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Immunochemical Studies on Lysozyme

1. Comparative Studies of Lysozyme and Lysozyme Methyl Ester*

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SUMMARY

Lysozyme, crystallized at its isoelectric point, was further purified by Amberlite IRC-50. The methyl ester derivative (L. M. E.) was prepared from the main component. The quantitative precipitin reaction and neutralization of lysozyme were studied with lysozyme antiserum and lysozyme methyl ester antiserum.

In the precipitin reaction one determinant group of lysozyme in five was found to be inactivated through esterification and had a different specificity from the others. L. M. E. did not gain any new antigenic determinant group.

Lysozyme antiserum as well as L. M. E. antiserum neutralized lysozyme completely. By cross absorption experiments lysozyme antiserum was found to contain two specific neutralizing antibodies, one of which was directed to the antigenic site in the neighborhood of the active centre. The other was directed to the combining site of lysozyme with the substrate.

Anaphylactic reactions of lysozyme and L. M. E. were also investigated.

INTRODUCTION

Recently immunochemists have been interested in enzymes, because enzymes have specific activities and antigenic determinant groups. Egg white lysozyme is especially suitable material for immunochemical studies, because the lysozyme molecule is a single polypeptide chain of low molecular weight (Fraenkel-Conrat, 1951). Moreover, it is quite stable and easily crystallized and its amino acid sequence will soon be clarified by Jollés and Fromageot (1958). Although immunochemical studies on lysozyme have been reported by several workers (Robert, 1937; Smollens *et al.*, 1947; Wetter *et al.*, 1951; Gonçalves *et al.*, 1956), the preparations of lysozyme used by these workers were not homogeneous chromatographically. Since Hirs, Stein & Moore (1953) described the chromatographic inhomogeneity of isoelectric lysozyme, no studies have been reported using the homogeneous main component separated by IRC-50 chromatography.

On the other hand, many workers have tried to determine the nature of the structures responsible for the biological activity of lysozyme through chemical

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modifications of the molecule (Fraenkel-Contrat, 1950; Geschwind *et al.*, 1957; Josefsson *et al.*, 1957). Among these workers, Frieden (1956) found that lysozyme methyl ester lost enzymatic activity but could still combine with substrate (*Micrococcus lysodeikticus*). He assumed that the site of combination and the active centre of lysozyme were different and separated from each other. To confirm his assumption immunologically, the authors studied the immunochemical properties of lysozyme and lysozyme methyl ester comparing both their quantitative precipitin reactions and their neutralizations.

Materials and Methods

1. Egg white lysozyme: 2.0 gr. of egg white lysozyme recrystallized three times, prepared by the method of Alderton and Fevold (1946), were separated chromatographically on an IRC-50 (XE-64) ion-exchange column (10×30 cm) according to the method of Hirs and Stein (1953). The main component was pooled, dialysed for 3 days against several changes of cold distilled water, concentrated using a flash-evaporator and finally lyophilized. When this preparation was rechromatographed on an analytical scale using the same procedure, only one homogeneous component was obtained. This homogeneous preparation was used throughout the experiments.

2. Lysozyme methyl ester (L. M. E.): L. M. E. was prepared as described by Fraenkel-Conrat (1945) and Frieden (1956). 500 mg. of finely powderd lysozyme purified chromatographically were suspended in 50 ml. of absolute methanol containing 0.4 ml. of conc. HCl. The gelatinous suspension formed was agitated at 25°C. After 48 hrs., it was diluted with five volumes of chilled distilled water, dialysed against 5 liters of cold distilled water for 24 hrs., concentrated and lyophilized. Dialysis and lyophilization were repeated twice more to remove all the HCl and methanol.

3. Antisera: Antisera to lysozyme and L. M. E. were obtained as follows. 20 mg. of lysozyme or L. M. E., dissolved in 10 ml. of saline containing 0.02 per cent merthiolate, was added to a mixture of 9.0 ml. of liquid paraffin and 1.0 ml. of Arlacel A and homogenized to make an emulsion. Rabbits received an initial subcutaneous dose of 20 ml. of the antigen emulsion injected at four separated loci. Injections were repeated five times at 14 days intervals. The animals were bled three weeks after the last injection. Antisera were pooled after inactivated at 56°C for 30 min. and stored at −60°C.

The antisera used for neutralization experiments were pretreated with bentnite (Wako Pure Chemicals, Lot. No. 77. CD 74254) according to the method of Inai and Kishimoto (1958) to remove "serum lysozyme".

4. Quantitative precipitin reaction: The quantitative determination of the precipitable antibody nitrogen in lysozyme antiserum and L. M. E. antiserum was made as described by Heidelberger and Kendall (1935). The antigen nitrogen and precipitable nitrogen were determined by our modified direct Nesslerization method.

5. Direct Nesslerization for determination of protein nitrogen: The procedure used was essentially as described by Harold (1950).

The method for preparation of the acid digestion mixture was somewhat modified. 100 ml. of conc. sulfuric acid were diluted with 300 ml. of deionized water and cooled. 0.125 ml. of $SeOCl_2$ was added to the diluted sulfuric acid and the solution mixed well.

To the Kjeldahl tubes, 3.0 ml. of the Nessler reagents (Folin *et al.*, 1919) were added, in place of the 10.0 ml. used by Harold, to avoid precipitation of the colored product. The standard curve made by this modified method was linear up to 50.0 μg. N. Experimental error was in the range of ± 2.0 μg. N.

6. Determination of lysozyme activity: The preparation of *M. lysodeikticus* used as substrate in these experiments was made as described by Smolelis and Hartsell (1949). Prior to the assay

a cell suspension was prepared from U. V. irradiated and lysophilized cells in M/15 phosphate buffer at pH 7.0. This bacterial suspension was adjusted to the extinction of 1.0 at 540 mμ. in a Coleman universal spectrophotometer equipped with a PC-4 filter. A standard curve for the lysozyme activity was obtained as follows. 3.0 ml. of saline containing varying amounts of lysozyme were mixed with 3.0 ml. of the bacterial suspension, the mixtures were incubated at 30°C. After 13 minutes the turbidity was estimated and the reduction in turbidity plotted against the logarithm of the concentration of lysozyme.

7. Zone electrophoresis of lysozyme and L. M. E.: Zone electrophoresis of lysozyme and L. M. E. was performed essentially as described by Flodin (1956) at 20°C, using a (3×50 cm) column with ethanolized cotton cellulose as supporting medium and M/20 acetate buffer at pH 4.8 as eluent.

8. Acid-base titration of lysozyme and L. M. E.: Titrations were carried out by the continuous method using a Horiba pH meter model-P. An one per cent solution of the protein in M/15 KCl, was used for titration. The HCl and KOH were 1 N., and in alkaline range, the titration was performed in a stream of nitrogen.

9. Determination of methoxyl groups: Methoxyl groups were estimated by the method of Vieböck and Schwappach as modified by Clark (1932), using completely dialysed and dried L. M. E. and lysozyme.

10. Miscellaneous analytical methods: A manometric micro Van Slyke apparatus was used to estimate amino groups in protein (15 min. reaction period). Free phenolic groups were estimated by Herriott's modification (1935) of the Folin method. All samples were hydrolyzed prior to analysis by refluxing with 6 N. HCl for 6 hrs. Control experiments showed that phenol esters were stable under these conditions. Tryptophan was determined, after alkaline hydrolysis, by Graham's method (1947) with p-dimethylamino-benzaldehyde. The total nitrogen of lysozyme and L. M. E. was also determined by the micro Duma method. Amide nitrogen was estimated according to the method of Bailey (1937).

RESULTS

1. Enzymatic, physical and chemical properties of lysozyme methyl ester (L. M. E.)

Though the enzymatic, physical and chemical properties of L. M. E. have already been well studied by Frieden (1956), it is necessary in this report to describe the properties of the preparation used.

i) Enzymic activity of L. M. E.

Frieden stated that the carboxyl groups of lysozyme are important for its catalytic reaction, but are not required for the formation of the intermediate enzyme-substrate complex, and hence he concluded that L. M. E. is an "enzymoid", a name first proposed by Bayliss (1911). The authors confirmed the enzymoid nature of our L. M. E. preparation.

2.0 ml. aliquots of M/15 phosphate buffer (pH 7.0) containing varying amounts of L. M. E. (50, 100, 150 and 200 μg.) were put into four tubes. 3.0 ml. of the bacterial suspension (0.5 mg. dry weight per ml.) was added to each tube. Two additional tubes served as controls. The one (C2) contained 2 ml. of buffer containing L. M. E. (200 μg.) and 3 ml. of bacterial suspension. The other (C1) contained 2 ml. of the buffer and 3 ml. of bacterial suspension. The six tubes were incubated for 10 min. at 38°C. 1.0 ml. of buffer containing 6.7 μg. of lysozyme was added to each of the experimental tubes and to tube C1. 1.0 ml. of buffer was added to tube C2. The tubes were reincubated for 13 minutes at 30°C. The turbidity of the tubes was determined in a Coleman universal spectrophotometer at 540 mμ.

Table 1. The competitive inhibition of lysozyme by L. M. E.

Tube No.	Lysozyme μg./ml.	L. M. E. μg./ml.	Lytic Activity %
1	6.7	0	100
2	0	100	0
3	6.7	100	60.7
4	6.7	75	85.5
5	6.7	50	91.5
6	6.7	25	95.7

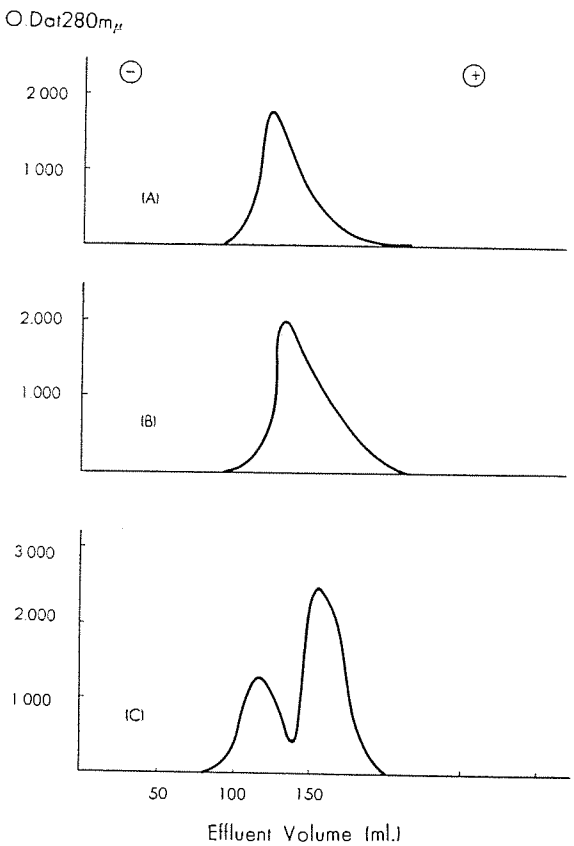

Fig. 1. Zone electrophoresis of lysozyme and L.M.E.
(A): Lysozyme (30 mg.). 22 hours at 21 mA (640 V)
(B): L.M.E. (30 mg.). 17 hours at 16 mA (500 V)
(C): Lysozyme (15 mg.).+L.M.E. (15 mg.). 21 hours at 16 mA (610 V)

2 ml. of a 1.5% solution in M/20 acetate buffer (pH 4.80) of each sample was applied on a column (3 × 50 cm.) buffered with the same solution and zone electrophoresis was carried out at 20°C. The samples were eluted from the column in 5 ml. fraction using the fraction collector. The protein concentration of each fraction was estimated by U.V. absorption at 280 mμ.

As can be seen from the results in Table 1, the competitive inhibition of L. M. E. against lysozyme described by Frieden was confirmed.

ii) Electrophoretic properties of L. M. E. and lysozyme.

Since the carboxyl groups of lysozyme were esterified, the isoelectric point of L. M. E. must be more alkaline than that of lysozyme. To confirm this, zone electrophoretic studies were performed using ethanolized cotton cellulose as a supporting medium, as recommended for basic proteins by Flodin and Kupke (1956).

As shown in Fig. 1, lysozyme and L. M. E. are electrophoretically homo-

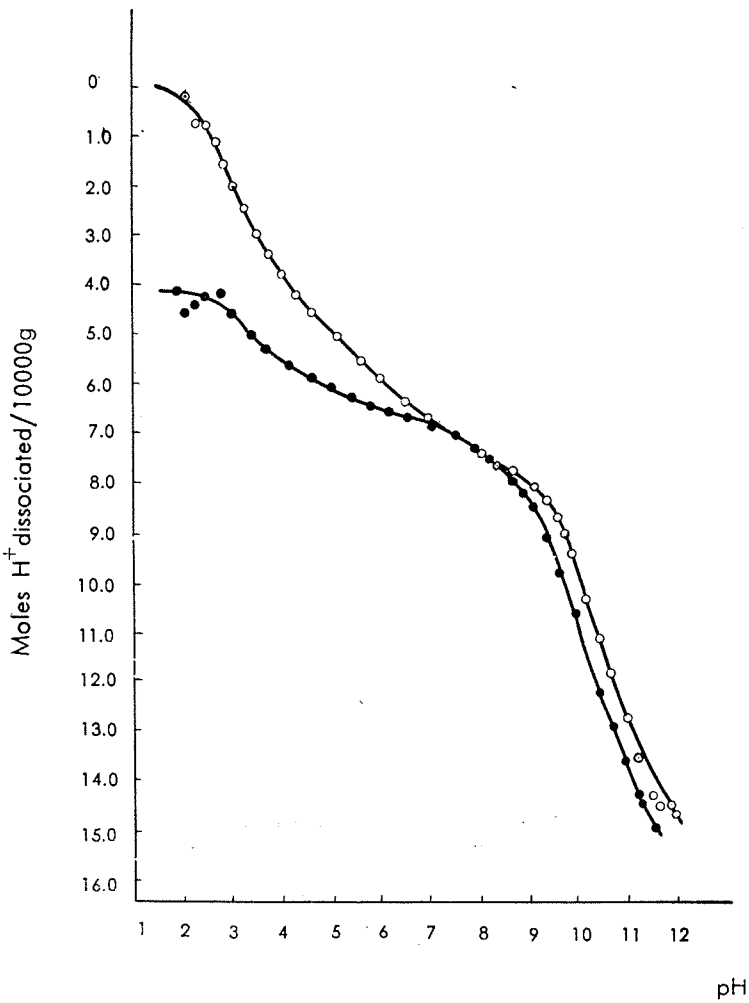

Fig. 2. Titration curves of lysozyme ond L.M.E.
—○—○— Isoelectric lysozyme
-●-●-●- L. M. E.

geneous. In the third pattern of Fig. 1, the faster moving fraction was shown to be L. M. E. because it was enzymatically inactive and because the slower moving fraction had lysozyme activity. Therefore, the isoelectric point of L. M. E. is more alkaline than that of lysozyme.

iii) Acid-base titration curves of lysozyme and L. M. E.

Titrations were made with 10 ml. aliquots of one per cent solution. As shown in Fig. 2, the number of moles of dissociated hydrogen ion between pH 3~5 is certainly diminished in L. M. E. In other words, L. M. E. contains less free carboxyl groups than lysozyme.

On the other hand, there was almost no difference in the titration curves in the alkaline range. From the results of titration experiments the number of carboxyl groups of L. M. E. and lysozyme were calculated, assuming that molecular weight of L. M. E. and lysozyme was 14,500 and that at pH 8.0 the carboxyl and imidazol groups of lysozyme were completely dissociated (Tanford, 1954) and 90 per cent of α-amino groups and 20 per cent of more basic groups were also dissociated at this pH. The calculation showed that one mole of lysozyme contained 7.8 free carboxyl groups and one mole of L. E. M. 1.7. The L. M. E. was calculated to be 78 per cent methylated.

iv) Determination of methoxyl groups in L. M. E.

Methylation of L. M. E. was directly estimated by determination of methoxyl groups according to the method of Vieböck (1932). The results together with those of the previous experiment, are summarized in Table 2. One mole of L. M. E. contained 5.8 methoxyl groups and lysozyme none. L. M. E. was calculated to be 76 per cent esterified. This is in good agreement with results obtained from acid-base titration.

Table 2. The numbers of free carboxyl groups per molecule of lysozyme and L. M. E. and extent of methyl esterification of L. M. E.

		Lys.	L. M. E.	
Free carboxyl groups/mole*		7.8	1.7	Acid-base titration method
Methoxyl groups/mole*		0	5.8	Vieböck method
Per cent esterification	Acid-base titration method	0	78	
	Vieböck method	0	76	

*These values were calculated assuming the molecular weight of lysozyme and lysozyme methyl ester to be 14,500.

v) Chemical analyses of lysozyme and L. M. E.

As the conditions for methyl esterification of lysozyme seemed to be rather drastic, it was necessary to see whether other comparatively unstable groups as well as the carboxyl groups were modified by the treatment. Chemical analyses of total N, amide N, amino N and the contents of tyrosine and tryptophane in L. M. E. and lysozyme were made.

As shown in Table 3, the values obtained from L. M. E. were in good agreement with those of lysozyme. Thus there were no detectable modification in L.

Table 3. Analyses of lysozyme and L. M. E.

	Lysozyme		L. M. E.	
	%	groups/mole*	%	groups/mole*
Total N	18.52	—	18.58	—
Amide N	1.88	19.5	1.93	20.0
Amino N	0.63	6.5	0.64	6.6
Tyrosine	4.8	3.8	4.5	3.7
Tryptophan	8.4	6.0	8.6	6.1

*These valuse were calculated assuming the molecular weight of lysozyme and lysozyme methyl ester to be 14,500.

M. E. molecule other than esterification of carboxyl groups.

This is consistent with the fact that the titration curves of L. M. E. and lysozyme in the alkaline range were almost identical.

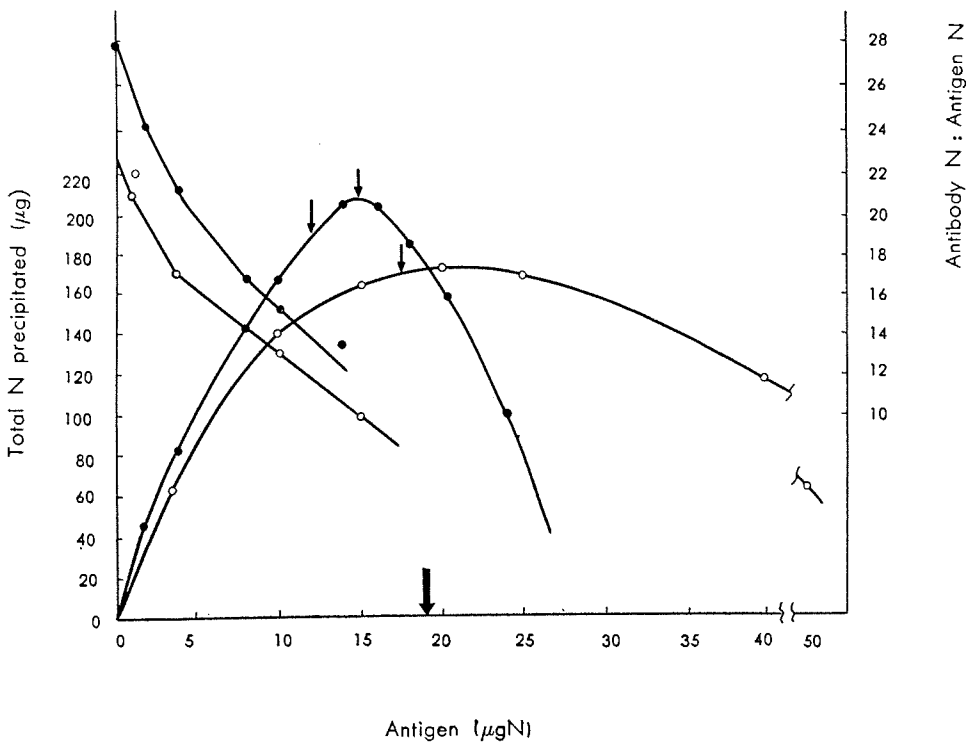

Fig. 3. Quantitative precipitin reactions of lysazyme and L. M. E. in lysozyme antiserum.
Note: Antiserum: 1.0 ml.
↓ Equivalence zone
⬇ Point where the minimum lytic activity was detectable in the supernatants of the lysozyme-antilysozyme system
—●—●— Lysozyme and antilysozyme system
—○—○— L. M. E. and antilysozyme system

2. Quantitative precipitin reaction of lysozyme antiserum with lysozyme and L. M. E.

On zone electrophoresis, lysozyme purified by IRC-50 chromatography, showed only one homogeneous component. To investigate the nature of the antibodies formed by lysozyme, quantitative precipitin reactions of lysozyme and L. M. E. with lysozyme antiserum were made.

Varying concentrations of lysozyme (1.0 to 12.0 μg.N) and L. M. E. (0.5 to 25.0 μg.N) in 0.5 ml. of saline containing 0.02 per cent merthiolate, were pipetted into 5 ml. graduated centrifuge tubes. 0.5 ml. of undiluted lysozyme antiserum was added to each tube. A serum control

Table 4. Quantitative precipitin reaction of lysozyme in lysozyme antiserum

(lysozyme antiserum : 0.5 ml.)

Lysozyme added	Precipitated protein N	Test on supernatant	
		Antibody excess test with lysozyme	Antigen excess test with lysozyme antiserum
1 μg.N	22.5 μg.N	+++	−
2	40.5	+++	−
4	71.3	++	−
5	83.6	±	−
6	93.4	±	−
7	106.7	−	−
8	106.0	−	+
9	97.7	−	++
10	79.0	−	++
12	49.8	−	++

+++ : Heavy precipitation.
++ : Moderate precipitation.
+ : Slight precipitation.
± : Questionable precipitation.
− : No precipitation.

Table 5. Quantitative precipitin reaction of L. M. E. in lysozyme antiserum

(lysozyme antiserum : 0.5 ml.)

L. M. E. added	Precipitated protein N	Test on supernatant		
		Antibody excess test		Antigen excess test with lysozyme antiserum
		with L. M. E.	with lysozyme	
0.5 μg.N	9.2 μg.N	++	+++	−
1.5	28.3	++	+++	−
5.0	70.5	+	++	−
7.5	82.6	±	±	−
10.0	86.8	−	−	+
12.5	84.2	−	−	++
20.0	59.7	−	−	++
25.0	32.0	−	−	++

containing 0.5 ml. of lysozyme antiserum and 0.5 ml. of saline was also set up. All tubes were set up in duplicate. The contents of the tubes were mixed and the tubes were stoppered. They were incubated at 37°C for 1 hour and then at 2°C for 14 days, agitating daily. At the end of the incubation they were centrifuged for 30 minutes at 3000 r.p.m. at 0°C. The precipitates were washed twice with 2.0 ml. of chilled saline and recentrifuged at 0°C. 0.5 ml. of 0.5 N NaOH was added and when the precipitates were dissolved, the solutions were poured into micro-Kjeldahl tubes. Each tube was washed five times with 1.0 ml. aliquots of deionized water and the washings were also put into the micro-Kjeldahl tubes. The Kjeldahl tubes were digested and nitrogen determined by our modified direct Nesslerization method as described above. The supernatants were tested for antigen and antibody excess. The results of these experiments are presented in Fig. 3, Table 4 and 5. The precipitated antibody-antigen nitrogen ratios are shown in Fig. 3.

From Fig. 3 it can be seen that the precipitin curve of the lysozyme-anti-lysozyme system declines much more steeply in the antigen excess zone than does that of the L. M. E.-antilysozyme system. The maximum precipitable antibody nitrogen of the L. M. E.-antilysozyme system is 80 per cent of that of the homologous lysozyme-antilysozyme system.

As seen from the curve of the L. M. E.-antilysozyme system, there was a fairly large amount of nitrogen precipitated in the antigen excess region. It is probable that, for some reason, the L. M. E. solution was unstable and so some precipitated during the 14 days incubation and that this precipitated L. M. E. could still react with antibodies. Therefore the precipitated protein nitrogen in the antigen control was not subtracted from the total precipitated protein nitrogen.

When extrapolated to zero ratios of the precipitated antibody nitrogen to added antigens were 28 for the lysozyme-antilysozyme system and 23 for the L. M. E. -antilysozyme system. If the valency of precipitated antibody is assumed to be 2, the valency of lysozyme is calculated as 5 and that of L. M. E. as 4.

As shown in Table 5 it is remarkable that the supernatants in the antigen excess region of the L. M. E.-antilysozyme system did not give precipitates with lysozyme, although 20 per cent of the lysozyme antibody remained in solution in the L. M. E.-antilysozyme system and a similar fraction of antibody was precipitated in the lysozyme-antilysozyme system. If all the determinant groups of lysozyme have the same specificity, this cannot be explained.

3. Quantitative precipitin reaction of L. M. E. antiserum with L. M. E. and lysozyme.

Analogous experiments were carried out using L. M. E. antiserum. The method used was as in the previous experiments.

From Fig. 4, Table 6 and 7, it can be seen that the maximum precipitable antibody nitrogen of the lysozyme-anti L. M. E. system was almost equal to that of the homologous L. M. E. system. Extrapolated ratios of precipitated antibody nitrogen to the zero point of added antigen was also the same (23) in both systems. Thus lysozyme behaved towards L. M. E. antiserum as if it were an antigen with 4 valencies, while towards homologous antiserum it seemed to have 5 valencies. As shown in Table 7, when a small amount of L. M. E. was added to the supernatant in the equivalence zone of the lysozyme-anti L. M. E. system and the L. M. E. antibody left in the supernatant was examined, no precipitate was detected. Therefore L. M. E. gained no new antigenic site and lost one ant-

igenic site and enzymic activity during esterification.

4. Neutralization of lysozyme with lysozyme antiserum and L. M. E. antiserum.

In previous experiments it was impossible to investigate the neutralization of lysozyme in antigen-antibody mixtures because lysozyme activity is measured by the reduction in optical density of the bacterial suspension and very turbid antigen-antibody mixtures disturbed it. To avoid this the neutralization experiment was made with diluted antisera. However the difficulty arose that both

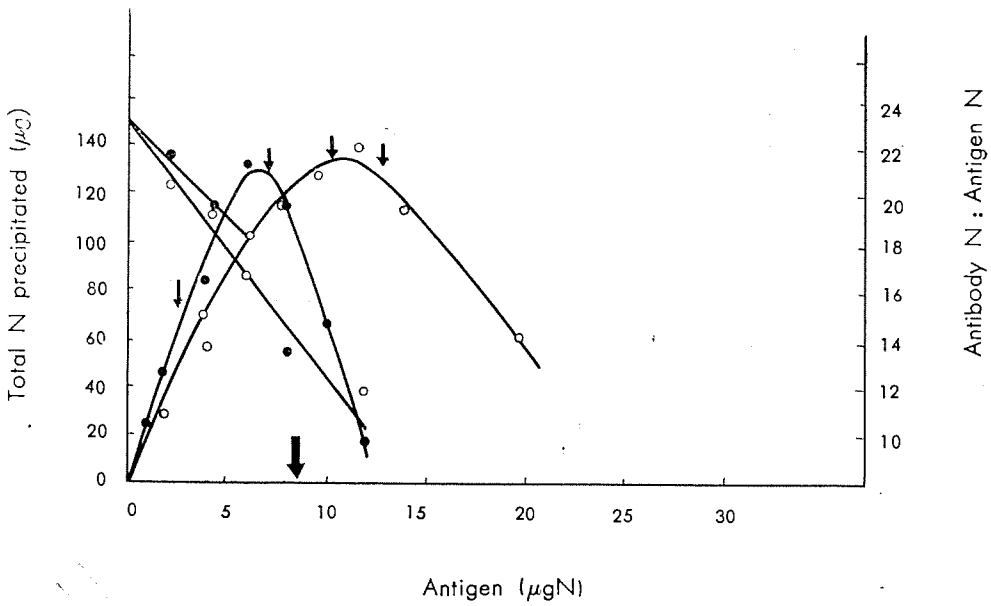

Fig. 4. Quantitative precipitin reactions of L. M. E. and lysozyme in L. M. E. antiserum.
Note : L. M. E.-antiserum : 1.0 ml.
—●—●— Lysozyme and antiL. M. E. system
—○—○— L. M. E. and antiL. M. E. system

Table 6. Quantitative precipitin reaction of L. M. E. in L. M. E.-antiserum
(L. M. E. antisetum : 0.5 ml.)

L. M. E. added	Precipitated protein N	Test on supernatant	
		Antibody excess test with L. M. E.	Antigen excess test with L. M. E. antiserum
1.0 μg.N	18.9 μg.N	++	—
2.0	35.3	++	—
3.0	50.2	+	—
4.0	60.1	+	—
5.0	65.0	±	—
6.0	67.9	—	—
7.0	56.5	—	+
10.0	30.4	—	++

Table 7. Quantitative precipitin reaction of lysozyme in L. M. E. antiserum
(L. M. E.-antiserum : 0.5 ml.)

Lysozyme added	Precipitated protein N	Test on supernatant		
		Antibody excess test		Antigen excess test with L. M. E. antiserum
		with L. M. E.	with lysozyme	
0.5 μg.N.	11.3 μg.N.	++	++	−
1.0	24.5	±	±	−
2.0	42.4	−	−	−
3.0	65.7	−	−	−
4.0	57.0	−	−	+
5.0	33.3	−	−	+
6.0	7.2	−	−	+

normal rabbit serum and antiserum contained serum lysozyme at a concentration of 3 to 10 μg. per ml. Hence the serum lysozyme had to be removed without loss of antibobies. Inai and Kishimoto (1958) succeeded in doing this by adsorbing serum lysozyme onto bentnite.

5 mg. of bentnite was suspended in 1.0 ml. of serum. After shaken for 10 min. at room temperature, the bentnite was removed by centrifugation. In this way more than 96 per cent of serum lysozome was removed. The maximum precipitable

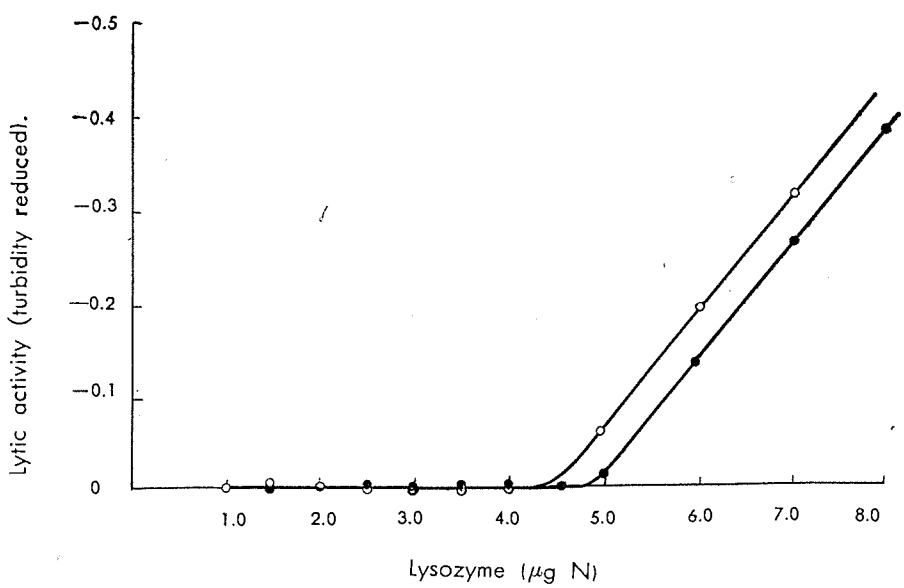

Fig. 5. Neutralization of lysozyme activity by lysozyme antiserum and L. M. E. antiserum.
(A) Neutralization with varying amounts of antigen.
—●—●— : Lysozyme-antilysozyme system
—○—○— : Lysozyme-antiL. M. E. system

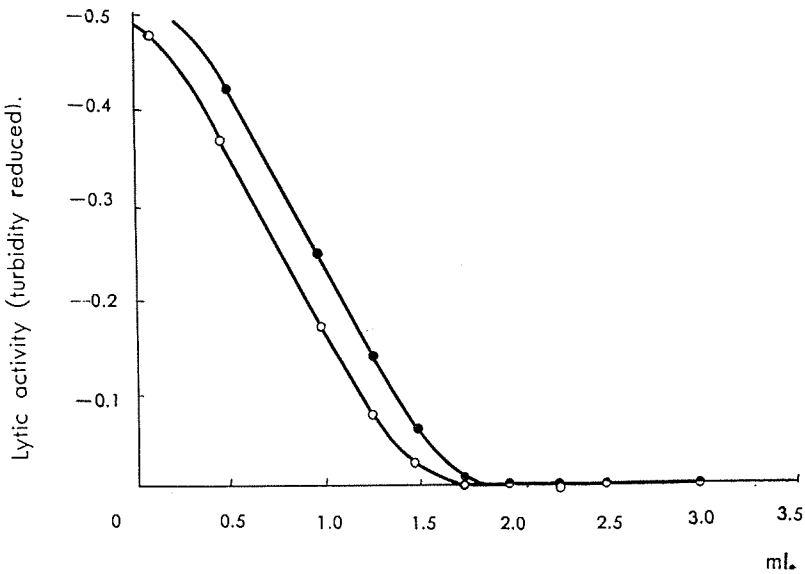

Fig. 6. Neutralization of lysozyme activity by lysozyme antiserum and L. M. E. antiserum.
(B) Neutralization with varying amounts of antisera.
Note: Lysozyme antiserum (1:12)
L. M. E. antiserum (1:6)
—●—●—: Lysozyme-antilysozyme system
—○—○—: Lysozyme-antiL. M. E. system

antibody nitrogen was not reduced and no difference was detected in antibody excess tests on the supernatants of treated antiserum and untreated antiserum.

Lysozyme neutralization experiments were made with this treated antisera varying either the amount of antigen or of antiserum.

i) Neutralization of varying amounts of antigen.

Varying quantities of lysozyme (1.0 to 8.0 μg.N), dissolved in 3.0 ml. of saline, were pipetted into test tubes. 3.0 ml. of a 1 : 12 diluted lysozyme antiserum solution or the same amount of a 1 : 6 diluted L. M. E. antiserum solution were then added. The solutions were mixed and the tubes were incubated at 37°C for 1 hour and then at 2°C overnight. 3.0 ml. aliquots from each tube were put in tubes containing 3.0 ml. of the bacterial suspension. 3.0 ml. of M/15 phosphate buffer was added to each of the former tubes, which served as controls for estimation of the reduction of bacterial density. All the tubes were incubated at 30°C for 13 min. and reduction of bacterial density was estimated.

ii) Neutralization with varying amount of antiserum.

Varying quantities (0.1 to 3.0 ml.) of a 1 : 12 diluted lysozyme antiserum solution or varying quantities (0.1 to 3.0 ml.) of a 1 : 6 diluted solution of L. M. E. antiserum were pipetted into tubes. The total volume was adjusted to 3.0 ml. with saline. 3.0 ml. aliquots of lysozyme containing 3.0 μg.N were then added to the tubes containing diluted lysozyme antiserum. To each of the tubes containing diluted L. M. E. antiserum 3.0 ml. of lysozyme solution containing 2.5 μg.N were added. Then the method was the same as described above. The results are presented in Fig. 5 and 6.

The results show lysozyme is completely neutralized by either lysozyme antiserum or L. M. E. antiserum. For neutralization the ratios of lysozyme to lysozyme antiserum by both assay methods were the same. The same was true in the lysozyme-antiL. M. E. system. For some reason it required approximately 1.5 times more antibody nitrogen of L. M. E. antiserum than of lysozyme antiserum to neutralize the lysozyme.

Assuming that for neutralization the ratio of lysozyme to lysozyme antiserum or to L. M. E. antiserum would be the same in diluted or undiluted antisera, the end points of the undiluted antisera were calculated and are shown by arrows in Fig. 3 and 4. The correctness of this assumption is proved in the next experiment. As can be seen in Fig. 3 and 4, complete neutralization of either antiserum was at a slight excess of antigen.

5. Two specific neutralizing antibodies of lysozyme antiserum.

It was demonstrated in the previous experiment that L. M. E. antiserum and lysozyme antiserum were neutralized by a slight excess of antigen, therefore the problem arose of whether the supernatants at the equivalence zone of each system contain neutralizing antibodies in spite of negative result of the antibody excess test in the precipitin reaction, or whether all the neutralizing antibody molecules were precipitated with antigen-antibody aggregates.

It has already been shown that lysozyme and L. M. E. molecules have an antigenic valency of 5 and 4 respectively and that both lysozyme antiserum and L. M. E. antiserum contained neutralizing antibodies, and a study was made of whether the antibody molecule directed to a determinant group of lysozyme lost in L. M. E. can neutralize or whether it only precipitates. Therefore the quantitative pricipitin reactions were repeated in the lysozyme-antilysozyme and L. M. E.-antilysozyme systems.

Varying quantities of lysozyme (2.0 to 25.0 μg.N) and L. M. E. (3.5 to 50.0 μg.N), dissolved in 1.0 ml. of saline containing 0.02 per cent merthiolate, were pipetted into 5 ml. graduated centrifuge tubes. 1.0 ml. of undiluted lysozyme antiserum was then added to each tube. A serum control containing 1.0 ml. of lysozyme antiserum and 1.0 ml. of saline was also set up. All the tubes were incubated at 37°C for 1 hour and at 2°C for 14 days. The tubes were agitated daily, and were finally centrifuged at 3000 r.p.m. for 30 min. at 0°C. After washing twice the precipitated proteins were estimated by direct Nesslerization. Remaining neutralizing antibody was estimated in each supernatant. Serial 1 : 2 dilutions (1 : 1, 1 : 2, 1 : 4, 1 : 8, and 1 : 16) were made from 1.0 ml. of each supernatant, leaving 0.5 ml. in each tube. 1.0 ml. of lysozyme solution (1 μg.N per ml.) and 0.7 ml. of buffered saline were added to the tubes which were incubated at 37°C for 1 hour and then at 2°C overnight. 2.2 ml. of bacterial suspension was then added. As controls, 0.5 ml. of each supernatant from the precipitin reaction was put in a tube containing 1.0 ml. of lysozyme solution (1.0 μg.N pre ml.) and 0.7 ml. of buffered saline. These tubes were also incubated. Then 2.2 ml. of M/15 phosphate buffer were added to each control tube. The experimental and control tubes were again incubated at 30°C for 13 minutes. The reduction of the bacterial density was estimated. The turbidity of the control tubes was measured in 1 : 1 diluted controls and then the contents were chilled and diluted 1 : 2 serially (1 : 2, 1 : 4, 1 : 8, and 1:16). The turbidities of these diluted controls were estimated. The readings of the serially diluted controls were inversely proportional to the dilution factor. The readings of optical density of the control tubes were subtracted from those of corresponding experimental tubes. From the corrected values of the reduction of bacterial density, the concentration of unneutralized lysozyme

in the experimental tubes was calculated from a standard curve of lysozyme. The free lysozyme concentrations of the tubes thus obtained were plotted against the corresponding dilution factors. The result was approximately linear. One unit of the lysozyme antibody was defined as the amount which can neutralize 0.1 μg.N of lysozyme. From this definition, the remaining neutralizing antibody in 0.5 ml. of the supernatants of the precipitin reaction is calculated by multiplying the dilution factors by the values of neutralized lysozyme (μg.N per tubes) divided by 0.1 μg.N. These antibody units were again multiplied by four, because the content of the original precipitin reaction was 2.0 ml. These calculated units of neutralizing antibody were plotted against the amounts of added antigen together with the precipitated total protein nitrogen.

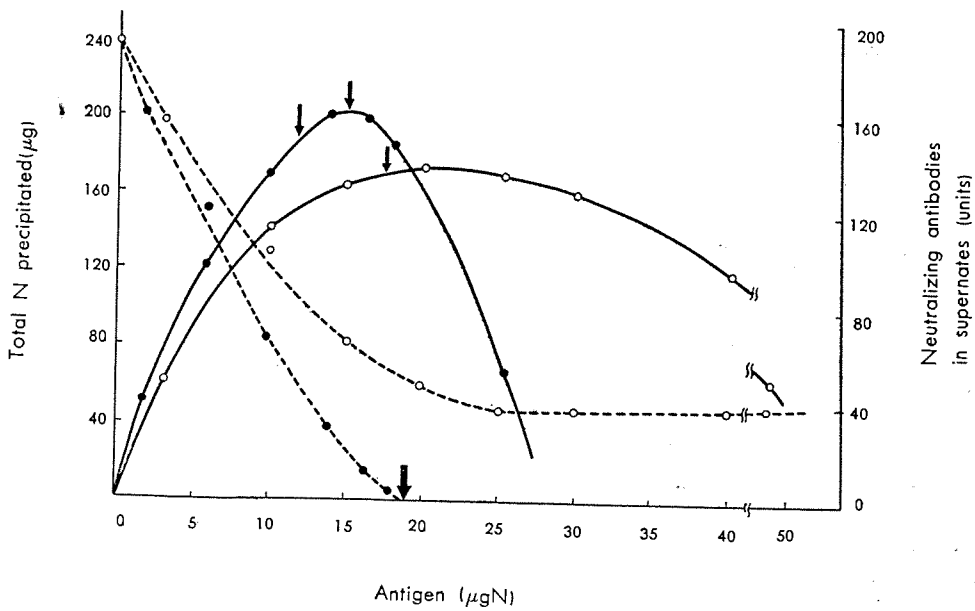

Fig. 7. Determination of neutralizing antibodies in the supernatants ofter the precipitin reactions.

↓ Equivalence zone

⬇ End point of complete neutralizotion of the lysozyme-antilysozyme system

—●—●—: Precipitin curve of the lysozyme-antilytozyme system

—○—○—: Precipitin curve of the L. M. E.-antilysozyme system

·····●·····●·····: Neutralizing antibodies in supernatants (units) of the lysozyme -antilysozyme system

·····○·····○·····: Neutralizing antibodies in supernatants (units) of the L. M. E. -antilysozyme system

The results are presented in Fig. 7. Results for neutralization in the antilysozyme-lysozyme system in this experiment were identical with those calculated from the neutralization of diluted antiserum (shown in Fig. 3). The assumption, that the ratio of antigen to antibody at the endpoint of the complete neutralization obtained in diluted antiserum is the same as in undiluted serum, was thus confirmed. As can be seen from the Fig. 7, the endpoint for complete neutralization is located in the slight antigen excess region. Even soluble antigen-antibody complexes present in the supernatants should be neutralized between equivalence

and complete neutralization. It is also clearly demonstrated that a certain amount of free neutralizing antibody was still left in the supernatants at the equivalence zone, though they gave no precipitate on addition of a small amount of lysozyme as shown in Fig. 7.

The results for the L. M. E.-antilysozyme system are also presented in Fig. 7. The curve of units of neutralizing antibody in the supernatants of the precipitin reaction gives a plateau with a large excess of antigen. Therefore even a large excess of L. M. E. did not absorb all the neutralizing antibody molecules elicited by lysozyme. In other words, lysozyme elicited two kinds of neutralizing antibodies: the one is specifically directed to the active centre, or to its neighboring determinant group which is inactivated through the esterification. The other is directed to the combining site (or sites) of lysozyme with the substrate, or to its neighboring determinant group (or groups), which is unaffected by esterification.

However, in opposition to this conclusion L. M. E. inhibited lysozyme competitively at the surface of the bacteria, so soluble L. M. E.-antibody complexes present in the supernatants in the antigen excess region may still inhibit the activity of added active lysozyme as if antibodies neutralized lysozyme. If so the curve showing the units of neutralizing antibody in the supernatants would increase from the plateau in proportion to the increment of added antigen. In this experiment there was a plateau till 50 μg.N of added L. M. E. which corresponds roughly to three times the amount at the equivalence point. The following experiment was made to disprove this contradictory theory.

20 μg.N aliquots of L. M. E. in 0.5 ml. of saline, were put into two tubes (No. 1, 2) containing 0.5 ml. of lysozyme antiserum. The tubes were incubated at 37°C for 1 hour and then at 2°C for 14 days. After washing twice with cold saline the precipitated protein nitrogen was estimated. 0.5 ml. of the supernatant of tube No. 1 was added to a tube containing 0.5 ml. of bacterial suspension. An equal volume of the supernatant of tube No. 2 was added to a tube containing 0.5 ml. of M/15 phosphate buffer. The two tubes were incubated at 30°C for 13 min. and centrifuged. 0.5 ml. of each supernate was added to a tube containing 0.7 ml. of lysozyme antiserum to precipitate the remaining antigen-antibody complex completely in the antibody excess zone. The tubes were incubated at 37°C for 1 hour and then at 2°C for two days. The precipitated total protein nitrogen was estimated.

As can be seen from Table 8, there was no difference in precipitated total protein nitrogen in tubes 1 and 2. Therefore soluble L. M. E.-antibody complexes in the antigen excess region are not adsorbed onto the surface of the bacteria and hence the complexes do not behave as though they were neutralizing antibodies. Lysozyme antiserum contains two neutralizing antibodies. The amount of the neutralizing antibody directed to the active centre or to its neighborhood accounts for 20 per cent of that of total neutralizing antibody. The remaining 80 per cent is directed to the combining site (or sites). However, it is not yet clear whether all the precipitating antibodies directed to L. M. E. can neutralize lysozyme, or whether some fraction or all of the anti L. M. E. antibodies can neutralize lysozyme, because the amount of neutralizing antibody could not be estimated quantitatively on a nitrogen basis.

Table 8. Examination of adsorption of soluble complex onto bacterial cells

Tube No.	1	2
L. M. E. (μg.N) in 0.5 ml.	20	20
Lysozyme antiserum (ml.)	0.5	0.5
Centrifuged after 14 days		
Precipitated protein N (μg.)	78.0	78.0
Supernatants after the centrifuge (ml.)	0.5	0.5
Bacterial suspension (ml.)	0.5	—
Saline (ml.)	—	0.5
Centrifuged after 13 min. at 30°C		
Supernatants after centrifugation (ml.)	0.5	0.5
Lysozyme antiserum (ml.)	0.7	0.7
Centrifuged after 2 days		
Precipitated protein N (μg.)	24.8	24.7

6. Antigen-antibody reaction *in vivo*

The specific antigen-antibody reaction of lysozyme and lysozyme methyl ester have been demonstrated by the precipitin reaction and neutralization. Further experiments were made on the *in vivo* antigen-antibody reaction. Passive anaphylaxis was used for this study.

Guinea pigs weighing about 250 gr., were given an intravenous injection of 1.0 ml. of lysozyme antiserum (containing 0.04 mg. of antibody N per ml.), which was also used in the

Table 9. Passive systemic anaphylaxis

Antigen used		Symptoms*			
		Death	Severe	Slight	No reaction
Lysozyme	0.20 mg.N	5	0	0	0
	0.10	2	0	0	0
	0.05	3	1	0	0
	0.03	3	1	0	0
	0.01	1	1	0	3
L. M. E.	0.10 mg.N	2	0	0	0
	0.05	5	2	1	0
	0.03	1	1	2	1
	0.01	1	0	0	3

*The numbers in each column show the number of animals with symptoms.

precipitin reaction and neutralization. After 48 hours, varying amounts of lysozyme or L. M. E. dissolved in saline were injected intravenously. The results are presented in Table 9.

Lysozyme and L. M. E. evoke a typical anaphylaxis, so the specific antigen-antibody reactions of lysozyme and L. M. E. were proved *in vivo*. The lowest concentrations of lysozyme and L. M. E. to evoke the anaphylactic shock were identical. When small amounts were used, the same amount of lysozyme and L. M. E. caused anaphylactic symptoms of the same intensity.

DISCUSSION

Wetter and Deutsch studied the quantitative precipitin reaction with lysozyme crystallized at its isoelectric point and found "a very narrow antibody excess region with no clearly defined equivalence zone". In their precipitin curve the antigen excess region covered the upper half of an ascending part of the curve. This curious phenomenon was not observed in our experiments. The reason can be explained by differences in experimental materials and procedures. The crystalline lysozyme preparation used in the present studies was purified further by an IRC-50 (XE-64) ion-exchange chromatogram and the antigen-antiserum mixtures were incubated at 2°C for 14 days, while Wetter and Deutsch incubated the antigen-antserum mixtures for only 5 days and did not purify the crystalline lysozyme further, so that it must have contained impurities as shown by Stein and Moore (1953).

Cinader (1956) studied the quantitative precipitin reaction with antisera evoked by crystalline ribonuclease with the main fraction, "RNase-A", of bovine crystalline ribonuclease purified by an IRC-50 ion-exchange chromatogram and found an equivalence zone. In addition, he described an antigen-antibody complex of ribonuclease which was still enzymatically active even in the antibody excess zone. In other words, there were no clearly defined endpoint for the neutralization of ribonuclease.

In the present studies, the endpoint of neutralization of lysozyme could be clearly demonstrated with both lysozyme antiserum and L. M. E. antiserum by assaying the enzymic activities of the antigen-antiserum mixtures. The endpoints of neutralization were located in the slight antigen excess region. Neutralizing antibody remaining in the supernatants was also estimated in the lysozyme-antilysozyme system. The neutralizing antibody was found to be exhausted in the supernatants just at the endpoint of the neutralization of the lysozyme-antilysozyme system. Soluble complexes were also neutralized as well as precipitates between the equivalence zone and the point of neutralization. Neutralizing antibody was detected in the supernatants at the equivalence point of the lysozyme-antilysozyme system, although excess precipitating antibody could not be detected. One of the authors (Amano) reported a similar and more extreme phenomenon on colicine K with Goebel and Miller-Smidth (1958). The problem is much complicated in the case of colicine K, because colicine K is a lipocarbohydrate protein complex, but it is interesting that the same phenomenon was seen with a simple protein like lysozyme. Whether the neutralizing antibody left in the supernatants at the equivalence zone is univalent or multivalent will be discussed

later.

The nature of the neutralizing antibody of lysozyme antiserum was studied by absorption with L. M. E., which in turn could evoke the antibodies capable of neutralizing lysozyme. Eighty per cent of the neutralizing units of lysozyme antiserum could be absorbed by L. M. E. Even excess amounts of L. M. E. could not absorb the remaining 20 per cent of neutralizing units and a graph of the remaining units of the neutralizing antibody showed a plateau in the region of excess antigen. Before concluding that the two neutralizing antibodies are different in specificity, the following possibility should be excluded.

Since the competitive inhibition of L. M. E. against lysozyme was demonstrated on the cell surface of the bacteria, the activity of additional lysozyme towards remaining neutralizing antibody may be competitively inhibited by soluble L. M. E. -antibody complexes. If so the curve showing units of neutralizing antibody in the supernatants would increase from the plateau in proportion to the amount of added L. M. E. However, there was a plateau until 50 μg. N of L. M. E., roughly corresponding to three times the amount at the equivalence point. Therefore the following possibilities are excluded that L. M. E. contains a residual amount of inactive but antigenically unaltered lysozyme, that the antigenic site (sites) of lysozyme corresponding to the neutralizing antibody is slightly modified through esterification but still capable of reacting with the neutralizing antibody with decreased affinity, and that the neutralizing antibodies of lysozyme antiserum are microheterogeneous in their affinity with L. M. E. The first possibility is also excluded by the experiments, in which, after the supernatants in the antigen excess region were mixed with the bacterial suspension and centrifuged, they were added to an excess of antiserum and the precipitated protein nitrogen compared with a control with saline in place of the bacterial suspension. The precipitated protein nitrogen was equal to that of the control. Therefore lysozyme elicited two kinds of neutralizing antibodies differing their specificities the one was directed to the combining site (sites) or to its neighboring determinant group (groups) of lysozyme and was unaffected in L. M. E. after esterification. The other was directed to the active site or to its neighboring determinant group, and was inactivated both enzymatically and antigenically by esterification. Although the enzymic activity of lysozyme could be completely neutralized by both lysozyme antiserum and L. M. E. antiserum, it is uncertain whether the neutralizing antibodies entirely correspond to the active centre or to the combining site (sites). However, it is assumed that the neutralizing antibodies correspond to the determinant group (groups) located in the neighborhood of the active centre or of the combining site (sites) and hence the breakdown of the substrate or the attachment of enzyme to the cell surface of the bacteria can be inhibited sterically by the antibody. As substrate for the enzyme, UV-inactivated lyophilized cells of *M. lysodeikticus* have been widely used, we have also used these cells. Although Meyer (1946) described the isolation from the bacteria of a polysaccharide as substrate, this was not used in the present study. If a synthetic substrate of lysozyme of low molecular weight were available and were used in the neutralization experiment, it could not be predicted whether complete neutralization would be observed, or whether there would be only partial

neutralization as in the case of ribonuclease.

As described above, after esterification one determinant group in five of lysozyme was lost in the neighborhood of the active centre and there was loss of lysozyme activity. As shown by the quantitative precipitin reaction, there was one determinant group less in L. M. E. Therefore it seems likely that the determinant group lost in L. M. E. is the active centre itself or a determinant group in the neighborhood of the active centre. If the valencies of the antigens are calculated, assuming that the valency of the precipitating antibody is two, the valencies of the neutralizing antibodies should be two. This has not yet been proved. Moreover if this is correct, the 20 per cent decrement in the maximum precipitable antibody nitrogen at the equivalence point of the L. M. E. -antilysozyme system in comparison with the lysozyme-antilysozyme system, should correspond to the ratio of the amount of the neutralizing antibodies of lysozyme antiserum unabsorbed by L. M. E. to the total amount of both precipitating and neutralizing antibodies. There is as yet no way to estimate the amount of neutralizing antibodies on a nitrogen basis.

The heterogeneity in specificity of determinant groups on a protein antigen is very fascinating to immunochemists. For instance, Hooker and Boyd (1934 and 1936), Osler and Heidelberger (1948) and Ouchterlony (1953) demonstrated it with hen egg albumin cross-reacted with duck egg albumin. Recently Lapresle (1955, 1957a, 1957b and 1958) showed differences in specificity between peptides of human serum albumin split by enzymic digestion. Raynaud (1958) investigated the differences in specificity of split peptides of diphtherial toxin with precipitating and neutralizing antibodies. Fujio *et al.* (1958) also demonstrated this with enzymic digestion products of horse diphtherial antitoxic T-globulin. Our present study is the first to demonstrate heterogeneity in specificity of neutralizing antibodies evoked by an enzyme. It is not known whether the determinant groups of a protein antigen are all specifically heterogeneous, or whether they can be classified into several groups of specificity. However, it seems unlikely that all the determinant groups of a protein antigen are specifically homogeneous. This was shown in the present study of the quantitative precipitin reaction. L. M. E. could precipitate 80 per cent of antibodies in lysozyme antiserum at the equivalence zone and the supernatants in the region of excess antigen gave no precipitates. As the difference of valency between lysozyme and L. M. E. was 1, only one specific antibody should be left in the supernatants in the antigen excess region. As lysozyme carries only one determinant group capable of reacting with antibody left in the supernatants in the antigen excess region of the L. M. E.-antilysozyme system, there was no precipitate.

Thus it can be assumed that there are at least two kinds of specific precipitating antibodies in lysozyme antiserum and the one determinant group of lysozyme, inactivated by esterification, was different in specificity from the other four determinant groups. It is also not yet known, whether the antibody which is not precipitated and remains in the supernatants at the equivalence zone of the lysozyme-antilysozyme system was univalent, or whether the five determinant groups of the lysozyme molecule were heterogeneous in specificity. Perhaps a di-

valent neutralizing antibody directed to one of them is occasionally produced in a large excess and hence some is not incorporated into the specific precipitate and remains in the supernatant. We are studying this possibility.

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