



HOKKAIDO UNIVERSITY

Title	BIOCHEMICAL STUDIES ON THE INCREASE AND FORMATION OF ANTIBODIES WITH CHYMOPAPAIN IN VITRO : III. STUDIES ON THE INCREASE OF DIPHTHERIAL ANTITOXIC TITERS FROM PRELIMINARILY DENATURATED ANTITOXIC GLOBULINS
Author(s)	ITO, Tokiya
Citation	Japanese Journal of Veterinary Research, 3(2), 105-110
Issue Date	1955-08-10
DOI	https://doi.org/10.14943/jjvr.3.2.105
Doc URL	https://hdl.handle.net/2115/1671
Type	departmental bulletin paper
File Information	KJ00002372932.pdf



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

III. STUDIES ON THE INCREASE OF DIPHTHERIAL
ANTITOXIC TITERS FROM PRELIMINARILY
DENATURATED ANTITOXIC GLOBULINS

Tokiya Itô

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

(Received for Publication, Feb. 23, 1954)

In the preceding papers¹⁾, studies on the increase of diphtherial antitoxic titers from the pepsin-or trypsin-pre-digested antitoxic equine serum globulins were reported.

In this paper are described negative studies on the increase of the antitoxic titers from preliminarily acid-heating-, alkali-heating-, urea- or sodium salicylate-denaturated antitoxic globulins in vitro.

MATERIALS AND METHODS

1. Preparation of Purified Native Antitoxic Equine Pseudoglobulin Solutions
Antitoxic pseudoglobulin solutions (Lf 15~2,000 u/ml; protein-N 13~14 mg/ml) were prepared from immune sera which were obtained from a horse (No. 1145) at the initial and at some advanced stages of diphtherial immunization by fractionating precipitation with ammonium sulfate as previously described¹⁾.
2. Pre-denaturation of Purified Native Antitoxic Globulins with Acid-heating
Antitoxic globulin solutions were made down to pH 1.5 with 10 N. HCl. They were heated gently to 58°C and then cooled rapidly to 40°C.
3. Pre-denaturation of Purified Native Antitoxic Globulins with Alkali-heating
Antitoxic globulin solutions were made up to pH 11.0 with 10 N. NaOH. They were heated to 58°C according to PAULING and CAMPBELL¹⁾ and then cooled rapidly to 40 C.
4. Pre-denaturation of Purified Native Antitoxic Globulins with Urea
Antitoxic globulin solutions were made up to 5 Mol. of urea at pH 7.3. They were stood still for 36~48 hours at room temperature and then were dialyzed against running distilled water for 72 hours to remove urea.

5. Pre-denaturation of Purified Native Antitoxic Globulins with Sodium Salicylate

Antitoxic globulin solutions were made up to 5 Mol. of sodium salicylate at pH 7.3. They were stood still for 36~48 hours at room temperature and then were dialyzed against running distilled water for 72 hours to remove sodium salicylate.

6. Incubation of the Pre-denaturated Antitoxic Solutions with Chymopapain and Diphtherial Toxin

Antitoxic globulin solutions, which were pre-denaturated with acid-heating, alkali-heating, urea or sodium salicylate, were adjusted to pH 7.3. Addition was made of a quantity of purified oxidized chymopapain²⁾ obtained from papaya fruit latex equivalent to 10% of the globulin 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 described¹⁾) equivalent to about 10~30 times as much as the Lf titers of the antitoxin. The mixtures were made up to Eh 450~500 mV by the addition of 0.6% hydrogen peroxide with a small amount of cystine, fumaric acid, CoCl₂ and Fe₂(SO₄)₃. These mixtures were incubated at 40°C with a continuous supply of hydrogen peroxide. During the incubation procedures, pH, Eh, amino-N and protein-N titers were determined every 12 hours. The highest titers of protein-N were found in 72~84 hours.

7. The Fractionation of Antitoxin from the Chymopapain-Incubated Mixtures

When the titers of protein-N in the incubated mixtures reached the highest, globulins were precipitated with 60% saturation of ammonium sulfate and electrodialed against running distilled water by PAULI's electro dialysis apparatus. Finally, they were concentrated to the volumes equivalent to each corresponding starting native pseudoglobulin solutions. The antitoxic titers of these final preparations were determined by RAMON'S flocculation test.

8. 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 4 progressive procedures, viz; O) the original antitoxic sera, A) the purified native antitoxic globulin solutions, B) pre-denaturated antitoxic solutions and C) the fractionated, concentrated antitoxic solutions finally prepared from the chymopapain-incubated mixtures, mixing with the equivalent titers 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:

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

RESULTS

In the pre-denaturation procedures, antitoxic titers of the immune equine serum globulins were remarkably reduced, sometimes almost all were lost, although

decrease of protein-nitrogen remained only 3~5%.

Incubating the pre-denaturated antitoxic globulin solutions with purified oxidized chymopapain, purified concentrated diphtherial toxic solution, hydrogen peroxide, cystine, fumaric acid, CoCl_2 and $\text{Fe}_2(\text{SO}_4)_3$, pH and Eh went gradually upwards, reaching the peak in 60~72 hours. Protein-nitrogen, increasing slightly at the beginning, attained the highest level in 72~84 hours.

FIG. 49. Antidiphtherial Serum 1145-1 Pseudoglobulin

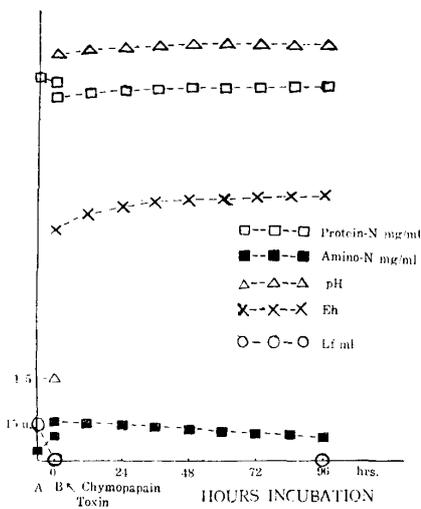


FIG. 50. Antidiphtherial Serum 1145-3 Pseudoglobulin

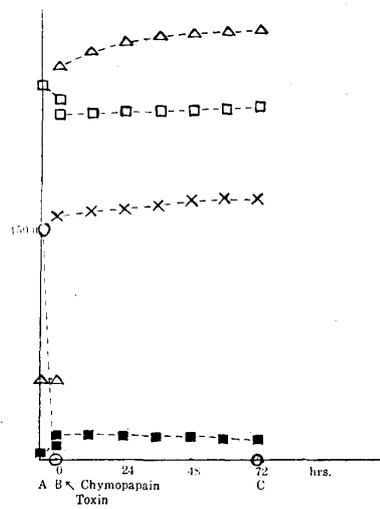


FIG. 51. Antidiphtherial Serum 1145-6 Pseudoglobulin

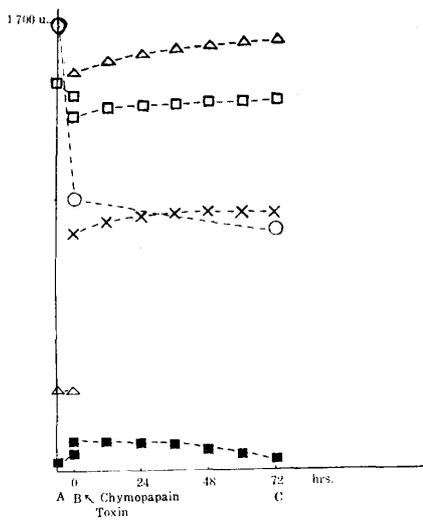
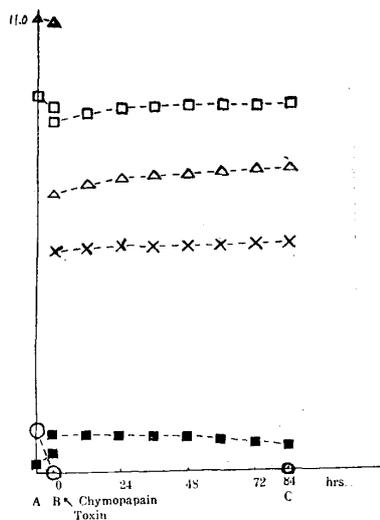


FIG. 52. Antidiphtherial Serum 1145-1 Pseudoglobulin



With the preparations, prepared from the mixtures after the final incubation by fractionating precipitation with 60% saturation of ammonium sulfate and following electro dialyzing concentration to the volumes equivalent to each corresponding starting native pseudoglobulin solutions, there were found no increase of the antitoxic titers (Figs. 49~58).

FIG. 53. *Antidiphtherial Serum*
1145-3. *Pseudoglobulin*

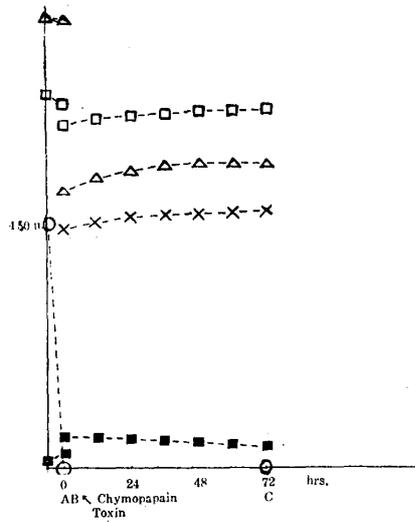


FIG. 54. *Antidiphtherial Serum*
1145-6 *Pseudoglobulin*

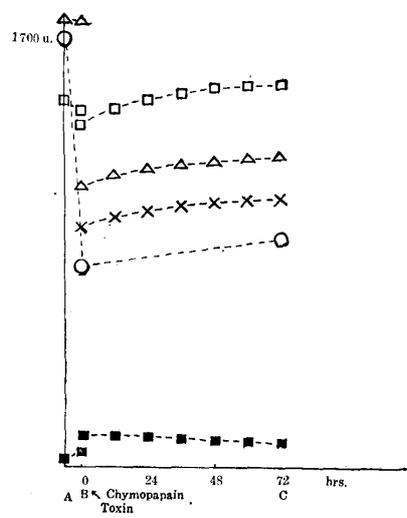


FIG. 55. *Antidiphtherial Serum*
1145-2 *Pseudoglobulin*

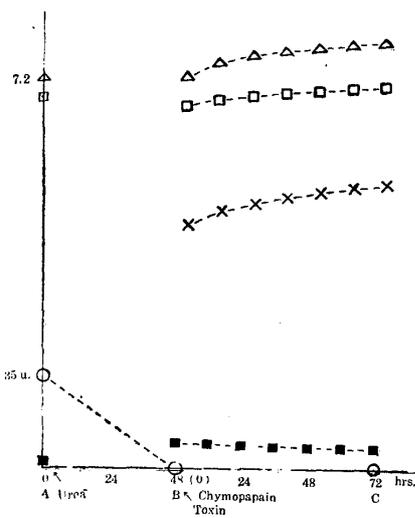


FIG. 56. *Antidiphtherial Serum*
1145-6 *Pseudoglobulin*

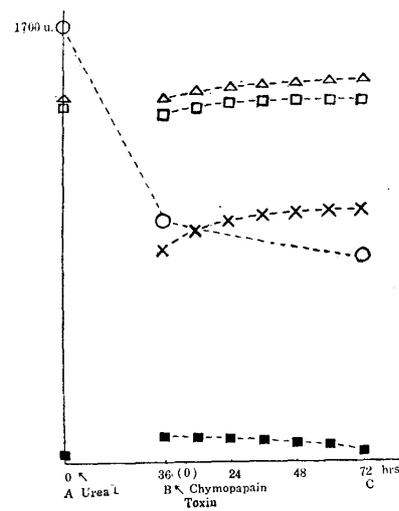


FIG. 57. Antidiphtherial Serum 1145-2 Pseudoglobulin

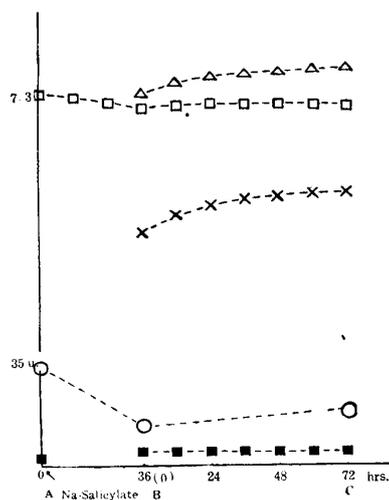


FIG. 58. Antidiphtherial Serum 1145-7 Pseudoglobulin

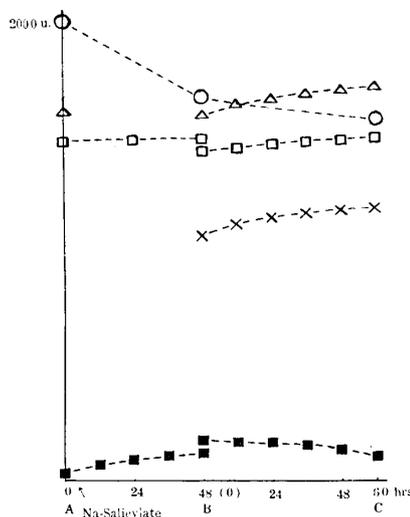


TABLE 3. Changes of Antitoxic Titers, Pure Antitoxic N mg/Lf during Incubation

SERUM NO.	ORIG. SERA		PURIFD. PSEUDOGLOBULINS			PRE-DENATED. GLOBULINS			FURTHER INCUBTD.			
	Lf u/ml	A.N. mg/Lf	P.N. mg/ml	Lf u/ml	A.N. mg/Lf	P.N. mg/ml	Lf u/ml	A.N. mg/Lf	Hrs.	Lf u/ml	A.N. mg/Lf	
1145	-1	10	0.0060	13.9	15	0.0034	13.6	0	.	96	0	.
	-3	150	0.0033	13.9	450	0.0034	12.8	0	.	72	0	.
	-6	700	0.0018	14.3	1700	0.0019	13.2	1000	0.0017	72	900	0.0014
	-1	10	0.0060	13.9	15	0.0034	12.9	0	.	84	0	.
	-3	150	0.0033	13.9	450	0.0034	12.8	0	.	72	0	.
	-6	700	0.0018	14.3	1700	0.0019	13.1	850	0.0014	72	950	0.0012
	-2	30	0.0038	13.8	35	0.0030	13.4	0	.	72	0	.
	-6	700	0.0018	14.3	1700	0.0019	14.1	950	0.0017	72	850	0.0015
	-2	30	0.0038	13.8	35	0.0030	13.3	15	0.0016	72	20	0.0013
	-7	800	0.0016	13.5	2000	0.0016	13.1	1500	0.0013	60	1400	0.0012

DISCUSSION AND SUMMARY

The present data indicate that the pre-denaturated diphtherial antitoxins produced by acid-heating, by alkali-heating, by urea or by sodium salicylate addition, from the large incomplete antitoxic molecules, those produced in a horse at the initial stages of diphtherial immunization, are not converted newly into small and complete antitoxic molecules in the incubating procedures with oxidized

chymopapain and diphtherial toxin, in which those produced with pepsin-pre-digestion were converted into.

In these studies, the fact was found that to increase the diphtherial antitoxic titers of immune equine serum globulins, which obtained from horse at the initial stages of immunization, with oxidized chymopapain and diphtherial toxin under suitable definite conditions of the incubation in vitro, the pre-digestion of large incomplete antitoxic globulins with pepsin is particularly necessary.

REFERENCES

- 1) ITÔ, T. (1954 & 1955): *Jap. J. Vet. Res.*, **2**, 143 & **3**, 33.
- 2) JANSEN, E. F. & A. K. BALLS (1941): *J. Biol. Chem.*, **137**, 459.
- 3) KABAT, E. A. (1943): *J. Immunol.*, **47**, 513.
KABAT, E. A. (1949): *Experimental Immunochemistry*, Th. Springfield, Illinois.
- 4) PAULING, L. & D. H. CAMPBELL (1942): *J. Exp. Med.*, **76**, 211.

Erratum for Vol. 3, No. 1

Page 35, Fig. 39, for Ehrlich's read Ehrlich's units/ml.