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The Antigenic Relationship between the T-component of Diphtheria Antitoxic Horse Serum and Antitoxic Globulin modified with Protease*

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SUMMARY

1. The T-component of diphtheria antitoxic horse serum (DAHS) was highly purified by zone electrophoresis. It was then homogenous by physico-chemical and immunological criteria.

2. From the same batch of the DAHS three types of enzymically modified antitoxic globulin (EAGL) were prepared. These were diphtheria antitoxic globulin digested with *Aspergillus niger* protease (DGL ANP), diphtheria antitoxic globulin digested by pepsin and dissociated from the specific conjugate with toxin by pepsin (DGL pep) and DGL pep further digested by *Aspergillus niger* protease (DGL pep·ANP).

Quantitative precipitin reaction of each EAGL with T-component antiserum were made. DGL ANP and DGL pep precipitated about 40% of the T-component antibody and DGL pep·ANP precipitated about 20% of the T-component antibody. The equivalence point of each EAGL found in the antibody excess region of the homologous T-component system and the supernatants of each EAGL and T-component antibody system always contained antibody precipitable by further addition of T-component.

3. The precipitin line of the T-component against T-component antiserum fused with that of each EAGL forming a "spur" in agar medium. But these EAGL could not be differentiated from each other by precipitation reaction in agar medium.

4. When the purified T-component was digested by *Aspergillus niger* protease (ANP), a precipitin line of the T-component was connected with two distinct precipitin lines forming a "spur" behind two lines of the digest. From these results it is suggested that the determinant groups of the T-component consist of two or more sub-groups differing from one other in their serological specificity and that because of loss of the determinant groups each EAGL can only react with some of the antibody fractions of the T-component antibody.

INTRODUCTION

Many workers has intended to reduce or eliminate the antigenicity of therapeutic diphtheria antitoxic horse serum. In such efforts, *Aspergillus niger* (Amano *et al.*, 1955b), acid-Taka protease (Amano *et al.*, 1953, 1954, 1955a; Isojima *et al.*, 1954) and pepsin (Pope, 1939a, 1939b; Levine *et al.*, 1952) were found suitable for modifing the antigenicity of antitoxic globulin.

Many workers have compared the antigenicity of the modified antitoxic globulin with that of normal horse serum (N.H.S.). However, Ando (1937a, 1937b, 1937c, 1937d, 1938a, 1938b) and Treffers & Heidelberger (1941) showed independently that the antigenicity of antitoxic globulin differs from that of other antibodies produced in the horse.

*This paper was presented at the 20th Annual Meeting of the Japanese Biochemical Society in July 1957 at Kyoto Prefectural University of Medicine.

In highly immunized diphtherial antitoxic horse serum, a new serum component, the T-component, was demonstrated by Van der Scheer and Wyckoff (1940). Amoureux and Yeu (1951) also reported that the T-component is a major fraction and contains a large part of the antitoxic activity of such sera.

In our previous report (Fujio *et al.*, 1958) it was stated that after digestion of the DAHS by ANP, the remaining antitoxin was derived from the T-component fraction and the antitoxin originally present in the γ-globulin had been almost completely destroyed. Kekwick *et al.* (1941) also showed that modified T-component was left after peptic digestion of the DAHS.

It is therefore necessary to compare the antigenicity of the antitoxic globulin (EAGL) modified by the enzyme with that of the T-component. For this study, the T-component of the DAHS was highly purified by zone electrophoresis and the antigenicity of the EAGL still retained was tested with T-component rabbit antiserum *in vitro*.

Materials and Methods

Aspergillus niger protease (ANP): *Aspergillus niger* strain Usami* produces a protease which can hydrolyze serum proteins in acid medium, as described in our previous report (Amano *et al.*, 1955). Further studies on ANP have shown that it is relatively stable in acid medium, but in the region of above pH 7.6 its activity is rapidly lost.

Therefore *Aspergillus niger* strain Usami was grown in an acid medium. The extract of a whole culture of *Aspergillus niger* strain Usami fractionated with cold 30%-50% ethanol and this fraction was further purified by zone electrophoresis at pH 5.0 according to the method of Flodin and Kupke (1956). The specific activity of the purified ANP was about 15 times greater than that of the crude extract. The purified ANP preparation was homogenous by zone electrophoresis at pH 5.0 and had a slight yellow color. To estimate the protease activity of ANP, horse serum albumin (H.S.A.) was used as a substrate at pH 3.5. The details of the determination of protease activity are as described previously except for a difference of substrate (Amano *et al.*, 1953).

Purified T-component: The pseudoglobulin, obtained by precipitation with 1/3 to 1/2 saturated ammonium sulfate, was further purified by zone electrophoresis at pH 8.6.

T-component antiserum: Antiserum to the T-component of the DAHS was obtained from rabbits after injecting purified T-component mixed with imcomplete Freund's adjuvant. 20 mg. of the purified T-component was dissolved in 10 ml. of saline** containing M/50 phosphate buffer (pH 7.6) and 0.01% merthiolate. 10ml. of a mixture of Arlacel A and paraffin oil (1;9) was added to the antigen solution.

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* *Aspergillus niger* strain Usami was kindly supplied by the Department of Brewery, Faculty of Engineering, Osaka University.

** 0.85 per cent NaCl and 0.01% merthiolate solution in M/50 phosphate buffer (PH 7.6) is designated as P.B.S. in this paper.

After mixing in a homogenizer, this antigen preparation was injected subcutaneously into rabbits once a week. After 4 injections of the antigen, 20 mg. of the T-component solution without adjuvant was injected subcutaneously one week later. Seven days after this last injection, the rabbits were bled. As antigen for the immunization two batches of T-component preparation described below were used. Three antisera (R-1, R-2 and R-3) were obtained from three rabbits by immunization with T-comp. I. Two antisera (R-4 and R-5) were obtained by injection of T-comp. II. These antisera were inactivated by heating at 56°C for 30 minutes and merthiolate was then added at a concentration of 0.01% as a preservative. They were stored at -60°C.

Zone electrophoresis: A vertical cellulose (acid-ethanol treated) column was prepared according to the method of Flodin and Kupke (1956) and zone electrophoresis was carried out using borate-phosphate buffer (pH 8.6, $\mu=0.05$) at 4°C. The column was developed with the same buffer and 2.5 ml aliquots collected by fraction collector. The protein concentration of each fraction was estimated from the ultraviolet absorption at 277 mμ.

Ultracentrifugation: Ultracentrifugation of the purified T-component was carried out using a Spinco Model E centrifuge.

Quantitative precipitin analysis: A quantitative precipitin reaction was made by the method of Heidelberger and Kendall (1935). The antigen was dissolved in saline containing M/50 phosphate buffer (PH 7.6) and 0.01% of merthiolate.

Determination of total nitrogen in the specific precipitate was made in a micro-Kjeldahl apparatus or by direct Nesslerization. The total nitrogen estimation of the specific precipitate by direct Nesslerization was as follows: 2 drops of N/2 NaOH solution was added to each test tube containing the specific precipitate and the later was washed into a calibrated Kjeldahl flask. 0.2 ml. of acid digestion mixture* was added to each flask. Then the flask was gently heated until the digest becomes almost colorless.

After cooling for a few minutes one drop of 30% H_2O_2 was added to the flask. Again each flask was gently heated until the digest becomes colorless. 10 ml. of distilled water was added to each flask and the flasks were placed in an ice bath for 20 minutes. 3 ml. of previously cooled Nessler's reagent (Folin, 1919) were added to the flasks and the contents diluted to 20 ml. with distilled water. After standing at 25°C for 30 minutes, the optical density of each sample was measured at 415 mμ in a Coleman Universal Photometer. The amount of total nitrogen of each sample was deduced from a standard curve obtained with ammonium sulfate.

Agar diffusion reactions: The double diffusion technique of Ouchterlony (1949) was used. One per cent agar (Difco) and 0.01% merthiolate in M/50 phosphate buffer at PH 7.6 was employed. In each well were placed 0.2 ml. of antigen solution (dissolved in phosphate saline buffer at pH 7.6) and 0.2 ml. of undiluted antiserum. These plates were incubated at 37°C and photographed on between the 7th and 12th day of incubation.

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*Acid digestion mixture: 100 ml. of conc. H_2SO_4 were diluted with 300 ml. of deionated water and 0.125 ml. of $SeOCl_2$ were added to the solution.

RESULTS

1. Purification of the T-component of DAHS by zone electrophoresis.

DAHS was diluted with an equal volume of water and precipitated with 1/3 to 1/2 saturation of ammonium sulfate. The precipitate was dissolved in a small quantity of water and dialyzed against running tap water for 3 days and then twice against distilled water for 3 days. The dialyzed sample was lyophilized. Two hundred mg. of lyophilized globulin was dissolved in 3 ml. of borate-phosphate buffer (PH 8.6, $\mu = 0.05$) and applied to the top of a column (3×40 cm) of buffered cellulose powder. Zone electrophoresis was carried out at 900 Volt and 26 mA. at 4°C for 24 hours. The fractions were eluted using a fraction collector. The protein concentration of each fraction was estimated from absorption at 277 mμ using a Beckman spectrophotometer. The T-component was collected and dialyzed against running tap water for 3 days and against 50 volumes of distilled water for 2 days. The dialyzed sample was lyophilized.

When purification was carried out with DAHS of lower antitoxin content, the T-component fraction obtained by one run of zone electrophoresis was not homogenous, still containing small quantities of β_1-and γ-globulin. Therefore in these cases zone electrophoresis was repeated. The antitoxin content of various purified T-component preparations obtained by zone electrophoresis were appreciably different. Higher antitoxin content was found in the T-component when more potent DAHS was used initially. A T-component preparation (T-comp. I) purified from DAHS containing 1900 AU. per ml. had 205 AU. per mg. N. while a T-component preparation (T-comp. II) from DAHS containing 960 AU. per ml. had 140 AU. per mg. N.

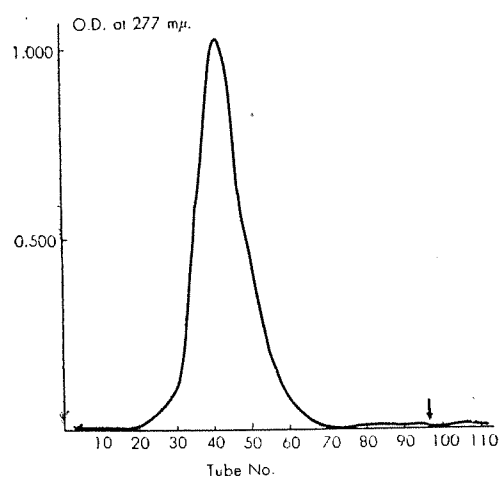

Fig. 1 Electrophoresis of purified T-component (T-comp. 1) on a cellulose column.
Note : Borate-phosphate buffer, PH 8.6, ionic strength 0.05; 25 hours at 40 mA. 70 mg applied in 2ml. Cellulose column, 3×40cm. Optical densities were read at 277 mμ. Fraction size about 2.5 ml.
To check the starting position of the material 2, -4 dinitrophenylethanolamine was used. It appeared in the effluent at the point indicated by the arrow.

2. Physico-chemical homogeneity of the purified T-component preparation.

The physico-chemical homogeneity of T-component preparations purified in this way was examined by zone electrophoresis and by ultracentrifugation.

1) *Electrophoreiic properties.*

Purified T-component preparations were examined by zone electrophoresis at pH 8.6 as described above. The electrophoretic pattern of the T-comp. I is shown in Fig. 1.

As can be seen, this preparation was homogenous. Although the patterns of other preparations are not shown, these were also electrophoretically homogenous.

2) *Ultracentrifugation of the purified T-component preparation.*

The ultracentrifugal pattern of T-comp. I is shown in Fig. 2.

No. 1.

Fig. 2 Ultracentrifugal patteln of purified T-component (T comp. 1) The experiment was carried out using a Spinco Model E. at 12°C and 52, 640 r.p.m. Sample : 0.34 % T-comp. 1 solution dissolved in 1% NaCl in M/15 phosphate buffer pH. 7.02)

No. 1 Photograph at 32 minutes.

No. 2.

No. 2 Photograph at 48 minutes.

No. 3.

No. 3 Photograph at 64 minutes.

As seen in Fig. 2, it was homogenous T-comp. II was also homogenous.

These T-component preparations were therefore homogenous by both physico-chemical criteria.

3. Immunological properties of the purified T-component.

An examination was made of the purity by immnuological criteria.

The quantitative aspects of the antigenicity of the T-component have not yet been reported because of difficulties in its purification.

Physico-chemically homogenous preparations of the T-component were injected into rabbits and the antisera obtained. Quantitative precipitin analyses were carried out.

The increasing amounts of T-comp. I dissolved in P.B.S. were added to test tubes containing 1 ml. of T-component antiserum (R-3). As an antigen control, 0.306 mg. of T-comp. I dissolved in 1 ml. P.B.S. was introduced into a test tube containing 1 ml. of P.B.S. and as an antiserum control, 1 ml. of P.B.S. was introduced into a test tube containing 1 ml. of T-component antiserum(R-3). These tubes were incubated in a water bath at 42°C for 3 hours and stored at 0°C for 48 hours. Then each tube was centrifuged at 3000 r.p.m. for 30 minutes at 0°C, and

the specific precipitate obtained was washed twice with the P.B.S. The total nitrogen of the specific precipitates was estimated by micro-Kjeldahl analysis. As neither control tube had an appreciable amount of nitrogen by micro-Kjeldahl analysis, they were neglected.

The results of this experiment are shown in Table I and Fig. 3.

Table I. Quantitative precipitin reaction of T-component in homologous antiserum

Antigen added	Total N. precipitated	Antibody N. precipitated	Antibody N./Antigen N. in specific ppt.	Test on supernatant*
mg.	mg.	mg.		
0.019	0.159	0.140	7.2	Excess antibody
0.039	0.302	0.262	6.7	〃
0.058	0.413	0.354	6.0	〃
0.078	0.500	0.421	5.4	Antigen(−),Antibody(−)
0.100	0.585	0.484	4.8	Antigen(−),Antibody(−)
0.120	0.625	0.505	4.2	Excess antigen
0.139	0.635			〃
0.154	0.643			〃
0.169	0.635			〃
0.184	0.639			〃
0.245	0.622			〃
0.306	0.602			〃

Antigen : T-comp. I
Antiserum : R-3 (1 ml.)
*Test on supernatant: detection of antigen.....0.2 ml. of T-component antiserum was added to 0.2 ml. of each supernatant.
detection of antibody.....0.2 ml. of antigen solution containing 0.2μgN. of T-component was added to 1 ml. of each supernatant.

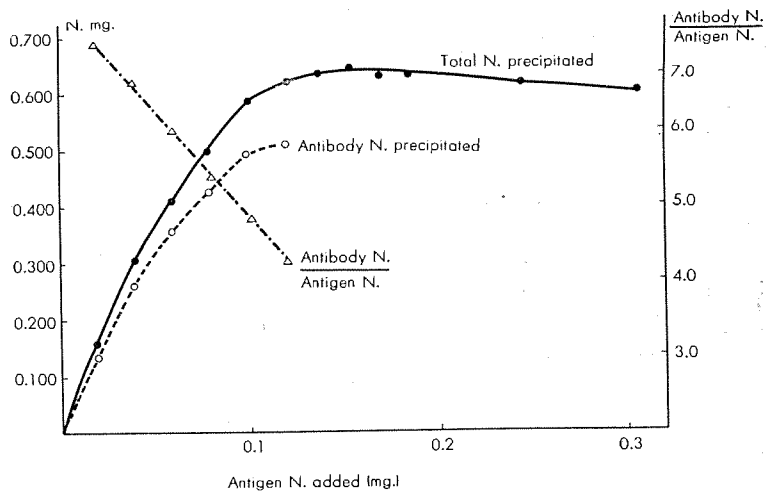

Fig. 3 Quantitative precipitin reaction of T-component in homologous antiserum.
Antigen.....T-comp. I
Antiserum...R-3 (1ml.)

A distinct equivalence zone was observed from the results of a test on supernatant.

The ratio of the precipitated antibody N to antigen N added was plotted against the antigen N added, as shown in Fig. 3.

As the result was linear, it can be concluded that the purified T-component preparation was also homogenous on the criterion of the quantitative precipitin analysis.

As the antitoxin contents of the T-component preparations were appreciably different, their antigenic properties were examined by quantitative precipitin analysis using individual rabbit antisera.

The results are summarized in Table II.

Table II. Comporative study of quantitative precipitin reaction using various preparations of T-component and of T-component antiserum

Precipitinogen	Antiserum		Antibody N. mg./ml.	Molecular composition* of specific precipitate at antigen excess end of equivalence zone
	Preparation No.	Immunizing antigen		
T-comp. I	R-1	T-comp. I	0.738	5.3
T-comp. II	R-1	〃	0.729	4.8
T-comp. I	R-2	〃	0.652	5.4
T-comp. II	R-2	〃	0.643	5.4
T-comp. I	R-3	〃	0.505	5.4
T-comp. II	R-4	T-comp. II	0.133	5.1
T-comp. II	R-5	〃	0.323	5.3

* These values were calculated from the results of the quantitative precipitin reaction assuming the molecular weight of T-component to be 18×10^4 and that of the rabbit antibody to be 16×10^4.

Though the two T-component preparations differed from each other in their antitoxin contents, they precipitated almost the same amount of antibody nitrogen from a batch of T-comp. I antiserum. Therefore it can be assumed that antigenic properties of T-component were not correlated with their antitoxicity.

The molecular ratio of antibody to antigen in the specific precipitates formed at the antigen excess end of the equivalence zone was calculated from the results of the quantitative precipitin analysis, assuming that the molecular weight of the T-component was 18×10^4 and that of the rabbit antibody was 16×10^4. As can be seen in column 5 of Table II, the ratios calculated were fairly consistent with a mean value of 5.2.

It is concluded that the two preparations of the T-component were both homogenous in their immunological behaviours. Further analysis of their immunological homogeneity were made using Ouchterlony's agar diffusion technique. As shown from the results described below, both preparation were homogenous by this analysis too.

4. Quantitative precipitin reaction of EAGL with T-component antiserum.

Digested globulins prepared from DAHS by pepsin or by ANP were shown to be derived from the T-component, as described above. These digested

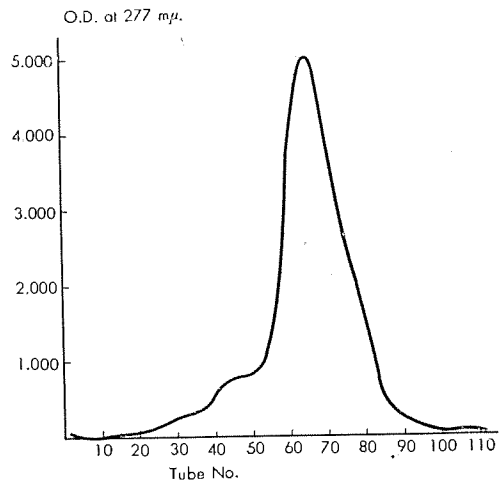

Fig. 4 Electrophoresis on cellulose column of antitoxic globulin digested by ANP.
Note : Borate-phosphate buffer, PH 8.6, ionic strength 0.05 ; 43 hours at 35 mA at 4°C 400 mg. applied in 6 ml. Cellulose column 3×40cm. Optical densities were read at 277 mμ. Fraction size about 2.5 ml.

by zone electrophoresis.

Three kinds of EAGL were prepared. To compare their antigenicities, these were prepared from a batch of DAHS containing 960 AU. per ml.

a) DGLANP : DAHS containing 960 AU. per ml. was digested with ANP for 72 hours as described in our previous report (Amano *et al.*, 1955) and then the globulin fraction was obtained by precipitation with half saturated ammonium sulfate. The major component of this fraction was further purified by zone electrophoresis at pH 8.6 as shown in Fig. 4.

The antitoxic activity of the purified antitoxic globulin was 200 AU. per mg. N.

globulins (DGLpep and DGLANP) still gave a faint reaction with T-component antiserum when tested by the ring test. There are two explanations for this residual precipitation by T-component antiserum. The one is that digested globulins might still be contaminated with a small amount of undigested T-component. The other explanation is that large peptides which have been split off might still be precipitated by T-component antibody. To decide which alternative is correct quantitative precipitin reaction were carried out with the digested globulins and T-component antiserum.

1) *Preparation of enzyme-modified antitoxic globulins and their purification*

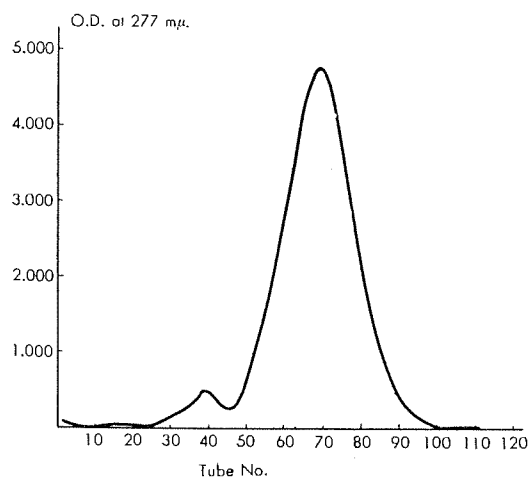

Fig. 5 Electrophoresis on cellulose column of antitoxic globulin obtained by peptic digestion of the specific precipitates.
Note : Borate-phosphate buffer, PH 8.6, ionic strength 0.05 ; 42 hours at 36 mA at 4°C. 500 mg. applied in 6 ml.

b) DGLpep : The same batch of DAHS was digested with pepsin according to the method of Pope (1939a and 1939b). The antitoxic globulin digested by pepsin was precipitated with an optimal ratio of crude diphtherial toxin (Pope *et al.*,

1939c). The DGLpep was obtained by peptic digestion of the specific precipitates. It was purified by zone electrophoresis as shown in Fig. 5.

The purified DGLpep contained 710 AU. per mg. N.

c) DGLANP : To eliminate more completely the antigenicity of the antitoxic globulin, further digestion of DGLpep was attempted. However no increase in antitoxicity was found on further digestion of DGLpep with pepsin at various concentrations, and the more digested DGLpep still formed a white ring with T-component antiserum by ring test.

Next DGLpep was digested by ANP. A preliminary experiment, showed that a large quantity of ANP rapidly destroyed the antitoxin in the DGLpep. Therefore a very small amount of ANP was added to the DGLpep solution.

Two hundred mg. of DGLpep was dissolved in to 20 ml. of M/15 acetate buffer (PH 3.5) containing 0.25% phenol and M/2000 $ZnSO_4$ and the pH was adjusted to 3.5 with N-HCl. Four tenth ml. of ANP (protease activity 9.2 units) was added to this solution. The ratio of the enzyme nitrogen to the substrate nitrogen was about 1 : 320.

At the beginning of the enzymic action there were 1050 antitoxin units per ml. of DGLpep. After 72 hours incubation there were about 560 antitoxin units per ml. of digest. After 72 hours the pH of the medium was 3.8. The precipitates formed during the digestion were filtered off and the pH of the filtrate was adjusted to 7.6 with N-NaOH. This filtrate was dialyzed three times against distilled water for 4 days at 5°C and then lyophilized. Further purification of DGLpep·ANP was also performed by zone electrophoresis, as seen in Fig. 6. There were 780 antitoxin units per mg. N in the purified DGLpep·ANP. A slight increase in antitoxin units per mg. N was observed.

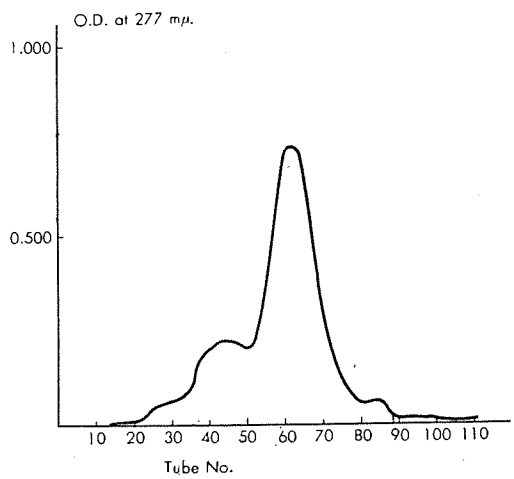

Fig. 6 Electropherogram on cellulose column of antitoxic globulin obtained by further digestion of DGLpep with ANP.
Note : Borate-phosphate buffer, PH 8.6, ionic strength 0.05 ; 24 hours at 30 mA at 4°C. 40mg. applied in 3ml.

2) *Quantitative precipitin analysis of DGLANP, DGLpep and DGLpep·ANP with T-component antiserum.*

The residual antigenicity of three type of EAGL preparation was examined with a sample of T-component antiserum.

0.5 ml. of T-component antiserum (R-4) was put into a series of test tubes and mixed with a series of increasing amounts of each type of EAGL dissolved in 0.5ml. of P.B.S. Four series of controles were also prepared containing the same amount of each antigen and P.B.S. In addition, a serum control containing 0.5 ml. of T-component antiserum (R-4) and 0.5 ml. of saline was prepared. These tubes were incubated in a water bath at 42°C for 3 hours and stored at 0°C

for 48 hours. The specific precipitates from each tube were washed twice with P.B.S. and the total nitrogen was then cstimated by the direct Nesslerization. Since the antigen and the serum control did not give any appreciable color with Nessler reagent, they were neglected.

The results of the N. determinations on the precipitates are shown in Fig. 7 and the results of tests on the supernatants are summarized in Table. III.

An equivalence zone was found in each series of supernatant tests in which the supernatant formed no precipitate with the T-component antiserum or with the same antigen used. The value of the antibody N. precipitated by each sample of EAGL was calulated by subtracting the amount of added antigen N. from the total precipitated N. in the region of antibody excess and equivalence zone. The precipitable antibody N. of each system is shown in Fig. 8, calculated as described by Heidelberger and Kendall (1935).

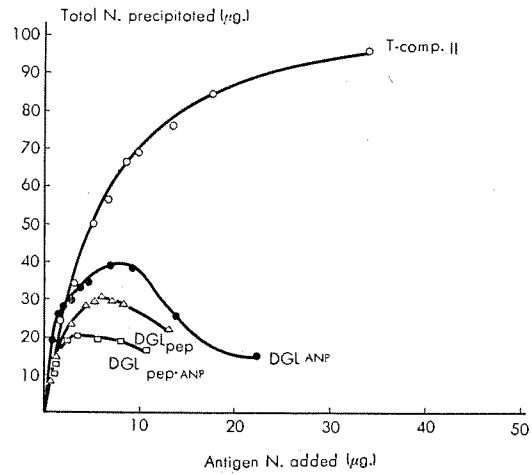

Fig. 7 Comparative study of quantitative precipitin reaction of each EAGL and of T-component in T-component antiserum. (Total nitrogen precipitated)

These values are not very accurate and somewhat more antibody N. may be precipitated, because there are no means of detecting univalent or non-precipitable peptides which may have been split off and remain in the supernatant. However it is obvious that all the T-component antibodies were not precipitated even at the equivalence zone of each EAGL systems, because the supernatants of each series of EAGL tubes gave appreciable precipitates when they were mixed with T-component.

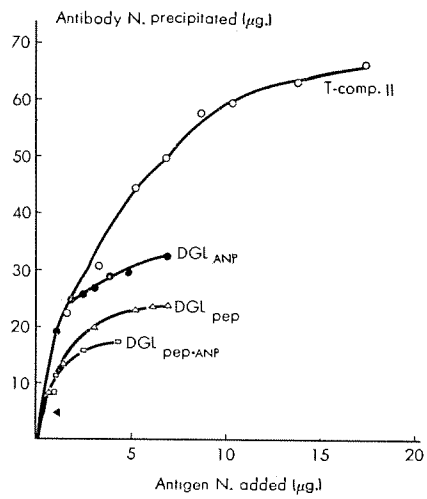

Fig. 8 Comparative study of quantitative precipitin reaction of each EAGL and of T-component in T-component antiserum. (Antibody nitrogen precipitated)

This mean that some fractions of the T-component antibody could be precipitated with EAGL but the other fractions could not be bound by EAGL. These facts suggest that the T-component antibodies are heterogenous in specificity. The DGLANP and the DGLpep could precipitate about 40% of the T-component antibody.

When the DGLpep was further digested with ANP, the precipitating ability of the resulting globulin as an antigen

Table III. Test on supernatants in quantitative precipitin reaction of T-component and of each EAGL against T-component antiserum

A. T-comp. II — R-4 system

Antigen N. added μg. per 0.5 ml. antiserum		1.7	3.5	5.2	6.9	8.6	10.3	1.38	17.3	34.5	86.2
Test on supernatant	T-comp. II	+	+	+	±	−	−	−	−	−	−
	T-component antiserum	−	−	−	−	−	−	−	±	+	+

B. DGLANP — R-4 system

Antigen N. added μg. per 0.5 ml. antiserum		1.1	1.5	2.3	3.0	3.8	4.6	6.8	9.1	13.7	22.7
Test on supernatant	DGLANP	+	±	−	−	−	−	−	−	−	−
	T-comp. II	+	+	+	+	+	+	+	+	+	+
	T-component antiserum	−	−	−	−	−	−	±	±	+	+

C. DGLpep — R-4 system

Antigen N. added μg. per 0.5 ml. antiserum		0.7	1.5	2.9	4.4	5.1	5.8	7.3	8.7	13.1
Test on supernatant	DGLpep	+	+	+	−	−	−	−	−	−
	T-comp. II	+	+	+	+	+	+	+	+	+
	T-component antiserum	−	−	−	−	−	±	+	+	+

D. DGLpep·ANP — R-4 system

Antigen N. added μg. per 0.5 ml antiserum		0.5	0.7	0.9	1.0	2.2	3.3	4.3	5.4	8.2	10.9
Test on supernatant	DGLpep·ANP	+	+	+	−	−	−	−	−	−	−
	T-comp. II	+	+	+	+	+	+	+	+	+	+
	T-component antiserum	−	−	−	−	−	±	+	+	+	+

Detection of antigen in supernatant: 0.2 ml. of T-component antiserum was added to each 0.1 ml. of supernatant

Detection of antibody in supernatant: 0.2 μg. N. of each EAGL (0.1 ml.) was added to each 0.3 ml. of supernatant

was appreciably reduced. Only 20% of the T-component antibody could be precipitated. The supernatant was taken at the equivalence zone of this system and mixed with DGLpep or DGLANP. Though it was calculated that another 20% (40% minus 20%) of antibody N. could be precipitated by DGLpep or DGLANP, no precipitate was detected.

Essentially the same phenomenon was observed in gel diffusion analysis, namely when DGLpep·ANP and DGLpep or DGLANP were compared on Ouchterlony plates with T-component antiserum no "spur" formation was observed as described below. The exact explanation can not be given from our experimental results. However the authors feel that DGLpep·ANP may be heterogenous in antigenic valency and so a part of added DGLpep·ANP would be left in

the supernatant blocking the combining sites of antibodies, which could be precipitated from the T-component antiserum by DGLpep or DGLANP. However these supernatants gave appreciable precipitates when they were mixed with the T-component.

From the resluts described here it can be concluded that the peptide having antitoxic activity split by protease still retains some of the determinant groups of the T-component.

3) Precipitin reaction in agar medium

T-component purified by zone electrophoresis was proven as homogenous by physico-chemical criteria and also in the quantitative precipitin reaction. However, it remained to confirm the homogeneity of the T-component by agar diffusion analysis. The precipitin reaction in agar medium was developed by Oudin and Ouchterlony and its high sensitivity for detecting small amounts of antigenic impurities has been recognized by many workers. The double diffusion technique in agar medium according to the method of Ouchterlony was employed in this study to test the homogeneity of the purified T-component and to clarify the antigenic relationships between the T-component and each type of EAGL.

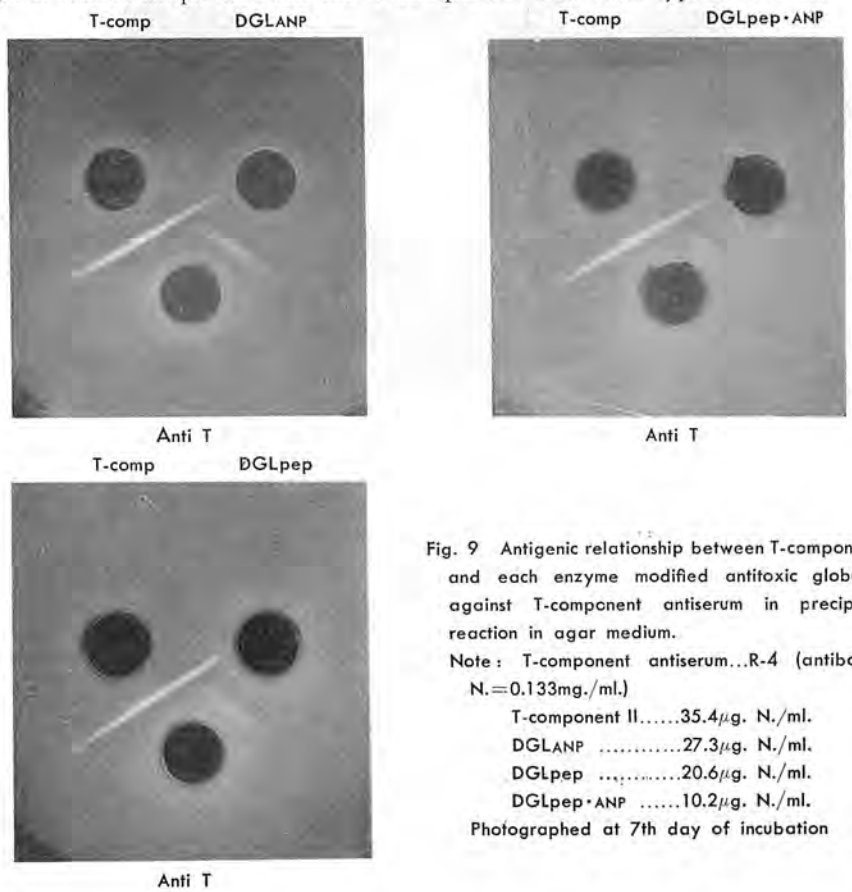

Fig. 9 Antigenic relationship between T-component and each enzyme modified antitoxic globulin against T-component antiserum in precipitin reaction in agar medium.
Note: T-component antiserum...R-4 (antibody N.=0.133mg./ml.)
T-component II.....35.4μg. N./ml.
DGLANP .....27.3μg. N./ml.
DGLpep .....20.6μg. N./ml.
DGLpep·ANP .....10.2μg. N./ml.
Photographed at 7th day of incubation

As seen in Fig. 9, the T-component always gave only one precipitn line against the T-component antiserum. The precipitin lines of EAGL differed from

that of the T-component in their vague and diffuse appearance. Each EAGL gave only one precipitin line against the same serum also. When the T-component was compared in its behaviour with T-component antiserum with each EAGL, it was found that its line fused with that of each EAGL forming a "spur". These results are consistent with those of the quantitative precipitin reaction and suggest that the determinant groups of each EAGL is the same as some derminant groups of the T-component.

To show the difference of the antigenicity between the T-component and each EAGL more clearly, each EAGL solution was mixed with the T-component in different proportions and diffused against T-component antiserum. Two distinct precipitin lines were observed as shown in Fig. 10. Fig. 10 shows the results of DGLpep. In the case of DGLANP and DGLpep•ANP, essentially the same phenomenon as in the case of DGLpep have been observed.

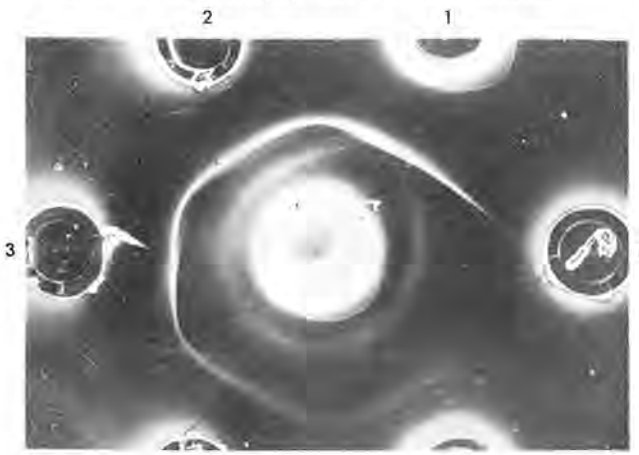

Fig. 10 Precitpitation in agar medium by a mixture of the T-component and DGLpep against T-component antiserum.
Note : T-component antiserum...0.2 ml. of R-3 (antibody N.= 0.505mg./ml.)
Antigen: 1 ... 0.2 ml. of T-comp. II solution (0.13mg. N./ml.)
2 ... 0.1 ml. of T-comp. II (0.13mg.N./ml.)
+0.1 ml. of DGLpep solution (0.12mg. N./ml.)
3 ... 0.1 ml. of T-comp. II solution (0.06mg. N./ml.)
+0.1 ml. of DGLpep solution (0.12mg. N./ml.)
4 ... 0.1 ml. of T-comp. II solution (0.03mg. N./ml.)
+0.1 ml. of DGLpep solution (0.12mg. N./ml.)
5 ... 0.1 ml. of T-comp. II solution (0.015mg. N./ml.)
+0.1 ml. of DGLpep solution (0.12mg. N./ml.)
6 ... 0.2 ml. of DGLpep solution (0.06mg. N./ml.)
Photograph at 7th day of incubation

It is concluded that the residual antigenicity of each EAGL was due not to a small amount of residual T-component after digestion, but to peptides having antitoxic activity which were split off.

As the three types of EAGL precipitated different amounts of antibody from T-component antiserum, the combinations of the three kinds of EAGL with the

T-component antiserum was examined using the gel diffusion technique to see if there was any differences in their antigenicities.

The results are shown in Fig. 11. No distinct difference was not observed and the precipitin lines of each EAGL fused without forming any "spur".

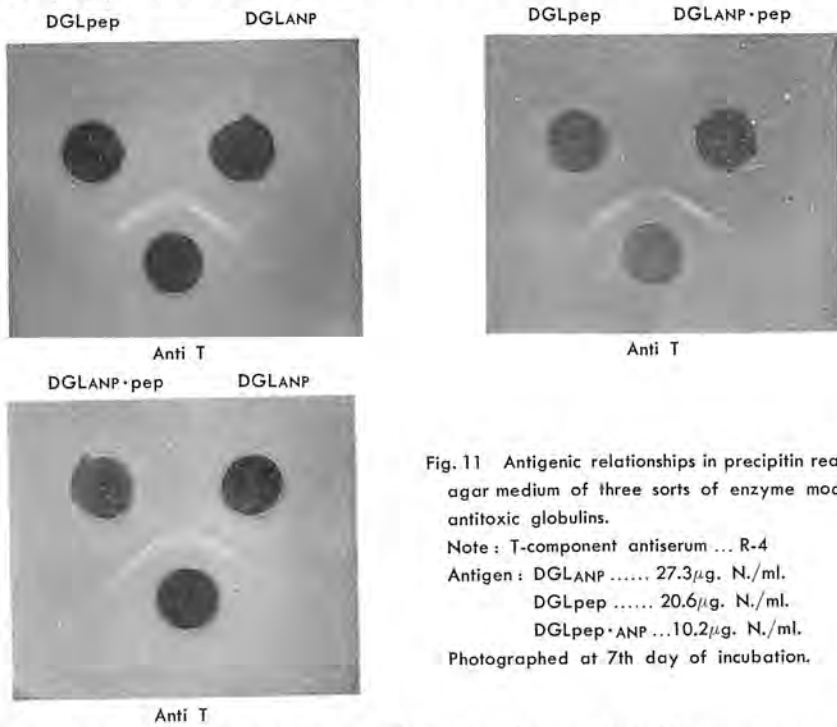

Fig. 11 Antigenic relationships in precipitin reaction agar medium of three sorts of enzyme modified antitoxic globulins.
Note : T-component antiserum ... R-4
Antigen : DGL_{ANP} ..... 27.3μg. N./ml.
DGLpep ..... 20.6μg. N./ml.
$DGLpep \cdot_{ANP}$ ...10.2μg. N./ml.
Photographed at 7th day of incubation.

4) The antigenic modification of previously purified T-component during digestion with ANP.

To clarify the mechanism of the reduction or elimination of antigenicity of the T-component by ANP, antigenic variations during digestion of T-component with ANP were studied by the precipitin reaction with T-component antiserum in an agar medium.

One hundred mg. of the T-component (T-comp. II) was dissolved in 10 ml. of M/15 acetate buffer (pH 3.5) containing 0.25% phenol and M/2000 $ZnSO_4$. The pH was adjusted to pH 3.5 with N-HCl. 0.14 ml. of ANP (Protease actibity, 9.6 units) was added to the T-component solution and the mixture incubated at 37°C for 72 hours. The ratio of enzyme N. to substrate N. was about 1 : 310 in this experimental. Initially and at 1, 3, 9, 24, 48 and for 72 hours during the incubation, an 1 ml. aliquot of the digest was taken. One drop of N-NaOH was added to this sample to stop enzymic action. Then it was diluted to 4 ml. with 1% NaCl and 0.01% merthiolate in M/50 phosphate buffer at pH 7.6. The antitoxic activity remaining was estimated in each sample and each was examined by the gel precipitin reaction. The digests were stored at -60°C before use. As a control, denatured ANP heated at 60°C for 1 hour at PH 7.6 was added to the T-component solution and incubated at 37°C for 72 hours. Each sample was put into a well made in a agar plate and made to react with T-component antiserum.

The results are shown in Table IV and in Fig. 12.

Table IV. Course of digestion of purified T-component by ANP.

Sample taken at (hr)	0	1	3	9	24	48	72	Control
Antitoxin* unit/ml.	50	40	35	30	30	30	30	50
Number of precipitin line	1	2	2	1	1	1	1	1

* Antitoxin units of each sample expressed as the value obtained after 4 fold dilution.

The destruction of antitoxin proceeded for 9 hours of incubation but then the antitoxicity remained constant.

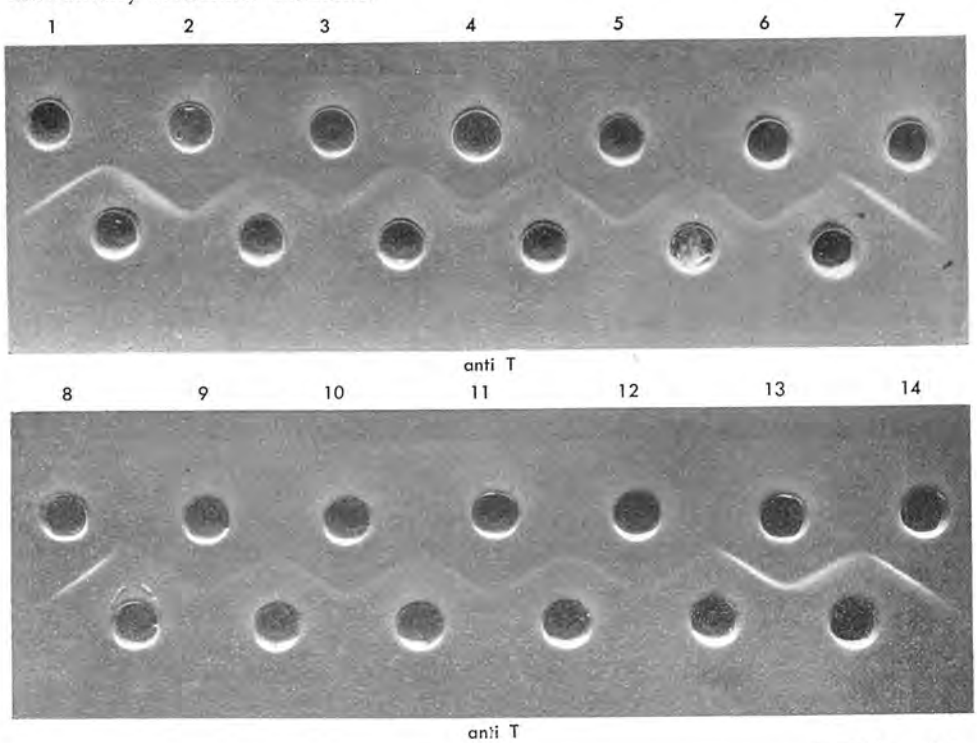

Fig. 12 Variations of precipitin line in the course of digestion of purified T-component by ANP against T-component antiserum.

Note : T-component antiserum ... R-5 (antibody N.=0.323mg./ml.)

Antigen: 1 ..... T-comp. II (2.5mg./ml.)
2 ..... digest after 0 hr.
3 ..... // // 1 hr.
4 ..... // // 3 hr.
5 ..... // // 9 hr.
6 ..... // // 24 hr.
7 ..... T-comp. II (2.5mg./ml.)
8 ..... // //
9 ..... digest after 9 hr.
10 ..... // // 24 hr.
11 ..... // // 48 hr.
12 ..... // // 72 hr.
13 ..... T-comp. II (2.5mg./ml.)
14 ..... Control

Photographed at 10th day of the incubation.

The appearance of the precipitin lines varied considerably during the first 9 hours of incubation. After one hour two precipitin lines appeared and after 3 hours the two precipitin lines were clearly separated from each other. In later stages of digestion only a faint precipitin line was observed. As shown in Fig. 12, the precipitin line of the T-component fused with that of samples of antigen solution after 9, 24 and 72 hours forming a "spur". The intial precipitin line of the digest was relatively vague and diffuse as compared with that of the native T-component. Though the initial sample was taken as soon as the ANP had been added to the T-component solution, the T-component could have been digested to some degree by the ANP untill the NaOH was added.

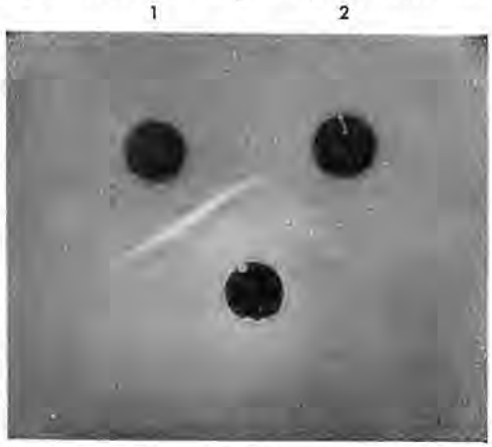

Fig. 13 .Antigenic relationship between T-component digested for 3 hours by ANP and T-component against T-component antiserum in precipitin reaction in agar medium.
Note : T-component antiserum ... R-5 (antibody N.= 0.323mg./ml.)
Antigen : 1 ..... T-com. II (2.5mg./ml.)
2 ..... digestion product at 3 hr.
Photgraphed at 10th day of the incubation

The precipitin line of the control solution of T-component treated with denatured ANP had the same appearance as that of native T-component in spite of some decrease in antitoxicity. To see if one of the two precipitin lines could be due to undigested T-component remaining in the solution a sample digested by ANP for 3 hours and native T-component were put into antigen-wells and diffused against T-component antiserum.

As shown in Fig. 13 the single line of native T-component fused with both two precipitin line of sample of antigen solution after 3 hours digestion forming a "spur" against T-component antiserum. Therefore the two precipitin lines of the later sample must would be formed by two types of peptides split from the T-component.

It is concluded that the determinant groups of the T-component can be differentiated into two or more sub-groups differing in their serological specificity.

DISCUSSION

In past years, it has been supposed that, the residual antigenicity of the enzyme modified antitoxic globulin may be due to contaminating undigested serum component remained after enzymic digestion. If the residual antigenicity of EAGL is due only to contaminating undigested T-component, all precipitable antibodies would be precipitated in the extreme antigen excess region in the quantitative precipitin reactions of the EAGL with T-component antiserum. However, the equivalence zone of each sample of EAGL was always found in the antibody excess region of the orginal T-component and the maximal amount of each antibody precipi-

tated was always less than the actual amount of the antibody precipitated by the T-component. Moreover, the supernatants of these systems contained antibody which was precipitated by further addition of the T-component.

Moreover, in the supernatants of the quantitative precipitin reaction of the EAGLs with T-component antiserum, there was a region in which the supernatants did not give any precipitates on further addition of each EAGL or of T-component antiserum. This suggests that the antibody produced against homogenous T-component is essentially heterogenous in its serological specificity. If different antibodies were produced against each determinant group in terms of their specificity, our results could easily be explained by supposing that each EAGL lost some determinant groups during digestion by protease, so that it could not precipitate some of the fractions of the T-component antibodies. Such a possibility was emphasized by the fact that the precipitin line of the T-component fused with that of each EAGL forming a "spur".

Moreover, during digestion of purified T-component by ANP, the precipitin line of the native T-component was fused with two lines of digested T-component forming a "spur" behind two lines. From this fact it is evidenced that T-component had been split into two or more types of peptides differed in its serological specificity.

Recently Lapresle (1955, 1957a, 1967b, and 1958) and Pope (1958) independently observed a similar phenomenon with highly purified protein antigen. Pope (1958) reported that highly purified crystalline diphtherial toxin gave a number of precipitin lines treated with protease or under conditions of denaturation. He therefore assumed that there was microheterogeneity in the molecules of crystalline toxin and these different molecules were digested or denatured in different ways.

Though our experimental conditions employed were supposed to be drastic for the T-component and the antitoxicity of the control without enzyme decreased to about 90%, the precipitin line formed by this sample could not be differentiated from that of the native T-component. Therefore the multiplicity of the precipitin lines of the T-component partialy digested by ANP could only be explained as being the lines formed by the precipitation of the peptides split off with their respective antibodies.

Lapresle (1955, 1957a, 1957b, and 1958) and Raynaud (1958) had found essentially the same phenomenon using human serum albumin and diphtherial toxin respectively. To elucidate more precisely the multiplicity of precipitin lines it would be necessary to separate the various peptides which were split off in pure state and to study the structure of each determinant group in relation to the chemical structure of the peptide.

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