Title	BIOCHEMICAL STUDIES ON THE INCREASE AND FORMATION OF ANTIBODIES WITH CHYMOPAPAIN IN VITRO : I. STUDIES ON THE INCREASE OF DIPHTHERIAL ANTITOXIC TITERS FROM THE PEPSIN-DIGESTED ANTITOXIC GLOBULINS
Author(s)	ITO, Tokiya
Citation	Japanese Journal of Veterinary Research, 2(4), 143-156
Issue Date	1955-01-31
DOI	https://doi.org/10.14943/jjvr.2.4.143
Doc URL	https://hdl.handle.net/2115/1662
Type	departmental bulletin paper
File Information	KJ00002372907.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

BIOCHEMICAL STUDIES ON THE INCREASE AND FORMATION OF ANTIBODIES WITH CHYMOPAPAIN IN VITRO

I. STUDIES ON THE INCREASE OF DIPHTHERIAL ANTITOXIC TITERS FROM THE PEPSIN-DIGESTED ANTITOXIC GLOBULINS

Tokiya ITÔ

Laboratory of Biochemistry, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan

(Received for Publication, Feb. 21, 1954)

PAULING has published an interesting theory on the structure and process of formation of antibodies which was developed from available informations about immunology and physico-chemistry. The prediction was made from this theory that antibodies could be manufactured in vitro from normal serum γ-globulins by use of a suitable physico-chemical method.

PAULING and CAMPBELL believed that they succeeded to manufacture antibodies in vitro by mild denaturation and renaturation of normal bovine serum γ-globulins in the presence of certain dyes (methyl blue and 1, 3-dihydroxy—2, 4, 6-tri (p-azophenylarsonic acid) benzene) and of antigens including the type III pneumococcal polysaccharide. The precipitates formed during these procedures were considered to contain antibodies, and those authors stated that on separating the antigens, they obtained the antibodies which react specifically with the antigens used to manufacture them. Later, FRIEDRICH-FREKSA and LOISELEUR have published the papers studying on the synthesis of antibodies. CAMPBELL has stated that these trials have been mostly negative although a few preparations gave slight specific reactivity with the antigens used.

As was the case with the much earlier works[1] reviewed in the Jonrnal of the American Medical Association, these studies do not present the full details of the control experiments necessary for a proper evaluation of their data, so that the identity of their preparations and unique "antibodies" is far from established.

The present author[4] has carried out and communicated his works on the increase of diphtherial antitoxic titers from low potent immune equine serum globulins, which were obtained at regular intervals during the progressive immunization process with diphtherial toxoid and toxin and on the formation of antitoxin from normal serum globulins using enzymatic methods.

Incubating the preliminarily pepsin-digested incomplete antitoxins, which were prepared from the immune sera, obtained at the initial stages of immunization, with addition of suitable amounts of purified oxidized chymopapain[5] and purified concentrated diphtherial toxin under suitable conditions of pH, Eh, temperature, and the presence of suitable amounts of oxidizing agents with continuous supply of hydrogen peroxide, the increase of the antitoxic titers has been proved not only by RAMON's flocculation test but by protective test using guinea pigs.

Moreover, incubating the heat-weak-alkali treated equine serum globulins, prepared from normal sera preliminarily containing natural antitoxins, under the essential same conditions and treatments as the above, it has been found that more or less 5% of the starting globulins is made into large molecular incomplete antitoxins, which specifically protect guinea pigs from the challenge of large amounts of the toxin.

On the other hand, preparing the normal equine globulins, those prepared from normal sera containing none of natural antitoxin, these trials were entirely negative.

In this paper, the studies on the increase of the antitoxic titers from the preliminarily pepsin-digested antitoxic globulins in vitro are described.

MATERIALS AND METHODS

1. Preparations of the Purified Native Equine Antitoxic Eu- and Pseudoglobulin Solutions

Five horses were subcutaneously injected daily with diphtherial toxoid solutions (Lf 20~35 u/ml) in the initial three weeks and with toxic solutions (Lf 30~45 u/ml) for the following 5 to 9 weeks. Every weeks 300~1,000 ml of sera (Lf 10~1,700 u/ml) were obtained throughout the progressive immunization. Antitoxic eu- and pseudoglobulin solutions (Lf 10~3,600 u/ml; protein-N 13~14 mg/ml) were prepared from every pooled sera by five-repeated fractionating precipitation with 1/3 and 1/2 saturation of ammonium sulfate, following dialysis against distilled water and concentration to the volumes equivalent to the corresponding starting serum protein concentrations.

2. Pre-digestion of Purified Native Equine Antitoxic Eu- and Pseudoglobulins with Pepsin

Antitoxic eu- and pseudoglobulin solutions were made down to pH 4.4 with 10 N. HCl. Adding a quantity of pepsin (Merck, 1:3,000) equivalent to 5% of the globulins with small amounts of ascorbic acid and cysteine, they were made down to Eh 200~250 mV. Incubating these mixtures at 37°C, the pH, Eh, amino-N, protein-N (10% trichloracetic acid precipitable) and RAMON's flocculation titers were

determined every 12 hours. In these experimental procedures, the proteolytic activity of the enzyme deceases after 72～108 hours, and a tendency of reversing turnover appears at the hour.

3. Incubation of the Pepsin-Digested Antitoxic Solutions with Chymopapain and Diphtherial Toxin

Catching the chance of the turnover, the mixtures were made up to pH 7.3 with NaOH. Adding a quantity of purified oxidized chymopapain[5] equivalent to 10% of the globulins with the purified concentrated diphtherial toxic solution (Lf 1,500～2,000 u/ml; protein-N 13～14 mg/ml; prepared with repeated fractionating precipitation with 3/4 to 1/1 saturation of ammonium sulfate from the culture filtrates consisting of broth and trypsin-digested beef media inoculated with *Corynebacterium diphtheriae* P.W. No. 8 Dairen for 9 days at 34°C, and further dialysis and concentration) equivalent to 10～30 times as much as the Lf titers of the antitoxin, the mixtures were made up to Eh 450～500 mV by the further addition of 0.6% hydrogen peroxide with small amounts of cystine, fumaric acid, $CoCl_2$ and $Fe_2(SO_4)_3$. These mixtures were incubated at 40°C with the continuous supply of hydrogen peroxide. During the incubation procedures, the same determinations (except only flocculation titers) as in the pre-digestion procedures were carried out every 12 hours. The highest titers of the protein-N were found in 48～84 hours.

4. The Fractionation of Antitoxin from the Papain-Incubated Mixtures

When the titers of the protein-N in the incubated mixtures reached the highest, these were made up to 60% saturation of ammonium sulfate at pH 8.0. The precipitates formed were collected and electrodialyzed using PAULI's electrodialysis apparatus, finally they were concentrated to the volumes equivalent to the corresponding starting native globulin solutions. The antitoxic titers of these final preparations were determined by RAMON's flocculation test and by protective test using guinea pigs.

5. The Determination of Nitrogen Values of the Toxin-Antitoxin Floccules

The determinations of the N values of the pure toxin-antitoxin floccules which precipitated incubating the preparations of every four progressive procedures, viz.: 0) the original antitoxic sera, A) the purified native antitoxic globulin solutions, B) pre-digested antitoxic solutions and C) the fractionated concentrated antitoxic solutions finally prepared from the chymopapain-incubated mixtures, mixing with the equivalent titers (preliminarily titrated by RAMON's quantitative flocculation test) of the standard toxic solutions (Lf 60 u/ml; L_+ 0.16 ml; toxic N 0.00046 mg per Lf) were made by Micro-Kjeldahl method, washing the formed floccules with physiological saline three times. Pure antitoxic N were calculated out by the following formula:

$$\text{Antitoxic N mg} = \frac{\text{Floccules N mg}}{\text{Lf}} - 0.00046\ \text{mg}^{(6)}$$

6. The Determination of the Antigenicity of Equine Serum Globulin of Each Preparations

Increase and Formation of Antibodies 147

TABLE. 1 *Changes of Antitoxic Titers, Pure Antitoxic N mg/Lf and Antigenicity during the Incubation Procedures*

SERUM No.	ORIG. SERA Lf u/ml	ORIG. SERA EHRLICH's u/ml	ORIG. SERA A. N. mg/Lf	FRACTION	PURIFD. GLOBULINS P.N. mg/ml	PURIFD. GLOBULINS Lf u/ml	PURIFD. GLOBULINS EHRLICH's u/ml	PURIFD. GLOBULINS A. N. mg/Lf	PURIFD. GLOBULINS Antigenicity*	PRE-DIGSTD. GLOBULIN Hrs.	PRE-DIGSTD. GLOBULIN P. N. mg/ml	PRE-DIGSTD. GLOBULIN Lf u/ml	PRE-DIGSTD. GLOBULIN EHRLICH's u/ml	PRE-DIGSTD. GLOBULIN A.N. mg/Lf	PRE-DIGSTD. GLOBULIN Antigenicity*	FURTHER INCUBTD. Hrs.	FURTHER INCUBTD. Lf u/ml	FURTHER INCUBTD. EHRLICH's u/ml	FURTHER INCUBTD. A.N. mg/Lf	FURTHER INCUBTD. Antigenicity*
1130-1	10	15	0.0064	Ps.	13.8	10	20	0.0066	--	108	13.4	10	20	0.0016	—	72	70	80	0.0011	—
1130-2	25	—	0.0067	Ps.	14.4	70	—	0.0063	2400	96	13.3	70	—	0.0017	80	96	400	—	0.0008	16
1130-3	45	—	0.0063	Ps.	13.8	150	—	0.0062	2400	84	13.2	150	—	0.0017	64	72	950	—	0.0011	16
1130-4	65	100	0.0053	Ps.	13.7	200	350	0.0038	2400	96	13.3	200	350	0.0015	64	72	1300	1400	0.0011	16
1130-5	150	200	0.0054	Ps.	14.2	550	650	0.0033	2000	72	13.3	550	600	0.0015	64	72	1550	1450	0.0012	8
1130-6	400	—	0.0020	Ps.	13.9	750	—	0.0017	—	84	13.6	700	—	0.0014	—	48	650	—	0.0012	—
1130-7	500	—	0.0022	Ps.	13.8	1250	—	0.0016	2400	84	13.3	1200	—	0.0014	64	72	1300	—	0.0011	16
1130-8	900	—	0.0016	Ps.	13.9	2500	—	1.0016	—	72	15.5	2500	—	0.0010	—	84	2300	—	0.0010	—
1135-1	10	—	0.0072	Ps.	14.0	10	—	0.0063	—	96	13.4	10	—	0.0018	—	84	70	—	0.0013	—
1135-2	20	—	0.0074	Ps.	13.8	20	—	0.0066	2400	96	13.4	10	—	0.0019	80	72	130	—	0.0013	16
1135-5	400	350	0.0035	Ps.	13.9	350	400	0.0016	2400	84	13.4	350	400	0.0011	64	72	500	350	0.0012	16
1135-8	1100	—	0.0016	Ps.	14.2	2900	—	0.0015	2400	84	13.8	2800	—	0.0011	—	84	2500	—	0.0009	—
1138-1	10	25	0.0065	Ps.	14.4	15	30	0.0063	2400	96	13.8	15	30	0.0015	64	84	95	105	0.0010	12
1138-3	30	—	0.0052	Ps.	13.9	30	—	0.0038	—	96	13.3	30	—	0.0017	—	72	180	—	0.0011	—
1138-5	150	—	0.0034	Ps.	14.2	550	—	0.0032	2400	96	13.8	550	—	0.0009	120	84	2100	—	0.0009	12
1138-7	450	—	0.0018	Ps.	14.2	1500	—	0.0018	—	96	13.6	1500	—	0.0013	—	48	1600	—	0.0011	—
1141-1	15	—	0.0072	Ps.	14.4	10	—	0.0066	2400	96	13.4	10	—	0.0018	—	72	60	—	0.0013	12
1141-2	30	—	0.0066	Ps.	13.9	25	—	0.0064	—	72	13.5	25	—	0.0020	—	84	150	—	0.0013	—
1141-6	1700	—	0.0016	Ps.	13.8	3600	—	0.0016	2400	72	13.3	3600	—	0.0010	—	72	3200	—	0.0009	12

Rabbit	Sample																				
1141	-1	15	—	0.0072	Ps.	14.2	15	—	0.0067	2400	96	13.6	15	—	0.0019	64	84	120	—	0.0012	16
	-4	60	—	0.0034		14.2	70	—	0.0033	—	84	13.5	70	—	0.0014	—	84	190	—	0.0011	—
	-8	1400	—	0.0016		13.9	3800	—	0.0017	—	72	13.3	3800	—	0.0011	—	60	3500	—	0.0011	—
1130	-1	10	15	0.0064	Eu.	14.2	20	—	0.0086	—	96	13.3	20	—	0.0023	—	84	160	—	0.0014	—
	-2	25	—	0.0067		14.2	30	—	0.0082	—	96	13.5	30	—	0.0020	—	72	180	—	0.0013	—
	-4	65	100	0.0053		13.8	70	90	0.0063	2400	96	13.7	70	90	0.0019	64	84	500	470	0.0012	12
	-7	500	—	0.0022		13.7	70	—	0.0034	—	84	13.6	70	—	0.0018	—	72	200	—	0.0012	—
	-8	900	—	0.0016		13.6	90	—	0 0030	—	84	13.6	90	—	0.0014	—	84	150	—	0.0011	—
1135	-1	10	—	0 0072	Eu.	14.3	15	—	0.0090	—	96	13.1	15	—	0.0016	—	84	120	—	0.0014	—
	-2	20	—	0.0074		14.2	35	—	0.0085	—	96	13.6	35	—	0.0021	—	72	210	—	0.0009	—
	-5	400	—	0.0016		13.8	200	300	0.0038	2400	108	13.2	200	300	0.0013	64	72	1100	1200	0.0009	12
1138	-1	10	25	0.0065	Eu.	14.1	10	—	0.0072	—	108	13.4	10	—	0.0017	—	84	80	—	0.0012	—
	-5	150	—	0.0034		13.8	200	—	0.0038	—	84	13.4	200	—	0.0012	—	84	950	—	0.0010	—
1135	-1	10	—	0.0072	Ps.	14.0	10	—	0.0063	—	—	—	—	—	—	—	96	10	—	0.0060	—
	-5	400	350	0.0035		13.9	350	400	0.0016	2400	—	—	—	—	—	—	120	350	—	0.0016	—
1138	-2	30	—	0.0052	Ps.	13.9	30	—	0.0038	—	—	—	—	—	—	—	120	30	—	0.0032	—
1135	-2	20	—	0.0074	Eu.	14.2	35	—	0.0085	—	—	—	—	—	—	—	144	35	—	0.0076	—
1138	-3	30	—	0.0052	Ps.	13.9	30	—	0.0032	—	96	13.3	30	—	0.0017	—	96	20	—	0.0014	—
1135	-2	20	—	0.0074	Eu.	14.2	35	—	0.0085	—	96	13.6	35	—	0.0021	—	96	20	—	0.0042	—

* These values indicate the dilution degree of the preparations stating the positive limits of precipitin reaction with anti-equine serum globulin rabbit serum.

By the determination of the dilution degree of these prepared solutions stating the positive limits of precipitin reaction with anti-equine serum globulin rabbit serum, the antigenicity of equine serum globulin of those preparations was compared with at the same concentration of protein.

RESULTS

During the incubation procedures of pre-digestion of purified native equine antitoxic eu- and pseudoglobulins with pepsin, pH and Eh went downwards gradually at the beginning, and turned slightly upwards in 72~108 hours. Amino-N increased to 4~6 times within these hours. The protein-N, decreasing gradually by about 5% at the beginning, displayed a slight tendency toward a reversing turnover in 72~108 hours. There were no decrease in Lf titers during the procedures.

Incubating the pepsin-digested antitoxic solutions added with purified oxidized chymopapain, purified concentrated diphtherial toxic solution, hydrogen peroxide, cystine, fumaric acid, $CoCl_2$ and $Fe_2(SO_4)_3$, pH and Eh went gradually upwards, reaching the peak 48~84 hours.

With the preparations, which, after the final incubation with chymopapain and toxin equivalent to 20~30 times as much as the Lf titers of the antitoxic solutions, were prepared from the mixtures by the fractionating precipitation with 60% saturation of ammonium sulfate and following electrodialysis and concentration to the equivalent volumes of the corresponding starting native globulin solutions, it was found that the antitoxic titers have increased to 4~7 times as great values as those of the starting native antitoxic globulin solutions prepared from low potent immune sera (Lf 10~150 u/ml; 20~200 EHRLICH's units/ml) obtained at the initial stages of the immunization (in 1~5 weeks) (Figs. 1~5, 9, 10, 17, 18, 20, 21, 23~32).

Pure antitoxic nitrogen values were found:

0) 0.003 ~0.009 mg/Lf A) 0.003 ~0.009 mg/Lf
B) 0.0014~0.002 " C) 0.0009~0.0013 "

The comparative antigenicity of equine serum globulin of those preparations showed:

B) 1/16~1/35* C) 1/130~1/150*

*The antigenicity of every A) was signified as 1.

On the other hand, with the preparations, which were prepared by the same procedures as the above, starting from the antitoxic solutions prepared from the higher potent immune sera (Lf 400~1,700 u/ml; 400~1,700 EHRLICH's units/ml) obtained at the advanced stages of the immunization (in 6~9 weeks), no increase of the antitoxic titers was found (Figs. 6~8, 11, 12, 16, 19 and 22).

Incubating the purified native antitoxic globulin solutions without pepsin-pre-digestion, with oxidized chymopapain, diphtherial toxic solutions and hydrogen peroxide with other oxidizing agents under the routine procedure, no increase of the antitoxic titers was found (Figs. 33~36).

FIG. 1. *Antidiphtherial Serum No. 1130-1 Pseudoglobulin*

FIG. 2. *Antidiphtherial Serum No. 1130-2 Pseudoglobulin*

FIG. 3. *Antidiphtherial Serum No. 1130-3 Pseudoglobulin*

FIG. 4. *Antidiphtherial Serum No. 1130-4 Pseudoglobulin*

FIG. 5. *Antidiphtherial Serum No. 1130-5 Pseudoglobulin*

FIG. 6. *Antidiphtherial Serum No. 1130-6 Pseudoglobulin*

FIG. 7. *Antidiphtherial Serum No. 1130-7 Pseudoglobulin*

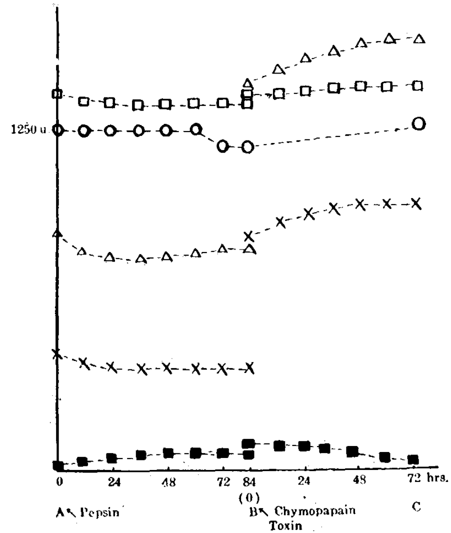


FIG. 8. *Antidiphtherial Serum No. 1130-8 Pseudoglobulin*

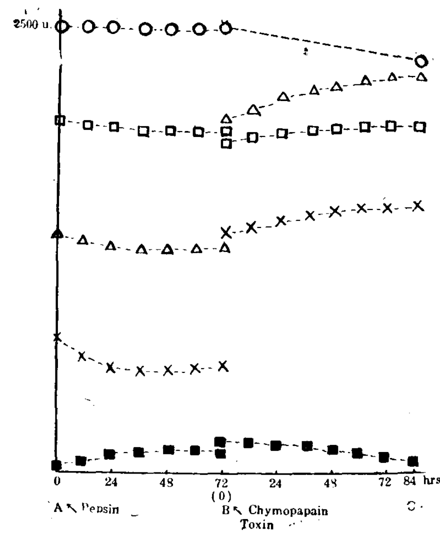


FIG. 9. *Antidiphtherial Serum No. 1135-1 Pseudoglobulin*

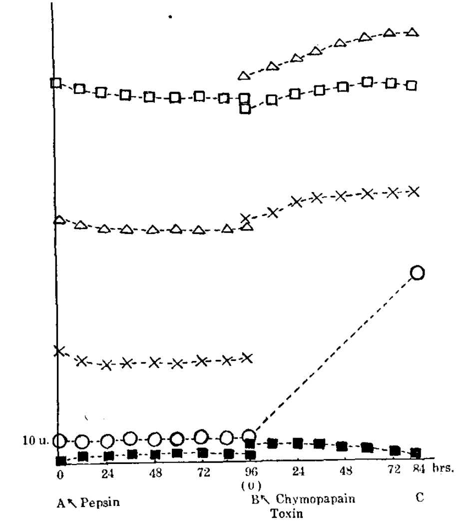


FIG. 10. *Antidiphtherial Serum No. 1135-2 Pesudoglobulin*

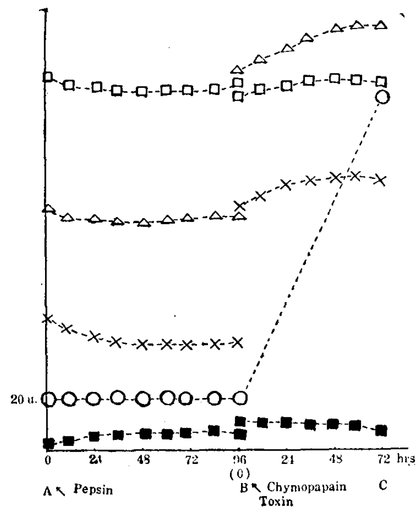


FIG. 11. *Antidiphtherial Serum No. 1135-5 Pseudoglobulin*

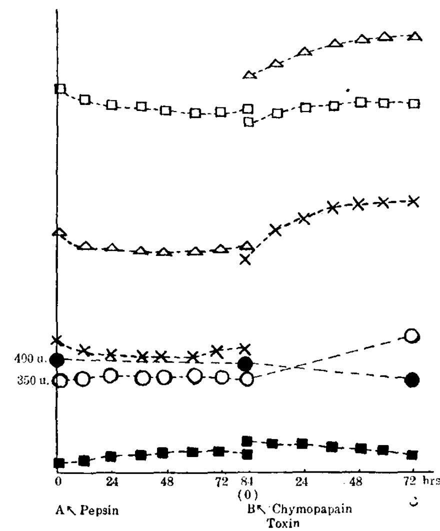


FIG. 12. *Antidiphtherial Serum No. 1135-8 Pseudoglobulin*

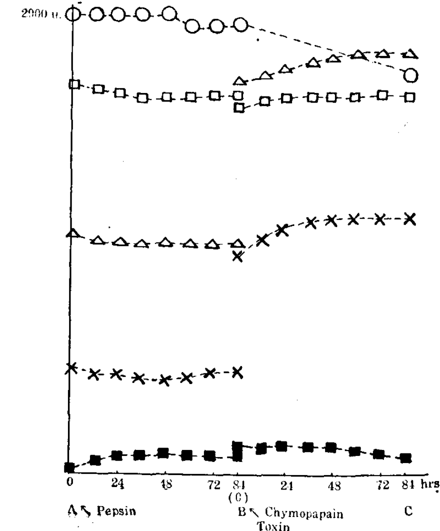


FIG. 13. *Antidiphtherial Serum No. 1138-1 Pseudoglobulin*

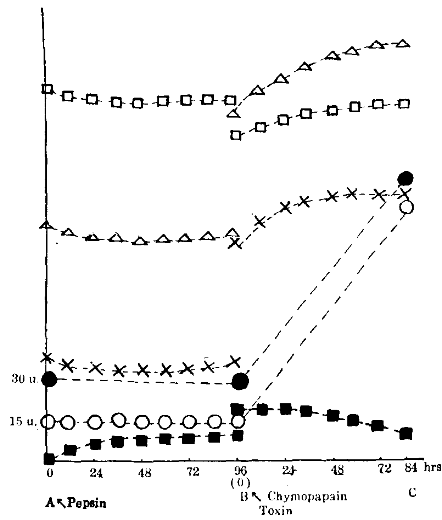


FIG. 14. *Antidiphtherial Serum No. 1138-3 Pseudoglobulin*

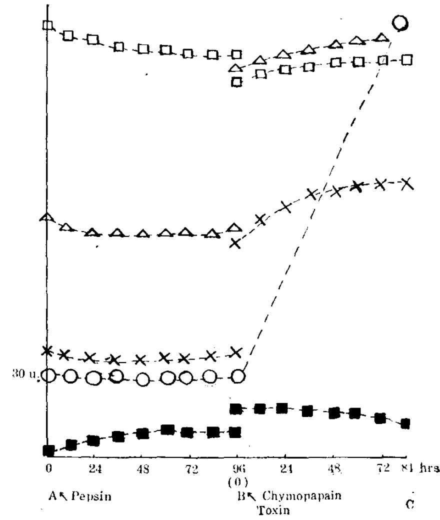


FIG. 15. *Antidiphtherial Serum No. 1138-5 Pseudoglobulin*

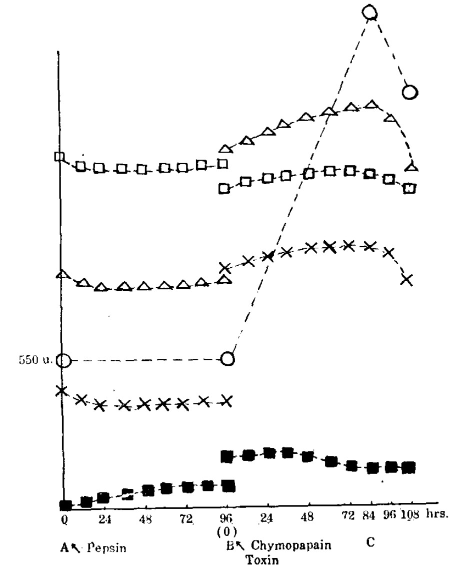


FIG. 16. *Antidiphtherial Serum No. 1138-7 Pseudoglobulin*

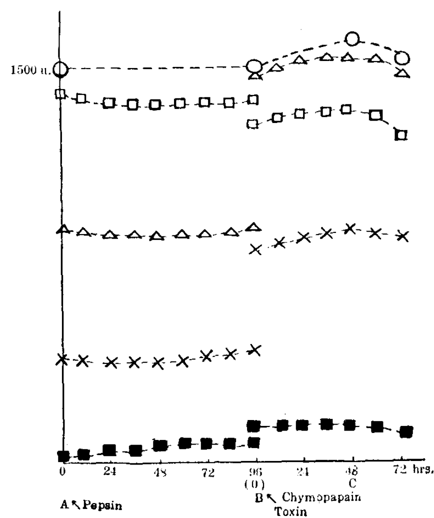


FIG. 17. *Antidiphtherial Serum No. 1141-1 Pseudoglobulin*

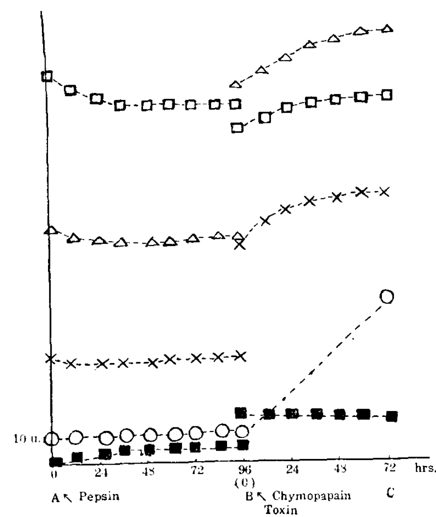


FIG. 18. *Antidiphtherial Serum No. 1141-2 Pseudoglobulin*

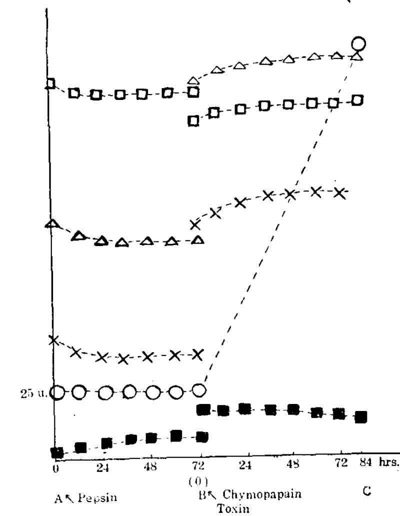


FIG. 19. *Antidiphtherial Serum No. 1141-6 Pseudoglobulin*

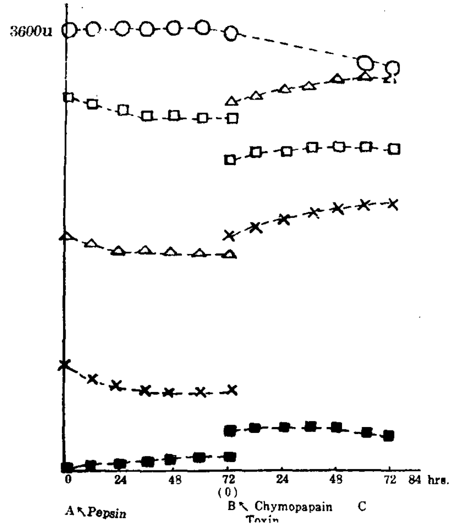


FIG. 20. *Antidiphtherial Serum No. 1144-1 Pseudoglobulin*

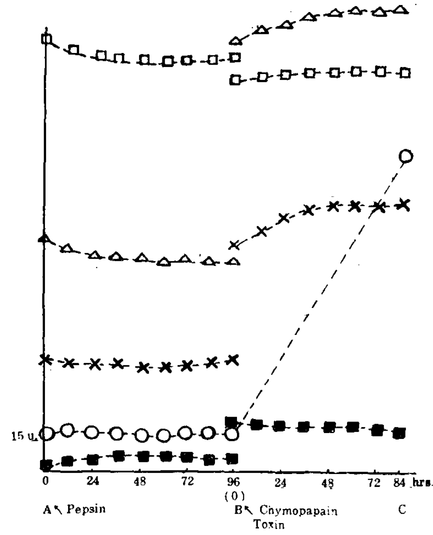


FIG. 21. *Antidiphtherial Serum No. 1144-4 Pseudoglobulin*

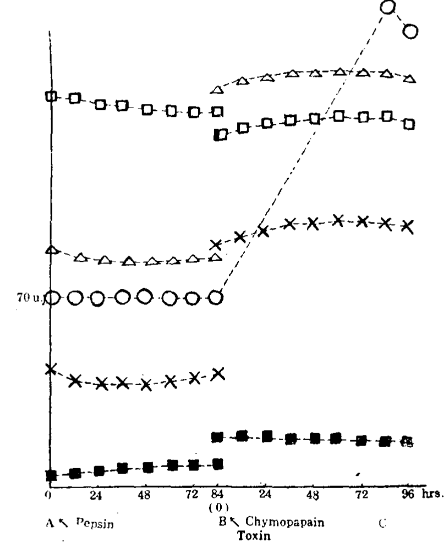


FIG. 22. *Antidiphtherial Serum No. 1144-8 Pseudoglobulin*

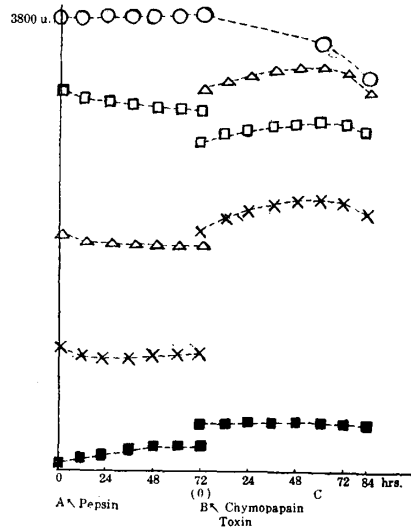


FIG. 23. *Antidiphtherial Serum No. 1130-1 Euglobulin*

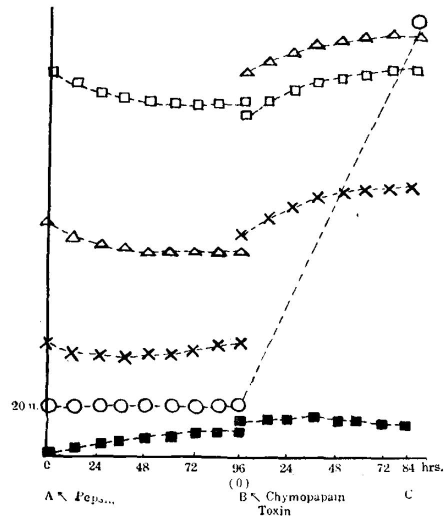


FIG. 24. *Antidiphtherial Serum No. 1130-2 Euglobulin*

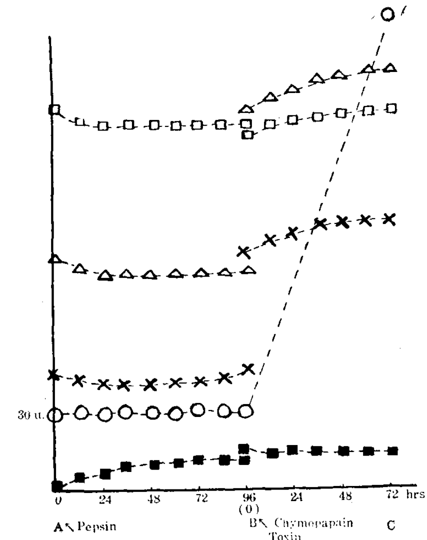


FIG. 25. *Antidiphtherial Serum No. 1130-4 Euglobulin*

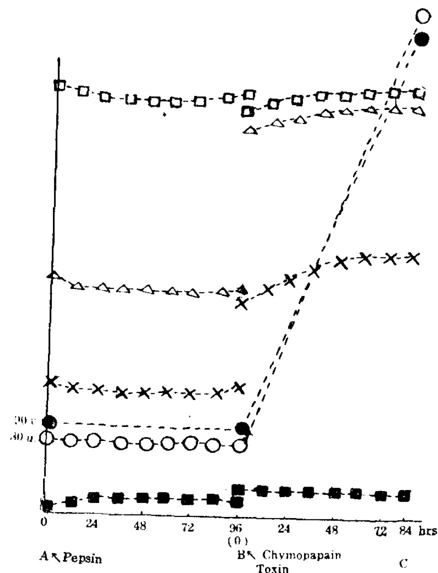


FIG. 26. *Antidiphtherial Serum No. 1130-7 Euglobulin*

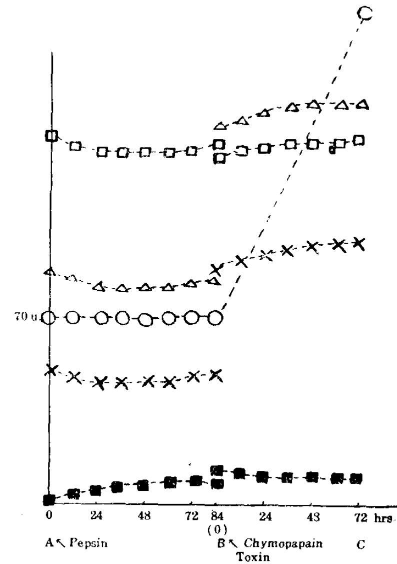


FIG. 27. *Antidiphtherial Serum No. 1130-8 Euglobulin*

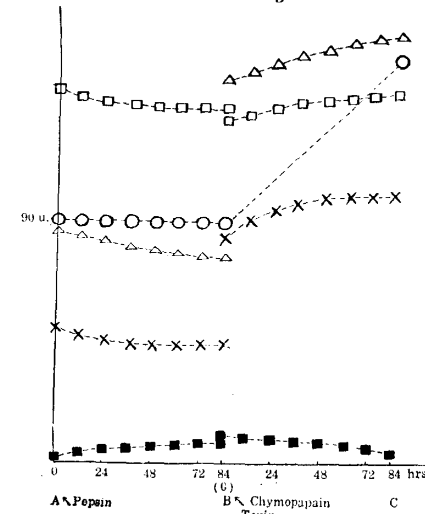


FIG. 28. *Antidiphtherail Serum No. 1135-1 Euglobulin*

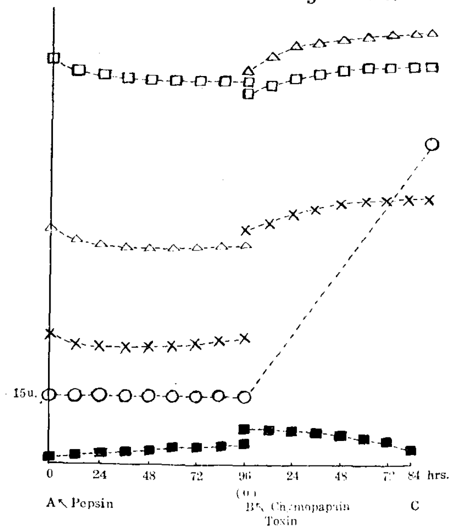


FIG. 29. *Antidiphtherial Serum No. 1135-2 Euglobulin*

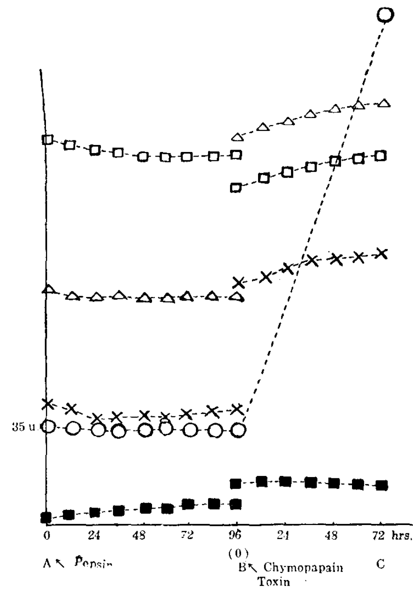


FIG. 30. *Antidiphtherial Serum No. 1135-5 Euglobulin*

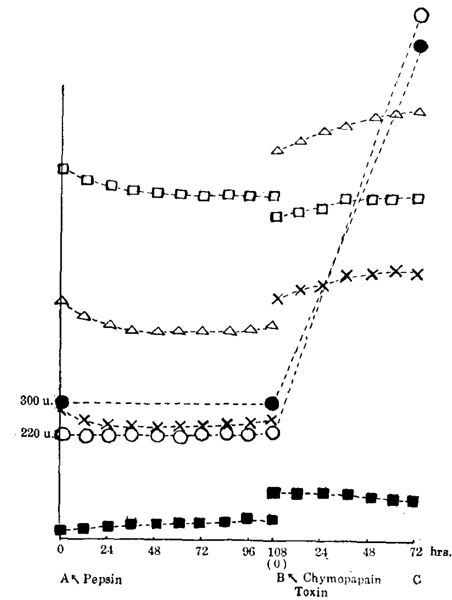


FIG. 31. *Antidiphtherial Serum No. 1138-1 Euglobulin*

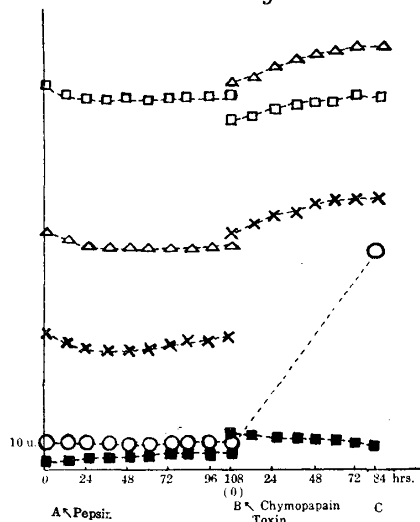


FIG. 32. *Antidiphtherial Serum No. 1138-5 Euglobulin*

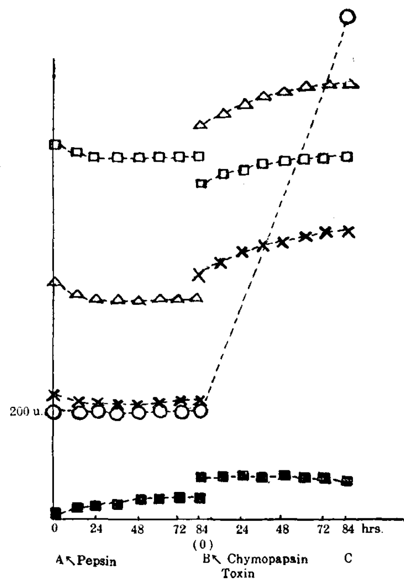


FIG. 33. *Antidiphtherial Serum No. 1135-1 Pseudoglobulin*

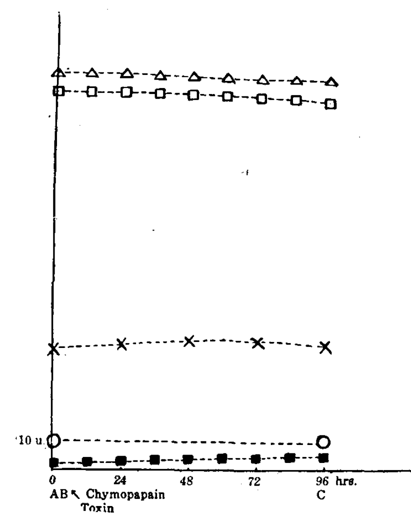


FIG. 34. *Antidiphtherial Serum No. 1135-5 Pseudoglobulin*

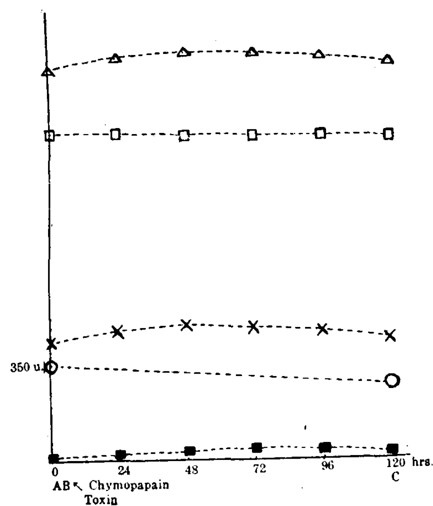


FIG. 35. *Antidiphtherial Serum No. 1138-2 Pseudoglobulin*

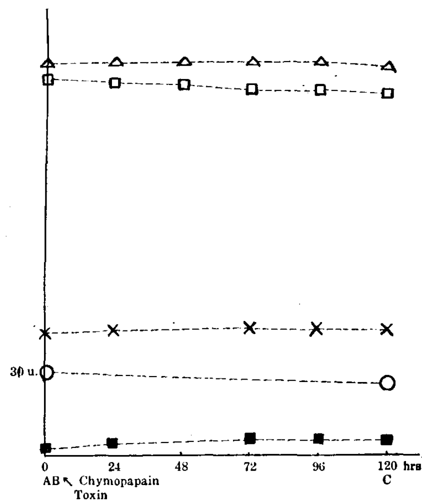
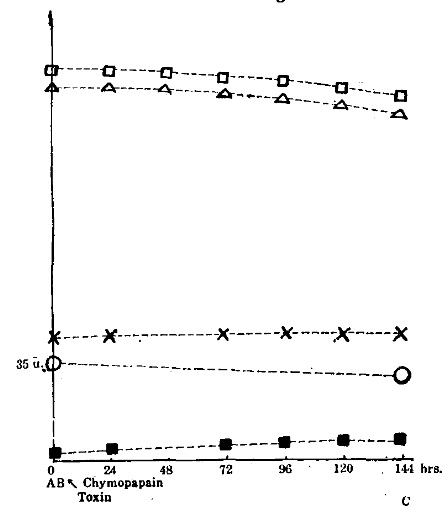


FIG. 36. *Antidiphtherial Serum No. 1135-2 Euglobulin*



Incubating the pepsin-digested antitoxic globulin solutions without the presence of oxidized chymopapain under the routine procedure, also no increase of antitoxic titers was found (Figs. 37 and 38).

FIG. 37. *Antidiphtherial Serum No. 1138-3 Pseudoglobulin*

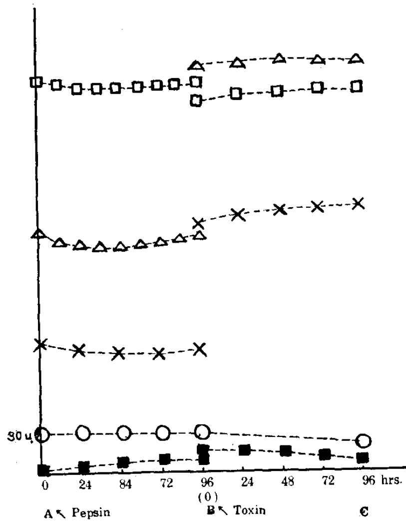
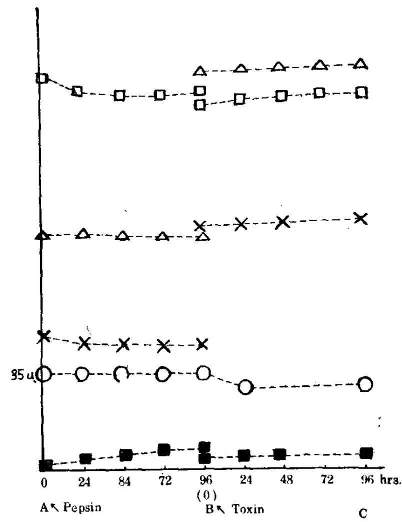


FIG. 38. *Antidiphtherial Serum No. 1135-3 Euglobulin*



DISCUSSION

The present data indicate the fact that fragmented small diphtherial antitoxins produced from the large incomplete antitoxin molecules with pepsin digestion, those produced in horses at the initial stages of the immunization with diphtherial toxoid and toxin, are converted into small complete antitoxins by incubating procedures with suitable amounts of oxidized chymopapain and toxin, under certain definite convenient conditions of pH, Eh and temperature in the presence of oxidizing agents.

It is considered that the fragments, produced from the large incomplete antitoxins with pepsin digestion, are converted into small complete antitoxins being affected by the antigenic action of the toxin and polymerizing action of the enzyme in the further incubating procedures.

Also, it is considered that the fragments, produced with pepsin digestion from the small complete antitoxins (antitoxic N 0.0016～0.0020 mg/Lf), those produced at the advanced stages of the immunization, have no other spaces to be converted into antitoxin newly in the further incubating procedures.

It is concluded that incubating the fragmented antitoxins produced with pepsin digestion from large incomplete antitoxins produced at the initial stages of the diphtherial immunization, in order to convert them into small complete antitoxin molecules, it is essentially necessary to prepare antigenic activity of toxin, polymerizing activity of enzyme under the suitable conditions of pH, Eh, temperature with supply of hydrogen peroxide and other oxidizing agents.

SUMMARY

Purified eu- and pseudoglobulin solutions of equine diphtherial antitoxic sera obtained at the initial stages of the immunization, were incubated with pepsin at 37°C, pH 4.4 and Eh 200~250 mV. In 72~108 hours, pH was adjusted to 7.3, and the solutions were incubated further mixed with purified oxidized chymopapain, purified concentrated diphtherial toxin being supplied hydrogen peroxide and other oxidizing agents at 40°C and Eh 450~500mV. In 48~84 hours, when the protein-N values reached their highest, the mixtures were made up to 60% saturation of ammonium sulfate at pH 8.0. The formed precipitates were electrodialyzed and finally concentrated to the volumes equivalent to those of the corresponding starting native globulin solutions. The antitoxic titers of these preparations were found to be 4 to 7 times as great as those of the each starting native globulins by RAMON's flocculation test and by protective test using guinea pigs. These preparations, prepared by these procedures, are consisted of largely small but complete avid and less antigenic antitoxins.

The author wishes to thank Prof. Dr. Koji ANDO for his aid and helpful suggestions, and Dr. K. MANAKO and Dr. R. OYAMA for their technical assistance.

REFERENCES

1) ANONYMOUS, (1942): *J. Am. Med. Assoc.*, **119**, 1376.
2) CAMPBELL, D. H. (1948): *Ann. Rev. Microbiol.*, **2**, 269.
3) FRIEDRICH-FREKSA, A. H. (1946): *Z. Naturforsch.*, **1**, 44.
4) ITÔ, T. (1950): *Med. & Biol.*, **17**, 42 (Japanese).
5) JANSEN, E. F. & A. K. BALLS (1941): *J. Biol. Chem.*, **137**, 459.
6) KABAT, E. A. (1943): *J. Immunol.*, **47**, 513; KABAT, E. A. (1949): Experimental Immunochemistry, Springfield, Illinois.
7) LOISELEUR, J. (1947): *Compt. rend. Acad. Sci.*, **224**, 687.
8) PAULING, L. (1940): *J. Am. Chem. Soc.*, **62**, 2643.
9) PAULING, L. & D. H. CAMPBELL (1942): *J. Exp. Med.*, **76**, 211.