BIKEN JOURNAL 5, 201-226 (1962)

Immunochemical Studies on Lysozyme

III. Cross Reactions of Hen and Duck Lysozymes and Their Methyl Esters

HAJIME FUJIO, YOSHIHIKO SAIKI, MICHIKO IMANISHI,
SOHEI SHINKA AND TSUNEHISA AMANO

Department of Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka

(Received for publication, October 14, 1962)

SUMMARY

Duck and hen egg white lysozyme (DL and HL) were purified to the same degree. The homogeneity of the preparation from duck was proved by ultracentrifugation and gel diffusion experiments. Its methyl ester (DLME) was prepared and shown to inhibit hen and duck lysozymes competitively.

The immunological cross reactions of hen lysozyme, the methyl ester of HL (HLME), DL and DLME were studied by the quantitative precipitin reaction and neutralization of the two lysozymes, using their four antisera. The three heterologous antigens cross reacted with the four antisera in the precipitin reaction. In the cross reaction of DL with HL-antiserum and also of HL with DL-antiserum it was found that at least two antigenic sites are common to both antigens and at least two antigenic sites are specific for each antigen. By comparing the reactions of DLME and DL with DL-antiserum, DLME was shown to have lost only one of the antigenic sites of DL during methylation. This is also true of the antigenic sites of HLME in comparison with those of HL.

With regard to neutralizing antibodies, it was shown by studies on a special batch of HL-antiserum, that some HL-non-neutralizing antibodies can cross-neutralize DL. Four fractions of neutralizing antibodies of pooled HL-antisera were distinguished by absorption experiments. Fractions A_{HD} and C_{HD} were classified as HL- and DL-neutralizing antibodies. Fraction B_H was shown to be specific for HL only and fraction E_D was assumed to be HL- non-neutralizing but DL-cross-neutralizing. The neutralizing antibodies of the other three antisera were also distinguished into fractions.

INTRODUCTION

The immunochemical nature of hen egg white lysozyme has been studied in this laboratory for several years. Studies have been made on the different specificities of the antibodies (Fujio *et al.*, 1959) as well as on the behavior of lysozyme in immunochemical reactions (Shinka *et al.*, 1962). In our first paper it was reported that HL-antiserum contained two kinds of neutralizing antibodies of different specificities, the one being absorbed by the methyl ester of HL and the

The following abbreviations are used in this report: hen egg white lysozyme (HL), duck egg white lysozyme (DL), methyl ester of HL (HLME) and methyl ester of DL (DLME).

other not. However in this work it was uncertain whether all the antibodies elicited by HL in rabbits could neutralize HL. This problem was partially solved, as reported in our second paper, for it was demonstrated that in some batches of HL-antisera not all HL antibodies were neutralizing. In the present work duck egg white lysozyme was purified and its methyl ester was prepared. The immunological cross reactions of HL, HLME, DL and DLME with the four respective antisera were studied by the quantitative precipitin reaction and by neutralization of HL and DL.

MATERIALS AND METHODS

1. *Hen egg white lysozyme*

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

2. *Lysozyme methyl esters*

Methyl ester derivatives were prepared according to the method of Fraenkel-Conrat (1945) and Frieden (1956), as described in our first report.

3. *Antisera*

The immunization schedule and the preparation of antigens in incomplete Freund's adjuvant were as described in our first report. Each batch of antiserum from a single rabbit, was tested for the presence of an equivalence zone, and homologous antisera showing the immunological homogeneity were pooled. A special batch of HL-antiserum from a rabbit (LF-32) was also used in this study. In this paper when the batch number is not specified, pooled HL-antiserum was used. After inactivation at 56°C for 30 minutes, serum lysozyme was removed by bentnite (Wako Pure Chemicals, Lot No. 77CD 74254) according to the method of Inai and Kishimoto (1958) and then stored at −20°C.

4. *Quantitative precipitin reaction*

The quantitative determination of precipitated antibody nitrogen in antiserum was made as described by Heidelberger and Kendall (1935). The antigen nitrogen and precipitated antibody nitrogen were determined by the modified direct Nesslerization method (Yokoi and Akashi, 1955).

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 within ± 2.0 μg N.

6. *Determination of lysozyme activity*

The *M. lysodeikticus* suspension used as substrate in these experiments was prepared as described by Smolelis and Hartsell (1949). Prior to the assay, a cell suspension was prepared from UV irradiated and lyophilized cells in M/15 phosphate buffer at pH 7.0. This bacterial suspension was adjusted to an extinction of 1.08 at 540 mμ using a Beckman spectrophotometer. A standard curve for lysozyme activity was obtained as follows: 2.0 ml of saline containing varying amounts of lysozyme (0.1 to 1.0 μg N) in M/50 phosphate buffer, pH 7.0, were mixed with 2.0 ml of the bacterial suspension and the mixtures were incubated at 30°C. After 13 minutes, the turbidity was estimated and the reduction in turbidity was plotted against the logarithm of the lysozyme concentration. A straight line was obtained between 0.1 and 1.0 μg N of added lysozyme. To determine the lysozyme activity of a sample, 2.0 ml of the sample was added in place of the standard solution of lysozyme.

7. *Assay of neutralizing antibodies in antisera and in the supernatants of the precipitin reaction*

Serial 1:2 dilutions (1:1, 1:2, 1:4, 1:8 and 1:16) were made from 1.0 ml of the supernatants or antisera and 0.5 ml volumes were put in each tube. One ml of lysozyme solution (1 μg N per ml) and 0.5 ml of buffered saline were added to the tubes, which were then incubated at 37°C for 30 minutes and at 2°C overnight. Two ml of bacterial suspension was then added. As controls, two additional series of serial dilutions were made. To one series of tubes 1.0 ml aliquots of M/50 phosphate buffered saline was added in place of 1.0 ml of lysozyme solution. After the incubation, bacterial suspension was added to each tube. To the other series, 1.0 ml aliquots of lysozyme solution were added and after the incubation, 2.0 ml of M/15 phosphate buffer was added in place of the bacterial suspension. All the tubes were incubated at 37°C for 13 minutes and the reduction in bacterial density was estimated in the experimental and the first control series. The turbidity was estimated in the tubes of the second control series. From the value of the reduced optical density of the experimental tube the optical density of the corresponding tube of the first control series and the turbidity of the corresponding tube of the second control series were subtracted. From the corrected values for the reduction in bacterial density, the concenration of unneutralized lysozyme in the experimental tubes was calculated, using a standard curve of HL or DL. Thus the free lysozyme concentration could be plotted against the corresponding dilution factors. The result was approximately linear except at very low values. The linear portion of the plot was extrapolated to the abscissa. The dilution of the sample at the intercept was regarded as that which completely neutralized the added lysozyme. One unit of lysozyme antibody is defined as the amount which can neutralize 0.1 μg N of lysozyme. Therefore, the neutralizing antibody remaining in 1.0 ml of the supernatant was calculated as follows:

μg N of lysozyme $\times$ dilution factor $\times$ 1/0.1 μg N $\times$ 1.0/0.5

Usually the supernatants of the precipitin reaction had a volume of 2.0 ml. Therefore, the value was doubled when the total amount of neutralizing antibody in the supernatants was calculated.

8. *Gel diffusion experiment*

Ouchterlony's double diffusion technique was employed. Difco agar was dissolved to give a 1 per cent solution in M/50 phosphate buffered saline, pH 7.0, containing 0.01 per cent merthiolate. The distance between the wells was approximately 1 cm.

9. *Ultracentrifugal analysis*

A Spinco model E ultracentrifuge was used. The centrifuge was rotated at 20°C.

RESULTS

1. *Purification and nature of duck egg white lysozyme*

As the lysozyme content of duck egg white is very low as compared to that of hen egg white (about 1/10), DL was first concentrated by adsorption-elution with bentnite according to the method of Alderton et al. (1945). The concentrated solution was fractionated by ammonium sulfate and further purified by IRC-50 (CG-50) column chromatography in the same way as HL (Hirs *et al.*, 1953).

One liter of fresh duck egg white was homogenized, filtered through gauze and added to an equal volume of 1 per cent bentnite suspension (Wako Pure Chemicals) containing 1 per cent KCl. The mixture was stirred for 15 minutes and then centrifuged at 3,000 rpm for 30 minutes. The bentnite was washed three times with 1 l of 0.5 M phosphate buffer, pH 7.5, and then three times with 1 l of 5 per cent pyridine solution at pH 9.2. Then the DL was eluted with 900 ml of 5 per cent pyridine solution at pH 5.0 (adjusted with conc. H_2SO_4). The elution was completed within 24 hours. To the eluate was added solid ammonium sulfate to a concentration of 2.6 M and the mixture was kept

at 5°C overnight. The precipitate was dissolved in a small amount of water and dialyzed against water in the cold for five days using a cellophane membrane from a selected batch. The dialyzed solution was centrifuged and the supernatant was lyophylized.

The crude material was further chromatographed on an IRC-50 (CG-50) column (10 × 30 cm) equilibrated with 1/5 M phosphate buffer, pH 7.8, containing 0.01 per cent merthiolate. A volume containing 2.7 g of protein were applied to the column and eluted with the same buffer at a rate of 96 ml per hour at 22-25°C. The eluate was collected in 16 ml fractions. The protein content of each fraction was determined by the Biuret reaction and the optical density was read at 555 mμ in a Beckman spectrophotometer. Results from a typical chromatogram are shown in Fig. 1.

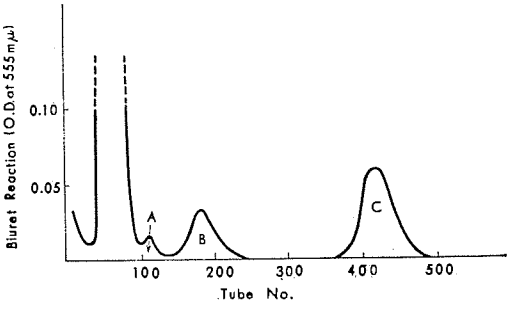

Fig. 1. Chromatography of Crude DL Preparation

As can be seen, three lysozyme fractions were found and fraction C was the main component. The main component was lyophilized, after dialysis against water. It was rechromatographed on a 1.5 × 34 cm column of the same resin in the same buffer. A volume containing 28.4 mg of protein was applied and the flow rate was 4.5 ml per 1 hour. Fractions of 3 ml were collected. The concentrations of protein and lysozyme activity in the effluent are shown in Fig. 2.

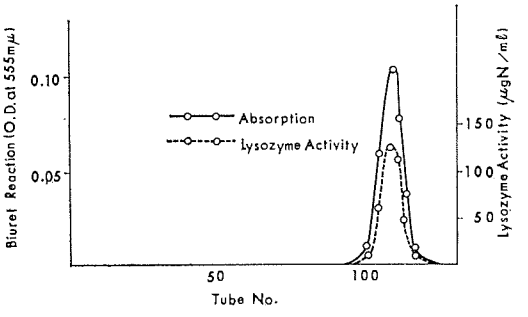

Fig. 2. Rechromatography of Fraction C

The purity of the main component was estimated, by ultracentrifugal analysis. A single homogeneous component of $S_{20} = 1.8$ was seen (Fig. 3). As another criterion of purity, gel diffsion experiments were made on Ouchterlony plates. To the center well, 0.2 ml of DL-antiserum was added and to side wells were added

(in 0.2 ml volumes): No. 1, 4 μg N of DL; No. 2, 8 μg N of DL; No. 3, 16 μg N of DL; No. 4, 32 μg N of DL; No. 5, saline. According to the results of the quantitative precipitin reaction, the equivalence point of DL was 2.88 μg N per 0.2 ml of DL-antiserum. As can be seen in Fig. 4, the amount of antigen added to well No. 4 was far in antigen excess (more than 11 times) and still only one precipitation line was observed. Therefore the main component was antigenically homogeneous.

Fig. 3. Ultracentrifugal Pattern of DL

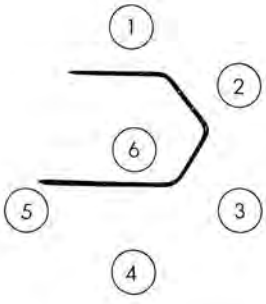

Fig. 4. Gel Diffusion Experiments with Varying Concentrations of DL

The yield and purity at each step of purification are presented in Table 1. The enzyme activity per μg N at the last step was almost the same as that of purified HL.

Table 1. Recovery of DL

	Protein (gN)	Lysozyme (gN)	Recovery (%)	Purification index
Duck egg white	244	1.56	100	1
Washing bentnite with 0.5 M P-buffer	6.0	—	—	—
Washing bentnite with 5% pyridine	2.85	—	—	—
Eluate with pyridine at pH 5.0	3.62	1.0	64.1	43.2
Crude DL	2.02	0.57	36.6	55.5
Purifid DL	0.55	0.55	35.2	194

Methylated duck lysozyme was prepared according to the method of Fraenkel-Conrat and Frieden. The sedimentation patterns of DLME, HL and HLME are shown in Figs. 5, 6, 7, respectively. A single component was found in each and their sedimentation constants were approximately 1.8.

Fig. 5. Ultracentrifugal Pattern of DLME

Fig. 6. Ultracentrifugal Pattern of HL

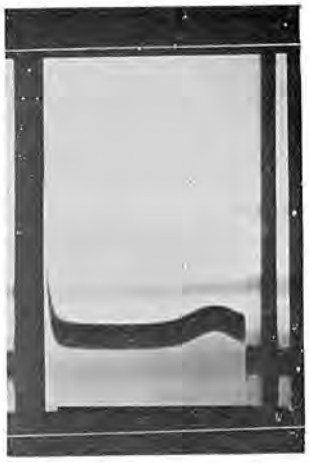

Fig. 7. Ultracentrifugal Pattern of HLME

2. *Competitive inhibition of DL and HL with DLME and HLME*

As HLME competitively inhibited HL, as already described (Fujio *et al.*, 1959), the inhibiting effects of DLME and HLME upon DL and HL were studied.

1.0 ml aliquots of M/15 phosphate buffer (pH 7.0) containing varying amounts of DLME (128.8, 193.4, 258.2 and 515.6 μg) or HLME (132.0, 198.0, 264.0 and 737.2 μg) were put into eight tubes. Two ml of a suspension of *M. lysodeikticus* (1.0 mg/ml) were added to each tube. Three additional tubes served as controls. One control (C_1) contained 1.0 ml of buffer in place of the inhibitor solution and 2.0 ml of bacterial suspension. The others (C_2 and C_3) contained 1.0 ml of buffer containing DLME (515.6μg) and HLME (737.2μg), respectively, and 2.0 ml of bacterial suspension. The eleven tubes were incubated at 30°C for 10 minutes. One ml of prewarmed buffer, containing 5.85 μg of DL, was added to each of the experimental tubes and to tube C_1. One ml aliquots of prewarmed buffer were added to tubes C_2 and C_3. The tubes were reincubated at 30°C for 13 minutes. The turbidity of the tubes was estimated in a Beckman spectrophotometer at 540 mμ. The degree of inhibition was calculated as shown in Table 2.

Table 2. Inhibitory Effect of HLME and DLME on DL

DLME final conc. (μgN)	Inhibition %	HLME final conc. (μgN)	Inhibition %
32.2	43.8	33.0	43.2
48.35	56.8	49.5	55.6
64.55	67.9	66.0	64.2
128.9	100.0	184.3	100.0

As can be seen from the table, DLME and HLME competitively inhibited DL and HL to the same degree.

3. *Quantitative precipitin reactions of the four antisera with the four antigens and the fate of neutralizing antibodies in the supernatants*

To study the nature of the antibodies formed by HL, HLME, DL and DLME, comparative studies were made of cross preciptin reactions of the four antigens with each of the four antisera and also of the cross neutralization reactions.

1) *HL-antiserum*

i) *Precipitin reaction*

Varying amounts of antigens (as shown in Table 3a, b, c and d in μg N) dissolved in 1.0 ml of saline, were added to tubes containing 1.0 ml of HL-antiserum. Control tubes were also included. The tubes were incubated at 37°C for 1 hour and then at 1°C for 5 days, and centrifuged. After removal of the supernatants, the precipitates were washed with chilled saline and the total precipitated protein N was estimated. The results are shown in Table 3a, b, c and d including the results of tests on supernatants for excess antigen and antibody.

In each of the four systems an equivalence zone was observed. The maximal amount of antibody precipitated was greatest with that of the homologous antigen (HL) and decreased in the order HLME>DL>DLME (tube 7 of Table 3a, b and c, tube 6 of Table 3b, and tube 4 of Table 3d).

Table 3. Quantitative Precipitin Reactions of the Four Antigens in HL-Antiserum

a.

Tube No.	HL (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: HL antiserum added	Tests on supernatants: HL added
1	4.2	133	128.8	30.7	—	2+
2	6.5	167.4	160.4	24.5	—	2+
3	9.8	237.4	227.6	23.1	—	±
4	11.8	257.6	245.8	20.8	—	—
5	12.6	264.0	251.4	20.0	—	—
6	16.4	283.6	267.2	16.3	—	—
7	19.6	295.6	276.0	14.1	±	—
8	26.2	275.6			+	—

(Table 3) b

Tube No.	HLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: HL antiserum added	HLME antiserum added	HL	HLME
1	6.0	114.6	108.6	18.1	—	—	+	4+
2	12.0	198.0	186.0	15.5	—	—	+	4+
3	14.4	226.4	212.0	14.7	—	—	+	3+
4	16.4	239.4	223.0	13.6	—	—	+	2+
5	19.0	268.6	249.0	13.1	—	—	+	+
6	24.0	287.0	263.0	11.0	—	—	±	—
7	30.0	303.0	273.0	9.1	+	+	—	—
8	40.0	274.0			2+	2+	—	—

(Table 3) c.

Tube No.	DL (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants				
					HL antiserum added	DL antiserum added	HL added	HLME added	DL added
1	0.5	15.5	15.0	30.0	—	—	3+	3+	±
2	1.0	27.8	26.8	26.8	—	—	3+	3+	±
3	2.0	52.6	50.6	25.3	—	—	3+	3+	±
4	3.0	61.9	58.9	19.6	—	—	2+	2+	—
5	4.0	78.8	74.8	18.7	—	—	2+	2+	—
6	6.0	111.0	105.0	17.5	—	—	2+	2+	—
7	8.0	119.0	111.0	13.9	±	±	2+	2+	—
8	12.0	99.8			3+	3+	2+	2+	—
9	20.0	77.8			3+	3+	2+	2+	—
10	24.0	40.8			3+	3+	+	+	—

(Table 3) d.

Tube No.	DLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants			
					DLME anti-serum added	DL added	DLME added	HL added
1	1.0	21.9	20.9	20.9	—	2+	+	3+
2	2.0	34.2	32.2	16.1	—	+	—	3+
3	4.0	59.1	55.1	13.8	—	—	—	3+
4	6.0	71.2	65.2	10.9	—	—	—	3+
5	8.0	67.0			±	—	—	2+
6	12.0	64.0			+	—	—	2+
7	16.0	59.0			2+	—	—	2+

As can be seen in Table 3b, the supernatants in the antigen (HLME) excess zone gave no precipitate with the slight amount of HL added, although there was slightly but definitely less precipitated antibody N at the equivalence point (tube 6 of Table 3b) than in the homologous HL~anti-HL system (tube 7 of Table 3a) and approximately 15 per cent of neutralizing antibodies for HL remained unabsorbed in the supernatants in the zone of antigen (HLME) excess, as described below (Table 4, Fig. 9). These facts were consistent with those reported previously from this laboratory (Fujio *et al.*, 1959). The results were also consistent with the findings on an Ouchterlony plate, in which HL and HLME were tested against HL-antiserum and the two single precipitation lines fused with each other without spur-formation, as can be seen in Fig. 8. Since the maximal amount of precipitated antibody of HL and HLME antigens differed, the antibodies corresponding to this difference must be mainly multivalent. Although a multivalent antibody could give precipitates with the antigen, no precipitation of the supernatants of the HLME~HL-antiserum system was found with a minute amount of HL and no spur-formation could be detected. These negative results may be explained

by assuming that the supernatant contained antibodies with only one kind of specificity and that these antibodies could react with only one of the antigenic sites of each HL molecule resulting in the formation of a soluble complex.

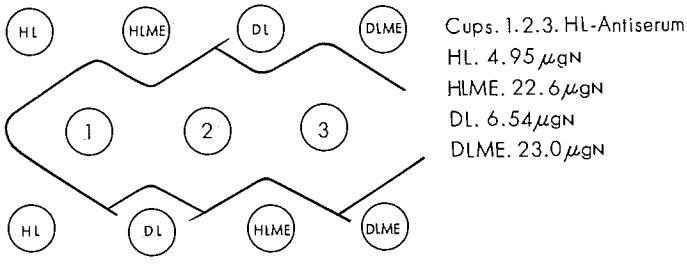

Fig. 8. Gel Diffusion Experiments of the Four Antigens with HL-Antiserum

In tests on the supernatants of the tubes shown in Table 3c, HL or HLME gave precipitates with all the supernatants, which were obtained after the precipitin reaction of DL with HL-antiserum. Therefore, according to the lattice theory, the supernatants contained antibodies of at least two different specificities with regard to their antigenic sites. The implication was confirmed by a gel diffusion experiment on an Ouchterlony plate, in which DL, HLME and HL were tested against HL-antiserum and the single line, between HLME (or HL) and the antiserum, fused forming a spur with the single line appeared between DL and the antiserum (Fig. 8).

When the supernatants of the DLME～HL-antiserum system were tested, as shown in Table 3d, HL and HLME gave precipitates throughout the range tested. Thus results are very similar to those with the DL～HL-antiserum system. However DL gave no precipitate in the antigen (DLME) excess zone, although the amount of antibody N precipitated in the equivalence zone of the DL～HL-antiserum system (tube 7 of Table 3c) was considerably larger than that in the DLME～HL-antiserum system (tube 4 of Table 3d). These results are also consistent with the findings in gel diffusion experiments (Fig. 8).

ii) *Neutralization*

The supernatants obtained in the quantitative precipitin reaction of the four systems were also analyzed for neutralizing antibodies against homologous antigen, HL and the heterologous antigen, DL. The results are presented in Figs. 9 and 10. As can be seen, the curves for unabsorbed neutralizing activity in the supernatants have plateaus in the antigen excess zone of the precipitin reactions. The percentage activities of these plateaus of neutralizing activity for the two lysozymes to that of the original HL-antiserum were calculated, as shown in Table 4. The neutralizing antibodies remaining after absorption by the excess of HLME, represented about 15 per cent of the original activity for HL and 23 per cent for DL. This fraction of neutralizing antibodies was capable of neutralizing HL as well as

DL and it is designated as A_{HD}. When the supernatants were further absorbed with an excess of DLME, the antibodies still unabsorbed represented 14 per cent of the original activity for HL and 23 per cent for DL, as shown in Table 5. The results

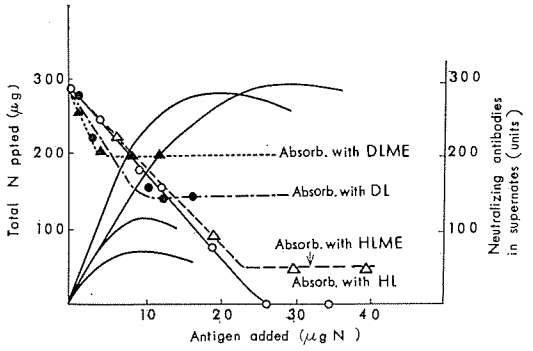

Fig. 9. Fate of HL-neutralizing Antibodies of HL-Antiserum in Supernatants of Precipitin Reactions

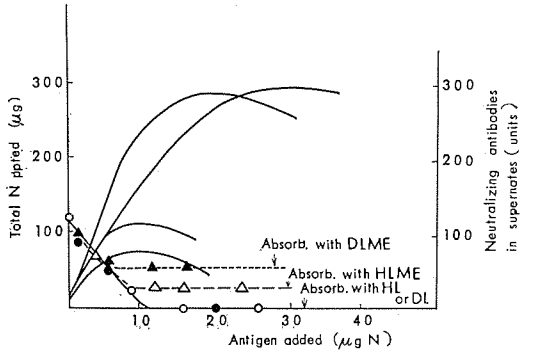

Fig. 10. Fate of DL-neutralizing Antibodies of HL-Antiserum in Supernatants

Table 4. Maximal Absorption of HL Antiserum

Antigen used for absorption	HL-neutralizing antibodies absorbed		DL-neutralizing antibodies absorbed	
	N.U.	%	N.U.	%
HL	290	100	121	100
HLME	245	84.7	93.8	77.5
DL	145	50	121	100
DLME	90	31	66.6	55

indicate that the second absorption removed no additional antibodies and the supernatant gave no precipitation with a trace of DLME. Therefore, 31 per cent (for HL) and 55 per cent (for DL) of the neutralizing antibodies directly absorbed

by an excess of DLME apparently belong to the same fraction, C_{HD}, and this fraction is assumed to have been included in the 85 per cent (for HL) and 78 per cent (for DL) absorbed by HLME. The neutralizing antibodies of HL corresponding to the difference between 85 and 31 per cent must be specific for HL and this fraction, representing 54 per cent of the total activity, was named B_H.

Table 5. Repeated Absorption of HL-Antiserum with HLME and DLME

	HL-neutralizing antibodies remaining		DL-neutralizing antibodies remaining	
	N.U.	%	N.U.	%
HL antiserum	290	100	121	100
after absorption with HLME	44.4	15.3	27.2	22.5
further absorbed with DLME	40.4	13.9	27.3	22.6

HL-antiserum was absorbed with a slight excess of DL and the remaining neutralizing antibodies were estimated for HL. In this estimation the excess DL activity was always subtracted in the calculation. After absorption, 50 per cent of HL neutralizing antibodies were detected and therefore the remaining 50 per cent of the original activity was absorbed by DL. The unabsorbed fraction must be B_H and the absorbed fraction must represent the sum of A_{HD} and C_{HD}(46 per cent). This can be regarded as identical with the experimental value (50 per cent) when the experimental error is taken into consideration.

If these assumptions are true and fraction B_H can only neutralize HL, the neutralizing antibodies for DL, which are absorbed by HLME and DLME, would be identical. However, the difference between the values (94 and 67 neutralizing units) can not be considered as within the range of experimental error. The difference can only be explained by postulating the presence of another fraction

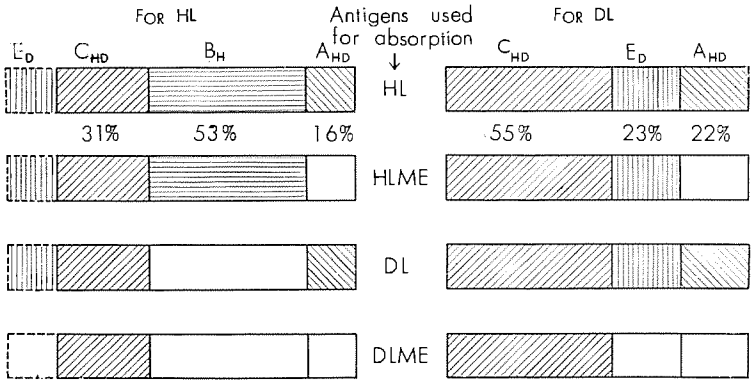

Fig. 11. Composition of Neutralizing Antibodies of HL-Antiserum

Shadowed parts represent fractions lost after absorption. Parts enclosed in dotted lines represent fraction which apparently neutralize only heterologous antigen and not homologous antigen.

(E_D) of antibodies elicited by HL. Fraction E_D has no neutralizing ability for HL but it neutralizes DL. This assumption is supported by results described below. Thus the neutralizing antibodies elicited by HL can be classified as shown in Fig. 11.

To reconfirm the above results, similar experiments were performed using individual HL-antisera of rabbits and a very interesting batch (LF-32) was found. The equivalence point of this antiserum for HL was 22 μg N per ml and it contained 254 units of neutralizing antibodies for HL and 83 units for DL per ml. When it was absorbed with DLME, there was no absorption of HL neutralizing antibodies (no C_{HD}) but DLME absorbed 51 per cent of DL neutralizing antibodies. This was named fraction F_D. The results indicate that some HL-non-neutralizing antibodies produced by HL are capable of neutralizing DL, as shown in Table 6.

Table 6. Absorption Experiments on Batch LF-32 of HL-Antiserum

	HL-neutralizing antibodies remaining		DL-neutralizing antibodies remaining	
HL antiserum	254 N.U.	100%	83 N.U.	100%
after absorption with HLME	98 N.U.	39%	22.1 N.U.	27%
further absorbed with DLME	110 N.U.	43%	25.5 N.U.	31%
after absorption with HLME, further absorbed with DL	0			
after absorption with DLME	255 N.U.	100%	40.4 N.U.	49%
further absorbed with HLME,	114 N.U.	45%	25.2 N.U.	30%
after absorption with DLME, further absorbed with DL	161 N.U.	63%		
after absorption with DL	168 N.U.	66%		
further absorbed with HLME	0			

As the presence of HL-non-neutralizing antibodies was proved by Shinka *et al.* (1962), the facts can easily be understood. When the absorbed antiserum was further absorbed by HLME, 55 per cent of the HL neutralizing antibodies (fraction B_H) and the 19 per cent fraction (G_D) of DL neutralizing antibodies were removed. When the antiserum was directly absorbed with HLME, 57 per cent of HL antibodies (fraction B_H) and 70 per cent of DL (fraction F_D and G_D) were absorbed. When the absorbed antiserum was treated in addition with DLME, no additional absorption could be detected. It is obvious that the 57 per cent fraction of HL neutralizing antibodies is identical with fraction B_H found in the two step absorption experiment with DLME and HLME. The value of 70 per cent for DL neutralizing antibodies absorbed by HLME was in good accord with the sum of fraction F_D and G_D. The problem here is whether all or an appreciable amount of fraction G_D was present in fraction B_H of HL neutralizing antibodies. Fraction G_D was shown to neutralize only DL and not HL in the following way. When the antiserum was first absorbed with DL, all the DL neutralizing antibodies were blocked and 34 per

cent of the HL neutralizing antibodies (fraction A_{HD}) were absorbed. The remaining 66 per cent of HL antibodies were completely absorbed by HLME. This 66 per cent can be regarded as equal to 57 per cent (fraction B_H) when the experimental error is taken into consideration. If fraction G_D could also neutralize HL, fraction B_H found after absorption with DL would be much smaller. Therefore fraction G_D can be considered to neutralize DL but not HL. Thus the neutralizing antibodies of this antiserum can be classified as shown in Fig. 12.

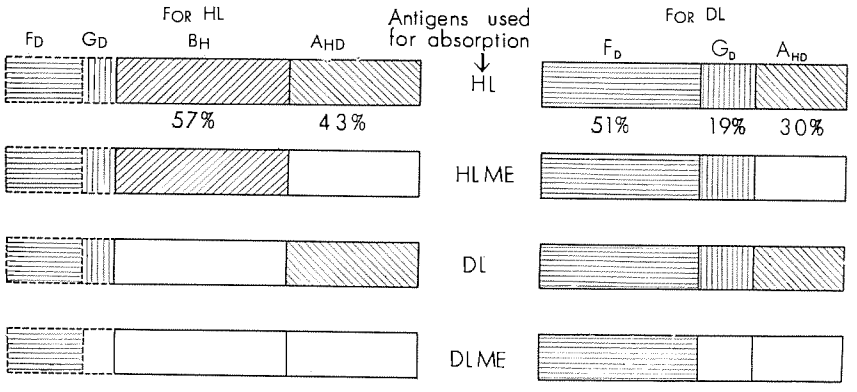

Fig. 12. Composition of Neutralizing Antibodies of Batch LF-32 of HL-Antiserum

Shadowed parts represent fractions lost after absorption. Parts enclosed in dotted line represent fraction apparently neutralizing only heterologous antigen and not homologous antigen.

2) *HLME-antiserum*

i) *Precipitin reaction*

In studies of HLME antiserum, HLME, DL and DLME were used as antigens. HL was omitted because it had been studied previously (Fujio *et al.*, 1959) and it

Table 7. Quantitative Precipitin Reactions in HLME-Antiserum

a.

Tube No.	HLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: HLME antiserum added	Tests on supernatants: HLME added
1	2	30.9	26.9	14.4	—	2+
2	4	56.6	52.6	13.2	—	±
3	6	75.7	69.7	11.6	—	—
4	10	121.0	111.0	11.1	±	—
5	13	145.0			2+	—
6	15	149.0			2+	—
7	18	143.0			2+	—
8	30	130.0			2+	—

(Table 7) b.

Tube No.	DL (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: HLME antiserum added	HLME added	HL added	DL added
1	2.0_3	27.6	25.5_7	12.6	−	2+	2+	+
2	4.0_5	37.2	33.1_5	8.1_8	−	+	+	+
3	5.7	47.8	42.1	7.3_9	±	+	+	−
4	8.1_1	54.0	45.9	5.6_6	±	+	+	−
5	10.4_6	/			+	+	+	−
6	13.3_1	32.9			3+	+	+	−
7	16.2	/			3+	+	+	−
8	26.7	33.5			3+	+	+	−
9	30.4	32.7			3+	+	+	−

(Table 7) c.

Tube No.	DLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: DLME antiserum added	HLME antiserum added	DLME added	DL added	HLME added
1	0.9_6	11.5_2	10.5_6	11	−	−	+	+	3+
2	1.0_2	.....	.....	.....	−	−	+	+	3+
3	1.9_2	26.9	24.9_8	13	−	−	+	+	3+
4	3.0_6	34.9	31.8_4	10.6	−	−	−	−	2+
5	3.8_2	36.0	32.2	9.4_3	−	−	−	−	2+
6	5.7_2	45.1	39.3_4	6.8_2	±	±	−	−	2+
7	7.7	55.3	(47.6)		+	+	−	−	2+
8	9.6	48.2			+	+	−	−	2+
9	13.5	46.8			+	+	−	−	2+
10	19.2	45.2			+	+	−	−	2+

was found that HL behaved in the same manner as HLME. The experimental procedures used were similar as those used for HL-antiserum. The results of quantitative precipitin reactions are shown in Table 7a, b, c.

Table 7b shows that a minute amount of HLME or HL gave precipitates with the supernatants of the precipitin reaction of the DL~HLME-antiserum system throughout the range tested. Similar results were also observed in tests on supernatants of the DLME~HLME-antiserum system (Table 7c) when a minute amount of HLME was added. In the DLME~HLME-antiserum system, an additional result should be described here. When the excess antibodies in the supernatants were tested with a minute amount of DL, precipitates formed only in the antibody excess zone (tubes 1 - 3 of Table 7c).

ii) *Neutralization*

In neutralization experiments, as shown in Table 8 and Figs. 13 and 14, DL

and DLME could absorb the same amount (71 per cent) of neutralizing antibodies for HL and those for DL were completely removed by DL but only 73 per cent was absorbed by DLME.

Table 8. Degree of Maximal Absorption of HLME-Antiserum

Antigens used for absorption	HL-neutralizing antibodies absorbed		DL-neutralizing antibodies absorbed	
	N.U.	%	N.U.	%
HLME	212	100	121	100
DL	150	70.8	121	100
DLME	150	70.8	89	73.8

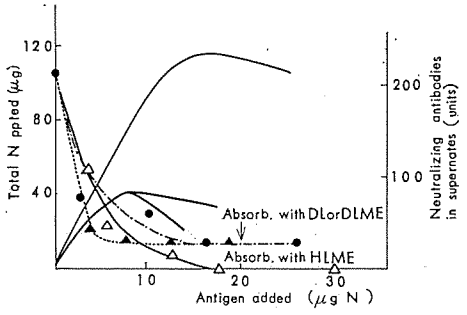

Fig. 13. Fate of HL-neutralizing Antibodies of HLME-Antiserum in Supernatants of Precipitin Reactions

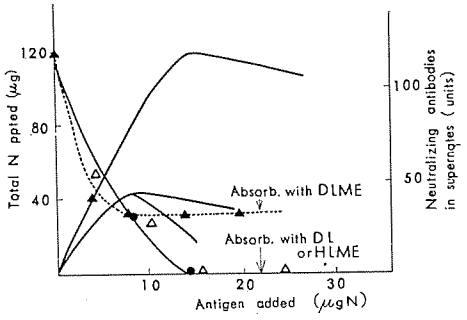

Fig. 14, Fate of DL-neutralizing Antibodies of HLME-Antiserum in Supernatants of Precipitin Reactions

The HL-neutralizing antibodies absorbed by DL or DLME (71 per cent) can be regarded as identical and this fraction is termed C_{HD}. It can neutralize both HL and DL. However, fraction C_{HD} does not represent all the DL-neutralizing antibodies, because DLME could absorb only 73 per cent. Therefore, this 73 per cent of DL-neutralizing antibodies must represent fraction C_{HD} and the remaining 27 per

cent can be regarded as HL-non-neutralizing and only DL-neutralizing. This is called fraction E_D. The 29 per cent of HL-neutralizing antibodies which were unabsorbed by DL is named fraction B_H and it neutralizes only HL. It is not certain whether fraction C_{HD} of HL-neutralizing antibodies contained another fraction, which neutralized only HL, because there was too great a difference between the neutralizing units of HL (150 N.U.) and DL (89 N.U.) absorbed by DLME. The neutralizing antibodies evoked by HLME can be summarized as shown in Fig. 15.

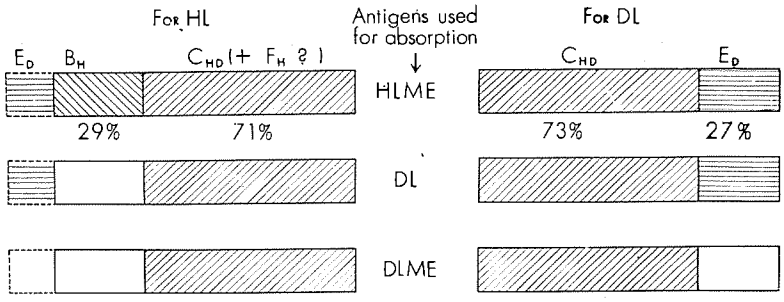

Fig. 15. Composition of Neutralizing Antibodies of HLME-Antiserum

Shadowed parts represent fractions lost after absorption. Parts enclosed in dotted lines represent fraction apparently neutralizing only heterologous antigen and not homologous antigen.

3) *DL-antiserum*

i) *Precipitin reaction*

The antigens used in this experiment were DL, DLME, HL and HLME. The experimental procedures were the same as those for HL-antiserum and the results are shown in Table 9a, b, c, d. The amount of antibody precipitated was greatest with the homologous antigen, DL, and decreased in the order of DLME>HL >HLME.

Table 9. Quantitative Precipitin Reactions of the Four Antigens in DL-Antiserum

a.

Tube No.	DL (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: DL antiserum added	Tests on supernatants: DL added
1	2.0_3	27.1	25.0	12.3	—	2+
2	3.9_1	45.4	41.5	14.2	—	+
3	6.1_1	80.4	74.7	12.2	—	±
4	7.9_4	118.0	110.1	13.9	—	—
5	12.2_2	158.0	145.8	11.9	—	—
6	14.4	162.0	147.6	10.3	— or ±	—
7	20.1_6	116.6			2+	—
8	28.8	100.3			3+	—

(Table 9) b.

Tube No.	DLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: DLME antiserum added	DL antiserum added	DLME added	DL added
1	1.7_2	18.2	16.4_8	9.5_6	—	—	+	+
2	3.4_4	45.3	41.8_6	12.1_5	—	—	±	±
3	5.1_6	71.7	66.5_4	12.9	—	—	—	—
4	8.3_5	117.7	109.3	13.1	—	—	—	—
5	11.6_9	142.5	130.8	11.2	—	—	—	—
6	15.4_8	144.3			+	+	—	—
7	25.8	126.8			+	+	—	—
8	33.4	83.3			+	+	—	—

(Table 9) c.

Tube No.	HL (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: HL antiserum added	HL added	HLME added	DL added
1	1.4_2	14.2_2	12.8	9.0_1	—	±	2+	+
2	2.2_6	18.0	15.7_4	6.9_5	—	—	+	+
3	2.8_3	19.9_8	17.1_5	6.0_5	—	—	+	+
4	3.4	18.0			±	—	+	+
5	3.9_6	15.7_6			+	—	+	+
6	4.9_9	12.7			2+	—	+	+
7	5.6_6	12.3_2			3+	—	+	+

(Table 9) d.

Tube No.	HLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: HLME antiserum added	HLME added	HL added	DL added	DLME added
1	1.3_3	11.2_5	9.9_5	7.5_5	—	+	+	+	+
2	1.6_6	13.6_5	12.0	7.2_4	—	±	±	+	+
3	2.6_5	.....	.....	.....	—	—	—	+	+
4	3.0	17.3_2	14.3_2	4.7_8	—	—	—	+	+
5	3.3_2	18.5	15.1_8	4.5_8	±	—	—	+	+
6	4.1_5	15.8			+	—	—	+	+
7	5.8_1	14.4			+	—	—	+	+

In tests on the supernatants of the DLME~DL-antiserum system, excess antibody was found only in the first two tubes when tested with traces of DLME and DL, although DL precipitated more antibody than DLME (tube 6 of Table 9a and tube 5 of Table 9b) and the supernatants in the antigen excess zone of the DLME~DL-antiserum system still contained neutralizing antibodies for DL and HL (Table 10, Figs. 18 and 19). This result is very similar to results with the

HLME~HL-antiserum system. The result was consistent with that obtained in a gel diffusion experiment on an Ouchterlony plate, on which DL and DLME were tested against DL-antiserum. In this experiment the line given by DL fused with that of DLME without spur-formation, as shown in Fig. 16. The cross reaction of HL with DL-antiserum is also shown in Fig. 17. The precipitation line given by

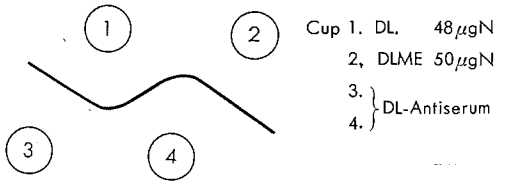

Fig. 16. Comparison of DL and DLME against DL-Antiserum in Ouchterlony Plate

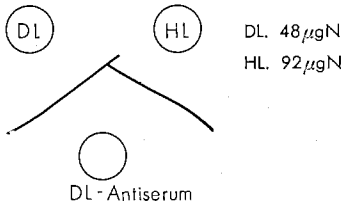

Fig. 17. Comparison of DL and HL against DL-Antiserum in Ouchterlony Plate

HL fused with that of DL with spur-formation. The result is consistent with that obtained in tests on supernatants of the HL~DL-antiserum system shown in Table 9c, in which excess antibody was detected throughout the range tested with a minute amount of DL.

ii) *Neutralization*

The neutralizing antibodies were also estimated in the supernatants of the precipitin reactions and the results are presented in Figs. 18 and 19 and Table 10.

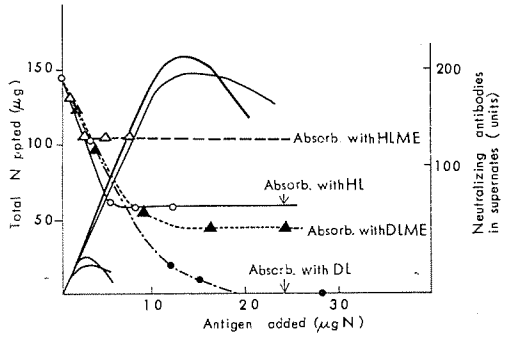

Fig. 18. Fate of DL-neutralizing Antibodies of DL-Antiserum in Supernatants of Precipitin Reactions

Excess DLME could absorb 69 per cent of neutralizing antibodies for DL and 78 per cent of those for HL, and excess HLME could remove 28 per cent of those for DL and 20 per cent of those for HL. To test whether the antibodies absorbed by HLME were included in those absorbed by DLME, the neutralizing antibodies remaining after the absorption by DLME were further absorbed with excess of

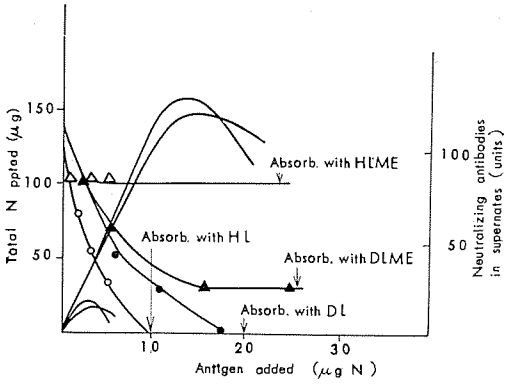

Fig. 19. Fate of HL-neutralizing Antibodies of DL-Antiserum in Supernatants of Precipitin Reactions

Table 10. Degree of Maximal Absorption of DL-Antiserum

Antigens used for absorption	DL-neutralizing antibodies absorbed		HL-neutralizing antibodies absorbed	
	N.U.	%	N.U.	%
DL	234	100	102	100
DLME	160	68.5	79.6	78
HL	126	54	102	100
HLME	64.4	27.5	20	19.6

HLME. No further absorption was seen as shown in Table 11. Therefore, the neutralizing antibodies absorbed by HLME must be included in those absorbed by DLME. DL could exhaust all the neutralizing antibodies of homologous antiserum for DL as well as for HL, and HL absorbed 54 per cent of those for DL and 100 per cent of those for HL.

Table 11. Repeated Absorption of DL-Antiserum with DLME and HLME

	DL-neutralizing antibodies remaining		HL-neutralizing antibodies remaining	
	N.U.	%	N.U.	%
DL Antiserum	234	100	102	100
after absorption with DLME	74	31.6	22.4	22
further absorbed with HLME	71	30.4	24.5	24

The neutralizing antibodies absorbed by HLME can neutralize both DL and HL and this fraction was designated as C_{DH} (28 per cent for DL and 20 per cent for HL). After absorption of DL antiserum with HL, 54 per cent was removed and 46 per cent was left, and this 46 per cent fraction neutralized only DL. Though no further absorption experiments were performed, this 46 per cent fraction can

be assumed to be identical with the difference between 69 per cent of DL-neutralizing antibodies and the 28 per cent of fraction C_{DH}. This is called fraction B_D. The remaining DL-neutralizing antibodies, after absorption with DLME, could neutralize both DL and HL and were called fraction A_{DH}. The sum of A_{DH} and C_{DH} cannot account for the total neutralizing power for HL. The difference in the HL-neutralizing activity absorbed by DLME and C_{DH} must be regarded as antibodies neutralizing only HL but not DL and it was named fraction E_H (58 per cent). In summarizing the above results, the neutralizing antibodies produced by DL can be classified as shown in Fig. 20.

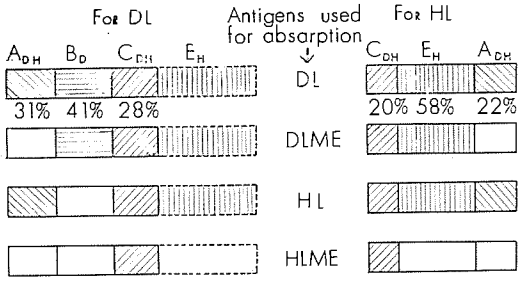

Fig. 20. Composition of Neutralizing Antibodies of DL-Antiserum

Shadowed parts represent fractions lost after absorption. Parts enclosed in dotted lines represent fraction apparently neutralizing only heterologous antigen and not homologous antigen.

4) *DLME-antiserum*

i) *Precipitin reaction*

The antigens used were DLME, DL, HL and HLME and the results of the precipitin reactions are shown in Table 12a, b, c, d.

Table 12. Quantitative Precipitin Reactions of the Four Antigens in DLME-Antiserum

a.

Tube No.	DLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: DLME anti-serum added	Tests on supernatants: DLME added
1	3.1_4	55.5	52.4	16.7	—	2+
2	4.7_1	69.5	64.8	13.7	—	±
3	7.0_6	84.8	77.4	11.0	—	—
4	10.0	98.8	88.8	8.8_8	—	—
5	13.0	113	100.0	7.7	—	—
6	14.9	123	108	7.2_5	— or ±	—
7	17.2	120			+	—
8	20.4	119			+	—
9	25.1	117			+	—
10	31.4	113			+	—

(Table 12) b.

Tube No.	DL (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: DL antiserum added	DL added	DLME added
1	1.0	26.0	25.0	25.0	—	+	2+
2	2.1	49.4	47.3	22.5	—	+	±
3	2.9_4	64.2	61.3	20.8	—	±	—
4	4.2	77.3	73.1	17.4	—	—	—
5	5.5	95.4	90.9	16.3	—	—	—
6	7.0_6	118.0	110.9	15.7	—	—	—
7	8.4_1	61.1			+	—	—
8	13.4_5	29.9			2+	—	—
9	18.5	19.4			2+	—	—

(Table 12) c.

Tube No.	HL (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on Supernatants: HL antiserum added	HL added	DLME added	DL added	HLME added
1	1.5_2	10.6	9.0	5.9_7	—	+	2+	2+	—
2	2.4_3	20.8	18.4	7.5_8	—	±	2+	+	—
3	3.8	24.8	21.4	5.5_4	—	—	2+	+	—
4	5.3_2	22.5			±	—	2+	+	—
5	8.3	20.0			+	—	2+	+	—
6	9.1_2	.....			+	—	2+	+	—
7	12.6	19.6			2+	—	2+	+	—
8	15.2	17.9			3+	—	2+	+	—

(Table 12) d.

Tube No.	HLME (μgN)	Total ppted protein (μgN)	Ppted antibody (μgN)	Antibody N / Antigen N	Tests on supernatants: HLME anti-serum added	HLME added	HL added	DL added	DLME added
1	1.6_6	20.7	19.0	11.4_3	—	2+	+	+	+
2	3.3_2	24.8	21.5	6.4_6	—	—	±	+	+
3	5.8_1	27.2	21.5	3.6_9	±	—	—	+	+
4	8.3	24.3			+	—	—	+	+
5	11.6_2	19.8			+	—	—	+	+
6	16.6	17.2			+	—	—	+	+
7	19.9_2	9.1_4			+	—	—	+	+

DL precipitated approximately the same amount of antibody N at the equivalence point as the homologous antigen (DLME) did (tube 6 of Table 12a and b), and HL also precipitated the same amount of antibody N at the equivalence point as HLME did (tube 3 of Table 12c and d). The latter result was very remarkable when compared with results on the three antisera described above. The supernatants of the systems of HL and HLME contained excess precipitins for DL as wellas for DLME throughout the range tested.

ii) *Neutralization*

The fate of the neutralizing antibodies in the supernatants of the four preci-

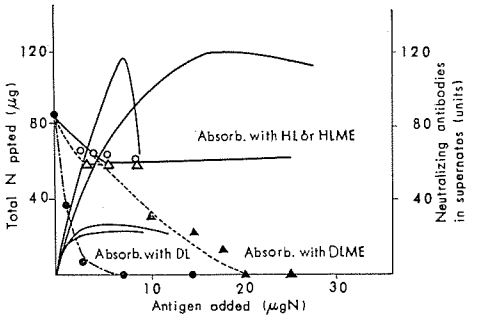

Fig. 21. Fate of DL-neutralizing Antibodies of DLME-Antiserum in Supernatants of Precipitin Reoctions

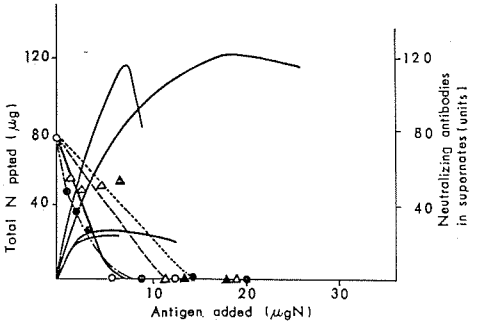

Fig. 22. Fate of HL-neutralizing Antibodies of DLME-Antiserum in Supernatants of Precipitin Reactions

Table. 13. Degree of Maximal Absorption of DLME-Antiserum

Antigens used for absorption	DL-neutralizing ontibodies absorbed		HL-neutralizing antibodies absorbed	
	N.U.	%	N.U.	%
DLME	89	100	76.2	100
DL	89	100	76.2	100
Hl	26.6	30	76.2	100
HLME	28.2	31.6	76.2	100

pitin reactions were also studied. The results are shown in Figs. 21 and 22 and Table 13. As can be seen, DLME and DL completely removed all the neutralizing antibodies for DL and HL, and HLME absorbed about 30 per cent of those for DL and 100 per cent of those for HL. In these neutralization experiments, DL and DLME behaved as the same antigen, as did HL and HLME. These facts are consistent with the facts that no difference could be found in the behaviors of DL and DLME or of HL and HLME as precipitinogens. The DL-neutralizing antibodies absorbed by HL or HLME (30 or 32 per cent) can be regarded as identical and this fraction could neutralize both DL and HL. Hence it is termed fraction C_{DH}. The 70 per cent fraction of DL-neutralizing antibodies could neutralize only DL and it is designated as fraction B_D. As all four antigens could absorb all the HL-neutralizing antibodies, HL-neutralizing antibodies may be due to C_{DH}. However, it is conceivable that another fraction, E_H, which can neutralize only HL and can be absorbed by all four antigens, was present in addition to C_{DH}, because there was too much difference between the HL-neutralizing activity of C_{DH} (28—26 N.U.) and the total DL-neutralizing activity of DLME-antiserum (76 N.U.). In reviewing the above results, the neutralizing antibodies can be classified as shown in Fig. 23.

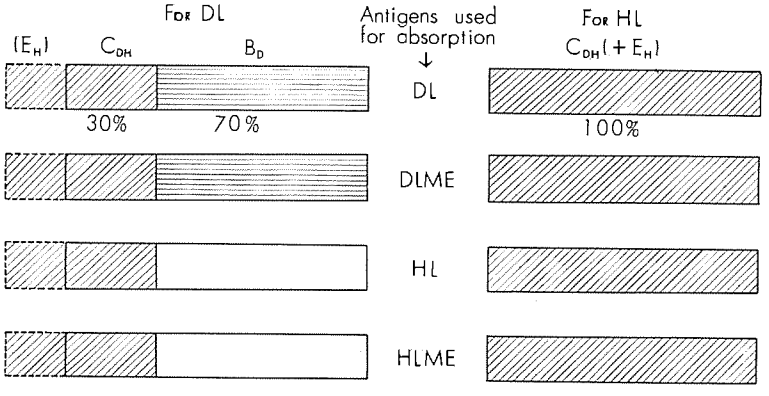

Fig. 23. Composition of Neutralizing Antibodies of DLME-Antiserum

Shadowed parts represent fractions lost after absorption. Parts enclosed in dotted lines represent fraction apparently neutralizing only heterologous antigen and not homologous antigen.

DISCUSSION

Though DL was not obtained in crystallin form, it was purified to almost the same specific activity as HL. The homogeneity of the DL preparation was proved by ultracentrifugation and gel diffusion experiments.

The cross precipitin reactions demonstrated on four antigens with their four respective antisera indicate the presence of some common antigenic sites on HL and DL, which are retained after esterification with methyl alcohol. As the super-

natants of the precipitin reaction of HL-antiserum with DL gave precipitates when excess antibody was tested with a minute amount of HL and *vice versa*, HL can be assumed to contain at least two antigenic sites specific for HL when interpreting results in the light of the lattice theory. The same assumption can be made for DL. In addition, HL and DL bear at least two common antigenic sites because they could give precipitates in cross reactions. Such assumptions were consistent with results of gel diffusion experiments, in which the two precipitation lines given by HL and DL with DL-antiserum fused forming a spur.

In our first report (Fujio *et al.*, 1959) it was assumed that HLME lost one antigenic site when HL was esterified and HLME acquired no new antigenic site as an artifical antigen, because HL precipitated the same amount of antibody as HLME itself with HLME-antiserum and some fraction (15 to 20 per cent) of HL antibodies could not be precipitated by HLME. Moreover the supernatants of the HLME~HL-antiserum system in the equivalence and antigen excess zones gave no precipitates when excess antibody was tested with an appropriate amount of HL. If the antigenic sites of HL molecules are all the same in specificity, some precipitate must be found in tests for excess antibody. The same phenomenon could be seen in the DLME~DL-antiserum system. In gel diffusion experiments, the precipitation line of HLME with HL-antiserum fused with that of HL against HL-antiserum without spur-formation. The same was true in tests on DLME and DL using DL-antiserum. To interpret these phenomena, the authors assume that the antigenic sites of a single protein molecule are different in specificity from each other. Such a concept was proved in studies on the proteolytic digests of human serum albumin (Lapresle, 1955; Lapresle *et al.*, 1957a, b, 1958), the proteolytic digests of diphtheria toxin (Raynaud, 1958) and the proteolytic digest of T-globulin of horse diphtheria antitoxin serum (Fujio *et al.*, 1958), and considered to be reasonable by Grabar and Marrack (1960). This concept was supported by our second report, in which some batches of HL-antisera contained non-neutralizng but precipitating antibodies together with neutralizing antibodies (Shinka *et al.*, 1962). This problem is discussed again below in relation to the heterogeneity of the neutralizing antibodies.

The heterogeneity in specificity of the neutralizing antibodies of HL-antiserum was described in our first report (Fujio *et al.*, 1959). Further extensive studies were made on this subject. At first we could not understand our results from absorption experiments. For instance, in the case of the pooled HL-antiserum, the HL neutralizing antibodies were classified into three fractions, among which C_{HD} (31 per cent) and A_{HD} (16 per cent) were capable of cross neutralizing DL, and B_H (53 per cent) was specific for HL. If the neutralizing powers of A_{HD} and C_{HD} antibody molecules are assumed to be equal, the ratio of the concentrations of C_{HD} and A_{HD} of DL-neutralizing antibodies must be 2:1. However, the results were not in good accord with this. If HL-antiserum was first absorbed by DLME, the only neutralizing antibodies for DL remaining should be A_{HD}, because B_H was incapable of

neutralizing DL. If the antiserum was first absorbed by HLME again, only A_{HD} should remain. In the former case 45 per cent remained and 23 per cent was unabsorbed in the latter case. This discrepancy was too great to be due to experimental error, and no reasonable explanation could be found for it. The results of absorption experiments with HL-antiserum from a rabbit (LF-32) threw light on this puzzling problem. When the antiserum was absorbed with DLME, there was no loss of HL-neutralizing antibodies but 51 per cent of DL-neutralizing antibodies were lost. At this time, the presence of non-neutralizing antibodies had already been demonstrated in some batches of HL-antisera (Shinka *et al.*, 1962), and hence the 51 per cent of antibodies neutralizing only DL produced by the antigen stimulation of HL, must be regarded as HL-non-neutralizing antibodies. If the pooled HL-antisera contained HL-non-neutralizing antibodies, the discrepancy between 45 and 23 per cent can be explained by the presence of another fraction (E_D), which could only neutralize DL and was produced by HL.

This substantial evidence for the presence of antibodies which are only cross-neutralizing and do not neutralize homologous antigen, is very important in considering the structure of HL and DL. It suggests that the active centre of DL is located in the neighbourhood of the antigenic site homologous to such an antibody, while the active centre of HL is not.

The antigenic valency of HL can be regarded as four from the molar ratio at the extreme antibody excess point. However, considering the results on the heterogeneity of HL antibodies we are forced to conclude that pooled HL-antisera contained at least six kinds of antibodies of different specificities, because fraction A_{HD} contained only one kind (A_{HD} did not give a precipitate with HL), fraction C_{HD} contained at least two kinds (C_{HD} was precipitated with DLME), fraction B_H consisted of at least two kinds (B_H gave a precipitate with HL), E_D contained at least one kind, and, in addition, the pooled sera may contain non-neutralizing antibodies. Consequently there is a discrepancy here, if results are interpreted by the lattice theory and different specificities are postulated for each antigenic site on the HL molecule. However, this discrepancy may be accounted for by assuming that six different antibody molecules can not all combine with a single HL molecule because of mutual steric hindrance, so that some of them are forced to combine with another molecule. Such a possibility is conceivable, because the molecular weight of lysozymes is very small (14,500) when compared to that of rabbit antibody (160,000). In addition the heterogeneity of the lysozyme molecules must be considered, because the hens and ducks used were not from pure stocks. As genetic allotypes have been found in human and rabbit γ-globulin (Grubb, 1956; Oudin, 1960), it is probable that genetic allotypes of lysozyme exist in hens and ducks. This subject requires further study.

ACKNOWLEDGEMENTS

The authors express their gratitude to Dr. Konosuke Fukai and Miss Kazuko Kawai of this

Institute for operating the ultracentrifuge.

REFERENCES

Alderton, G., Ward, W. H. and Fevold, H. L. (1945). Isolation of lysozyme from egg white. *J. Biol. Chem.* **157,** 43-58.

Folin, O and Wu, H. (1919). A system of blood analysis. *J. Biol. Chem.* **38,** 81-110.

Fraenkel-Conrat, H. and Olcott, H. S. (1945). Esterification of proteins with alcohols of low molecular weight. *J. Biol. Chem.* **161,** 259-268.

Frieden, E. H. (1956). Enzymoid properties of lysozyme methyl ester. *J. Am. Chem. Soc.* **78,** 961-965.

Fujio, H., Kishiguchi, S., Noma, Y., Shinka, S., Saiki, Y. and Amano, T. (1958). The antigenic relationship between the T-component of diphtheria antitoxic horse serum and antitoxic globulin modified with protease. *Biken's J.* **1,** 138-156.

Fujio, H., Kishiguchi, S., Shinka, S., Saiki, Y. and Amano, T. (1959). Immunochemical studies on lysozyme. I. Comparative studies of lysozyme and lysozyme methyl ester. *Biken's J.* **2,** 55-76.

Graber, P. and Marrack. J. R. (1960). *Immunochemical approaches to problems in microbiology.* p. 73. The Rutgers University Press. New Jersey.

Grubb, R. (1956). Agglutination of erythrocytes coated with "incomplete" anti-Rh by certain rheumatoid arthritic sera and some other sera. *Acta Pathol. Microbiol. Scand.* **39,** 195-197.

Heidelberger, M. and Kendall, F. E. (1935). A quantitative theory of the precipitin reaction. III. The reaction between crystalline egg albumin and its homologous antibody. *J. Exptl. Med.* **62,** 697-720.

Hirs, C. H. W., Moore, S. and Stein, W. H. (1953). Chromatographic studies on lysozyme. *J. Biol. Chem.* **200,** 507-514.

Inai, S., Kishimoto, S., Hirao, F. and Takahashi, H. (1958). Studies on serum lysozyme. II. Variations of the serum lysozyme level. Presented at the 11th meeting of the Kansai branch of the Society of Japanese Bacteriologists, on October 19, 1958.

Lapresle, C. (1955). Étude de la dégradation de la sérum albumine humaine par un éxtrait de rate de lapin. II. Mise en évidence de trois groupements spécifiques differents dans le motif antigénique de l'albumine humaine et de trois anticorps correspondants dans le sérum de lapin antialbumine humaine. *Ann. Inst. Pasteur* **89,** 654-655.

Lapresle, C. and Durieux, J. (1957a). Étude de la dégradation de la sérum albumine humaine par un éxtrait de rate de lapin. III. Modifications immunologiques de l'albumine en fonction du stade dégradation. *Ann. Inst. Pasteur* **92,** 62-73.

Lapresle, C. and Durieux, J. (1957b). Étude de la dégradation de la sérum albumine humaine par un éxtrait de rate de lapin. IV. Variations individuélles de la reaction de sérum de lapin antialbumine avec l'albumine dégradée. *Bull. Soc. Chim. Biol.* **39,** 833-841.

Lapresle, C. and Durieux, J. (1958). Étude de la dégradation de la sérum albumine humaine par un éxtrait de rate de lapin. V. Antigénicité de l'albumine dégradée. *Ann. Inst. Pasteur* **94,** 38-48.

Oudin, J. (1960). Allotype of rabbit serum proteins. I. Immunochemical analysis leading to the individualization of seven main allotypes. *J. Exptl. Med.* **112,** 107-124.

Raynaud, M. (1958). Hetorogeneity of diphtheria antitoxin. In *Mechanisms of hypersensitivity,* p.19, Little, Brown and Co.

Shinka, S., Imanishi, M., Kuwahara, O. and Amano, T. (1962). Immunochemical studies on lysozyme. II. On the non-neutralizing antibodies. *Biken J.* **5,** 181-200.

Smolelis, A. N. and Hartsell, S.E. (1949). The determination of lysozyme. *J. Bacteriol.* **58,** 731-736.

Yokoi, H. and Akashi, S. (1955). An improved Kjeldahl-Nessler method. *Nagoya Med. J.* **3,** 91-104.