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Immunochemical Studies on Lysozyme
II. On the Non-neutralizing Antibodies

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SUMMARY

Two polypeptides were isolated after the digestion of HL by pepsin at pH 1.62. These polypeptides were capable of inhibiting the precipitin reaction of some batches of HL antisera with HL but incapable of inhibiting the neutralization of HL by antibodies. The possibility, that the polypeptides were derived from some minor contaminants, was excluded by demonstrating the homogeneity of the antigen-antibody system by the gel diffusion test and by the recovery of active HL from neutralized precipitates but not from supernatants.

The presence of HL-non-neutralizing antibodies in HL antiserum was essentially proved by demonstrating an enzymatically active HL-antibody complex by gel filtration experiments with Sephadex G-75, G-200 and also by its ultracentrifugal separation in a linear sucrose density gradient using a swinging-bucket type rotor.

Suggestive evidence was obtained for the presencec of an antibody-like globulin of lower molecular weight 7S antibody which form an active complex with HL in HL antiserum.

INTRODUCTION

As lysozyme is the lowest molecular weight antigenic enzyme protein and consists of a single polypeptide chain (Fraenkel-Conrat, 1951), it is the most suitable for chemical analysis of protein structure as well as for immunochemical analysis of the determinant groups in its protein. For the latter study, results of the former analysis are necessary. In this point, lysozyme is the most suitable for immunochemical studies on its antigenic structure, because the amino acid sequence of hen egg white lysozyme had been studied by Jollès and Fromageot (1958) and almost all the sequence has recently been clarified by Jollès (1961). Therefore immunochemical analysis of this enzyme has been studied in this laboratory (Fujio *et al.*, 1959) and comparative studies have been made on lysozyme (HL) and lysozyme methyl ester (HLME), whose "enzymoid" nature was reported by Frieden (1956).

The following abbreviations are used in this report: hen egg white lysozyme (HL), methyl ester of HL (HLME), polyglutamate of *B. anthracis* (GPP)

It has been found that HL antiserum contains two kinds of specific neutralizing antibodies as well as precipitins.

Further attempts were made to isolate polypeptides split from HL which were capable of inhibiting corresponding neutralizing antibodies. However, neither treatment with pepsin nor trypsin resulted in the appearance of such polypeptides. In the course of this studies, it was found that pepsin digestion under certain conditions gave two polypeptide fractions, which inhibited the precipitin reaction of some batches of HL antisera but not at all the neutralization phenomena. As antisera were prepared by immunizing rabbits with a purified preparation of HL together with incomplete Freund's adjuvant and it was shown that immunization with Freund's adjuvant stimulates antibody formation against contaminants in the antigen preparation (Kabat, 1958), attempts were made to demonstrate the presence of non-neutralizing and precipitating antibodies in some batches of HL antisera. The present report is on the presence of a non-neutralizing, but precipitating antibodies and, in addition, an antibody-like globulin of low molecular weight capable of combining with HL without causing its neutralization.

MATERIALS AND METHODS

1. *Hen egg white lysozyme*

HL was prepared as described in our first report (Fujio *et al.*, 1959).

2. *HL antisera*

The immunization schedule and the preparation of the antigen in incomplete Freund's adjuvant were described in our first report. The batch numbers of the antisera used in this study were LF-13, LF-16, LF-18, LF-19 and SK-2. The total precipitable protein N at the equivalence point and the amounts of HL giving this point in each batch are listed in Table 1.

Table 1. Total Precipitable Protein N at Equivalence Point and Amount of HL giving this Point with Each HL-Antiserum

HL-antiserum	Total ppt protein	HL added
	μgN	μgN
LF-13	355	20
LF-16	626	37
LF-18	240	13
LF-19	470	27
SK-2	900	53

HL-antiserum : 1.0 ml

After inactivation at 56°C for 30 min., serum lysozyme was removed, when necessary, by gel filtration through a column of Sephadex G-75, a sieve for molecules of less than 40,000 molecular weight, equilibrated with saline containing M/50 phosphate buffer at pH 6.0. At this pH the association of lysozyme with serum proteins and its adsorption to Sephadex particles could be avoided. The antisera was adjusted to its original volume by adding an appropriate amount of dry Sephadex G-25 particles (molecular sieve for molecules of less than 3,500 - 4,500 molecular weight).

3. *Crystalline pepsin*

Difco pepsin was purified in crystalline form according to the method of Northrop (1930) and recrystallized twice.

4. *Quantitative precipitin reaction*

The procedures were carried out as described by Heidelberger and Kendall (1935). The antigen nitrogen and precipitated antibody nitrogen were determined by a modified direct Nesslerization method (Yokoi and Akashi, 1955). The pH of the mixture of antigen and antiserum was adjust to 6.0 in all experiments. The maximum precipitated protein N was the same as when experiments were carried out at pH 7.0.

5. *Direct Nesslerization for determination of protein nitrogen*

The procedure used was essentially as described by Yokoi and Akashi. The standard curve made by this modified method was linear up to 120 μg N. The experimental error was in the range of 2.0 μg N.

6. *Determination of lysozyme activity*

Two methods were used in this study.

i) *Turbidity method*

The *M. lysodeikticus* suspension used as substrate was prepared as described by Smolelis and Hartsell (1949). The bacterial suspension was made in saline buffered with M/15 phosphate buffer, pH 6.0, at a concentration of 1.4 mg of lyophilized cells per ml and was homogenized. A standard curve for lysozyme activity was obtained as follows. Two ml of prewarmed phosphate buffered saline containing varying amounts of lysozyme were mixed with 2.0 ml of prewarmed bacterial suspension, and the mixtures were incubated at 37°C for 5 minutes. The turbidity was estimated in a Beckman spectrophotometer at 540 mμ and the reduction in turbidity plotted against the concentration of lysozyme.

ii) *Capillary method*

When a very small amount of sample was to be assayed, this capillary method designated, as the micromethod, was used. The bacterial suspension was made in saline containing M/15 phosphate buffer, pH 6.0 at a concentration of 9 mg dry cells per ml and was homogenized. To one volume of the bacterial suspension were added 10 volumes of melted soft agar (0.67 per cent in saline containing M/15 phosphate buffer at pH 6.0) and the mixture was stirred thoroughly at 50°C. Glass capillaries (0.7 mm inner diameter) were filled with the mixture and then cut into about 5 cm lengths. When cool one tip of each capillary was sealed. The open tip of the capillary was inserted into the test sample and incubated at 37°C for 17 hours. The length of the transparent zone was measured. This assay method enabled us to estimate lysozyme in two drops of standard sample containing from 0.01 to 0.3 μg N per ml.

7. *Gelfiltration*

For the separation of proteins of different molecular weight, gel filtration at 4°C was employed. For this purpose a column of Sephadex G-75 or G-200 (Pharmacia) was used. In this experiment Sephadex particles were wetted with saline containing M/50 phosphate buffer, pH 6.0, and eluted with the same buffered saline.

8. *Concentration of protein solution by Sephadex G-25*

An appropriate amount of dry Sephadex G-25 was added to the protein solution to be concentrated. The particles were separated on a glass filter.

9. *Polyglutamate(A-GPP) of B. anthracis and A-GPP antiserum*

These materials were supplied by Dr. Utsumi of this laboratory, who used the same batches of materisls in his study (Utsumi *et al.*, 1959).

10. *Bovine serum albumin antiserum*

Immunization of rabbits to bovine serum albumin (Armour, powder) was accomplished in the same way as with HL.

11. *Gel diffusion experiment*

Ouchterlony's method (1949) was employed.

12. *Ultracentrifugation*

Ultracentrifugal analysis were performed in a Hitachi Model UCA-1 ultracentrifuge and substances with different sedimentation constants were separated in a linear sucrose density gradient in a Hitachi Model 40P ultracentrifuge with a swinging-bucket type rotor.

RESULTS

1. *Isolation of polypeptides which inhibited precipitation after peptic digestion of HL*

A purified preparation of HL was digested by pepsin at pH 1.62 and the split polypeptides were separated from intact HL by chromatography on IRC-50 (XE-64) ion-exchange resin at pH 7.18. The polypeptide fraction was rechromatographed at pH 6.6 and two fractions (Fr. 1 and Fr. 2) were obtained.

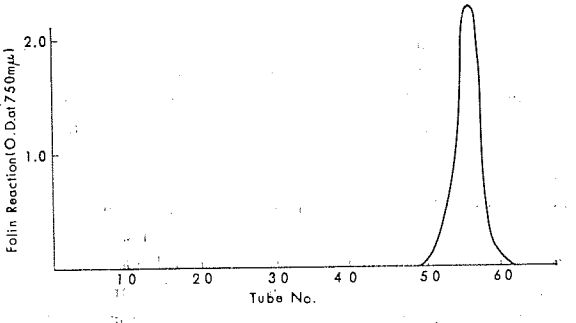


Fig. 1. Column Chromatography of Untreated HL
IRC-50 (XE-64): 1.5×30 cm column, M/5 P-buffer, pH 7.18, 25° C.
Fraction size, 2.8 ml; rate of elution, 4 ml per hour.

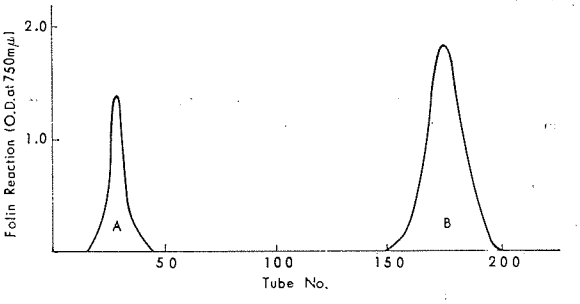


Fig. 2. Column Chromatography of Pepsin-digested HL
IRC-50 (XE-64): 9×32 cm column, M/5 P-buffer, pH 7.18, 25°C.
Fraction volume, 30 ml; rate of elution, 180 ml per hour.

Pepsin (Difco) was obtained in crystalline form and recrystallized according to the method of Northrop. 1864 [P.U.] cas.,275, γ-Tyr. of crystalline pepsin in a volume of 3.58 ml at pH 1.62 (adjusted with 0.1 N HCl) were added to 347 ml of 1.28 per cent HL solution at pH 1.62 (adjusted with 0.1 N HCl) and the mixture was incubated at 40°C for 1 hour. The pH was adjusted to 7.0 and the mixture centrifuged at 3,000 rpm for 20 min. The supernatant was applied to a column (9 × 32 cm) of IRC-50 (XE-64) equilibrated with M/5 phosphate buffer, pH 7.18, saturated with thymol. It was eluted with the same buffer at 25°C. The protein and polypeptides were measured with Folin reagent. Fig. 1 shows a chromatogram of untreated HL and Fig. 2 shows the polypeptide fraction separated from intact HL. This fraction was desalted according to the method of Dixon (1959): glacial acetic acid was added to the sample to 5 per cent and the solution was applied to a column (3 × 20 cm) of IRC-50 (CG-50) equilibrated with 0.1 N acetic acid. The phosphate buffer was removed by elution of the column with 0.1 N acetic acid until no sodium ion was detected by the flame reaction. Adsorbed polypeptides were eluted with 0.4 M ammonium carbonate buffer, pH 9.0. Fraction A containing polypeptides was lyophilized to remove ammonium carbonate.

The polypeptides were rechromatographed on a similar column equilibrated with M/5 phosphate buffer, pH 6.6, saturated with thymol and eluted with the same buffer. Two fractions were obtained, as shown in Fig. 3. These fractions were also desalted and lyophilized. Seventy seven mg of Fr. 1 and 305 mg of Fr. 2 were obtained.

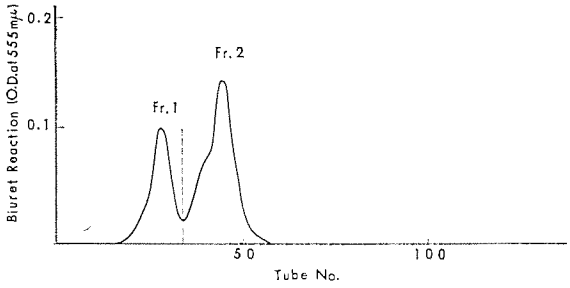


Fig. 3. Rechromatography of Fraction A of Fig. 2

IRC-50 (XE-64): 9×32 cm column, M/5 P-buffer, pH 6.6, 25°C. Fraction size, 30 ml; rate of elution, 180 ml per hour.

2. *Inhibitory effect of Frs. 1 and 2 on the precipitin reaction*

The polypeptides thus obtained inhibited the precipitin reaction of some but not all batches of HL antiserum with homologous antigen HL.

HL antiserum batch LF-16 was used and 7.3 μg N of HL was equivalent to 0.2 ml of the antiserum. Varying amounts (15, 30, 60, 120 μg N) of Fr. 1 or Fr. 2 in 1.0 ml of saline containing M/50 phosphate buffer, pH 6.0, were added to tubes containing 0.4 ml of 1:2 dilution of the antiserum. The tubes were set up in duplicate (A and B series) for each polypeptides fraction. As a control, a tube was set up containing 1.0 ml of buffered saline in place of the polypeptides solution.

All the tubes were incubated at 37°C for 30 min. and after 30 min. all were quite clear. Then 0.5 ml aliquots of buffered saline, containing 7.3 μg N of HL, were added to the tubes in series A and also to the control tube. To the tubes in series B 0.5 ml aliquots of buffered saline were added. All the tubes were again incubated at 37°C for 1 hour and then at 4°C for 48 hours. The amount of protein precipitate was estimated. The results are shown in Fig. 4. As the tubes in series B of Frs. 1

and 2 were quite clear and no significant precipitate was found, these results are not included in Fig. 4.

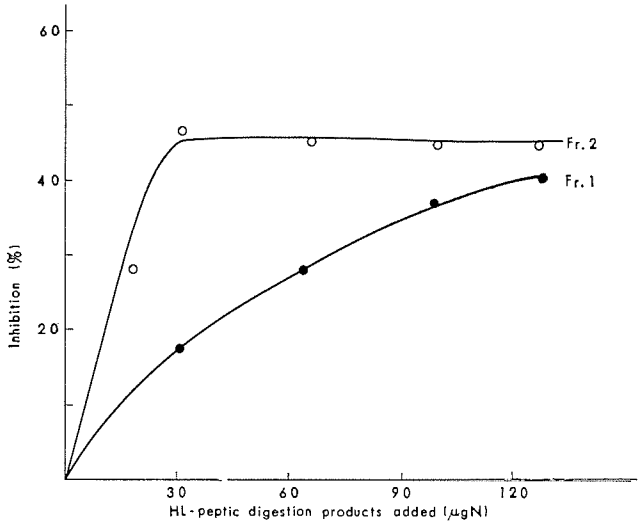


Fig. 4. Inhibition of Specific Precipitation in a System Containing HL and Anti-HL Serum by Two Polypeptide Fractions

HL: 7.3 μg N
Anti-HL Serum: 0.2 ml
(LF-16)

As can be seen in Fig. 4, Fr. 2 inhibited the precipitin reaction strongly and the maximal inhibition caused by the two fractions was about 40 per cent. As neither fraction gave a precipitate, they could be regarded to be univalent.

Table 2. Inhibition of Precipitation in a System Containing *B. anthracis* G.P.P. and Its Homologous Antiserum by Two Fractions of Polypeptides

	Tube No.							
	1	2	3	4	5	6	7	8
Fr.1 (60 μgN/0.5 ml)	0.5 ml	—	—	0.5 ml	—	0.5 ml	—	—
Fr.2 (60 μgN/0.5 ml)	—	0.5 ml	—	—	0.5 ml	—	0.5 ml	—
Buffered Saline ml	—	—	0.5	—	—	0.5	0.5	0.5
Anti G.P.P. Serum ml	0.5	0.5	0.5	0.5	0.5	—	—	0.5
Incubated at 37°C for 30 min								
G.P.P. (100 μg/ml) ml	0.5	0.5	0.5	—	—	0.5	0.5	—
Buffered Saline ml	—	—	—	0.5	0.5	—	—	0.5
Incubated at 37°C for 1 hr. and at 4°C for 48 hrs.								
Total ppt μgN	121.4	114.0	117.0	5.5	6.1	6.0	6.2	7.0

To demonstrate the specificity of the inhibitory effect of the two polypeptide fractions, the inhibitory effect was studied in the system composed of polyglutamate (GPP) of *B. anthracis* and its homologous antiserum.

The amount of antigen at the equivalence point was 50 μg per 0.5 ml of the antiserum. Sixty μg N of each fraction in 0.5 ml of buffered saline were added to two tubes containing 0.5 ml of GPP antiserum. After 30 min. incubation at 37°C, 50 μg of GPP in 0.5 ml of buffered saline were added to the tubes. The tubes were incubated at 37°C for 1 hour and then at 4°C for 48 hours. The precipitated protein N was estimated. The results are shown in Table 2.

As shown in Table 2, no significant inhibition was observed. From this fact it can be assumed that these polypeptides are specific univalent inhibitors for HL. Frieden (1956) described the competitive inhibition of HL by its inactive methylated derivative. Therefore the competitive inhibition of the two polypeptides against HL was studied. It was found that they exhibited no competitive inhibition.

3. *The inhibitory effect of the polypeptide fractions on the neutralizing activity of batch LF-16 of HL antiserum*

Varying amounts (7.5, 15, 30 and 60 μg N) of each polypeptide fraction in 0.5 ml of buffered saline, were added to tubes containing 1.0 ml of 1:20 dilution of the antiserum. A control tube was

Table 3. Specific Inhibition of the Neutralizing Activity of HL Antiserum by Two Polypeptide Franctions

	Tube No.												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Fr. 1 μgN in 0.5 ml	7.5	15	30	60			——		60	—	——		—
Fr. 2 μgN in 0.5 ml			——		7.5	15	30	60	—	60	——		—
Buffered Saline ml			——				——			——	0.5		2.0
HL-Antiserum LF-16 (1:20 diluted) ml			1.0				1.0			1.0	1.0		—
Incubated at 37°C for 30 min.													
HL μgN in 0.5 ml			3.65				3.65			3.65	3.65		—
Incubated at 37°C for 1 hr. and at 4°C for 24 hrs.													
Cell Suspension ml			2.0				2.0			——	2.0	—	2.0
Buffered Saline ml			——				——			2.0	—	2.0	—
Turbidity	0.581	0.582	0.595	0.592	0.590	0.575	0.598	0.595	0.013	0.012	0.598	0.010	0.731
HL Activity (—O.D.)	0.150	0.149	0.136	0.139	0.141	0.156	0.133	0.136	—	—	0.133	—	0

also included, in which the polypeptide solution was replaced by buffered saline. All the tubes were incubated at 37°C for 30 min. and 3.65 μg N of HL in 0.5 ml of buffered saline were added to them all. An amount of lysozyme was added corresponding to twice the equivalence point. After incubation at 37°C for 1 hour and at 4°C for 24 hours, 2.0 ml aliquots of *M. lysodeikticus* suspension were added to prewarmed tubes at 37°C and the tubes were further incubated at 37°C for 5 min. The optical density was read in a Beckman spectrophotometer at 540 mμ. The results are shown in Table 3.

As can be seen the decrease in optical density due to unneutralized HL was the same in the control and the experimental tubes. Similar results were obtained with two other batches of HL antiserum (LF-19 and -6) which were also inhibited by both polypeptides in the precipitin reaction. Therefore neither polypeptide contained an antigenic site corresponding to the neutralizing antibodies.

4. *Precipitin reaction on Ouchterlony plates*

Since Frs. 1 and 2 did not block the neutralizing antibodies for HL, it was possible that they were derived from minor contaminants. This is readily conceivable, because antisera prepared by immunizing rabbits with an antigen dissolved in Freund's adjuvant contains frequently antibodies to contaminating minor antigens. To test this possibility, it was desirable to study the precipitin reaction of HL with batch LF-16 of HL antiserum by the agar diffusion technique to determine whether detectable amounts of impurities were present.

As both Frs. 1 and 2 inhibited at maximum about 40 per cent of the precipitin reaction at the equivalence point and the yield of Fr. 2 was about 10 per cent of the HL digested, the amount of HL added in this experiment corresponded to the equivalence (well 1) and to ten times the equivalence (well 2). As can be seen in Fig. 5, only a single precipitation line was detectable. In addition, HL gave a single line when the other two batches (LF-19 and -6) were tested. In these batches precipitin reactions with HL were also inhibited by both Fr. 1 and Fr. 2. Therefore these antisera contained precipitins for a single antigen.

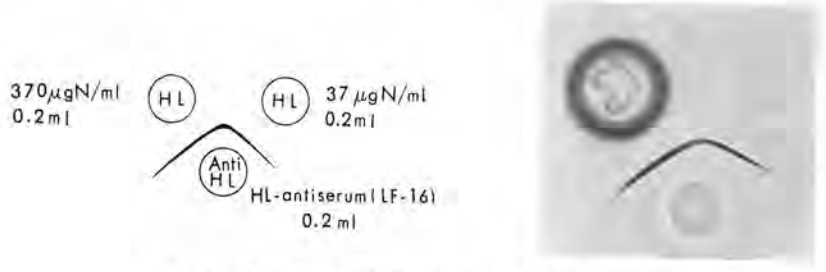


Fig. 5. Gel-diffusion of HL and Anti-HL Serum

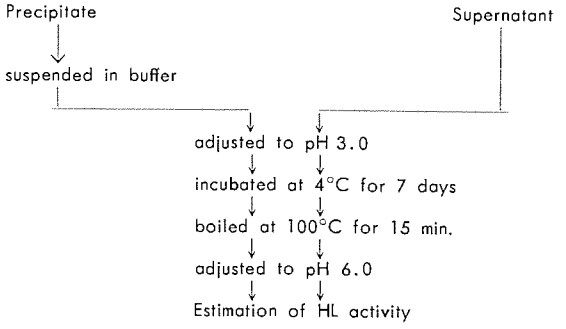
5. *Recovery of HL from the neutralized precipitates*

In the above experiment, batch LF-16 of HL antiserum was shown to contain precipitins for a single antigen. However, it was possible that the precipitins were

produced by a contaminant and that the neutralizing antibodies for HL present were non-precipitating. If so, the above results could easily be explained. To test this possibility attempts were made to denature the neutralizing antibodies leaving the HL intact and tests were made to see whether the HL was present in the supernatant of the precipitin reaction or in the precipitate. Heating the mixture at 100°C for 15 min. at pH 3.0 completely denatured antibodies in a 1:20 dilution of normal rabbit serum and this treatment destroyed only 20 per cent of the HL activity. Precipitin reactions were performed at the equivalence point and at three-fifths of the equivalence point. In both tubes HL was completely neutralized.

The HL antiserum used in this experiment was from batch LF-19, which behaved in the same way as batch LF-16 with Frs. 1 and 2. Varying amounts (32.5 and 50 μg N) of HL in 2.5 ml of saline containing M/50 phosphate buffer, pH 6.0, were added to tubes containing 2.5 ml of this antiserum. Two control tubes were also included, in which the antiserum was replaced by normal rabbit serum. The normal rabbit serum used in this study had previously been passed through a column of Sephadex G-75 in buffered saline, pH 6.0, to remove serum lysozyme. The tubes were incubated at 37°C for 1 hour and at 4°C for 7 days. After centrifugation, each precipitate was suspended in 4.0 ml of a 1:20 dilution of normal rabbit serum buffered at pH 3.0 with acetic acid. The pH of the supernatants and two control tubes was also adjusted to 3.0 with acetic acid. All the tubes were again incubated at 4°C for 7 days. Although a small portion of the suspended precipitates did not dissolve, all the tubes were heated at 100°C for 15 min. Then the pH was adjusted to 6.0 and the mixture were centrifuged. The activity of the recovered HL was estimated. In control tubes the recoveries of HL activity were 95 per cent of 50 μg N and 79 per cent of 32.5 μg N. Therefore, the recoveries in experimental tubes were corrected by multiplying their activities by 1/0.95 and 1/0.79, respectively. The experimental procedures and results are shown in Fig. 6.

Fig. 6. Recovery of HL Activity from Precipitates and Supernatants at Equivalence and an Antibody Excess Points (HL-Anti-HL Serum System)



Recovery from Ppt.		Recovery from Supernat.	
Ab. excess	Eq. point	Ab. excess	Eq. point
88.3%	72.7%	0%	0%

As shown in Fig. 6, 73 and 88 per cent of the HL activities were recovered from the two precipitates and no activity was recovered from either supernatant. Therefore neutralized HL was precipitated. Thus the single precipitation line seen between HL and HL antiserum of batch LF-16 on the Ouchterlony plate must be formed by HL and its antibodies. These results show moreover that Frs. 1 and 2 inhibited the precipitin reaction of HL and not of a contaminant.

6. *Demonstration of non-neutralizing antibodies for HL*

The results described above suggest the presence of non-neutralizing precipitins for HL in batches LF-16 and LF-19 of HL antiserum. If these really exist, it is conceivable that enzymatically active HL-antibody complexes are present in the supernatant in the zone of extreme antigen excess. To prove this, the supernatant of the precipitin reaction at the extreme of antigen excess was passed through a column of Sephadex G-75 equilibrated with saline containing M/50 phosphate buffer, pH 6.0, a pH at which there was no interaction of HL with Sephadex particles or association of HL with normal serum proteins.

536 μg N of HL in 8.9 ml of phosphate buffer, pH 5.65, (μ = 0.15) were added to a tube containing 4.5 ml of batch LF-13 HL antiserum, in which the precipitin reaction was also at maximum inhibited about 40 per cent by Frs. 1 and 2. The amount of HL added corresponded to 6 times the equivalence point. The pH of the mixture was 6.0. After incubation at 37°C for 1 hour and at 4°C for 3 days, the tube was centrifuged. Twelve ml of the supernatant were passed through a column (3 × 104 cm) of Sephadex G-75 equilibrated with saline containing M/50 phosphate buffer, pH 6.0. Material was eluted with the same buffered saline. The eluate was collected in 10 ml fractions. Their protein contents and HL activities were estimated.

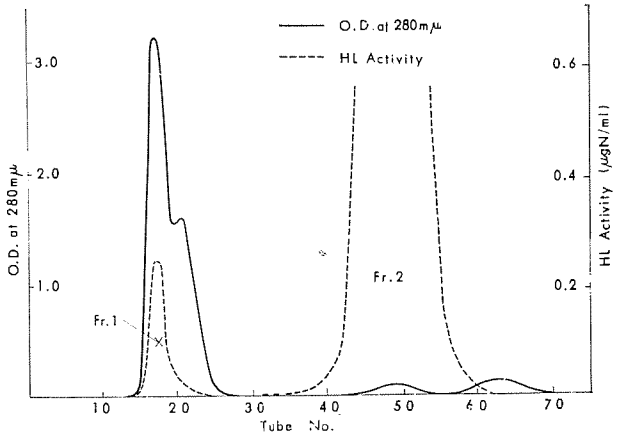


Fig. 7. Gel-filtration with Sephadex G-75 of the Supernatant at the Antigen Excess Zone of an HL-Anti-HL Serum System

Column: 3×104 cm, M/50 P-buffered saline, pH 6.0, 5-10°C. Fraction volume, 10 ml; rate of elution, 60 ml per hour.

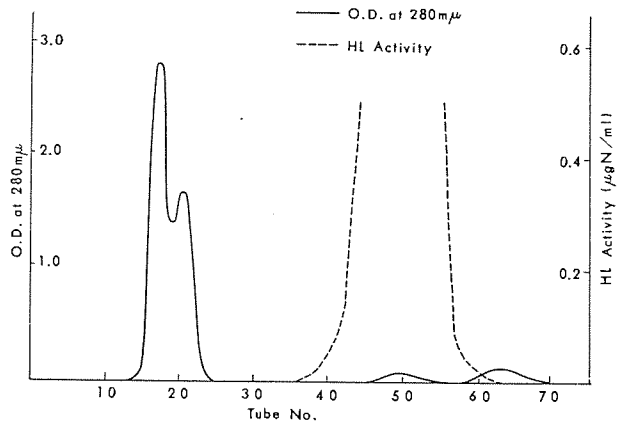


Fig. 8. Gel-filtration with Sephadex G-75 of a Mixture of HL and Normal Rabbit Serum

Column: 3×104 cm, M/50 P-buffered saline, pH 6.0, 5-10°C.
Fraction volume, 10 ml; rate of elution, 60 ml per hour.

As shown in Fig. 7, HL activity was found in two fractions. Fr. 1 was found at the end of the void volume of the column together with serum proteins. Fr. 2 was found at the end of the inner volume of the column and this fraction contained free, unneutralized HL. A control experiment was performed with normal rabbit serum in place of HL antiserum and no HL activity was found in the position of Fr. 1, as shown in Fig. 8. These facts indicate that the activity found in Fr. 1 was due to an active HL-antiboby complex. However it was possible that HL was dissociated from neutralized complexes by a shift in chemical equilibrium caused by the removal of free HL. If so, there would be no peak of HL activity in the first fraction. To test this possibility, Fr. 1 was concentrated by adding dry Sephadex G-25 particles and the mixture was then filtered through a glass-filter and

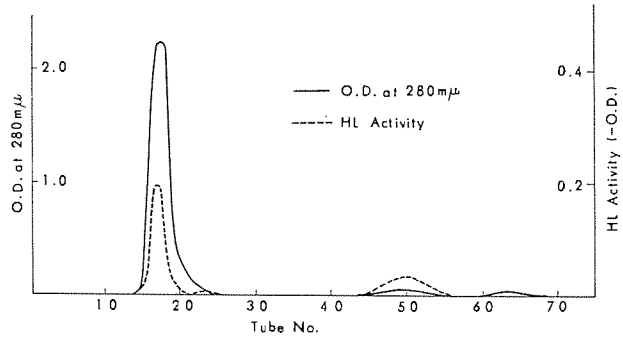


Fig. 9. Repeat of Gel-filtration with Sephadex G-75 of Fraction I of Fig. 7.

Column: 1.5×48 cm, M/50 P-buffered saline, pH 6.0.
Fraction volume, 2.5 ml; rate of elution, 15 ml per hour.

rechromatographed. At pH 6.0, HL was not adsorbed by Sephadex G-25 particles. A portion of the concentrated Fr. 1 was chromatographed on a column (1.5 × 48 cm) in buffered saline at pH 6.0. The results are shown in Fig. 9. Again, the first fraction showed a peak of HL activity and the peak of the second fraction was lower than that of Fr. 1 of the first run. Therefore enzymatically active HL-antibody complex was present in the supernatant and this antibody was definitely a non-neutralizing antibody.

7. *Ultracentrifugal studies on the enzymatically active complexes*

To reconfirm this, ultracentrifugal studies were made of Fr. 1 after concentration as described above. A Hitachi model 40P ultracentrifuge with a swinging-bucket type rotor was used.

A linear gradient of sucrose density in saline buffered with M/50 phosphate at pH 6.0 (from 20 to 5 per cent) was made in a tube (total volume 4.2 ml) and 0.2 ml of concentrated Fr.1 was overlayed on linearly varying sucrose concentration from 4 to 0 per cent (total volume 0.4 ml). As controls, 0.2 ml of a mixture of normal rabbit serum and HL, and 0.2 ml of HL were overlayed on two other tubes. The centrifuge was rotated at 38,000 rpm for 18.5 hours at 4°C. After centrifugation, a pin hole was made at the bottom of each tube and 2 drops of the contents were put into other tubes. The HL activity was estimated by the capillarly method as described in *Methods*. After esti-

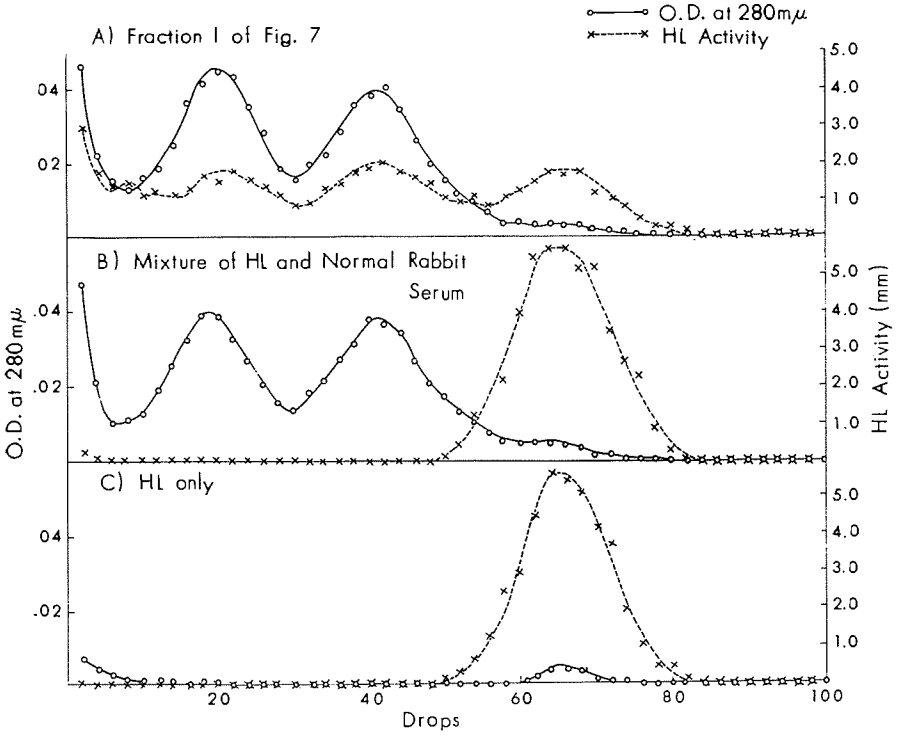


Fig. 10. Sucrose Density Gradient Centrifugation Analysis of the Fraction I of Fig. 7.

mation, 2.5 ml of saline buffered with M/50 phosphate buffer, pH 6.0, were added to the tubes and the protein concentration was estimated in a Beckman spectrophotometer at 280 mμ. The results are shown in Fig. 10A, B and C.

Fig. 10A shows three protein peaks. The slowest moving component was shown to be free HL when referred to the results shown in Fig. 10B and C. HL activity was found in both serum protein fractions shown in Fig. 10A. To identify the serum proteins, the following experiment was performed. Normal rabbit serum was fractionated with ammonium sulfate, and the globulin was precipiteted at 1/3 saturation and the albumin between 1/2 to 3/4 saturation. The albumin fraction was purified further by zone electrophoresis to remove traces of globulin. Using these serum protein fractions, ultracentrifugal studies were made under the conditions described above. The results are shown in Fig. 11A, B and C. The fast moving component and the slower moving component were shown to be globulin and albumin, respectively. The positions of the two serum proteins in Fig. 10A are superimposable with those in Fig. 11. Thus the globulin and albumin fractions in Fig. 10A contained HL activity. As shown in Fig. 10B, no HL activity was detected in globulin or albumin fractions of normal serum, so the HL activity fround in the globulin of antiserum can be regarded as an enzymatically active

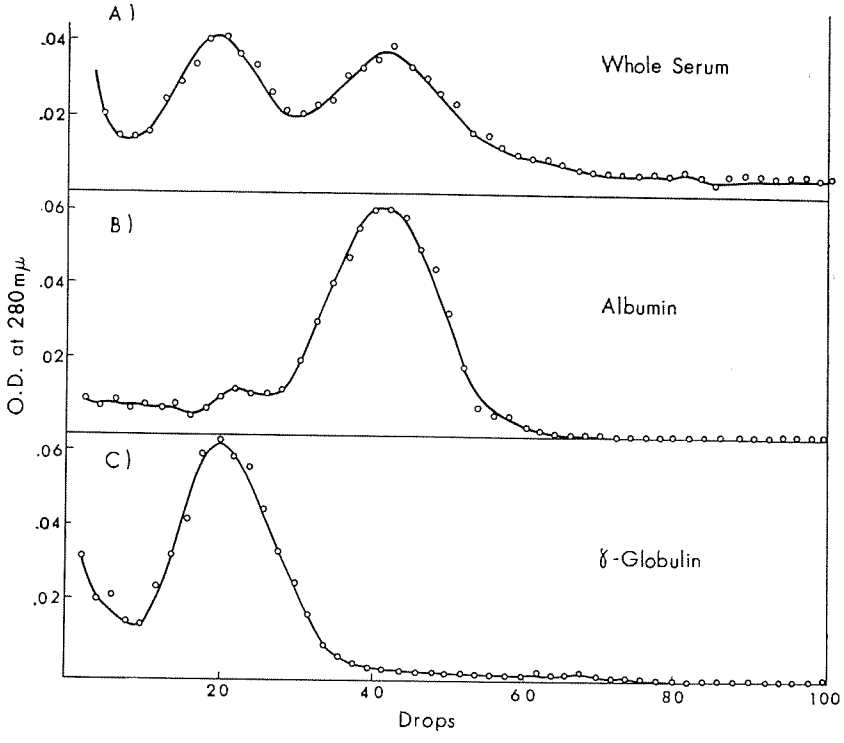


Fig. 11. Ultracentrifugal Analysis of Normal Rabbit Serum Components

complex, because the molecular weight of HL is very small (14,500). However, the reason why the albumin fraction had HL activity could not be explained.

To study this, batch LF-18 of HL antiserum of the same character was fractionated with ammonium sulfate in the same way as normal rabbit serum. The same excess of HL was added to tubes containing globulin and albumin. After incubation at 37°C for 1 hour and at 4°C for 2 days samples were centrifuged and the supernatants passed through a Sephadex G-75 column in buffered saline, pH 6.0. As shown in Fig. 12A and B, an active complex was found in the globulin

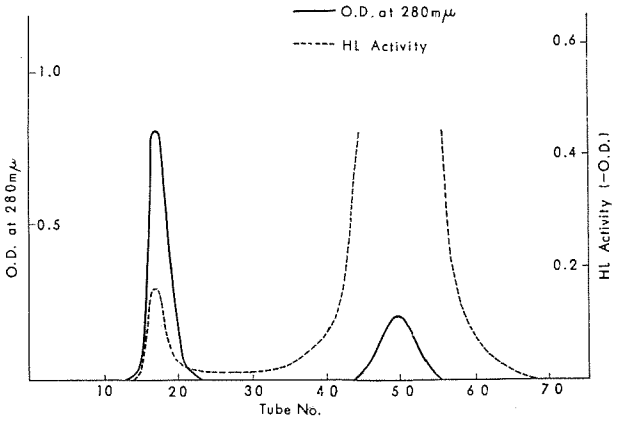


Fig. 12. A) Gel-filtration with Sephadex G-75 of Antigen Excess Supernatant of System of HL and Euglobulin of HL-Antiserum

Column: 3×104 cm, M/50 P-buffered saline, pH 6.0, 5-10°C.
Fraction volume, 10 ml; rate of elution, 60 ml per hour.

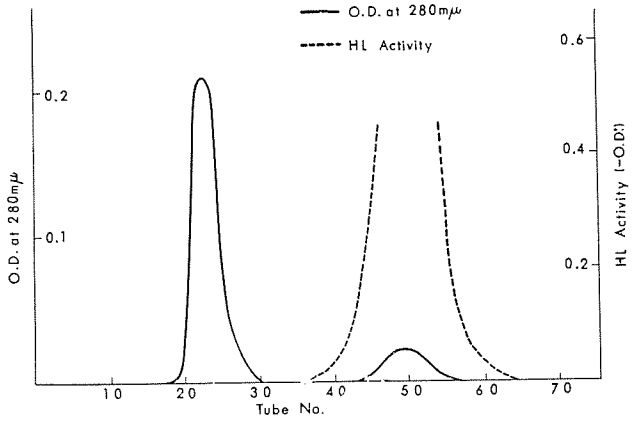


Fig. 12. B) Gel-filtration with Sephadex G-75 of a Mixture of HL and Albumin of HL-Antiserum

Column: 3×104 cm, M/50 P-buffered saline, pH 6.0, 5-10°C.
Fraction volume, 10 ml; rate of elution, 60 ml per hour.

fraction, but not in the albumin fraction. The globulin fraction with HL activity was concentrated, as described above, and analyzed by ultracentrifugation. The results are shown in Fig. 13A and B. Fig. 13A shows that HL activity appeared in 4 peaks. The fourth peak was shown to be free HL. The second peak was superimposed on the globulin fraction. The first peak of HL activity corresponded to the position of macroglobulin, calculated according to the method of Nomura, Hall and Spiegelman (1960). However it is not yet known whether HL was bound to the macroglobulin (19S) or whether the HL was still active in a complex with 6 molecules of 7S-antibody. The third peak was slightly slower than albumin, the peak of which could not be detected. A non-neutralizing antibody of smaller molecular weight than 7S may therefore have been present.

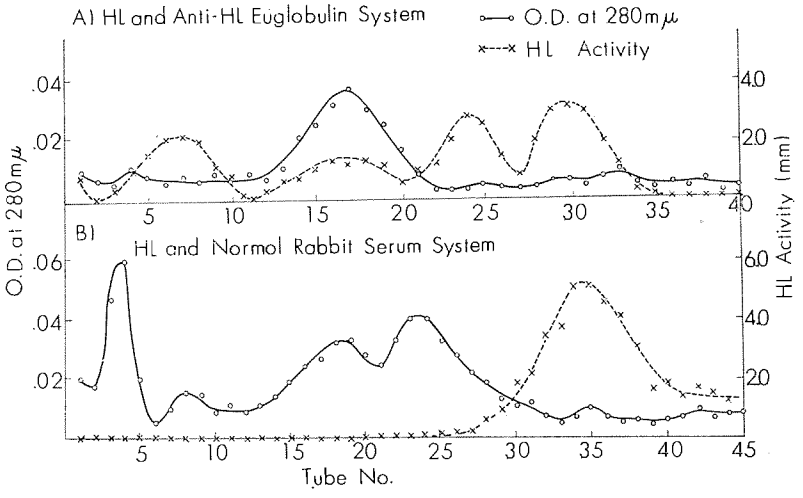


Fig. 13. Ultracentrifugal analysis of Antigen Excess Supernatant of System of HL and Euglobulin of HL-Antiserum

8. *Gel filtration of the soluble complex through Sephadex G-200*

Sephadex G-200, which is a molecular sieve for molecules of less than about 200,000 molecular weight was used for the analysis of active HL-antibody compexes. Sephadex column (1.5 × 106 cm) separated proteins of varying molecular weights of less than 200,000 very effectively.

The soluble complex was obtained at the antigen excess point when 6 times the amount of HL corresponding to the equivalence point of batch SK-2 of HL antiserum, was added. With batch SK-2 the precipitin reaction with HL was also inhibited by the polypeptides. The supernatant (2.0 ml) was filtered through a column of Sephadex G-75 (1.5 × 52 cm) to separate free lysozyme. The serum protein fractions were collected and concentrated by adding dry Sephadex G-25 particles and then filtered through a glass filter. The concentrated material (2.0 ml) was filtered through the column of Sephadex G-200. Material was eluted with saline containing M/50 phosphate buffer, pH 6.0. The results are shown in Fig. 14.

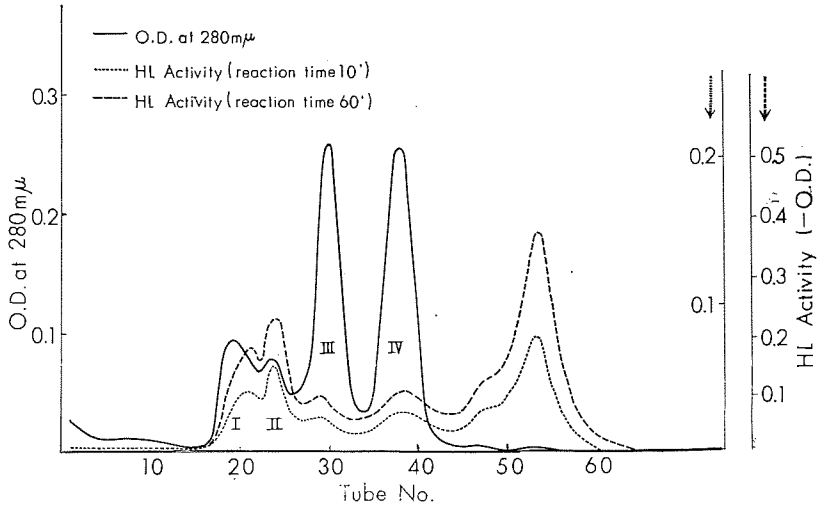


Fig. 14. Gel-filtration with Sephadex G-200 of Antigen Excess Supernatant (HL~SK-2 System)

Column: 1.5×106 cm, M/50 P-buffered saline, pH 6.0, 5°C.
Fraction volume, 3.3 ml; rate of elution, 3.3 ml per hour.

The first protein peak had previously been shown to contain the 19S component in addition to larger serum proteins and complexes with molecular weights of over 200,000, and the third and fourth peaks were also shown by ultracentrifugation to be globulin (7S) and albumin (4.2S). The second peak was assumed to be an antigen-antibody complex with a molecular weight of between 160,000 and 200,000. The lysozyme activity found in this fraction can be regarded as the active complex. The lysozyme activity in the peak superimposable with albumin clearly demonstrates the presence of an active complex of almost the same size as the albumin molecule.

As a control, a heterologous rabbit antiserum (anti-(bovine serum albumin)) was mixed with the same amount of HL and filtered directly through a similar sized Sephadex G-200 column. It was eluted with the same buffered saline (pH 6.0). No lysozyme activity was found in any serum protein fraction, as can be seen in Fig. 15.

The experiment was repeated using a column equilibrated with saline containing M/50 phosphate buffer, pH 6.5, eluted with the same buffered saline. In this case also no lysozyme activity could be seen in any serum protein fraction. If the small sized non-neutralizing antibody was really present in HL antiserum, it seemed also possible that the small sized neutralizing antibody could be demonstrated. To study this possibility, batch LF-18 of HL antiserum, which had an active complex which was the same size as albumin, was passed directly through a Sephadex G-200 column of the same size, at pH 6.0. The neutralizing activity against HL was measured in each fraction of eluate. As shown in Fig. 16,

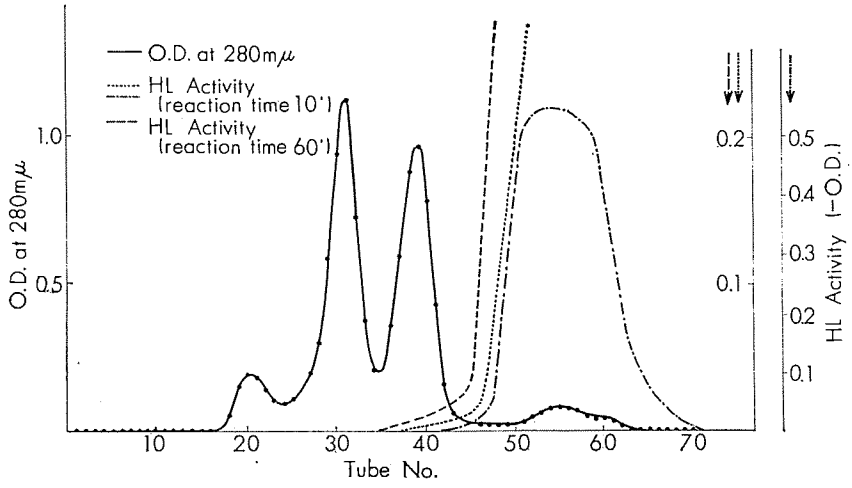


Fig. 15. Gel-filtration with Sephadex G-200 of a Mixture of HL and Heterologous Rabbit Antiserum (Anti-Bovine Serum Albumin)

Column: 1.5×106 cm, M/50 P-buffered saline,, pH 6.0, 5°C.
Fraction volume, 3.3 ml; rate of elution, 3.3 ml per hour.

the neutralizing activity was found only in the 7S globulin fraction. Therefore, it was necessary to see whether the active complex of small molecular weight was really a complex of HL with a fragment of non-neutralizing antibody.

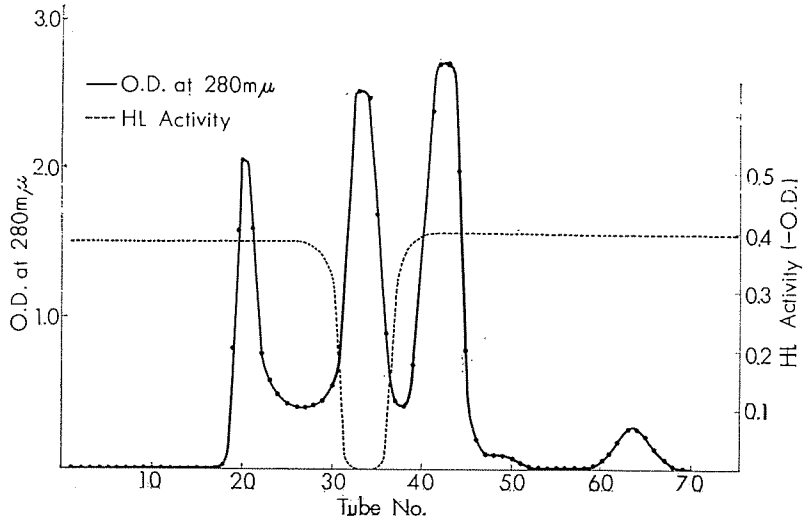


Fig. 16. The Neutralizing Activity against HL of HL-Antiserum Protein Fractions after Gel-filtration with Sephadex G-200

Column: 1.5×106 cm, M/50 P-buffered saline, pH 6.0, 5°C.
Fraction volume, 3.3 ml; rate of elution, 3.3 ml per hour.

1 μg N HL in 2.0 ml of M/50 P-buffered saline was added each tube of effluent. After the tubes had been incubated for 1 hour at 37°C, and overnight at 4°C, the lytic activities of the contents were estimated.

DISCUSSION

Since the HL preparation gave a single precipitation line against homologous antiserum, the preparation could be regarded immunologically homogeneous, if HL was precipited by antiserum. If HL were not precipited, the precipitation line must be due to an antigen which was immunologically distinct from HL. The polypeptides obtained by digesting the HL preparation with pepsin at pH 1.62, could easily have been shown to be derived from HL, if they had inhibited the neutralization of HL by homologous antiserum. However, they only inhibited the precipitin reaction of some batches of HL antisera and did not inhibit neutralization. Therefore, it could not be shown directly that they were derived from HL. To show that they were, attempts were made to demonstrate that HL was precipitated by homologous antiserum, the precipitin reaction of which with HL was inhibited by the polypeptides. As added HL was completely neutralized by antibodies at the equivalence point of the antiserum, the objective could only be achieved by recovering active HL from the precipitates after specific inactivation of the antibodies. For this purpose, the precipitates at the equivalence and at three-fifths of the equivalence and both supernatants were kept in the cold at pH 3.0 for 7 days to dissociate the antigen-antibody complexes and were then boiled. Lysozyme is quite stable on boiling at pH 3.0 but antibodies are inactivated. In this experiment, HL could be recovered from both precipitates but not from either supernatant. Therefore it was proved that HL was precipitated by the antiserum, and that the single precipitation line was due to HL and not to a separate contaminant. Since a mixture of the HL preparation and the antiserum contained only one precipitin system for HL, the precipitin-inhibiting polypeptides can be assumed to have been derived from HL. In addition, it can also be assumed that HL antiserum contained non-neutralizing antibodies for HL.

There was suggestive evidence for the presence of non-neutralizing antibodies in HL antiserum. So it was probable that an active HL-antibody complex could be found in the supernatant of the precipitin reaction in the antigen excess zone. This active complex was demonstrated by gel filtration experiments using Sephadex G-75. HL activity was found in the serum protein fraction. The fraction was concentrated and rechromatographed on another column of the molecular sieve and again HL activity was found in the serum protein fraction while the activity of free HL was much lower than that of the serum protein fraction in the first run. This showed that the activity was not due to free HL dissociated from the neutralized complexes.

Since the molecular weight of a complex consisting of two HL molecules and one antibody molecule must be 190,000, assuming that the molecular weight of antibody is 160,000, the active complex must behave very like normal γ-globulin on ultracentrifugation. To demonstrate this, ultracentrifugation was carried out

in a linear sucrose density gradient using a swinging-bucket type rotor. An active complex of almost the same size as γ-globulin was found. In additon, HL activity other than free HL, was found in albumin and macroglobulin fractions. The activity in the albumin fraction was shown to be unrelated to albumin but was found to have been due to HL associated with a globulin of lower molecular weight.

As the lysozyme activity in this ultracentrifugal experiment was estimated by the capillary method, the HL activity in each fraction was not comparable because free HL and active complexes must diffuse in soft agar at different rates. To obtain comparable results on the HL activity of the different fractions, gel filtration experiments were carried out using Sephadex G-200. In this experiment, much activity was found in a fraction with a molecular weight of between 200,000 and 160,000. This indicates the presence of an active complex consisting of one antibody molecule and one or two HL molecules. In addition, an active complex of similar size to albumin was again found. Neither normal rabbit sera nor heterologous immune rabbit sera formed an active complex with serum protein in gel filtration or ultracentrifugal experiments. However, it can not be stated at present that non-neutralizing antibody of low molecular weight was present in HL antiserum, because it was impossible to detect neutralizing antibody of low molecular weight.

The cause of the production of the non-neutralizing antibodies with the purified HL preparation by immunized rabbits must be considered here. There are two possible explanations for this. First, antigenic sites far from the active centre of HL may evoke non-neutralizing antibodies. Secondly, the HL preparation may contain some partially denatured HL molecules, which are enzymatically active and the denatured parts of these may bear antigenic sites of different specificity to the native molecule. The antibodies evoked by such antigenic sites may be non-neutralizing and such antibodies may form an enzymatically active complex with partially denatured HL. However, the first explanation is the more likely.

ACKNOWLEDGEMENTS

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