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

II. STUDIES ON THE INCREASE OF DIPHTHERIAL ANTITOXIC TITERS FROM THE TRYPSIN-DIGESTED ANTITOXIC GLOBULINS

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(Received for Publication, Feb. 22, 1954)

In the preceding paper[1], studies on the increase of diphtherial antitoxic titers from the pepsin-digested antitoxic equine serum globulins was reported. Purified eu- and pseudoglobulins of equine antitoxic sera obtained at the initial stages of diphtherial 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 450~500 mV. In 48~84 hours, when the protein-N values reached the highest, the mixtures were made up to 60% saturation of ammonium sulfate. The formed precipitates were dialyzed and concentrated to the volumes equivalent to those of the corresponding starting native antitoxic globulin solutions. The antitoxic titers of these preparations were found to be 4 to 7 times as great as those of each starting antitoxin.

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

MATERIALS AND METHODS

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

Antitoxic eu- and pseudoglobulin solutions (Lf 10~400 u/ml; protein-N 13~14 mg/ml) were prepared from immune sera which were obtained from 5 horses (No. 1130, 1135, 1138, 1141 and 1144) at the initial stages of diphtherial immunization by the fractionating precipitation with ammonium sulfate as previously described.[1]

2. Pre-digestion of Purified Native Antitoxic Globulins with Trypsin

Antitoxic eu- and pseudoglobulin solutions were made up to pH 8.0 with 10 N. NaOH. Adding a quantity of trypsin (Kahlbaum, purified) 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, pH, Eh, amino-N, protein-N and RAMON'S flocculation titers were determined every 12 hours. In those experimental procedures, proteolytic activity of the enzyme declined after 72~96 hours showing a slight tendency of reversing turnover.

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

Catching the chance of the turnover, pH values of the mixtures were adjusted to 7.3 with NaOH. Addition was made of a quantity of purified oxidized chymopapain[2] equivalent to 10% of the globulins with the purified concentrated diphtherial toxic solutions (Lf 1,500~2,000 u/ml; prepared from the culture filtrates inoculated with *Corynebacterium diphtheriae* P. W. No. 8 Dairen by the method previously described) 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 a continuous supply of hydrogen peroxide. During the incubation procedures, the same determinations (except flocculation titers) as in the pre-digestion were carried out every 12 hours. The highest titers of the protein-N were found in 48~72 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, antitoxic globulins were precipitated with 60% saturation of ammonium sulfate and electro-dialyzed. Finally, they were concentrated to the volumes equivalent to the each 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 determination of N values of the toxin-antitoxin floccules which were formed incubating the preparations of every four progressive procedures, viz; O) 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 flocculation reaction) of the standard toxic solution (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 three times with physiological saline. Antitoxic N values were calculated out by the following formula:

$$\text{Antitoxic N mg} = \frac{\text{Floccules N mg}}{\text{Lf}} - 0.00046\ \text{mg}^{3)}$$

6. The Determimation of the Antigenicity of Equine Serum Globulin of Each Preparation

By the determination of the degree of dilution of these prepared solutions of progressive procedures stating the positive limits of precipitin test with anti-equine serum

globulin rabbit serum, the antigenicity of equine serum globulin of these preparations was compared with at the same concentration of protein.

Results

In the incubation during pre-digestion of purified native antitoxic globulins with trypsin, pH went downwards rapidly at the beginning and remained stationary at 7.0～7.2. Eh went downwards gradually at the beginning, and turned slightly upwards in 72～96 hours. Amino-N increased to 5～7 times within the hours. The protein-N, decreasing gradually by about 6～10% at the beginning, displayed a slight tendency towards a reversing turnover in 72～96 hours. There were no significant decreases in Lf titers during these procedures.

Incubating the trypsin-digested antitoxic globulin solutions 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 values went gradually upwards, reaching the peak in 48～72 hours. The protein-N, increasing slightly at the beginnig, attained equilibrium in 48～72 hours.

With the preparations, prepared from the mixtures after the final incubation by fractionating precipitation with 60% saturation of ammonium sulfate and concentration to the volumes equivalent to the each corresponding starting native globulin solutions, there was no increase of antitoxic titers found (Figs. 39～48).

FIG. 39. *Antidiphtherial Serum No. 1130-1 Pseudoglobulin*

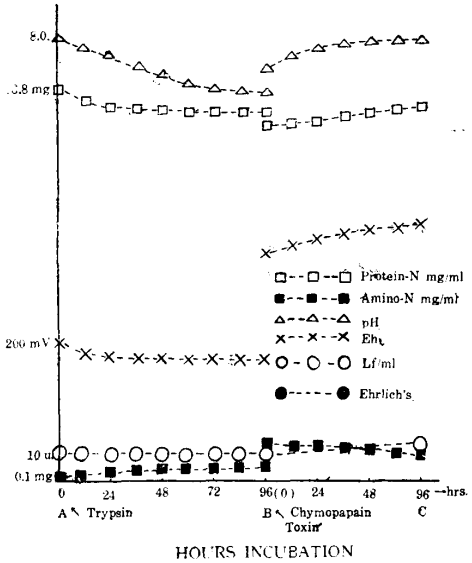
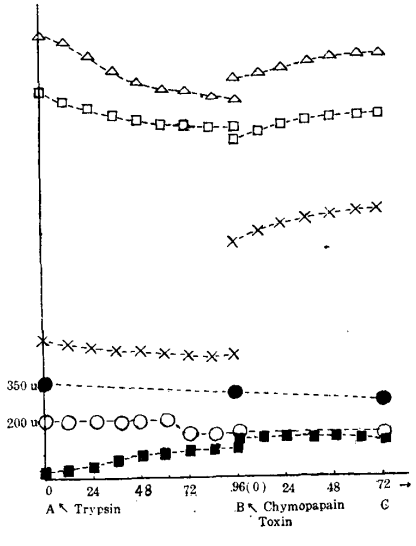


FIG. 40. *Antidiphtherial Serum No. 1130-4 Pseudoglobulin*



Antitoxic nitrogen values were found:

O) 0.0020～0.0068 mg/Lf　　A) 0.0017～0.0066 mg/Lf
B) 0.0011～0.0018 mg/Lf　　C) 0.0009～0.0014 mg/Lf

FIG. 41. *Antidiphtherial Serum No. 1130-6 Pseudoglobulin*

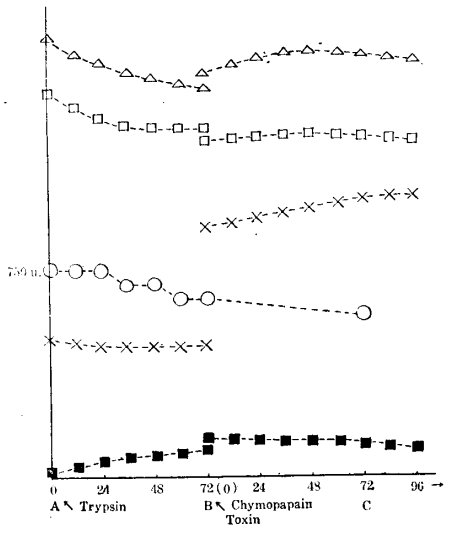


FIG. 42. *Antidiphtherial Serum No. 1135-4 Pseudoglobulin*

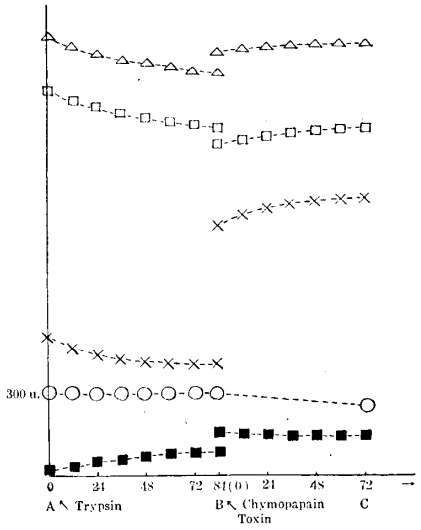


FIG. 43. *Antidiphtherial Serum No. 1138-1 Pseudoglobulin*

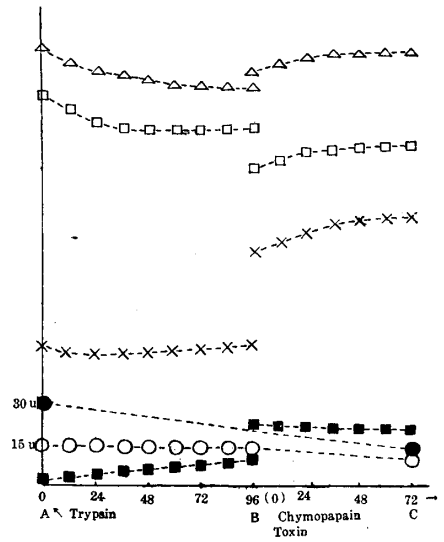
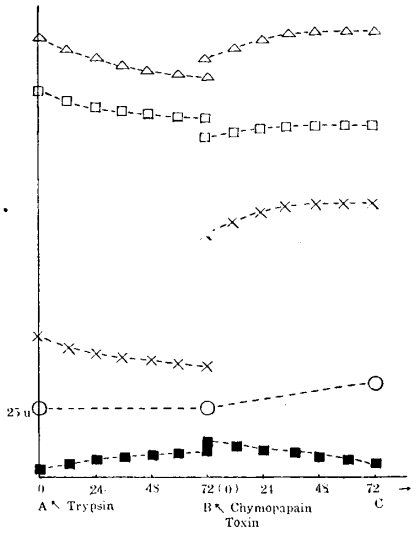


FIG. 44. *Antidiphtherial Serum No. 1141-2 Pseudoglobulin*



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

B) 1/40* C) 1/150*

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

FIG. 45. *Antidiphtherial Serum No. 1130-3 Euglobulin*

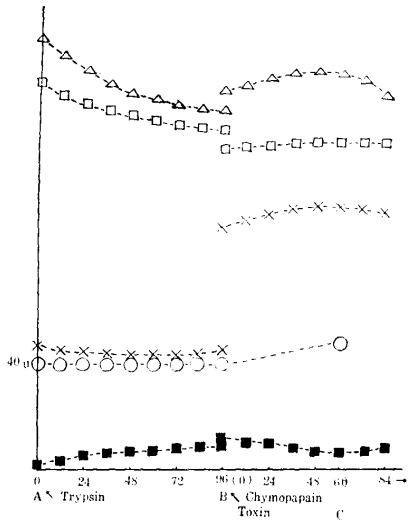


FIG. 46. *Antidiphtherial Serum No. 1130-5 Euglobulin*

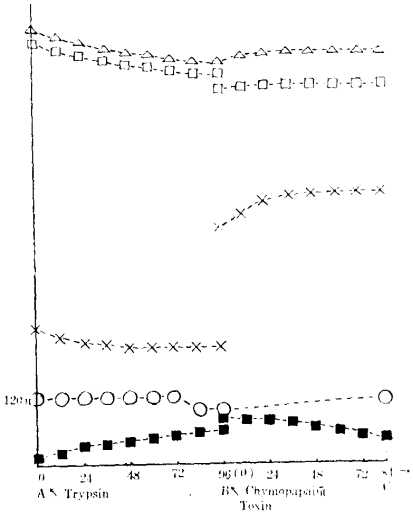


FIG. 47. *Antidiphtherial Serum No. 1135-4 Euglobulin*

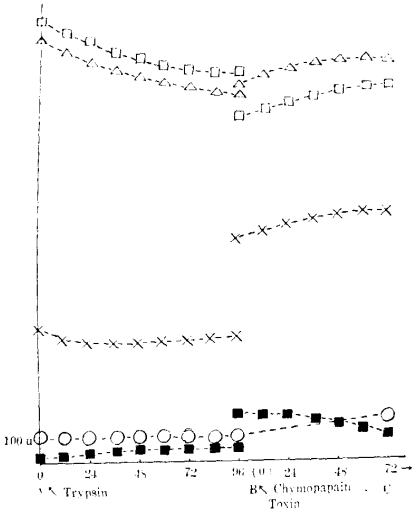


FIG. 48. *Antidiphtherial Serum No. 1144-2 Euglobulin*

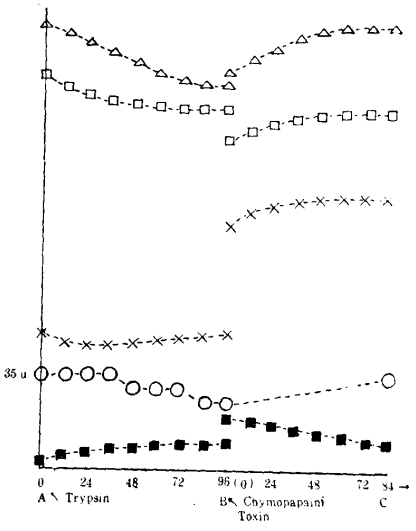


TABLE 2. *Changes of Antitoxic Titers, Pure Antitoxic N mg/Lf and Antigenicity during the Incubation*

SERUM NO.	ORIG. SERA			FRACTION	PURIFD. GLOBULINS					PRE-DIGSTD. GLOBULINS						FURTHER INCUBTD.				
	Lf u/ml	Ehrlich's u/ml	A. N. mg/Lf		P. N. mg/ml	Lf u/ml	Ehrlich's u/ml	A. N. mg/Lf	Anti-genicity*	Hrs.	P. N. mg/ml	Lf u/ml	Ehrlich's u/ml	A. N. mg/Lf	Anti-genicity*	Hrs.	Lf u/ml	Ehrlich's u/ml	A. N. mg/Lf	Anti-genicity*
1130—1	10	15	0.0064	Ps.	13.8	10	20	0.0066	·	96	12.8	10	·	0.0013	·	72	15	·	0.0010	·
1130—4	65	100	0.0053	Ps.	13.7	200	350	0.0038	2400	96	12.4	150	300	0.0011	64	72	150	250	0.0010	16
1130—6	400	·	0.0020	Ps.	13.9	750	·	0.0017	·	72	12.8	650	·	0.0012	·	72	600	·	0.0009	·
1135 —4	120	·	0.0045	"	14.0	300	·	0.0032	2400	84	12.5	300	·	0.0014	64	72	250	·	0.0010	16
1138 —1	10	25	0.0065	"	14.4	15	30	0.0063	·	96	12.9	15	·	0.0016	·	72	10	10	0.0012	·
1141 —2	30	·	0.0066	"	13.9	25	·	0.0064	·	72	12.9	25	·	0.0015	·	72	35	·	0.0012	·
1130—3	40	·	0.0064	Eu.	14.1	40	·	0.0062	·	96	12.5	40	·	0.0017	·	60	50	·	0.0013	·
1130—5	150	·	0.0035	Eu.	13.8	120	·	0.0033	·	96	12.4	100	·	0.0015	·	84	110	·	0.0010	·
1135 —4	120	·	0.0036	"	14.0	100	·	0.0038	·	96	12.5	100	·	0.0018	·	72	150	·	0.0012	·
1144 —2	20	·	0.0068	"	13.9	35	·	0.0066	·	96	12.9	25	·	0.0018	·	84	35	·	0.0014	·

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

Discussion and Summary

The present data indicate that the fragmental diphtherial antitoxins produced with trypsin digestion from the large incomplete antitoxic molecules, those produced in horses at the initial stages of the diphtherial immunization, are not converted newly into small and complete antitoxic molecules in the incubation with oxidized chymopapain and toxin, by which those produced with pepsin digestion were converted into.

It is considered that the fragments, produced with trypsin digestion, are not convenient to be converted into complete antitoxins by the incubating procedures with oxidized chymopapain and toxin. The fact suggests that the characteristic differentiation of the pepsin-digested globulins and trypsin-digested ones is due to the difference of the molecular fine structure of the two.

References

1) Itô, T. (1954): *Jap. J. Vet. Res.*, **2**, 143.
2) Jansen, E. F. & A. K. Balls (1941): *J. Biol. Chem.*, **137**, 459.
3) Kabat, E. A. (1943): *J. Imm.*, **47**, 513. Kabat, E. A. (1949): Experimental Immunochemistry, Th. Springfield, Illinois.

Errata for Volume 2

PAGE	LINE	DELETE	INSERT
2	10	flulid	fluid
4	TABLE 2	*S. aborutus equi*	*S. abortus equi*
5	9	inoculted	inoculated
14	6	adventitia slight	adventitia, slight
22	7	volume of	volume of 0.2%
29	27 (TABLE 4)	Hodehagi	Hidehagi
32	17	**21,** 146	**21,** 149
33	28	Ireland) and Scotland	Ireland and Scotland)
41	18	Unforfunately	Unfortunately
43	10	condider	consider
47	16	*Caniidae*	*Canidae*
51	1	*Lingualula*	*Linguatula*
55	26	Goverment	Government
66	7	stomachia	stomachica
68	TABLE 3	Settre	Setter
71	2	there are on	there are no
"	37	prongnoses	prognoses
74	17 (TABLE 2)	, S, HR, C,	, S, H, R, C,
89	24	1951 ; more	1951, more
95	3	stackfarms.	stockfarms.
98	FIG. 2	Ke :	Key :
103	4 (TABLE 1)	1.48	1.98
"	5 (")	1.95	1.85
"	6 (")	0.30	0.31
"	" (")	3.12	2.12
"	7 (")	3.94	2.94
"	9 (")	0.30	0.31
"	12 (")	0.28	0.27
"	13 (")	0.23	0.21
"	14 (")	0.24	0.23
"	15 (")	0.24	0.22
"	17 (")	1.50	1.60
"	19 (")	4.25	4.05
"	22 (")	6.3	6.4
"	" (")	0.40	0.41
115	11	(1914) :	(1941) :
118	17	capsula. was	capsula, was
122	25	structures was	structures were
130	FIG. 3	: Relapse	: Relapsed
146	12 (TABLE 1)	1.0016	0.0016
174	1 (TABLE 1)	PR/PB	PS/PB