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Quantitative Studies on Immune Bacteriolysis

II. The Role of Lysozyme in Immune Bacteriolysis*

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SUMMARY

Kinetic studies were made of the immune bacteriolysis of *Escherichia coli* B with limiting complement or antibody. Similar results were obtained to those of Mayer *et al.* (1948) on immune hemolysis.

Antiserum and complement free from lysozyme were prepared without loss of antibody or complement by adsorbing the enzyme onto bentnite. The effects of lysozyme on immune bacteriolysis and the bactericide process were studied using these sera. Such complement and antiserum showed a markedly decreased bacteriolytic activity. This activity was restored to the same level as that of untreated antiserum plus complement by the addition of crystalline egg white lysozyme. The concentration of lysozyme determined the rate and extent of immune bacteriolysis with constant excess of antibody and constant limited complement. The activity of lysozyme was indispensable for the conversion of bacterial cells into serum spheroplasts. On the other hand, the immune bactericide process could be induced only by antibody plus complement in the absence of lysozyme, but the addition of excess lysozyme, the concentration of which was approximately equivalent to that of serum or blood plasma, accelerated and enhanced the bactericidal action of antibody and complement.

INTRODUCTION

In the previous paper (Amano *et al.*, 1958), we presented a new quantitative method to estimate immune bacteriolysis. In this method, serum spheroplasts could be lyzed selectively by sodium desoxycholate and intact bacteria were removed by centrifugation. The centrifuged supernates were deproteinized by perchloric acid and the nucleic acid content was determined photometrically in the deproteinized supernates. This method is analogous in its principle to the routine method for immune hemolysis based on colorimetric determination of oxyhemoglobin. As the method permitted kinetic studies on immune hemolysis, so kinetic studies on immune bacteriolysis were accomplished by the former method. The results obtained in the complement limiting or antibody limiting conditions were analogous to those reported by Mayer *et al.* (1948).

As lysozyme was found to be present in sera (Inai *et al.*, 1958a, Yamamoto, 1954), we were forced to reinvestigate the already proven fact that the addition

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of extraneous lysozyme to an immune bacteriolytic system markedly accelerated immune bacteriolysis (Amano *et al.*, 1954). The immune bacteriolytic system itself contains a certain amount of lysozyme and hence the problem of whether the serum lysozyme plays a role in immune bacteriolysis must be studied. Fortunately, Inai *et al.* (1958b) succeeded in removing serum lysozyme by adsorbing it onto bentnite without loss of complement or antibody. We prepared lysozyme-free complement (RL) and antiserum by this technique, and clarified the significance of this enzyme upon immune bacteriolysis and the bactericide process. Thus, we examined the effect of lysozyme in such a lysozyme-free system with excess antibody and limited complement. Without lysozyme, the amount of nucleic acid extracted by desoxycholate was quite small, as compared to the original activity of the lysozyme containing system. In addition, the low activity of the lysozyme-free system was restored to the original level by readjusting the lysozyme concentration to that of the lysozyme containing system. The rate and final degree of immune bacteriolysis was accelerated and enhanced in proportion to the added lysozyme. Hence, lysozyme is indispensable for the conversion of bacilli into serum spheroplasts. The lysozyme-free system showed the same bactericidal activity as the lysozyme containing system, although the addition of a large excess of lysozyme, the concentration of which is approximately equivalent to that of serum or blood plasma, accelerated and enhanced the bactericidal action of antibody and complement.

Materials and Methods

1. *Bacteria:*

The medium used was yeast extract peptone water (Amano *et al.*, 1958). An overnight culture (about 3.8×10^8 cells/ml.) of *Escherichia coli* B was diluted eleven times with the medium, and incubated with aeration at 37°C for 2 hours. Cells (about 5.8×10^8 cells/ml.) were sedimented, washed once and resuspended in cold Mg-saline (Amano *et al.*, 1958) at an optical density of 0.12 at 550mμ. (approximately 2.5×10^8 cells/ml.) with a Coleman junior spectrophotometer using an 8 mm. diameter cuvette. Bacterial cells contained 50 per cent more nucleic acids than those used in the previous report (Amano *et al.*, 1958) when compared at the same optical density. In this report, living bacterial cells were used instead of UV-irradiated ones, though the latter was preferred in the previous paper (Amano *et al.*, 1958), since there were no differences between results with these two types of bacterial cells in the experiments. In the results reported previously (Amano *et al.*, 1958) the levels of lysis of living cells exceeded those of UV-killed ones, probably because surviving cells continued to synthesize further nucleic acid. However, the bacterial cells used in this report were harvested in the logarithmic growth phase and hence these cells were rich in nucleic acid. In addition, we could minimize the loss of complement during absorption of normal antibodies. For these two reasons we were able to use more highly diluted complement serum even in the experiments with excess complement. The higher dilution of sera minimized the new synthesis of nucleic acids of bacterial cells during incubation.

2. *Antiserum :*

Rabbit antiserum to *E. coli* B killed by heating at 56°C for 30 minutes was inactivated at 56°C for 30 minutes, and stored in the frozen state at −60°C without addition of any preservative. The antiserum had an agglutination titer of 1:12,800 against living homologous cells and a lysozyme content equivalent to 1.65 μg. of crystalline egg white lysozyme per ml.

3. *Complement :*

Fresh sera from more than twenty guinea pigs were pooled, and normal antibodies were absorbed without loss of hemolytic complement at 5°C for 1 hour by a packed cell wall preparation (3 mg. per ml. of serum) and zymosan (3 mg. per ml.) pre-heated at 100°C for 60 minutes as described previously (Amano *et al.*, 1958). The procedure to prepare cell walls was the same as described in the previous report (Amano *et al.*, 1958), except that bacterial cells were disrupted in a Kubota sonic oscillator (10 Kc/s) for 15 minutes. The treated sera were stored in sealed glass ampules frozen in dry ice and designated as " RA ". These RA preparations contained lysozyme activtiy equivalent to 1.2 to 3.7 μg. of crystalline egg white lysozyme per ml. Complement serum was inactivated by heating in a water bath at 56°C for 30 minutes.

4. *Removal of lysozyme from RA or antiserum :*

Following the method of Inai *et al.* (1958b) 10 mg. of bentnite (Wako Pure Chemicals Co.) were added to 1.0 ml. of serum (RA or antiserum), incubated at 5°C for 10 minutes and centrifuged. Lysozyme activity was undetectable by Smolelis' method (1949) in once treated sera. But the adsorption procedures were repeated from 2 to 5 times to remove traces of the enzyme, without appreciable loss of complement or antibody. These complement preparations were designated as " RL(n) ", in which n is the number of repetition of the bentnite treatment. RL was also stored in sealed glass ampules frozen in dry ice.

5. *Lysozyme :*

Crystalline egg white lysozyme was obtained by Alderton's method (Alderton *et al.*, 1946) and purified by means of IRC-50 column chromatography (Hirs *et al.*, 1953). Lysozyme activity was estimated by Smolelis' method (1949) just before use.

6. *Assay of immune hemolysis :*

Hemolytic activities of complement sera were assayed by colorimetric determination (Kabat and Mayer, 1948) and expressed as fifty per cent hemolytic untis (HU_{50}).

7. *Representation of degree of morphological change :*

The indices (−, ±, +, ++ and +++) used to indicate the degrees of conversion of bacilli into serum spheroplasts were described previously (Amano *et al.*, 1958). The indices (★, ★★ and ★★★) were used to indicate that about half, 2/3 and almost all of the bacterial cells, respectively, changed to scarcely stainable cell-wall like rods.

8. *Quantitative determination of immune bacteriolysis :*

The determination was carried out as described in the previous report (Amano *et al.*, 1958), with the exception that 100 per cent of immune bacteriolysis was

defined as the amount of nucleic acid directly extractable from bacterial cells with perchloric acid after the addition of desoxycholate.

9. *Sterilization of reagents for study of the immune bactericide process:*

With the exception of bacterial suspension the reagents were mixed and centrifuged at 16,000 rpm. for 30 minutes five times.

The centrifuged mixture was added to the bacterial suspension at time 0. As a control, samples were taken from the centrifuged supernates and a colony count was made on each sample. No colonies were ever detected in these samples.

RESULTS

1. *Kinetics of immune bacteriolysis:*

In the previous paper (Amano *et al.*, 1958), we followed the course of immune bacteriolysis with excess antibody and excess complement by our new quantitative method. But the mechanism of immune bacteriolysis can be better understood by study of its kinetics. Mayer *et al.* (1948) made kinetic studies on immune hemolysis and showed that the courses of immune hemolysis are different in complement limiting and antibody limiting systems. They concluded that limited hemolytic antibody possesses the ability to turn over without being exhausted, while limited complement can be rapidly exhausted. We performed similar experiments on immune bacteriolysis.

34 centrifuge tubes were placed in an ice bath. To these tubes previously chilled reagents as shown in Table 1 were added. The bacterial suspension was added last. The RA preparation employed contained 313 HU_{50} and lysozyme activity equivalent to 2.1 μg. of crystalline egg white lysozyme per ml. The final concentration of antiserum was 1:900 and those of RA were 1:33.7 in A series, 1:112.5 in B series, 1:225 in C series and 1:450 in D series. Tube No. 41 was directly deproteinized after the addition of desoxycholate and the extracted nucleic acids were estimated in a Beckman spectrophotometer using the content of tube No.40 as a control value. The reading was taken as 100 per cent nucleic acid content. Tubes No.2-8, 12-18, 22-28 and 32-38 were stoppered, incubated at 37°C and agitated occasionally. Tubes No.1, 11, 21 and 31 served as blank controls to each tube in the corresponding series and were not incubated at 37°C. At 5, 10, 30, 60, 120 and 150 minutes after incubation, one tube was taken out of each series, placed in an ice bath and 0.5 ml. of cold 0.5 per cent sodium desoxycholate were added. The mixture was centrifuged immediately. The supernates were treated with perchloric acid, and the nucleic acid contents were estimated using the control of each series. The results of this experiment are presented in Fig. 1. Parallel tubes were set up with the same reaction mixture for the microscopical observations. Results are shown in Table 2.

Table 1. Conditions for experiment shown in Fig. 1 and Table 2

	A		B		C		D		T	
	1	2-8	11	12-18	21	22-28	31	32-38	40	41
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	1.5	0.5	1.5	0.5	1.5	0.5	1.5	0.5	4.5	3.5
1:200 Antiserum	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—	—
1:15 RA	2.0	2.0	—	—	—	—	—	—	—	—
1:50 RA	—	—	2.0	2.0	—	—	—	—	—	—
1:100 RA	—	—	—	—	2.0	2.0	—	—	—	—
1:200 RA	—	—	—	—	—	—	2.0	2.0	—	—
Bacterial Suspension	—	1.0	—	1.0	—	1.0	—	1.0	—	1.0

This experiment was carried out with excess antibody and varying amounts of RA. With excess RA, the lytic process was completed within 60 minutes. In the systems with limited RA, plateaux were observed after about 60 to 90 minutes. The final level of lysis attained was dependent on the amount of RA

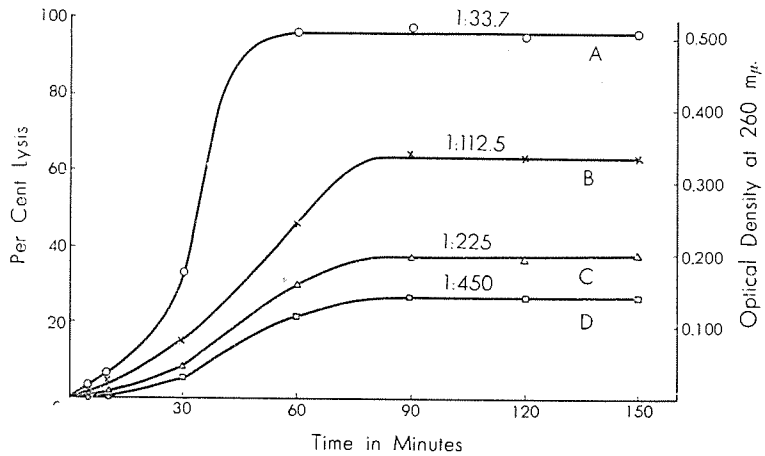

Fig. 1. Course of immune bacteriolysis with contant excess of antiserum and varying amounts of RA

Table 2. Immune bacteriolysis with constant excess of antiserum and varying amounts of RA

Time (min.)	A	B	C	D
15	—	—	—	—
30	±	—	—	—
60	++	—	—	—
90	+++	±	—	—
120	+++	++	—	—
150	+++	++	—	—

A second experiment was performed with excess RA and varying amounts of antibody.

Table 3. Conditions for the experiment shown in Fig. 2 and Table 4

	A		B		C		D		T	
	1	2-7	11	12-17	21	22-27	31	32-37	40	41
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	1.0	—	1.0	—	1.0	—	1.0	—	4.5	3.5
1:200 Antiserum	2.0	2.0	—	—	—	—	—	—	—	—
1:1,000 Antiserum	—	—	2.0	2.0	—	—	—	—	—	—
1:2,500 Antiserum	—	—	—	—	2.0	2.0	—	—	—	—
1:5,000 Antiserum	—	—	—	—	—	—	2.0	2.0	—	—
1:10 RA	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	—	—
Bacterial Suspension	—	1.0	—	1.0	—	1.0	—	1.0	—	1.0

The experimental procedures were similar to those of the first experiment. The tube contents are shown in Table 3. The final concentration of RA was 1 : 30 and those of antiserum were 1 : 450 in A series, 1 : 2,250 in B series, 1 : 5,625 in C series and 1 : 11,250 in D series. The RA preparation employed contained 323 HU_{50} and lysozyme activity equivalent to 2.4 μg. of crystalline egg white lysozyme per ml. The results are shown in Fig. 2 and Table 4.

The curves of bacteriolysis continue to increase throughout the experiment in the limited antibody system. These results are consistent with those obtained by Mayer *et al.* (1948).

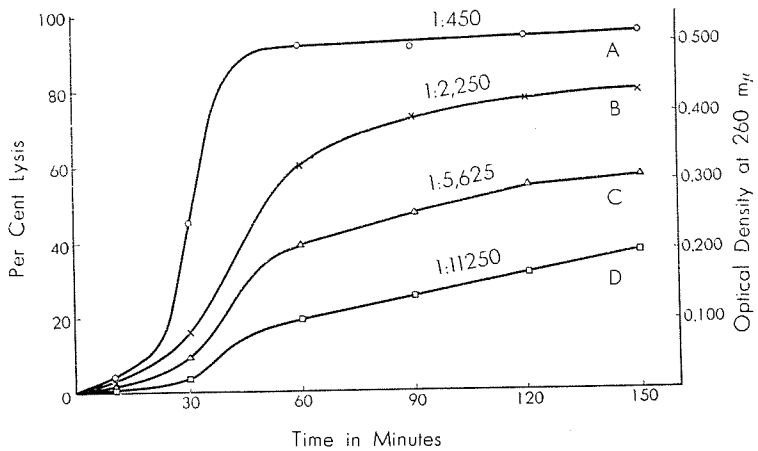

Fig. 2. Course of immune bacteriolysis with constant excess of RA and varying amounts of antiserum

Table 4. Immune bacteriolysis with constant excess of RA and varying amounts of antiserum

Time (min.)	A	B	C	D
10	−	−	−	−
30	±	−	−	−
60	##	+	±	−
90	##	##	±	±
120	##	##	+	±
150	##	##	##	+

The authors can not conclude, from the results of this experiment, that antibody can turn over from cell to cell. The reason will be described in the following experiments.

2. *The role of lysozyme in immune bacteriolysis :*

Since it was found that the addition of lysozyme to an immune bacteriolytic system results in marked acceleration of serum spheroplast formation (Amano *et al.*, 1954), the existence of lysozyme in serum was an important problem. Later, lysozyme was proved to be contained in sera (Inai *et al.*, 1958a ; Yamamoto, 1955), and Inai *et al.* succeeded in remoing lysozyme by adsorbing it with zymosan (1958a) or more easily with bentnite (1958b) without loss of hemolytic complement

and antibody. We confirmed these facts, and prepared lysozyme-free complement serum (RL) from RA by bentnite treatment. Using RL and lysozyme-free antiserum, we tried to clarify the significance of lysozyme in immune bacteriolysis.

The tube contents are shown in Table 5. The RA preparation employed contained $280HU_{50}$ of complement and lysozyme activity equivalent to 2.24 μg. of crystalline egg white lysozyme per ml., and RL(2) contained $280HU_{50}$ per ml. and no lysozyme activity. The final concentration of lysozyme-free antiserum was constant (1 : 900) and those of RA and RL(2) were also constant (1 : 112.5). Lysozyme was not added to series B. To series C was added lysozyme at the same concentration (0.02 μg./ml. in final concentration) as that of series A. The lysozyme concentration of series D and E was increased by adding lysozyme to 5.0 μg. per ml. in final concentration, which is approximately equivalent to that in undiluted serum. As the hemolytic activities of RA and RL(2) employed were equal, the tubes contained the same amounts of hemolytic complement. The results are presented in Fig. 3.

Table 5. Conditions for the experiment shown in Fig. 3

	A		B		C		D		E		T	
	1	2-8	11	12-18	21	22-28	31	32-38	41	42-48	50	51
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	2.5	1.5	2.5	1.5	1.5	0.5	1.5	0.5	1.5	0.5	4.5	3.5
1:200 Lysozyme-free Antiserum	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—	—
1:25 RA	1.0	1.0	—	—	—	—	1.0	1.0	—	—	—	—
1:25 RL(2)	—	—	1.0	1.0	1.0	1.0	—	—	1.0	1.0	—	—
0.09μg./ml. Lysozyme	—	—	—	—	1.0	1.0	—	—	—	—	—	—
22.5μg./ml. Lysozyme	—	—	—	—	—	—	1.0	1.0	1.0	1.0	—	—
Bacterial Suspension	—	1.0	—	1.0	—	1.0	—	1.0	—	1.0	—	1.0

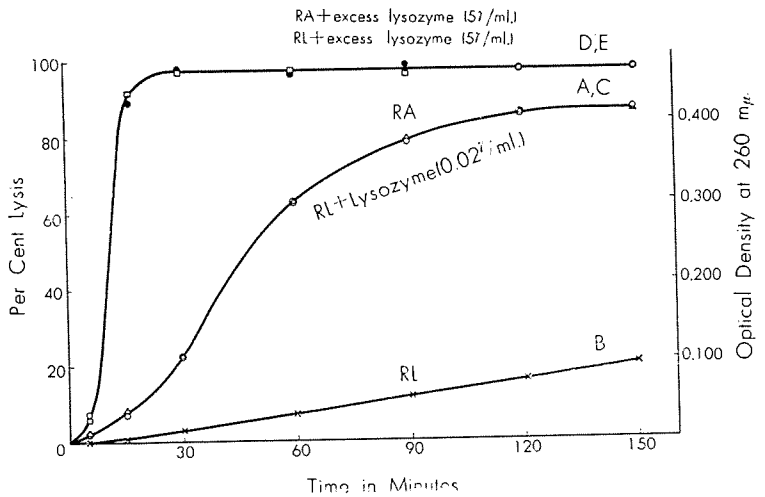

Table. 3. Reactivation of immune bacteriolysis by addition of egg white lysozyme to a lysoyme-free systesm

Fig. 3 shows the effect of lysozyme on immune bacteriolysis with excess antibody and limited complement. The removal of lysozyme decreased the extent of bacteriolysis, but the addition of egg white lysozyme to a concentration equivalent to that in the RA-system stored the full activity. The addition of a large excess of lysozyme markedly accelerated the lytic process. These facts indicate that lysozyme plays an important role in immune bacteriolysis. In addition, it can be stated that serum lysozyme can be replaced with equal effect by egg white lysozyme, though these two lysozymes differ from each other in antigenicity (Fujio *et al.*, 1959; Smolens *et al.*, 1947). The following experiment was performed to demonstrate more clearly that egg white lysozyme can replace serum lysozyme in immune bacteriolysis.

The contents of the experimental tubes are shown in Tables 6 and 7. 60 minutes after incubation, all the tubes were centrifuged and their nucleic acid concentration estimated. RL(4) contained 222 HU_{50} and lysozyme activity equivalent to 1.35 μg. of crystalline egg white lysozyme per ml. The final concentrations of RA and RL(4) were chosen as to contain the same hemolytic complement namely, 1 : 104 in the RA series and 1 : 90 in the RL(4) series. The final concentration of lysozyme-free antiserum was 1 : 900. To the RL(4) series with the exception of one tube varying amounts of lysozyme were added. The RA series initially contained lysozyme equivalent to 0.013 μg. of crystalline egg white lysozyme and except for one tube, in addition varying amounts of extraneous lysozyme were added. The results are shown in Fig. 4 with the final concentration of lysozyme as abscissa. The final concentrations of the RA series were calculated by adding the original content of lysozyme to the amount of added lysozyme. The arrow indicates the concentration of lysozyme in the tube containing RA and no added egg white lysozyme.

Table 6. Conditions for the experiment shown in Fig. 4. (I)

	1	2	3	4	5	6	7	8	9	10	11	12	13
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	2.0	1.9	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2	—	—
1:100 Lysozyme-free Antiserum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
1:20 RL(4)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.09 γ/ml. Lysozyme	—	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	—
11.25 γ/ml. Lysozyme	—	—	—	—	—	—	—	—	—	—	—	—	2.0
Bacterial Suspension	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

	1′	2′	3′	4′	5′	6′	7′	8′	9′	10′	11′	12′	13′
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	3.0	2.9	2.8	2.6	2.4	2.2	2.0	1.8	1.6	1.4	1.2	1.0	1.0
1:100 Lysozyme-free Antiserum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
1:20 RL(4)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.09 γ/ml. Lysozyme	—	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	—
11.25 γ/ml. Lysozyme	—	—	—	—	—	—	—	—	—	—	—	—	2.0

The curve of the RA series entirely overlapped that of the RL series. The results indicate the importance of lysozyme in immune bacteriolysis and that serum and egg white lysozymes are mutually and quantitatively replaceable.

Table 7. Conditions for the experiment shown in Fig. 4. (II)

	21	22	23	24	25	26	27	30
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	2.0	1.8	1.6	1.2	0.8	0.4	—	3.5
1:100 Lysozyme-free Antiserum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—
1:23.1 RA	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—
0.09 γ/ml. Lysozyme	—	0.2	0.4	0.8	1.2	1.6	2.0	—
Bacterial Suspension	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

	21′	22′	23′	24′	25′	26′	27′	30′
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	3.0	2.8	2.6	2.2	1.8	1.4	1.0	4.5
1:100 Lysozyme-free Antiserum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—
1:23.1 RA	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—
0.09 γ/ml. Lysozyme	—	0.2	0.4	0.8	1.2	1.6	2.0	—

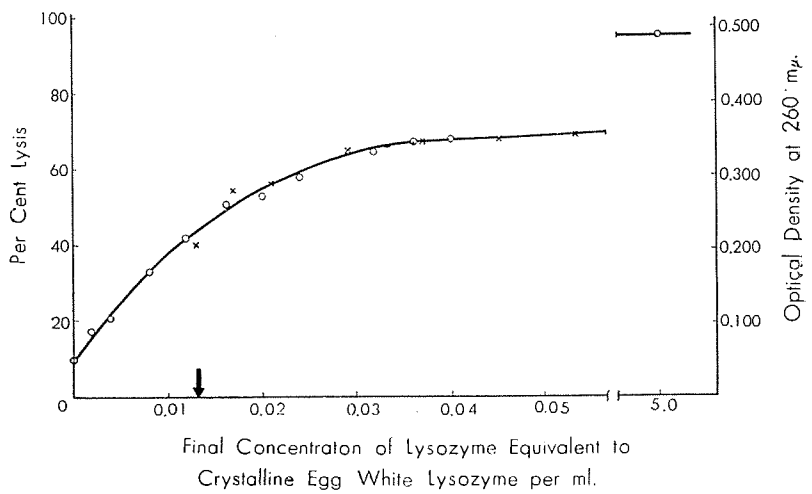

Fig. 4. Effect of lysozyme on the extent of immune bactriolysis

3. *Immune bacteriolysis in the absence of lysozyme :*

Though the authors expected in the last experiment that the curve of the RL series would converge to the origin of the co-ordinates, a small amount of nucleic acid was detected even in lysozyme-free conditions. This indicates that some fraction of the bacteria became sensitive to sodium desoxycholate treatment after the action of antibody and RL. However, the amount of nucleic acids extracted was markedly smaller than that after addition of lysozyme.

The following experiment was carried out to investigate the effect of lysozyme-free antiserum and RL. The tests on all the series were carried out using systems with constant excess of antibody and varying amounts of complement.

The contents of the experimental tubes are shown in Table 8. The final concentration of

lysozyme-free antiserm was 1:900. Those of RL were 1:360 in the A series, 1:180 in the B series, 1:90 in the C series and 1:45 in the D series. Parallel experiments were performed to record the morphological changes of the bacteria. The RL(2) employed contained 263 HU_{50} per ml. and no lysozyme activity. The results of nucleic acid determinations are persented in Fig. 5 and those of morphological investigations in Table 9.

Table 8. Conditions for the experiment shown in Fig. 5 and Table 9

	A		B		C		D		T	
	1	2-8	11	12-18	21	22-28	31	32-38	40	41
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	1.5	0.5	1.5	0.5	1.5	0.5	1.5	0.5	4.5	3.5
1:200 Lysozyme-free Antiserum	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—	—
1:160 RL(2)	2.0	2.0	—	—	—	—	—	—	—	—
1:80 RL(2)	—	—	2.0	2.0	—	—	—	—	—	—
1:40 RL(2)	—	—	—	—	2.0	2.0	—	—	—	—
1:20 RL(2)	—	—	—	—	—	—	2.0	2.0	—	—
Bacterial Suspension	—	1.0	—	1.0	—	1.0	—	1.0	—	1.0

The more concentrated was the RL, the more nucleic acid was extracted as shown in Fig. 5, and in each series the amount of nucleic acid increased with time. As shown in Table 9, some of the bacterial cells changed to scarcely stainable cell-wall like rods. Such cell-wall like rods increased with time and also with increasing concentration of RL. Though the mechanism of these two phenomena are not yet clear, it is possible that other factors than the four components of the complement play some role with the complement in such a lysozyme-free system.

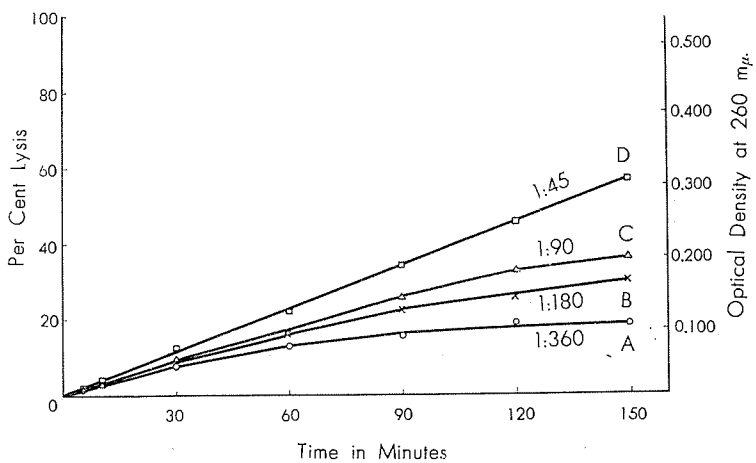

Fig. 5. Immune bacteriolysis in the absence of lysozyme activity

Table 9. Immune bacteriolysis in the absence of lysozyme activity

Time (min.)	A	B	C	D
60	—	—	—	—
120	—	—	↓	↓
150	↓	↓	↓	★

4. *Further analyses on the effect of lysozyme upon immune bacteriolysis:*

From the experiments described above it can be stated that at least three independent agents play roles in immune bacteriolysis, if the four complement components are regarded as a single agent. Hence it is necessary to investigate the effect of lysozyme upon the course of immune bacteriolsysis in a system with constant excess of antiserum and constant limited complement.

An experiment similarly as that of Fig. 1 was carried out. The contents of the experimental tubes are presented in Table 10. The tubes contained final concentration of RL(2) of 1:225 and of antiserum free from lysozyme of 1:900. Varying amounts of crystalline egg white lysozyme were added to these tubes. The RL(2) preparation used contained 357 HU_{50} per ml. and no lysozyme activity. Parallel experiments were performed to study the morphological changes of the bacteria. The results are presented in Fig. 6 and in Table 11.

Table 10. Conditons for the experiment shown in Fig. 6 and Table 11

	A		B		C		D		T	
	1	2-8	11	12-18	21	22-28	31	32-38	40	41
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	3.0	2.0	1.0	—	1.0	—	1.0	—	4.5	3.5
1:100 Lysozyme-free Antiserum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—	—
1:50 RL(2)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—	—
11.25 γ/ml. Lysozyme	—	—	2.0	2.0	—	—	—	—	—	—
0.09 γ/ml. Lysozyme	—	—	—	—	2.0	2.0	—	—	—	—
0.0225 γ/ml. Lysozyme	—	—	—	—	—	—	2.0	2.0	—	—
Bacterial Suspension	—	1.0	—	1.0	—	1.0	—	1.0	—	1.0

In the presence of excess lysozyme the lytic process rapidly reached to maximum. In the system with limiting amounts of lysozyme lysis increased slowly. Therefore, it can be stated that lysozyme is the rate limiting factor in this condition and that the limited complement can be exhausted, because the process does not reach complete lysis even when a large excess of lysozyme was used. Though the results are not shown, a lower plateau was found even in the presence of a large excess of lysozyme, when more diluted RL was employed.

In our previous reports (Amano *et al.*, 1955b, 1955a, 1956) it was showed that bacterial cells previously treated with excess egg white lysozyme were converted into serum spheroplasts by antiserum and complement at the same rate as untreated cells and that the cell walls were resistant to lysozyme alone. From these results it can be assumed that lysozyme attacks substrate which are exposed

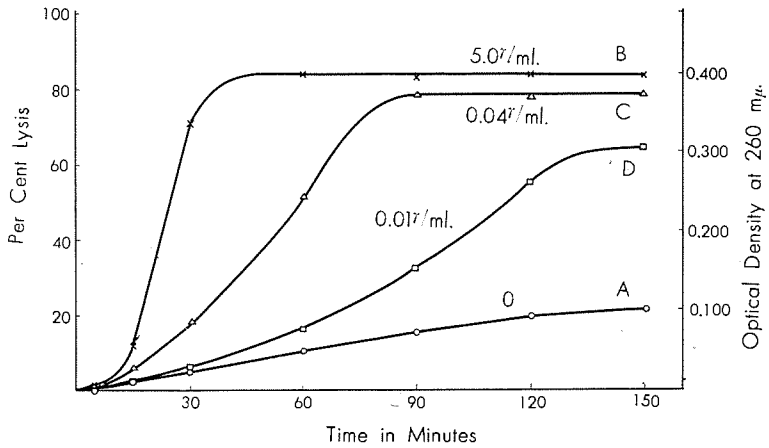

Fig. 6. Effect of lysozyme on the course of immune bacteriolysis

Table 11. Effect of lysozyme on the course of immune bacteriolysis

Time (min.)	A	B	C	D
10	−	±	−	−
30	−	+	−	−
60	−	++	±	−
90	−	++	+	−
120	−	++	++	±
150	−	++	++	++

Table 12. Conditions for the experiment shown in Fig. 7 and Table 13

	A		B		C		D		T	
	1	1-7	11	12-17	21	22-27	31	32-37	40	41
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	1.0	—	1.0	—	1.0	—	1.0	—	4.5	3.5
1:100 Lysozyme-free Antiserum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—	—
1:50 RL(2)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	—	—
Bacterial Suspension	—	1.0	—	1.0	—	1.0	—	1.0	—	1.0
incubated at 37°C for 60 minutes, and then the following reagents were added to the tubes										
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	1.0	1.0	—	—	—	—	—	—	—	—
0.045 γ/ml. Lysozyme	—	—	1.0	1.0	—	—	—	—	—	—
0.18 γ/ml. Lysozyme	—	—	—	—	1.0	1.0	—	—	—	—
22.5 γ/ml. Lysozyme	—	—	—	—	—	—	1.0	1.0	—	—

or activated through the action of antibody and complement. Therefore, we investigated the effect of lysozyme upon bacteria pre-treated with antiserum and RL.

All the tubes containing antiserum 1 : 700, RL(2) 1 : 87.5 and 1.0 ml. of the bacterial suspension were incubated at 37°C for 60 minutes. Then they were transferred to an ice bath, and varying amounts of lysozyme (0.01 μg. per ml. in the B series, 0.04 μg. per ml. in the C series and 5.0 μg. per ml. in the D series in final concentration) or Mg-saline in the A series were added. The contents of the tubes are shown in Table 12. Again they were incubated at 37°C. Thereafter, the experiment was carried out as described above. The RL(2) preparation contained 337 HU_{50} of hemolytic complement and no lysozyme. The results are presented in Fig. 7. Parallel experiments were performed to investigate morphological changes, the results of which are shown in Table 13.

Immediately after the addition of a large excess of lysozyme (curve D) the cells were converted into serum spheroplasts and a maximal level of lysis was attained. When less lysozyme was added, the cells were changed into serum spheroplasts relatively slowly (curve C). From this result it is obvious that the substrate of lysozyme was exposed or activated by antibody plus complement and that lysozyme is an independent factor from antibody and complement.

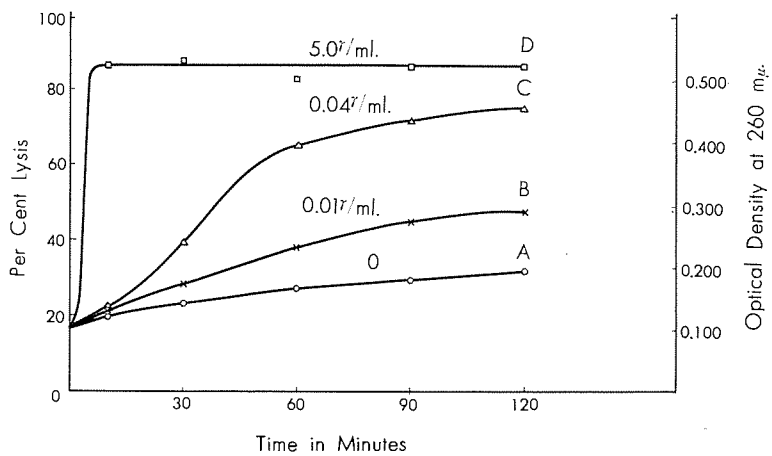

Fig. 7. Effect of lysozyme on the bacteria pre-treated with lysozyme-free antisesum and RL

Table 13. Effect of lysozyme on bacteria pretreated with lysozyme-free antiserum and RL

Time (min.)	A	B	C	D
0	—	—	—	—
5	—	—	—	##
10	—	—	—	##
30	—	—	—	##
45	—	—	+	##
60	✓	✓	##	##
90	✓	✓	##	##
120	★	★	##	##

The removal of lysozyme activity from the complement and antiserum does not affect immune hemolysis, and lysozyme activity is, on the other hand, indispensable for the conversion of bacterial cells to serum spheroplasts. However, such a conclusion was drawn from experiments with excess antiserum and limited complement. The following experiments were performed to validate this conclusion with excess RL and antiserum.

The contents of the experimental tubes are shown in Table 14. Each of the reagents was pipetted into test tubes placed in an ice bath, and the bacteria were added last. The tubes contained RA or RL(2) 1 : 36 and lysozme-free antiserum 1 : 900 in final concentration. The final concentration of added egg white lysozyme was 0.03 μg. per ml. in tube C, 0.061 μg. per ml. in tube D, 0.092 μg. per ml. in tube E, 0.122 μg. per ml. in tube F and 5.0 μg. per ml. in tubes G, I, L and M. The concentration in tube D is equivalent to that in tube A. The tubes were incubated at 37°C. At the intervals shown, smears were made from each of the tubes, fixed in absolute methanol and stained with Hucker's crystal violet solution. The RA contained 372 HU_{50} and lysozyme activity equivalent to 2.2 μg. of crystalline egg white lysozyme per ml. The RL(2) contained 372 HU_{50} per ml. and no lysozyme activity. The results are also presented in Table 14.

Toble 14. The indispensability of lysozyme for the formation of serum spheroplasts in a system with excess antibody and excess complement

		A	B	C	D	E	F	G	H	I	J	K	L	M
		ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline		2.0	2.0	1.5	1.0	0.5	—	1.0	3.0	2.0	2.5	2.5	1.5	2.5
1:100 Lysozyme-free Antiserum		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—	—	—	—
1:8 RA		1.0	—	—	—	—	—	—	—	—	1.0	—	—	—
1:8 RL(2)		—	1.0	1.0	1.0	1.0	1.0	1.0	—	—	—	1.0	1.0	—
0.275 γ/ml. Lysozyme		—	—	0.5	1.0	1.5	2.0	—	—	—	—	—	—	—
22.5 γ/ml. Lysozyme		—	—	—	—	—	—	1.0	—	1.0	—	—	1.0	1.0
Bacterial Suspension		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Morphological Changes	min. 5	—	—	—	—	—	—	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	++	—	—	—	—	—	—
	15	—	—	—	—	±	+	###	—	—	—	—	—	—
	30	+	—	—	+	+	+	###	—	—	—	—	—	—
	45	++	—	±	++	++	++	###	—	—	—	—	—	—
	60	++	—	+	++	###	###	###	—	—	—	—	—	—
	90	###	✓	++	###	###	###	###	—	—	—	—	—	—
	120	###	✓	###	###	###	###	###	—	—	—	—	—	—
	150	###	★	###	###	###	###	###	—	—	—	—	—	—

Serum spheroplasts could be formed only in the presence of lysozyme as shown in the Table 14, where ### shows that almost all the bacteria were changed to serum spheroplasts. The more lysozyme added, the more rapidly did serum spheroplasts form, when the amounts of both antiserum and complement were kept constant. In the absence of lysozyme, the shape of the bacteria was not changed, but scarecely stainable cell-wall like rods appeared after longer incubation. The addition of lysozyme equivalent to the concentration in the RA-system

brought about almost parallel changes as in the latter system. Lysozyme itself did not affect the shape of bacterial cells in the absence of either antibody or complement.

The effect of lysozyme on bacteria pre-treated with RL plus lysozyme-free antiserum was again examined.

Into a larger centrifuge tube were introduced 10.0 ml. of Mg-saline, 2.5 ml. of 1 : 100 diluted antiserum, 5.0 ml. of 1 : 8 diluted RL(2) and 5.0 ml. of bacterial suspension. The concentration of the reaction mixture was the same as that of tube B in Table 14. The tube was incubated at 37°C for 60 minutes, and then centrifuged in the cold and resuspended in 12.5 ml. of cold Mg-saline. 2.5 ml. aliquots of the suspension were added to each of four tubes placed in an ice bath. The contents of these four tubes are shown in Table 15. The tubes were incubated at 37°C. Smears were made from each tube at intervals. The results are also presented in Table 15.

Table 15. Effect of lysozyme on bacteria pre-treated with excess antiserum and excess complement

		A	B	C	D
		ml.	ml.	ml.	ml.
Mg-Saline		2.0	1.0	—	1.0
0.275 γ/ml. Lysozyme		—	1.0	2.0	—
22.5 γ/ml. Lysozyme		—	—	—	1.0
Pretreated E. coli*		2.5	2.5	2.5	2.5
Morphological Changes	min. 5	—	—	—	++++
	15	—	—	—	++++
	30	—	—	±	++++
	45	✦	±	+	++++
	60	✦	+	++	++++
	90	★	++	++++	++++

* See text.

When pre-treated cells were exposed to lysozyme, they were converted into serum spheroplasts. The conversion did not require additional complement and it is reasonable to assume that lysozyme exerts its action at the terminal step and that the substrate of lysozyme is already exposed or activated so as to be easily attacked by lysozyme. Therefore even in the absence of lysozyme, antibody and RL can cause some damage to the bacterial cell surface.

5. *The effect of lysozyme upon the immune bactericide forces:*

As elucidated in the last experiment, antibody plus complement can cause some damage on the bacterial surface even in the absence of lysozyme. It is necessary to investigate whether lysozyme is required in the immune bactericide process or not, because the damage of the bacterial surface may cause death of bacteria.

Reaction mixtures without the bacterial suspension of twice the volume of those shown in Table 16 were made in sterile centrifuge tubes and incubated at 4°C. These eight tubes were centrifuged at 16,000 rpm. for 30 minutes at 0°C. The supernates were transferred to fresh

sterile centrifuge tubes, and re-centrifuged. This centrifugation was repeated three times more in the same way. As a control, samples were taken from the last supernates and colony counts

Table 16. Conditions for the experiment shown in Fig. 8 and Table 17

	A	B	C	D	E	F	G	H
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	2.0	1.0	1.0	2.0	2.0	1.0	1.0	2.0
1:100 Lysozyme-free Antiserum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
1:20 RL(2)	1.0	1.0	1.0	—	—	—	—	—
1:20 Inactivated RL(2)	—	—	—	—	1.0	1.0	1.0	—
1:20 RA	—	—	—	1.0	—	—	—	—
1:20 Inactivated RA	—	—	—	—	—	—	—	1.0
0.18 γ/ml. Lysozyme	—	1.0	—	—	—	1.0	—	—
22.5 γ/ml. Lysozyme	—	—	1.0	—	—	—	1.0	—
Bacterial Suspension	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

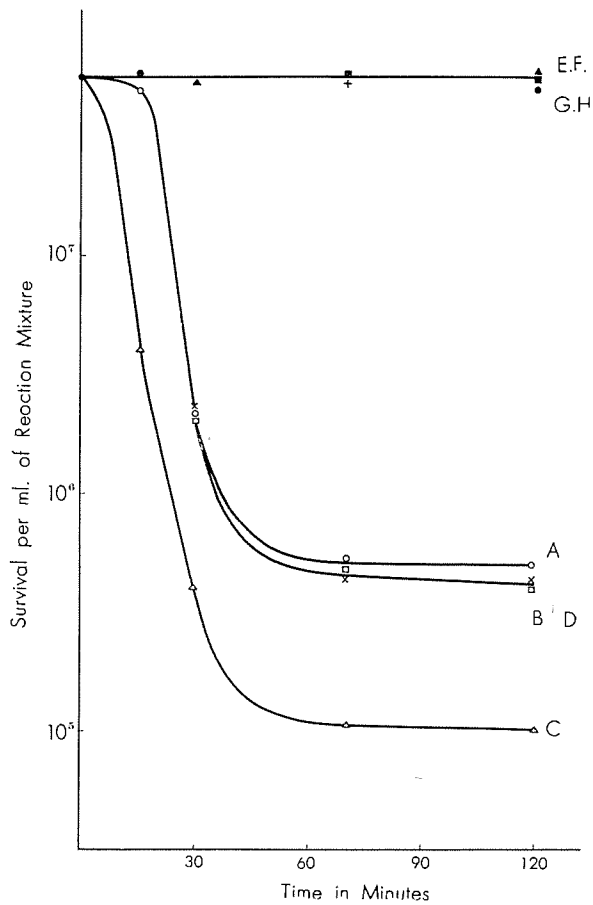

Fig. 8. Immune bactericide process with or without lysoyme

were made and the samples proved to be sterile. A 3.5 ml. aliquot of each sterile supernate was pipetted into a sterile tube and 1.0 ml. of bacterial suspension added. All the tubes were incubated at 37°C. At the intervals shown in Fig. 8, samples were taken and surviving bacteria assayed by the colony count method. All the reaction mixtures contained lysozyme-free antiserum at a final concentration of 1 : 900. The final concentration of RL(2) (tubes A, B, C) and of RA (tube D) was 1 : 90. The final concentration of lysozyme in tube D was equivalent to that of tube B. Tubes E, F, G and H served as controls. The RA preparation contained 295 HU_{50} and lysozyme activity equivalent to 2.52 μg. of crystalline egg white lysozyme per ml., and the RL(2) contained 295 HU_{50} per ml. and no lysozyme activity. The results are presented in Fig. 8. Parallel experiments were carried out to demonstrate the morphological changes of the bacteria. The results are shown in Table 17.

Table 17. Morphological changes observed in an experiment parallel to that shown in Fig. 8

(min.)	A	B	C	D	E	F	G	H
20	—	—	##	—	—	—	—	—
60	—	+	##	+	—	—	—	—
90	—	+	##	+	—	—	—	—
120	≠	##	##	##	—	—	—	—

As shown in Fig. 8, survival curves of the RA-system, the RL-system and the RA-system reconstructed with RL and an equivalent amount of egg white lysozyme were nearly identical. From these results, it can be stated that lysozyme does not appreciably affect the immune bactericide process.

However, excess lysozyme accelerated and enhanced the bactericidal action, as shown by curve C of Fig. 8. The lysozyme concentration of the serum is approximately equivalent to 5.0 μg. per ml. of egg white lysozyme. Therefore, lysozyme must play an important role in immune bacteriolysis and the bactericide process *in vivo*.

These effects of lysozyme on the immune bactericide process are demonstrated more clearly in Fig. 9, in which the initial concentration of bacteria was lowered to 4×10^3 cells per ml. to minimize the agglutination of cells and to eliminate dilution errors.

The contents of the tubes are shown in Table 18. The RL(2) preparation employed contained 278 HU_{50} per ml. and no lysozyme activity. The final concentration of lysozyme-free antiserum was 1 : 900 and that of RL(2) was 1 : 112.5. Lysozyme was added at a final concentration of 0.02 μg. per ml. to tube B or of 5.0 μg. per ml. to tube C.

Table 18. Conditions for the experiment shown in Fig. 9

	A	B	C	D	E	F
	ml.	ml.	ml.	ml.	ml.	ml.
Mg-Saline	2.0	—	—	2.0	—	—
1:200 Lysozyme-free Antiserum	2.0	2.0	2.0	2.0	2.0	2.0
1:25 RL(2)	2.0	2.0	2.0	—	—	—
1:25 Inactivated RL(2)	—	—	—	2.0	2.0	2.0
0.09 γ/ml. Lysozyme	—	2.0	—	—	2.0	—
22.5 γ/ml. Lysozyme	—	—	2.0	—	—	2.0
Bacterial Suspension (1.2×10⁴ cells/ml.)	3.0	3.0	3.0	3.0	3.0	3.0

Even in such conditions, the results are essentially the same as shown in Fig. 8.

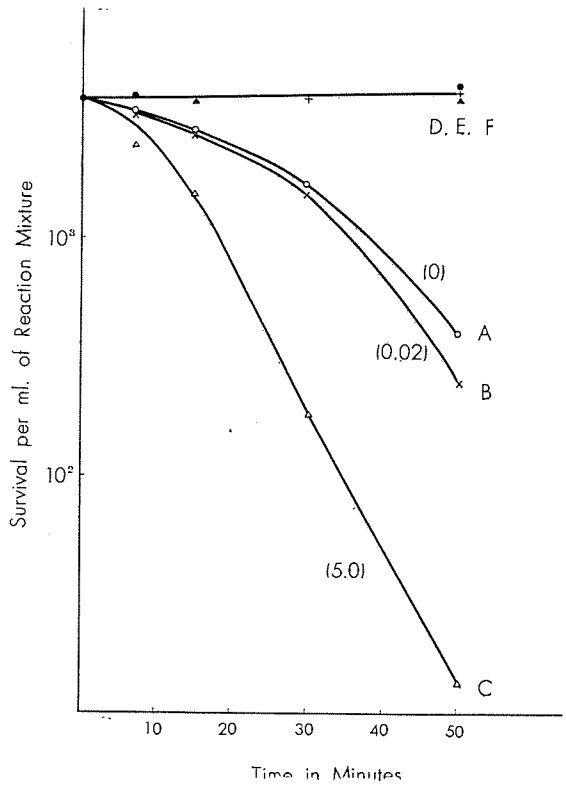

Fig. 9. Immune bactericide process with or without lysozyme using o low concentration of bacteria

DISCUSSION

Mayer *et al.* (1948) studied the kinetics of immune hemolysis in detail and found distinct differences between a limited complement and a limited antibody system. In the system with excess antibody and limited complement, the hemolysis-time curve was sigmoidal; the lytic process stops completely after a certain period. On the other hand, a plateau was not attained in a system with limited antibody and excess complement. Hence, they concluded that, with limited complement, the complement activity is rapidly exhausted, while with limited antibody, antibody molecules can turn over from cell to cell during the hemolyic reaction.

By means of our new quantitative technique (Amano, *et al.*, 1958), similar kinetic studies were made on immune bacteriolysis for the first time and similar results were obtained to those of Mayer *et al.* (1948). However, we were unable to show that antibody molecule can turn over from cell to cell, because it was already established that the serum contains lysozyme, which can also turn

over, and the addition of extraneous lysozyme to the immune bacteriolytic system markedly accelerated the conversion of Gram-negative bacilli into serum spheroplasts (Amano *et al.*, 1954).

Lysozyme can be removed from the serum by adsorption onto zymosan (Inai *et al.*, 1958a) or bentnite (Inai *et al.*, 1958b). Using latter method, we could obtain complement serum or antiserum free from lysozyme and study the role of serum lysozyme in immune bacteriolysis. As described in this paper, lysozyme plays an indispensable role in the conversion of bacilli to serum sphroplasts and exerts its action at the terminal step. Now it is obvious that the immune bacteriolytic system contains three independent agents, i. e., antibody, complement and lysozyme. Lysozyme alone can not attack the surface of *E. coli* B nor the cell walls of the bacteria (Amano *et al.*, 1955a and 1956). However the cell walls of the bacteria contain the substrate of lysozyme. From the results presented in this report, it can be stated that the substrate of lysozyme is exposed or activated through the action of antibody plus complement.

After the action of antibody plus complement without lysozyme, small amounts of bacterial nucleic acids were detectable. In such experiments, serum spheroplasts were never formed and some of the cells changes to scarcely stainable cell-wall like rods.

Therefore, it can be assumed that some damage had been caused to be bacterial surface. However, it is not known whether such damage was caused by antibody plus complement alone or whether substances other than lysozyme participated in causing such damage with antibody and complement.

The immunological reaction between bacteria and homologous antiserum plus complement has been studied on two facets of the reaction: The one, changes in morphology of the bacterial cells, and the other change in viability of the cells. Since antiboby plus complement without lysozyme could cause some damage on the bacterial surface, it is necessary to study the fate of bacteria in such a system by the viable count method.

The immune bactericide process can well be induced even in the absence of lysozyme. But excess lysozyme (5.0 μg. per ml. in final concentration) accelerated and enhanced the bactericidal action of antibody plus complement. Serum contains lysozyme approximately equivalent to 5.0 μg. of crystalline egg white lysozyme per ml., even when the serum is carefully prepared, as far as possible, without destruction of blood cells. Therefore, it is concluded that lysozyme plays an important role in the host's defence mechanisms even against Gram-negative bacteria.

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