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CHROMATOGRAPHIC PURIFICATION OF ANTIVENOMS AND ANTITOXINS +

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ABSTRACT: Commercial preparations of antivenoms and antitoxins produced in horses, that are essentially pure F (ab)2 immunoglobulin preparations, were submitted to ion-exchange chromatography. For anti-crotalic, anti-bothropic and anti-tetanic sera it is possible to remove 40-60% of the inactive globulins.

KEY WORDS: Antivenoms; tetanus; antisera; antitoxins.

INTRODUCTION

Antivenoms and antitoxins used for the treatment of snakebite, arthropods poisoning, diphteria or tetanus, are usually produced by immunization of horses, followed by treatment of the hyperimmune plasma with crude pepsin, and purification by heat denaturation and ammonium sulfate precipitation[3]. Previous investigations from this laboratory have shown that the product of crude pepsin digestion at pH 3.2 is a F $(ab)_2$ - like product from immunoglobulins hydrolyzed by para-pepsins present in the crude pepsin preparation, which is stable under the conditions used for heat denaturation[8].

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In the present paper we report further purification of the antisera by chromatography, that removes part of the inactive immunoglobulins.

MATERIALS AND METHODS

Hyperimmune plasma and serum: Hyperimmune plasma was prepared by the immunization of horses with snake venoms, purified diphteria or tetanus toxins. They constitute the sera supplied by the Institute for human use processed by the method of Harms.[3]

Chromatographic resins: Cibracon blue agarose and Blue Sepharose were obtained from Amicon, Pharmacia and Sigma Chemical Co, and S-Sepharose from Pharmacia.

Electrophoresis: SDS-acrylamide gel electrophoresis was carried out in 7% acrylamide[2]. Electrophoresis on cellulose acetate was carried out by the standard method used for human serum electrophoresis[4].

Immunodifusion was carried out according to Ouchterlony[9]. Identification of the heavy and light γ -immunoglobulin chains using immunodifusion was assayed by Prof. S. Kochwa at Mount Sinai School of Medicine.

Neutralizing activity: Antivenom activity was assayed by testing the effects of mixtures of a standard volume of the antiserum or one of its fractions with different (or several) dilutions of the venom in pigeons[1]. Tetanus and diphteria antitoxin activity was assayed in guinea-pigs by the methods established by the US National Institutes of Health[6,7].

Protein determination: was estimated by the absorbance at 280 nm[5].

RESULTS AND DISCUSSION

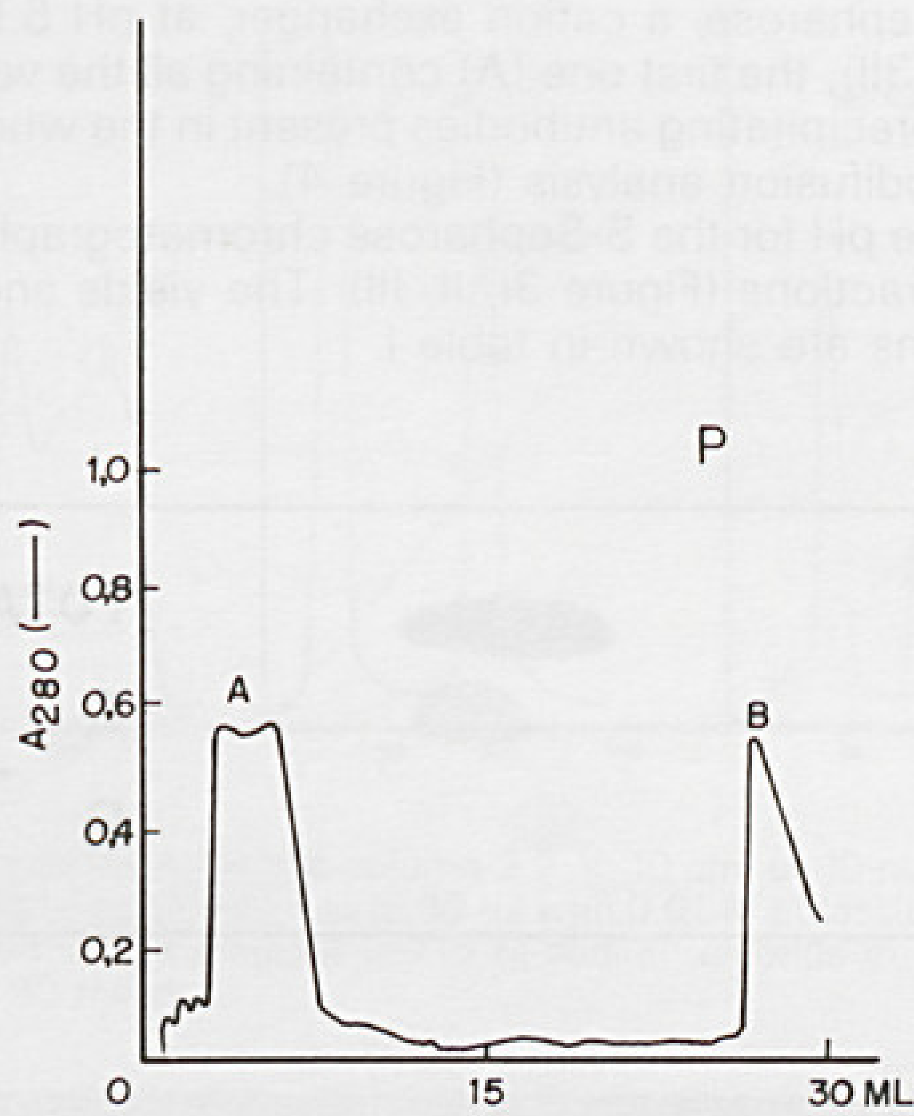
Chromatographic purification of hyperimmune anti-Crotalus venom plasma and serum

In attempting to purify anti-*Crotalus* venom plasma and serum, affinity chromatography was performed by using Cibracon blue-agarose, which is known to bind albumin but not globulins[10]. When using ''Matrex-blue'', the blue agarose produced by Amicon, two fractions were unexpectedly separated from the globulin fraction of plasma (P) and of serum (S) (Figure 1P and 1S) and only the second globulin fraction (B) had a venom neutralizing activity. When Matrex-blue was replaced by blue-agarose or blue-Sepharose from other sources, the separation of the globulins did not occur. Apparently, the difference among those resins is due to the Cibracon-blue used for their preparation, as this dye is not a chemically defined product.

On SDS-acrylamide gel electrophoresis the two serum globulin fractions seem to be identical, presenting two broad bands with an average molecular weight of 28.000-30.000 and 32.000-35.000 respectively.

Immunodifusion shows that the bands present in both fractions are heavy and light γ -globulin chains.

Electrophoresis on cellulose acetate showed that the two fractions have different mobility (Figure 2) thus differing in their electrical charges. This suggested that it would be possible to separate them by ion-exchange chro-



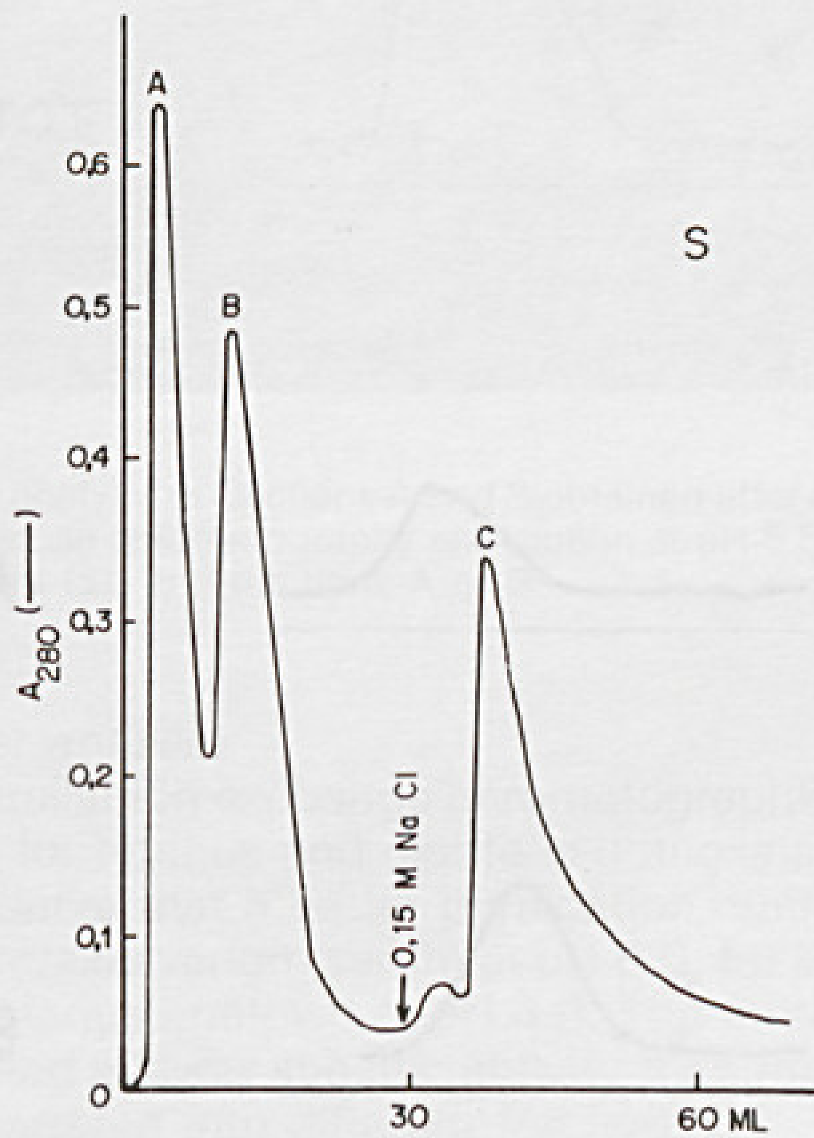


Fig. 1. Chromatography of plasma (P) or serum (S) on a Matrex-blue agarose column (2.5 x 10cm). 10 ml of horse hyperimmune plasma or serum containing 0.7 g and 0.32 g of protein respectively, were diluted to 30 ml with 1mM potassium phosphate buffer pH 6.8. Plasma and serum globulins were eluted with the same phosphate buffer, and plasma albumin with 0.15 M sodium chloride. A: globulin sub-fraction 1; B: globulin sub-fraction 2; C: albumin.

matography. S-Sepharose, a cation exchanger, at pH 5.5, separated two fractions (Figure 3II), the first one (A) containing all the venom neutralizing activity and the precipitating antibodies present in the whole serum, detectable by immunodifusion analysis (Figure 4).

By varying the pH for the S-Sepharose chromatography it was possible to obtain more fractions (Figure 3I, II, III). The yields and purifications of these preparations are shown in table I.

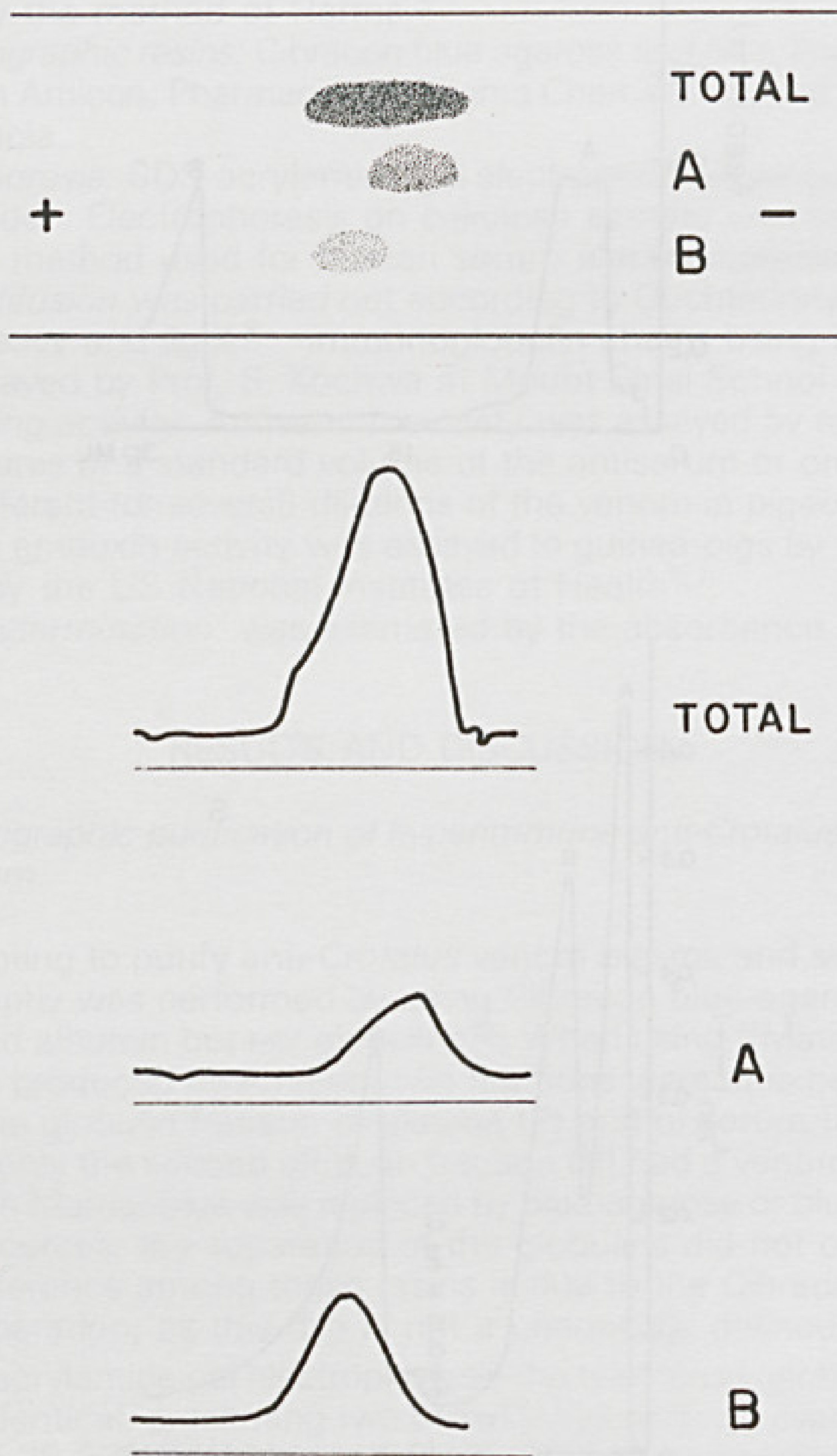


Fig. 2. Cellulose acetate electrophoresis of the two serum globulin fractions obtained after fractionation of anti-*Crotalus* venom serum by Matrex-blue agarose.

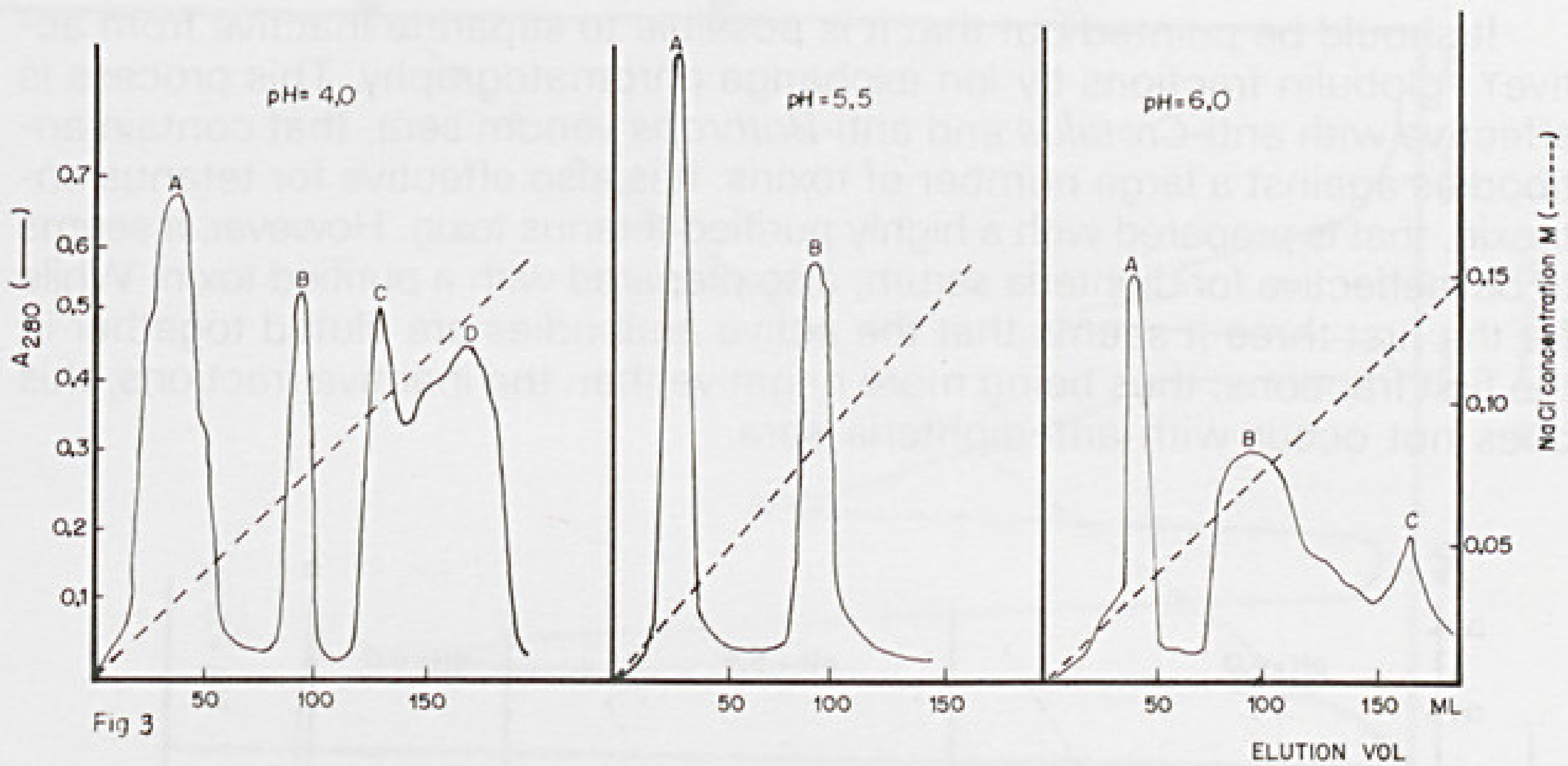


Fig. 3. Chromatogram on S-Sepharose column 2.2 × 10 cm, of 10 ml of anti-*Crotalus* serum, containing 0.32 g of protein diluted to 30 ml with 0.01 M potassium phosphate buffer at indicated pH, eluted with a gradient 0-0.15 M sodium chloride in the same buffer, for 4 hours, at a rate of 80 ml/hour.

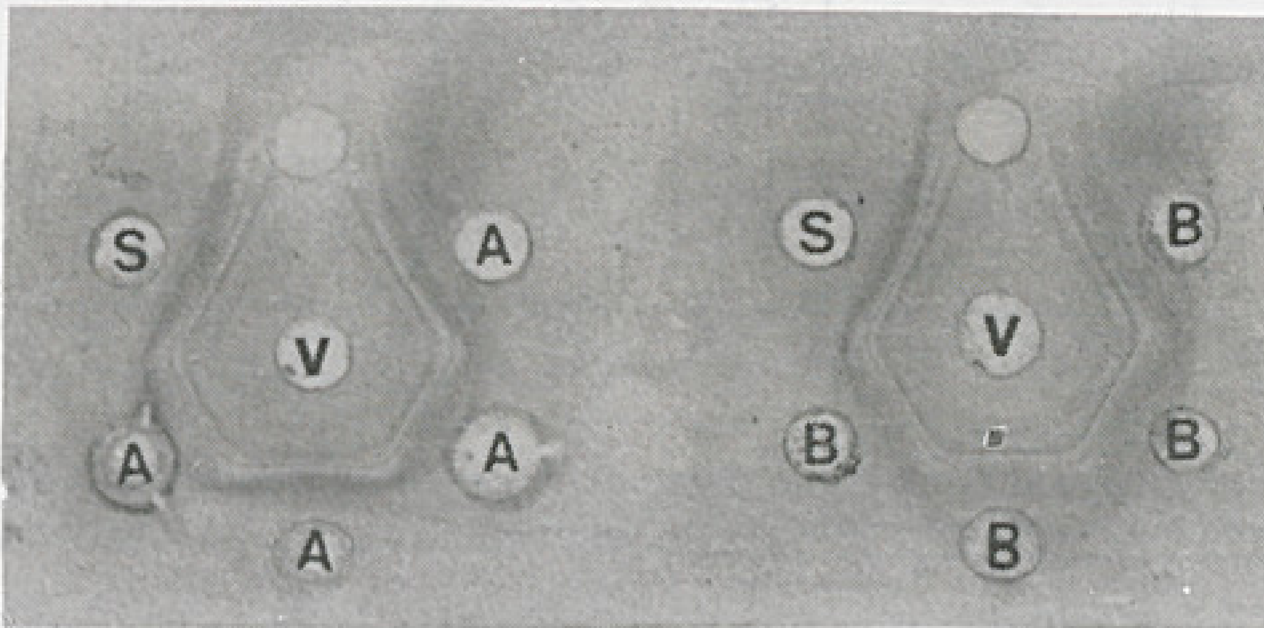


Fig. 4. Immunodifusion analysis of fractions A and B obtained after chromatography of anti-crotalic sera by S-sepharose chromatography and elution at pH 5.5 The wells contain venom (V), anti-crotalic sera (S), and fractions A or B.

Purification of other antisera

Table I shows similar ion exchange chromatographies for anti-*Bothrops* venom serum, and for tetanus and diphteria antitoxins (figures 5, 6, 7). It is interesting to observe that a better purification, with a good yield, was obtained for anti-*Crotalus* venom serum at pH 6.0, for anti-*Bothrops* serum at pH 5.5 and for tetanus antitoxin at pH 4.0. The fractions obtained from diphteria antitoxin had a lower specific activity than the whole serum, and the activity was scattered into different fractions.

The treatment of snakebites requires the administration of 50 to 100ml of antivenom. As shown in this paper, almost half of the protein administered is inactive and it can be removed by a single column chromatography, allowing not just to remove inactive foreign proteins, but to reduce the volume of the antivenom administration.

It should be pointed out that it is possible to separate inactive from active γ -globulin fractions by ion exchange chromatography. This process is effective with anti-*Crotalus* and anti-*Bothrops* venom sera, that contain antibodies against a large number of toxins. It is also effective for tetanus antitoxin, that is prepared with a highly purified tetanus toxin. However, it seems to be ineffective for diphteria serum, also prepared with a purified toxin. While for the first three it seems that the active antibodies are eluted together in the first fractions, thus being more negative than the inactive fractions, this does not occur with anti-diphteria sera.

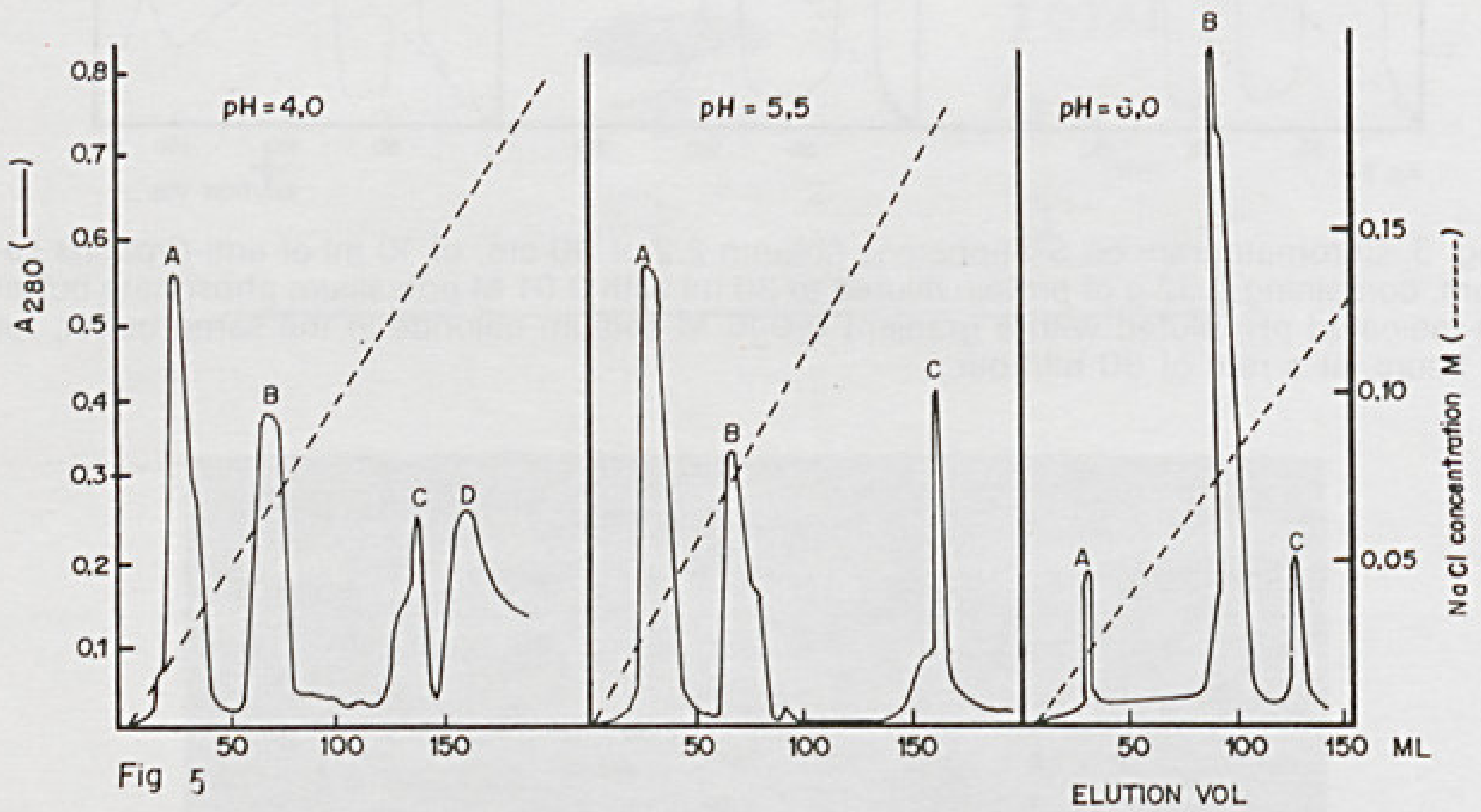


Fig. 5. Chromatography of 10 ml of anti-*Bothrops* venom serum containing 0.2 g of protein under the same conditions as described in Figure 3.

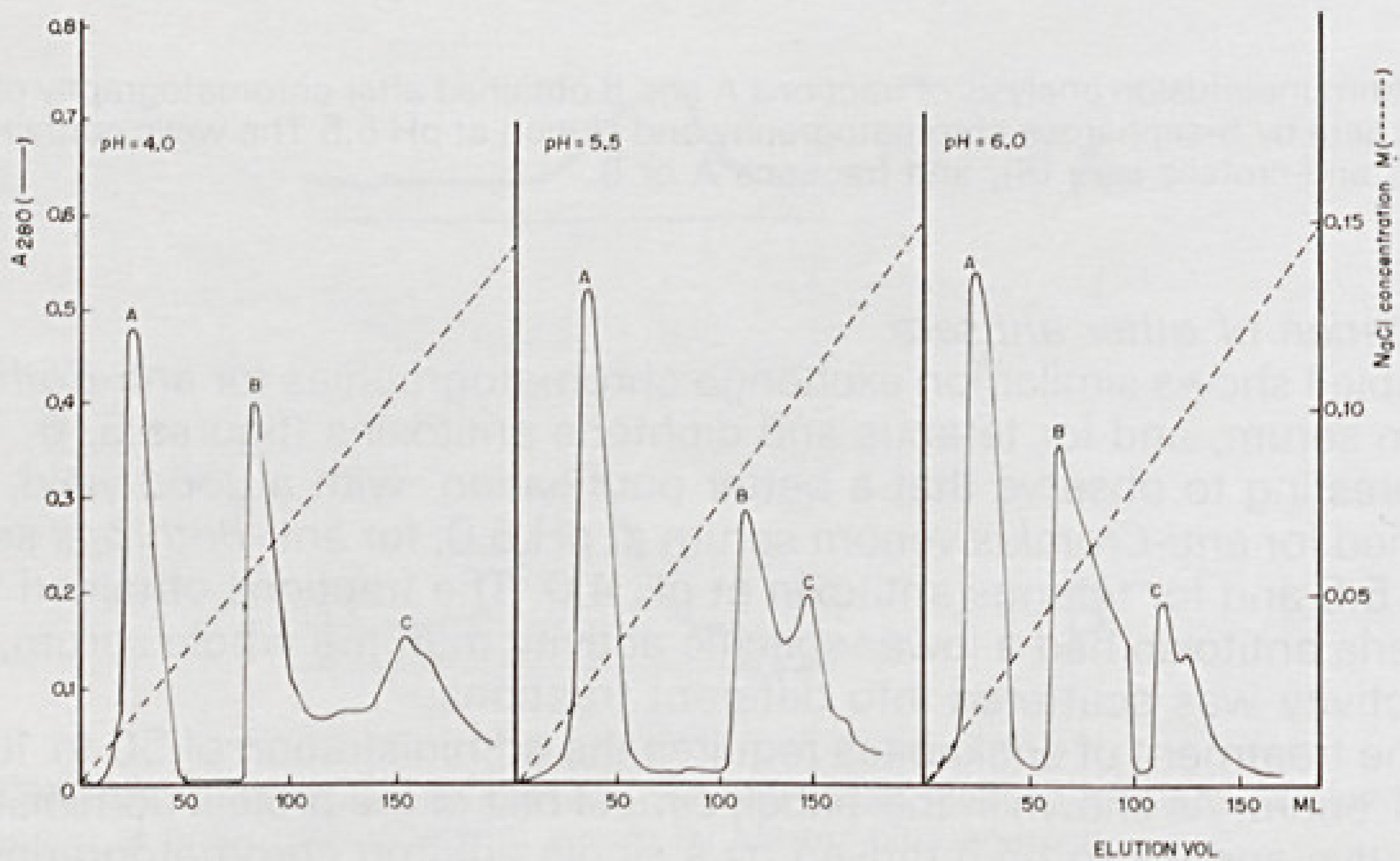


Fig. 6. Chromatography of 10 ml of anti-tetanus toxin serum containing 0.60 g of protein under the same conditions as described in Figure 3.

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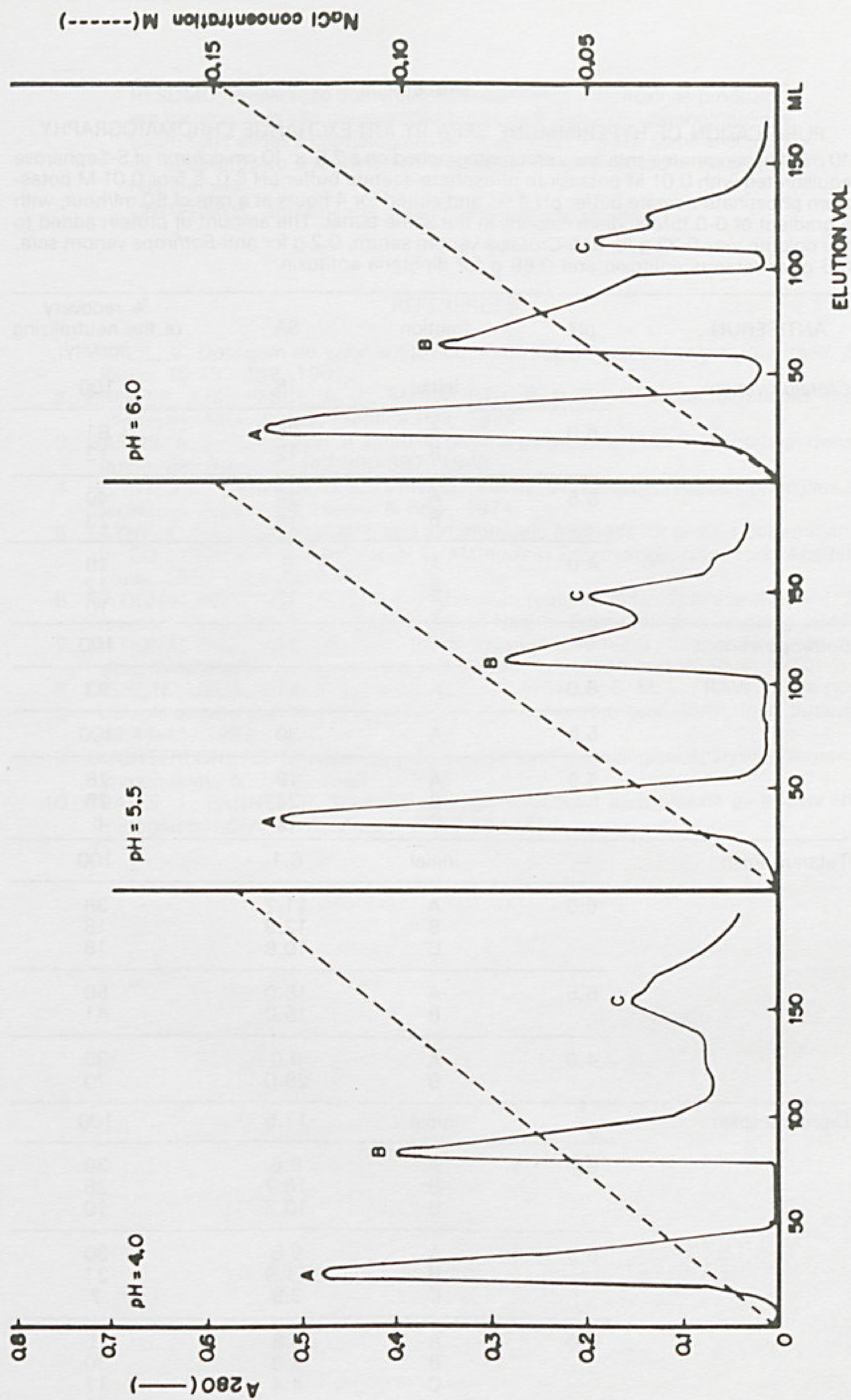

Fig. 7. Chromatography of 10 ml of anti-diphteria toxin serum containing 0.68 g of protein under the same conditions as described in Figure 3.

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TABLE 1

PURIFICATION OF HYPERIMMUNE SERA BY ION-EXCHANGE CHROMATOGRAPHY

10 ml of hyperimmune sera were chromatographed on a 2.5 × 10 cm column of S-Sepharose equilibrated with 0.01 M potassium phosphate-acetate buffer pH 6.0, 5.5 or 0.01 M potassium phosphate-acetate buffer pH 4.0.. and eluted for 4 hours at a rate of 80 ml/hour, with a gradient of 0-0.15M sodium chloride in the same buffer. The amount of protein added to the column was 0.32 g for anti-*Crotalus* venom serum, 0.2 g for anti-*Bothrops* venom sera, 0.6 g for tetanus antitoxin and 0.68 g for diphteria antitoxin.

ANTISERUN	pH	fraction	SA	% recovery of the neutralizing activity
Crotalus venom	—	initial	15	100
	6.0	A	25	81
		B	12	12
	5.5	A	28	49
		B	14	23
	4.0	A	6	19
		B	12	12
		C	3	10
Bothrops venom	—	initial	11	100
	6.0	A	11	93
	5.5	A	30	100
	4.0	A	19	28
		B	24	25
		C	12	5
Tetanus toxin	—	initial	8.1	100
	6.0	A	11.7	38
		B	12.9	18
		C	10.8	18
	5.5	A	15.0	50
		B	15.0	41
	4.0	A	9.0	30
		B	28.0	70
Diphteria toxin	—	initial	17.5	100
	6.0	A	9.6	30
		B	16.7	25
		C	10.3	10
	5.5	A	9.5	30
		B	11.9	21
		C	3.8	7
	4.0	A	6.8	15
		B	8.0	30
		C	4.4	17

* Fractions not tabulated had less that 30%

SA — specific activity is the venom neutralizing activity/A_{280nm}

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RESUMO: Preparação comercial de antivenenos e antitoxinas produzidas em cavalos, que são preparações puras de F (ab)2 de imunoglobulinas, foram submetidas à cromatografia de troca iônica. Para os soros anticrotálico, antibotrópico e antitetânico é possível remover 40-60% de globulinas inativas.

UNITERMOS: antivenenos, tétano, anti-soro, antitoxinas.

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