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Title	BIOCHEMICAL STUDIES ON THE INCREASE AND FORMATION OF ANTIBODIES WITH CHYMOPAPAIN IN VITRO : IV. STUDIES ON THE FORMATION OF DIPHTHERIAL ANTITOXIN FROM PRELIMINARILY PEPSIN-OR TRYPSIN-DIGESTED NORMAL EQUINE SERUM GLOBULINS
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BIOCHEMICAL STUDIES ON THE INCREASE AND FORMATION OF ANTIBODIES WITH CHYMOPAPAIN *IN VITRO*

IV. STUDIES ON THE FORMATION OF DIPHTHERIAL ANTITOXIN FROM PRELIMINARILY PEPSIN-OR TRYPSIN-DIGESTED NORMAL EQUINE SERUM GLOBULINS

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The previous papers[4] have reported studies on the increase of diphtherial antitoxic titers *in vitro* from the preliminarily pepsin- or trypsin-digested, acid- or alkali-heated, or with urea or with sodium salicylate pre-denaturated diphtherial antitoxic equine serum globulins, these globulins were obtained from immunized horses during the course of progressive diphtherial immunization, incubating with diphtherial toxin (antigen) and oxidized chymopapain (catalyzer) at pH 7.3, Eh 450~500 mV and 40°C.

The process and mechanism of antibody formation have invited numerous studies ever since effective techniques for immunization have been found. Although the studies of the investigators[1~3,5~9] to produce antibodies *in vitro* have brought remarkable advance in this field, lack of enzymechemical studies in the problem led the present authors to undertake some enzymic works on the formation and production of antibodies *in vitro*.

The present paper deals with negative results of studies on the formation of diphtherial antitoxin incubating preliminarily pepsin- or trypsin-digested normal equine serum globulins with toxic solutions and oxidized chymopapain.

MATERIALS AND METHODS

1. Preparation of Purified Native Normal Globulin Solutions

Normal serum globulin solutions (protein-N 13~14 mg/ml) were prepared from 7 normal equine sera by 5 times repeated fractionating precipitation with between 1/3 to 1/2 saturation of ammonium sulfate followed by dialysis against running water for 3 weeks, and final concentration to the volumes having equivalent titers of protein to each corresponding

starting serum protein concentrations respectively.

2. Pre-digestion of Purified Native Globulins with Pepsin

The normal serum globulin solutions (pH 6.5~7.0, Eh 300~400 mV) were made down to pH 4.4 with 10 N. HCl. They were made down to Eh 200~250 mV by the addition of small amounts of ascorbic acid and cysteine. Addition of a quantity of pepsin (Merck, 1 : 3,000) equivalent to 5% of the globulin was made. Incubating these mixtures at 37°C, pH, Eh, amino-N and protein-N were titrated every 12 hours. In these experimental procedures, the proteolytic activity of the enzyme deceased after 72~96 hours, showing a slight tendency of reversing turnover to increase portein-N titers hour by hour.

3. Pre-digestion of Purified Native Globulins with Trypsin

The normal globulin solutions were made up to pH 8.0 with 10 N. NaOH. Lowering to Eh 200~250 mV as above, addition of a quantity of trypsin (Kahlbaum, purified) equivalent to 5% of the globulin was made. The titrations similar to those in the pre-digestion procedures with pepsin were carried out. In these experimental procedures, similar decay of proteolytic activity of the enzyme was observed in 48~72 hours.

4. Incubation of Pepsin- or Trypsin-digested Globulins with Chymopapain and Diphtherial Toxic Solutions

Taking advantage of the turnover, those mixtures were made to pH 7.3. Addition was made of a quantity of purified oxidized chymopapain equivalent to 10% of the digested globulins with purified concentrated diphtherial toxic solutions (Lf 1,500~2,000 u/ml) equivalent amounts between 3~25 vol. % of the original globulin solutions. The mixtures were made up to Eh 450~500 mV by 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 0.6% hydrogen peroxide solution (1 drop per 5 minutes). During the incubating procedures, the titrations as in the pre-digestion were carried out every 12 hours. The highest titers of protein-N were found in 72~108 hours.

5. Fractionation of the Globulins from the Chymopapain-Incubated Mixtures

As soon as the highest titers of protein-N were found, the mixtures were made up to 60% saturation of ammonium sulfate at pH 8.0. The precipitated parts were collected by centrifuge (5,000 r. p. m. for 30 min.) and washed 3 times with 60% saturated ammonium sulfate solution. Removing ammonium sulfate by electrodialysis using PAULI's apparatus, the preparations were concentrated with fan to the volumes equivalent to the corresponding starting native globulin solutions respectively. Antitoxic titers of these final preparations were titrated by RAMON's flocculation test and occasional protective test using guinea pigs (b. w. 250~280 g).

RESULTS AND DISCUSSION

The present data (Figs. 59~68) indicate the fact that in these experimental procedures the pre-digested normal equine serum globulins are not suitable for the formation or production of diphtherial antitoxin *in vitro* independent of the titers of natural antitoxin in the original normal sera (less than 1/500 to 1/50

EHRLICH's unit/ml) and the amounts of added toxin, which would play an important *rôle* as antigen in the process of antibody formation.

From the present data, it is suggested that for the formation of diphtherial antitoxin from normal serum *in vitro*, more effective stimulating procedures and research of precise conditions are required *de nōvō*.

FIG. 59. *Normal Equine Serum No. 1149 (Less than 1/500 EHRLICH's u/ml) Pseudoglobulin*

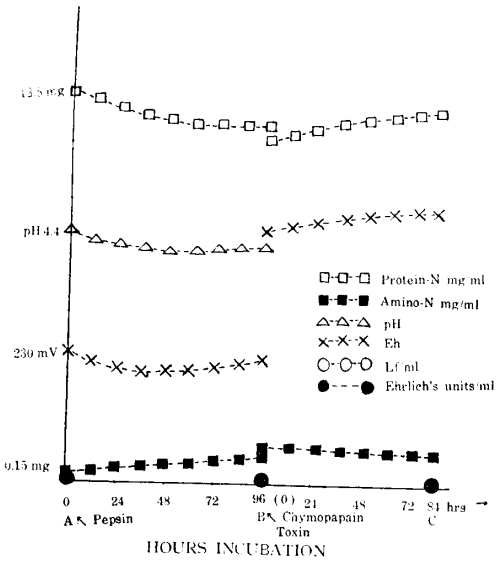


FIG. 60. *Normal Equine Serum No. 1151 (Less than 1/500 EHRLICH's u/ml) Pseudoglobulin*

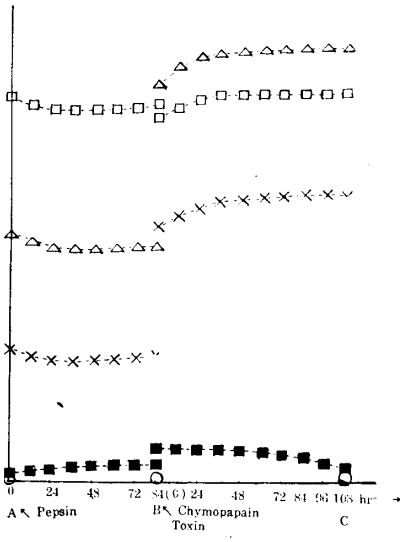


FIG. 61. *Normal Equine Serum No. 1156 (Less than 1/400 EHRLICH's u/ml) Pseudoglobulin*

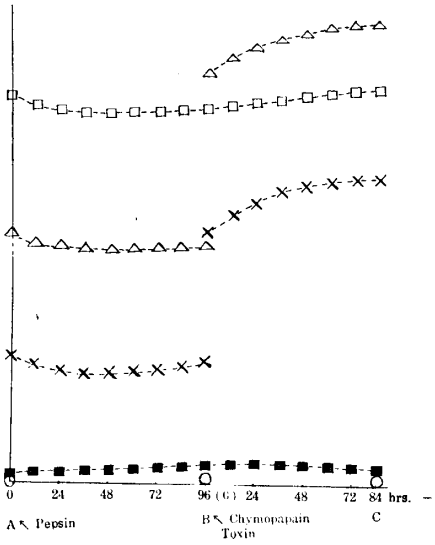


FIG. 62. *Normal Equine Serum No. 1162 (Less than 1/500 EHRLICH's u/ml) Pseudoglobulin*

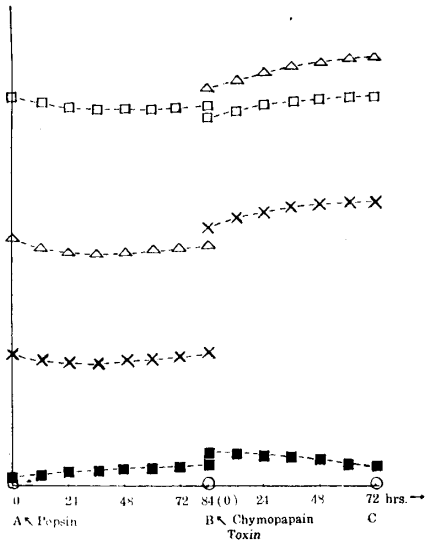


FIG. 63. *Normal Equine Serum No. 1153*
(Ca. 1/50 EHRLICH's u/ml)
Pseudoglobulin

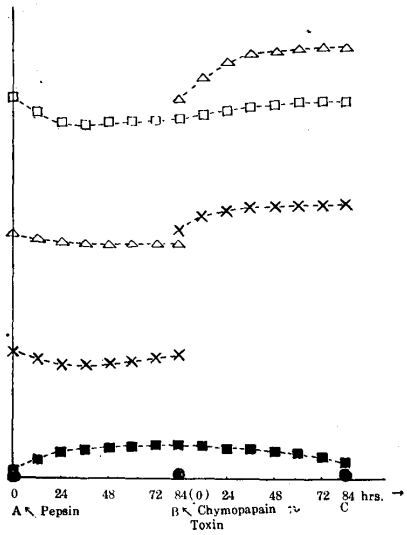


FIG. 64. *Normal Equine Serum No. 1158*
(Ca. 1/100 EHRLICH's u/ml)
Pseudoglobulin

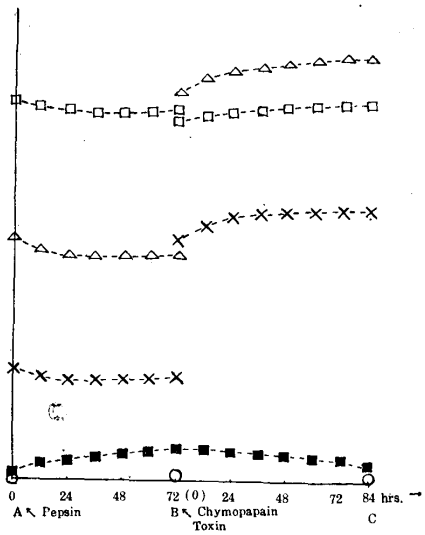


FIG. 65. *Normal Equine Serum No. 1151*
(Less than 1/500 EHRLICH's u/ml)
Pseudoglobulin

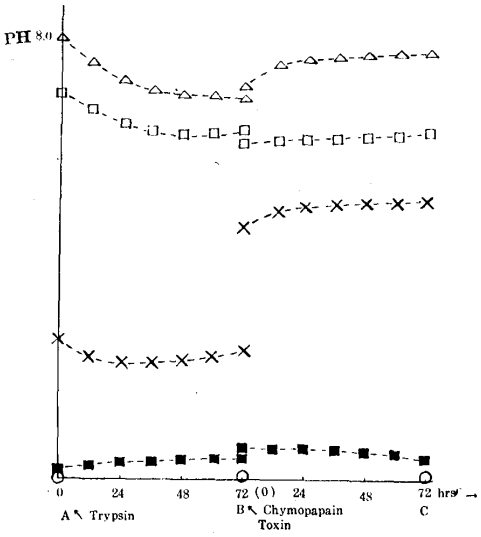


FIG. 66. *Normal Equine Serum No. 1156*
(Less than 1/400 EHRLICH's u/ml)
Pseudoglobulin

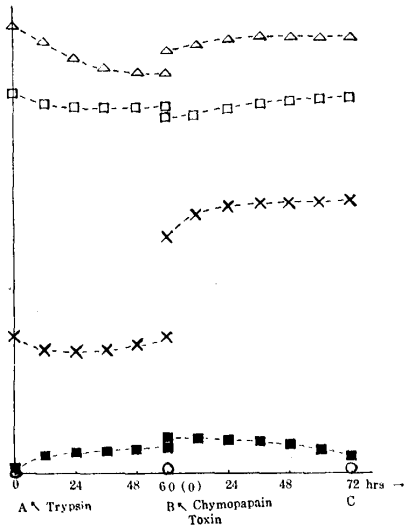


FIG. 67. *Normal Equine Serum No. 1153*
(Ca. 1/50 EHRLICH's u/ml)
Pseudoglobulin

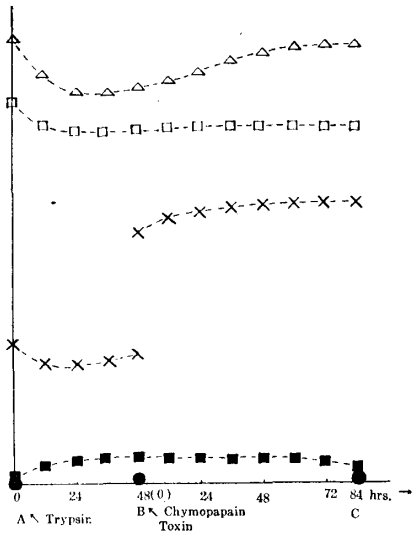
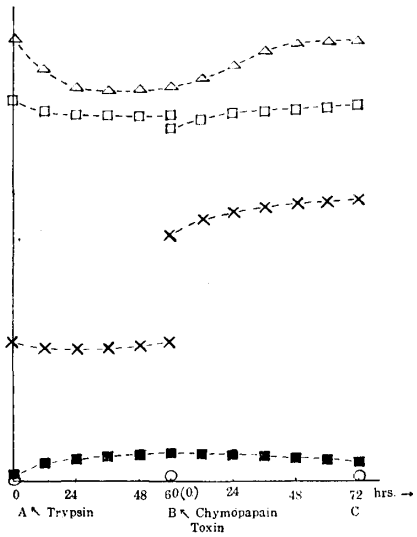


FIG. 68. *Normal Equine Serum No. 1164*
(Ca. 1/50 EHRLICH's u/ml)
Pseudoglobulin



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Erratum for Vol. 3. No. 2

The number indicating the open triangle in fig. 49, page 107 should be amended to read as follows:

Delete: 15 Insert: 1.5