. C., Chang, C. C. & Liu, I. F. (1974) Biochim. Biophys.

Acta 365, 1-14.

Yang, C. C., Lin, M. F. & Chang, C. C. (1977) Toxicon 15,

51-62.

Table I

Molar ratio of non-precipitating antibody to HNB-cobrotoxin
in soluble complex

Molar ratio of non-ppt Ab to HNB-cobrotoxin	Soluble complex			Unreacted non-ppt Ab (10^{-8} M)	Free HNB- cobrotoxin (10^{-8} M)
	Ab _s (10^{-8} M)	Ag _s (10^{-8} M)	Molar ratio of Ab to Ag		
1	10.6	12.9	0.82	3.5	-
2	13.7	14.1	0.97	14.8	-
0.5	11.8	14.9	0.79	1.8	13.5

Assuming the molecular weight of non-precipitating antibody is
150,000 and that of HNB-cobrotoxin is 7,086 (Yang et al., 1969).

Figure legends

Fig. 1. Production of precipitating and non-precipitating antibodies during the course of immunization.

The content of precipitating antibody (—●—) was determined by quantitative precipitin reaction and that of non-precipitating antibody (e---e) by affinity chromatography on cobrotoxin-Sepharose column and was calculated from the difference of amount of eluted antibody from that of precipitating antibody in the same sera.

Fig. 2. Stepwise elution of antibodies from a column of cobrotoxin-Sepharose.

Antibody preparations were applied by passage through a column (1.4 x 6.8 cm) of cobrotoxin-Sepharose (cobrotoxin 2 mg per ml of Sepharose, coupled at pH 9.0) three times, and washed with 50 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), 0.1 M glycine-HCl buffer-0.5 M NaCl (pH 3.0 and pH 2.5), and 0.53 M formic acid-0.15 M NaCl (pH 2.05).

Precipitating antibody (—●—) 20 mg

Non-precipitating antibody (e---e) 20.3 mg

Fig. 3. Ion-exchange chromatography on DEAE-cellulose.

The column (2 x 27 cm) was equilibrated with 0.0165 M phosphate buffer, pH 7.8, and eluted by four-step elutions as indicated. Precipitating antibody (—●—) obtained from cobrotoxin-Sepharose column at pH 2.5 and non-precipitating antibody (•---•) eluted at pH 3.0 from the same column were applied.

Fig. 4. Comparison of peptide maps of the peptic hydrolysates of RCM-Fab fragments of precipitating and non-precipitating antibodies.

About 5 mg each of the hydrolysate was applied on a sheet of Whatman No. 3 MM paper (45 x 74 cm).

Fig. 5. Effect of polyethylene glycol on quantitative precipitin reactions of cobrotoxin with non-precipitating antibody.

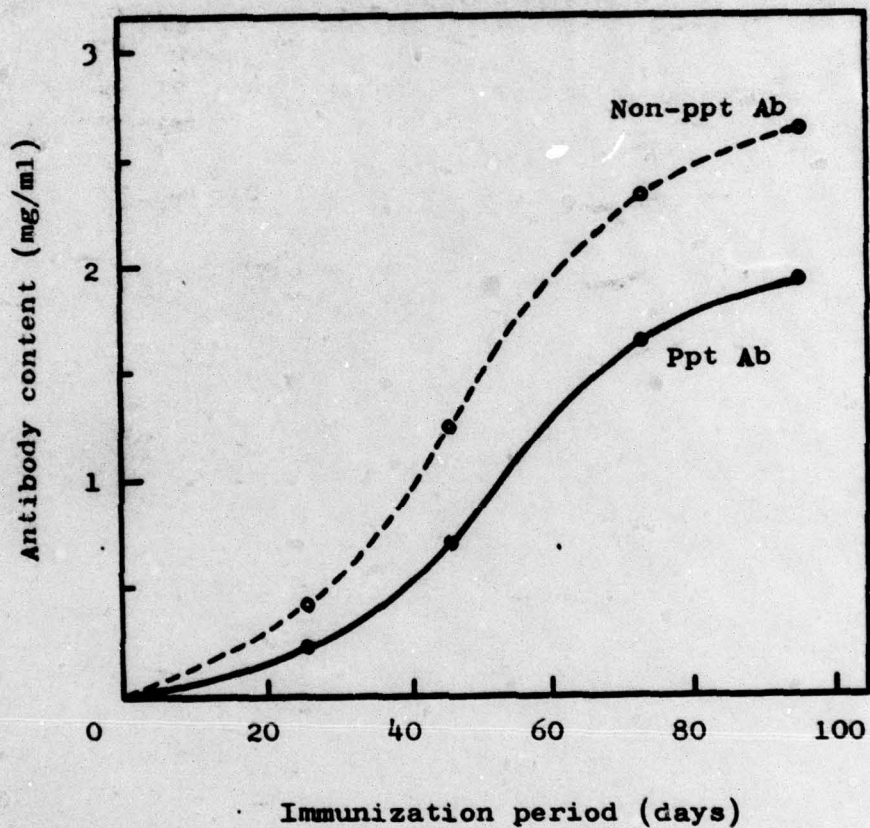
0.52 mg of precipitating antibody (—●—) and 0.48 mg of non-precipitating antibody (•---•) in the absence or in the presence of 4 % (▲---▲) and 8 % (—▲—) of polyethylene glycol were used in each determination. Polyethylene glycol was dissolved in 0.15 M NaCl and the concentration indicated was the final concentration in the reaction mixture.

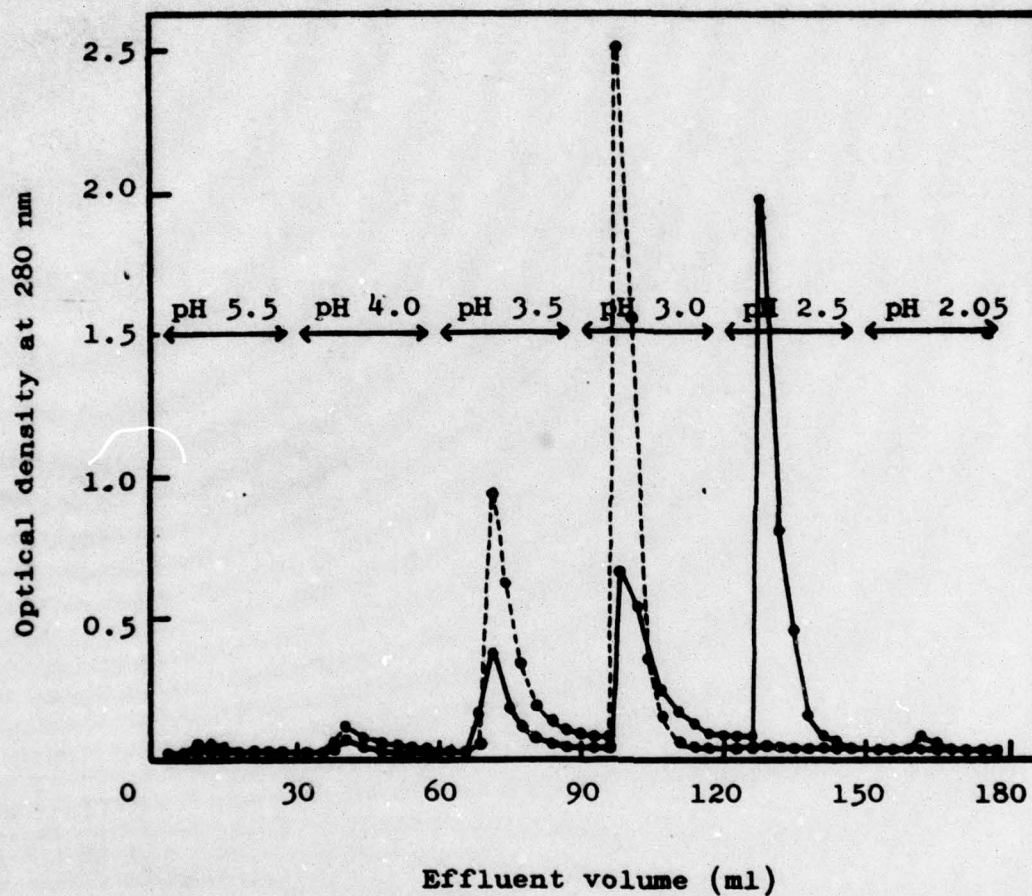
Fig. 6. Quantitative precipitin reactions of Fab of rabbit precipitating and non-precipitating antibodies with their goat antibodies.

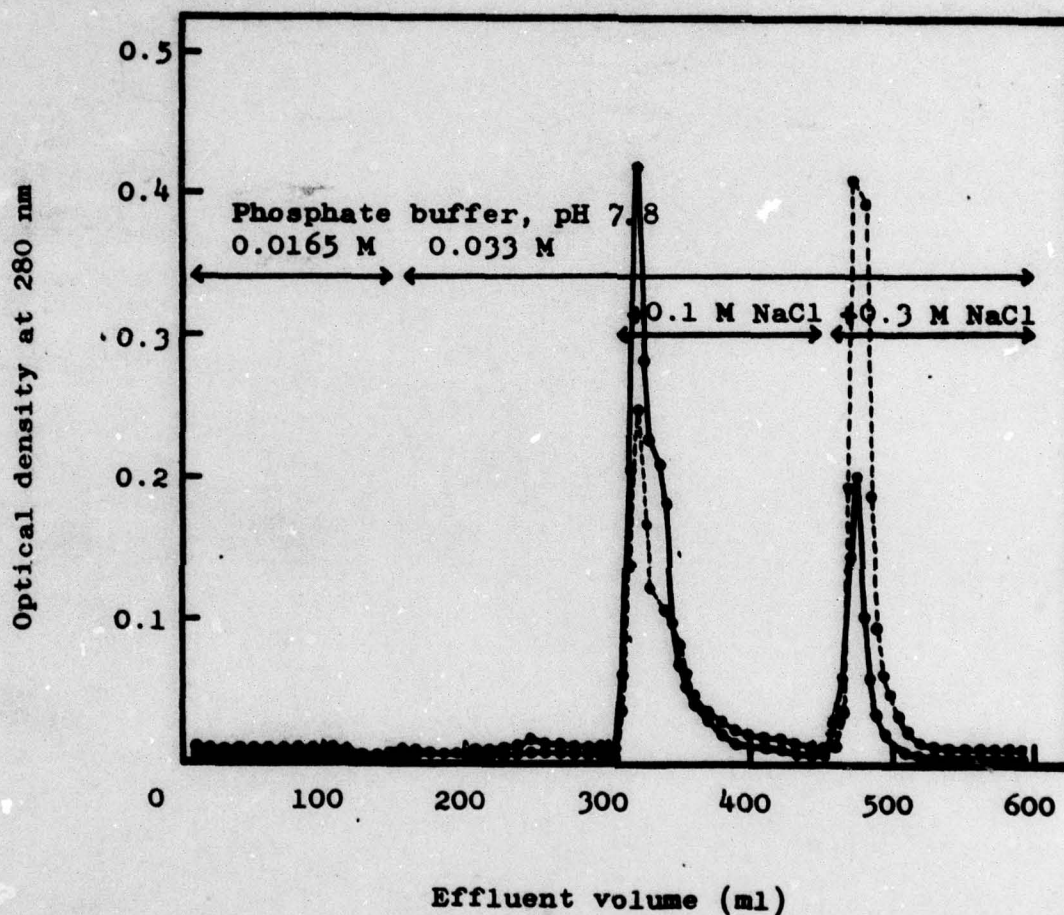
0.1 ml of the goat antisera obtained by immunization with Fab of precipitating (●—●) or non-precipitating antibody (●---●) were used in each determination.

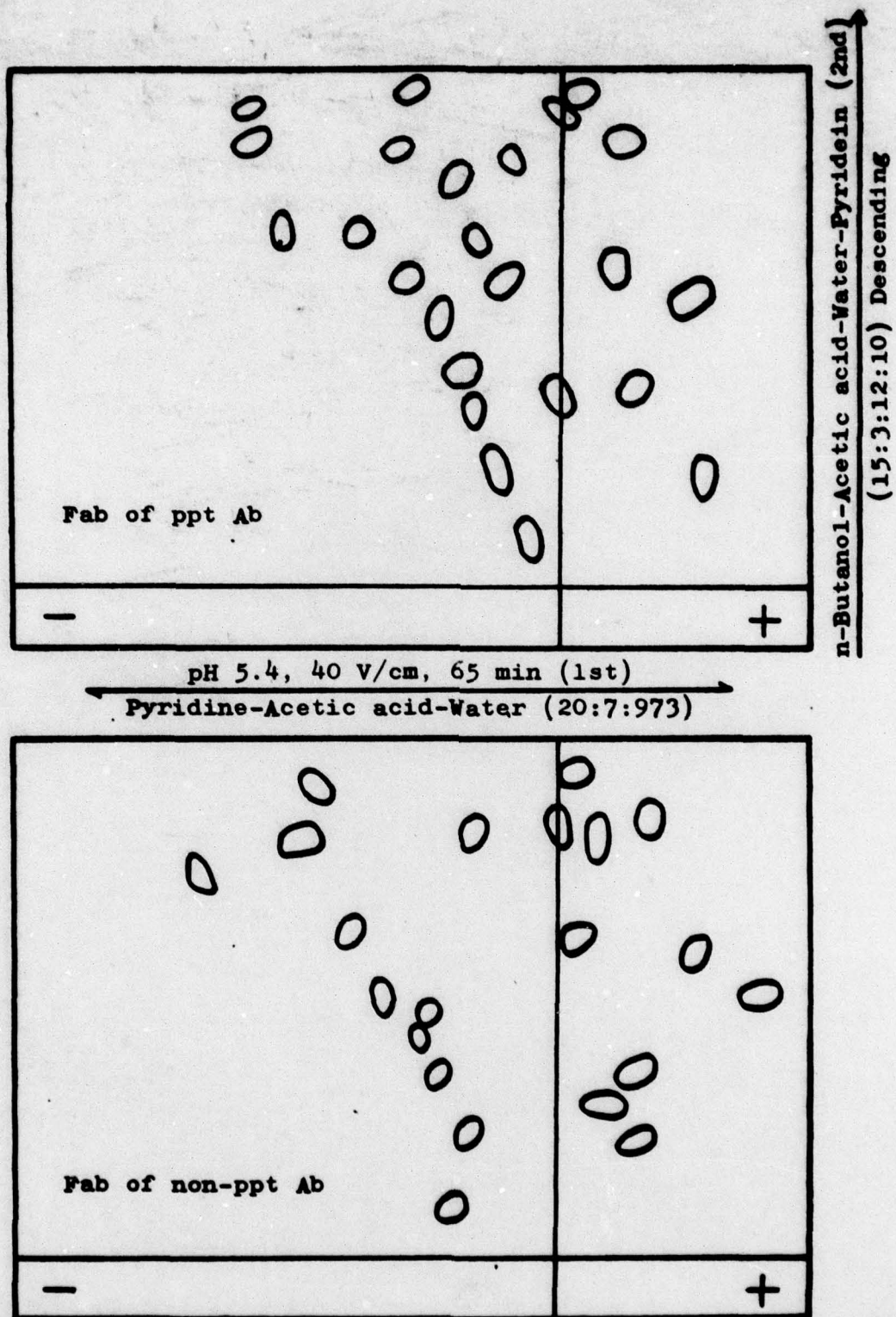
Fig. 7. Gel filtration of soluble complex formed from non-precipitating antibody and HNB-cobrotoxin on a column of Sepharose 6B.

The column was equilibrated with 0.02 M Tris-HCl buffer-0.5 M NaCl (pH 7.5) to a constant height (2.2 x 123 cm) and elution was performed with the same buffer at a flow rate of 20 ml per hr. Soluble complex (●---●) formed from non-precipitating antibody and HNB-cobrotoxin at a molar ratio of 1.0 (A), 2.0 (B) and 0.5 (C). The protein concentration was measured spectrophotometrically at 280 nm and the extinction coefficient, $E_{1\text{ cm}}^{280}$, was taken as 14 for IgG (----) and non-precipitating antibody (▲---▲), and 6.7 for HNB-cobrotoxin (●—●) at 410 nm.



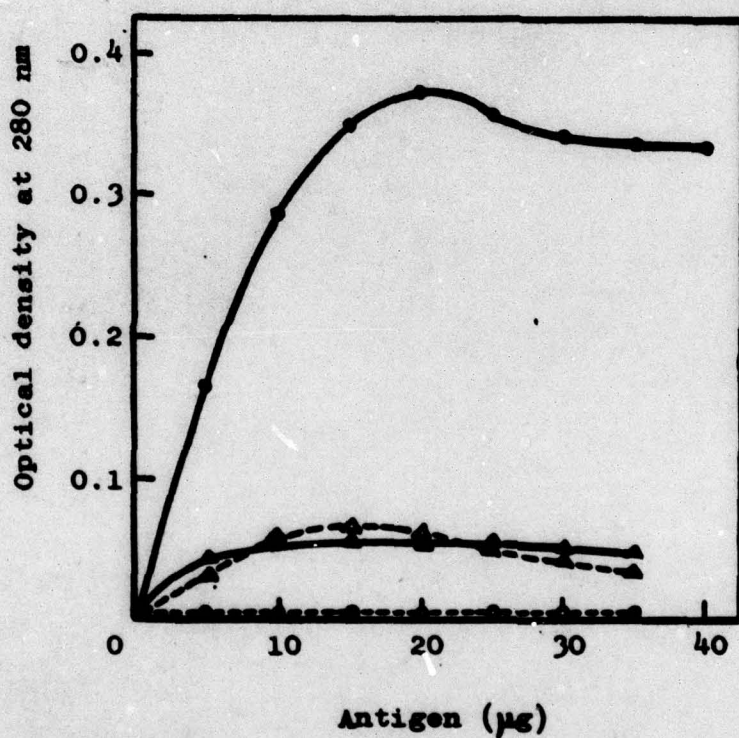


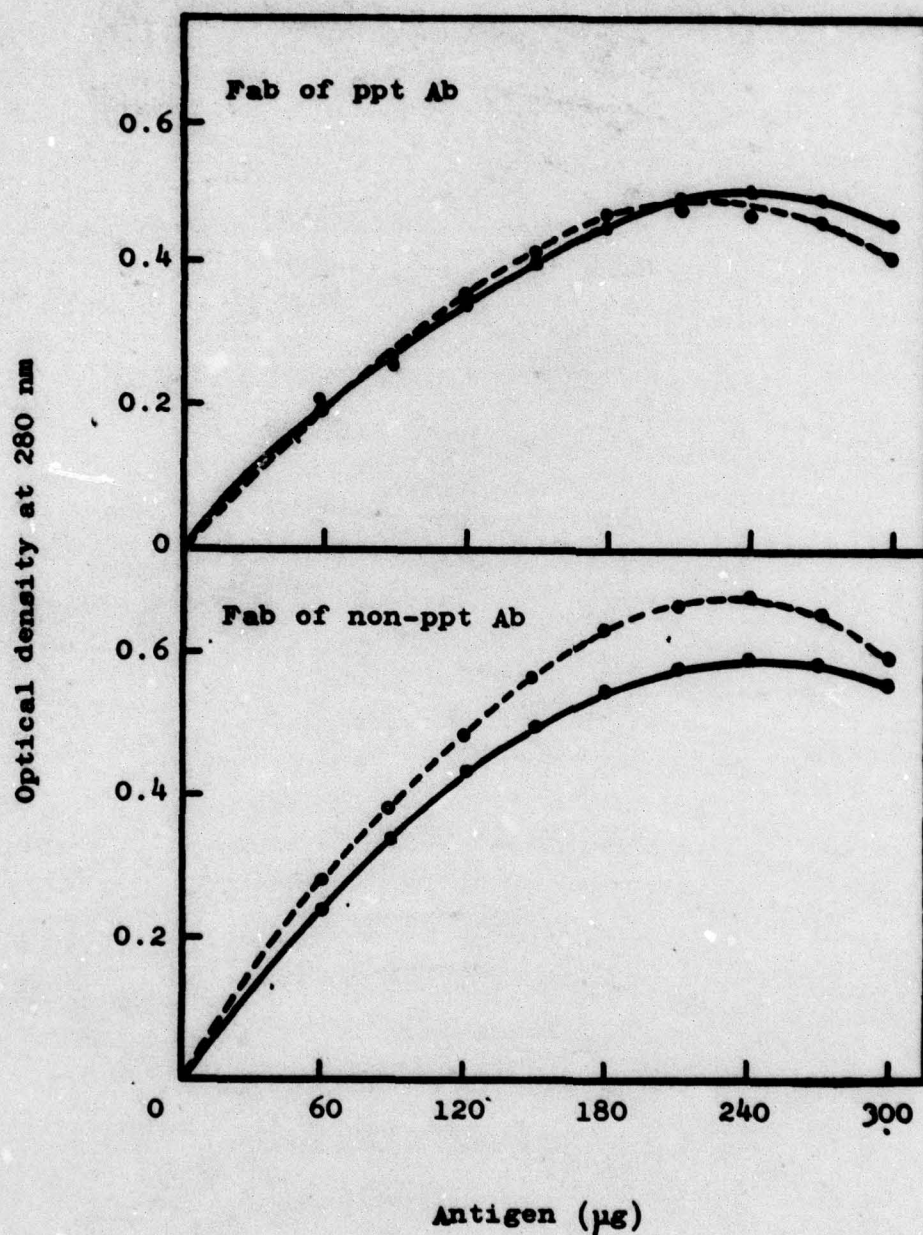




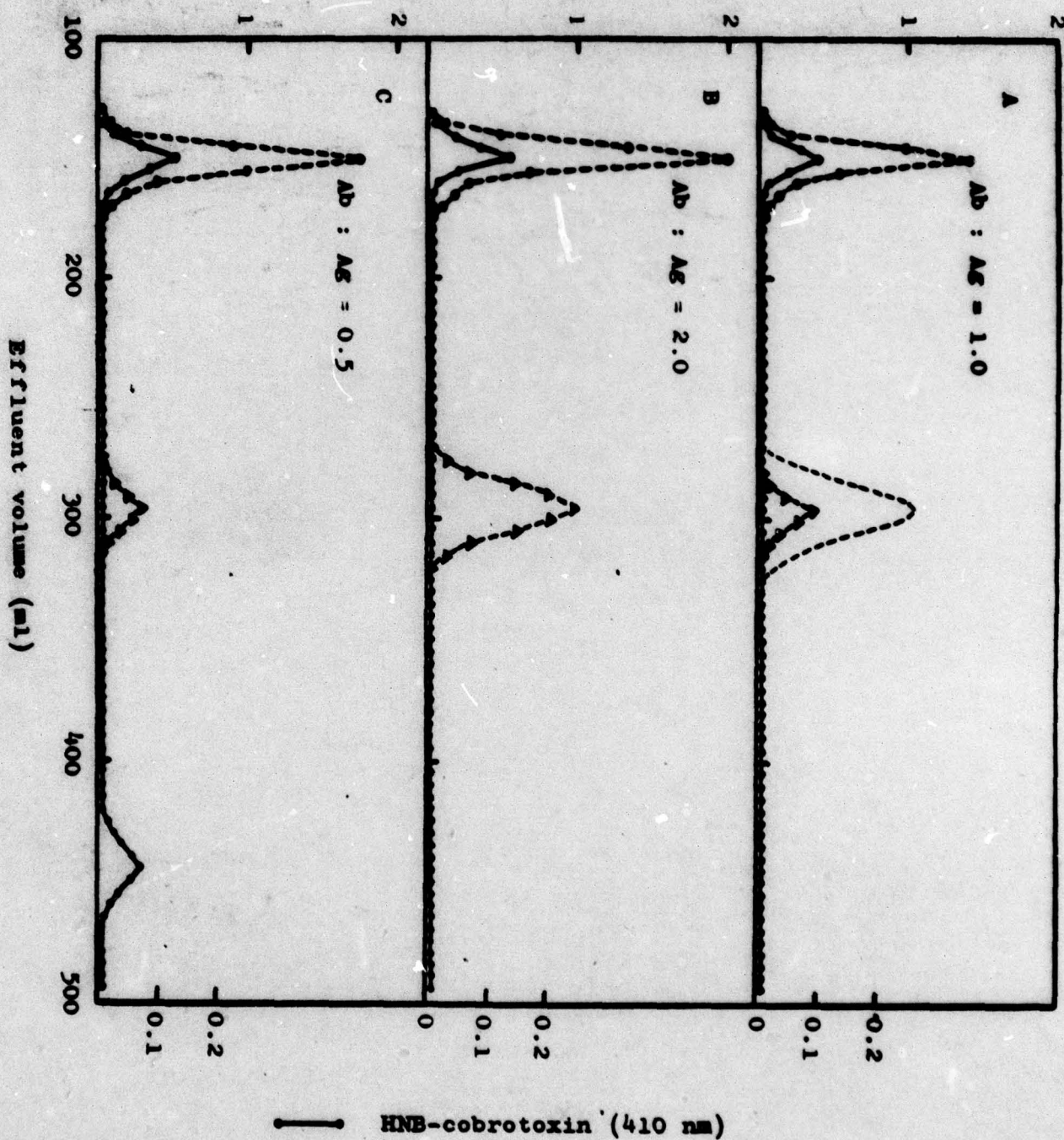
Intern. J. Peptide Protein Research
Purification and Properties of Non-precipitating Antibodies
to Cobrotoxin. C. C. Yang

Fig. 5





Optical density at 280 nm



List of Publications

1. Purification of anticobrotoxin antibody by affinity chromatography. Toxicon 15, 51-62 (1977).
2. Chemistry and biochemistry of snake venom neurotoxins. Toxins: Animal, Plant and Microbial Pergamon Press, Oxford, 261-292 (1978).
3. The reaction of cobrotoxin with the bifunctional reagent 4,4'-difluoro-3,3'-dinitro-diphenylsulfone (FNPS). Toxins: Animal, Plant and Microbial Pergamon Press, Oxford, 293-308 (1978).
4. Purification and properties of non-precipitating antibodies to cobrotoxin. Intern. J. Peptide Protein Research in press.

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