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Quantitative Studies on Immune Bacteriolysis

I. A New Method of Quantitative Estimation

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SUMMARY

A new method has been designed for the quantitative determination of immune bacteriolysis *in vitro*.

After incubation of the assay tubes containing antiserum, complement and a cell suspension of *Escherichia coli*, 0.05 per cent final concentration of Na-desoxycholate is added to the tubes. By this procedure only serum spheroplasts formed by the immune bacteriolysis are destroyed. After centrifugation of the lysates the supernatants are deproteinized with perchloric acid at a final concentration of 2 per cent, and recentrifuged. Ultraviolet absorption of these centrifuged supernatants is estimated in a Beckman spectrophotometer at 260 m μ . Thus the amount of nucleic acid involved in lysates is determined.

By this method, 50 per cent bacteriolytic units of complement could be estimated and the course of immune bacteriolysis was studied quantitatively.

INTRODUCTION

Since Pfeiffer discovered immune bacteriolysis, the bactericidal activity of sera has been studied quantitatively by survival cell counts. The serum bactericidal test was improved recently by Maaløe (1946) and by Muschel and Treffers (1956). However, the morphological change from bacilli to coccoid form, serum spheroplasts (Brenner *et al.*, 1958; McQuillen, 1958), has only been studied microscopically.

In the course of our previous works on immune bacteriolysis (Amano *et al.*, 1954a, b, c), it was necessary to use quantitative determination. For this purpose, it was necessary to separate serum spheroplasts from intact bacteria. We succeeded in the selective lysis of serum spheroplasts by the addition of a definite amount of sodium desoxycholate. Thus we could apply the principle of quantitative assay of Mayer, Croft and Gray (1948) on immune hemolysis to our bacteriolytic system. The nucleic acid formed from lysed bacteria was estimated after deproteinization.

*Materials and Methods*1) *Bacteria*

In all experiments *E. coli* B was used. Y medium used for *E. coli* B is as follows :

Peptone	20 g		
NaCl	5 g		
Yeast extract	1 g	Distilled water	1,000 ml. pH 7.2
Na ₂ HPO ₄	5 g		
(Agar	30 g)		

Overnight culture of *E. coli* B on agar slant were suspended in 10 ml. of Y medium and shaken at 37°C for 60 minutes. Following centrifugation and washing with saline, sedimented bacteria were resuspended in saline containing 0.02% MgSO₄·7H₂O. The bacterial suspension was adjusted to 0.12 O.D. (at 550 mμ) in a Coleman junior spectrophotometer.

2) *Antiserum*

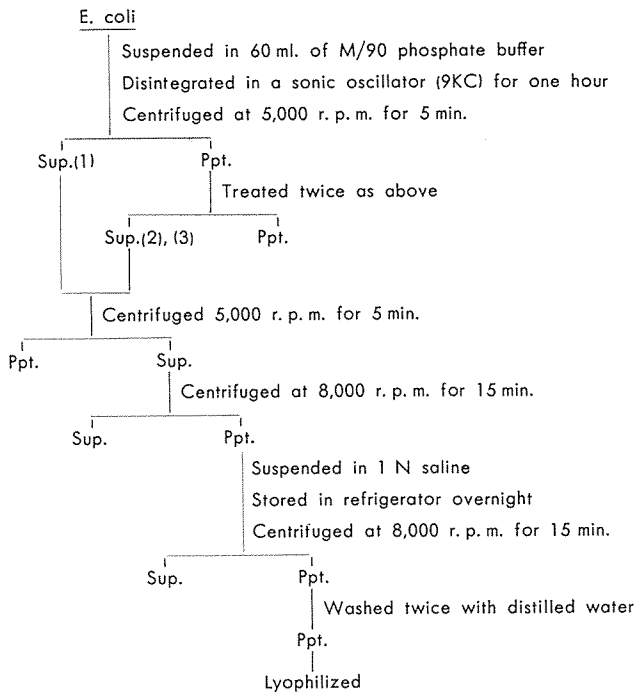
Rabbit anti-*E. coli* B serum was used. The agglutination titer of this antiserum was 1 : 12,800.

3) *Complement*

Fresh guinea pig sera were used as complement after treatment described later. They have a considerable bacteriolytic activity against *E. coli* B without added antiserum. For instance, when non-treated complement was used at a final dilution of 1 : 12 without antiserum, 86 per cent lysis was observed. For this reason we had to remove normal antibodies present in the complement. Fresh guinea pig serum could be absorbed in one hour by a large amount of heat killed cells at 0°C. The absorbed serum contained much nucleic acid liberated from dead cells. Therefore the cell wall preparation was used for the absorption in place of heated cells. The cell wall preparation was obtained as follows: The bacterial cells from 1 litre of overnight shaking culture were suspended in 60 ml. of M/90 phosphate buffer at pH 7.0 and mechanically disintegrated in a Raytheon sonic oscillator (9KC) for one hour. They were then centrifuged at 5,000 r.p.m. for 5 minutes. The supernatant containing cell walls was removed. The sediment was resuspended and the sonic disintegration was repeated twice in the same way. The supernatants were pooled and centrifuged at 8,000 r.p.m. for 15 minutes. The sediment was washed twice with physiological saline by centrifugation, resuspended in 1 N saline to extract nucleic acid from cell walls and stored in a refrigerator overnight. This suspension was centrifuged at 8,000 r.p.m. for 15 minutes, washed twice with distilled water and lyophilized. The whole procedure is summarized in Table 1.

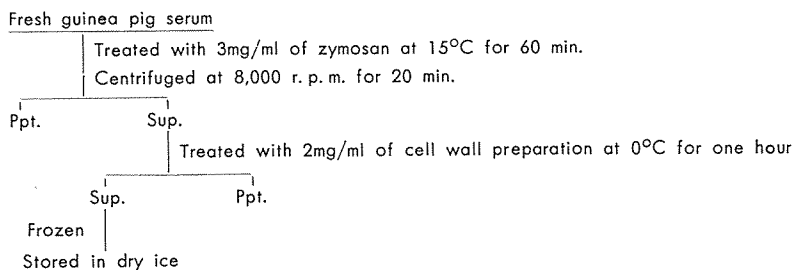
One ml aliquots fresh guinea pig serum were treated with 2 mg of the cell wall preparation at 0°C for one hour and centrifuged at 9,000 r.p.m. for 20 minutes. Even after this treatment 20 to 30 per cent bacteriolysis was observed without added antiserum. Though the reason is not yet known, we absorbed the cell wall treated serum with zymosan. Then no bacteriolysis was detected with this treated complement in the absence of added antiserum. Complement

Table 1. Preparation of cell wall



used in subsequent experiments was treated, first, with zymosan and then with cell walls as described in Table 2, and stored in sealed glass ampules in dry ice. The zymosan used in this treatment was extracted from Fleishman's yeast according to Pillemer's method (1956).

Table 2. Treatment of complement



4) *Diluent*

Physiological saline containing 0.02 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was used. Buffer solution was not used, because veronal buffer absorbs ultraviolet light strongly at 260 $\text{m}\mu$, phosphate buffer is anticomplementary and the pH of reaction mixture did not change in using Mg-saline during the reaction.

5) *Representation of degrees of morphological change*

The same indices were used as described previously (Amano *et al.*, 1954 c).

RESULTS

1) *Preliminary experiments to establish the assay method.*i) *Deproteinizing method for the supernatant of the reaction mixture.*

Perchloric acid was used for this purpose. It had been used by Ogur *et al.* (1950) to extract nucleic acid, because there was no loss of nucleic acid in extraction. When perchloric acid at an optimal concentration is used, almost all protein can be precipitated. Moreover, perchloric acid itself shows negligible absorption of ultraviolet light at $260\text{ m}\mu$. Other deproteinizing agents, i. e. trichloroacetic acid, tannic acid or heavy metals could not be utilized because of their intense absorption of ultraviolet light. The optimal concentration of perchloric acid for deproteinization under our experimental conditions was 2 per cent final concentration.

To find the optimal concentration of perchloric acid, the following experiment was performed. Experimental conditions are shown in Table 3. All tubes were heated at 70°C for 20 min. and then centrifuged. The nucleic acid content of each was estimated using distilled water as a blank. It can be seen from Fig. 1, that the supernatant of the tube containing 2 per cent perchloric acid shows the least absorption at $270\text{-}290\text{ m}\mu$. Though the supernatant deproteinized by 2 per cent perchloric acid shows some absorption at $260\text{ m}\mu$, it was to be remembered that 1 ml. of undiluted serum was used in this experiment and such absorption at $260\text{ m}\mu$ can be eliminated by using appropriate controls.

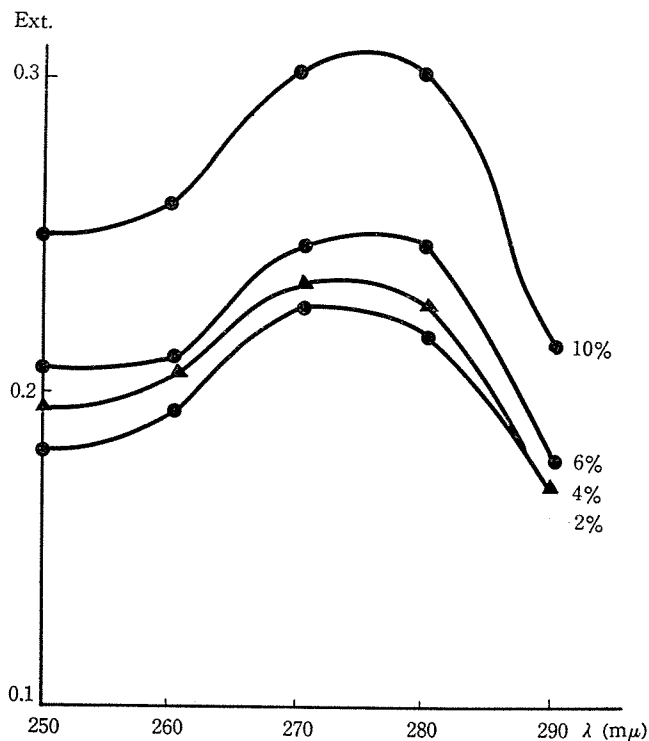


Figure 1. Ultraviolet absorption spectra of serum deproteinized with varying amounts of perchloric acid.

Table 3. Experimental conditions for the estimation of the ultraviolet light absorption spectra of serum deproteinized with varying amounts of perchloric acid

Guinea pig serum (1 : 1)	0.5	0.5	0.5	0.5
Saline	3.85	3.70	3.55	3.25
60% HClO ₄	0.15	0.30	0.45	0.75
Final concentration of HClO ₄ %	2	4	6	10

ii) *Recovery of nucleic acid added to serum*

In the preceding experiment the authors found the optimal concentration of perchloric acid for deproteinization. It was necessary to check the recovery of pentose-nucleic acid (RNA) during deproteinization. Table 4 shows the RNA content of the tubes. All tubes were heated at 70°C for 20 min. and centrifuged. Ultraviolet absorption of each supernatant was plotted, Fig. 2, using distilled water as the blank. The extinction of tube No. 1 was equal to the sum of the extinctions of tube No. 3 and No. 6. The discrepancy between that of tube No. 2 and that of tube No. 6 can be explained by the presence and absence of perchloric acid. Such an effect has already been reported by Ogur and Rosen (1950).

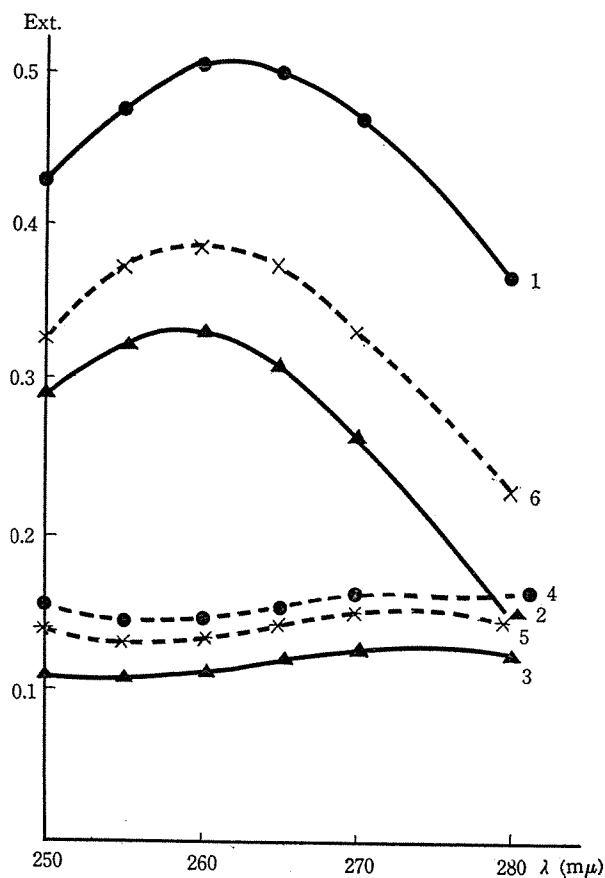


Fig. 2. Effect of perchloric acid on the ultraviolet absorption spectra of RNA solutions in serum.

Table 4. Experimental conditions to test the effect of perchloric acid on the ultraviolet absorption spectra of RNA solutions in serum

	1	2	3	4	5	6
Serum (1:3)	0.9	—	0.9	0.9	0.9	—
RNA solution (100 γ /ml)	0.9	0.9	—	—	—	0.9
Mg-saline	2.55	3.6	3.45	3.30	3.15	3.45
HClO ₄ (60%)	0.15	—	0.15	0.30	0.45	0.15
Final concentration of HClO ₄ (%)	2		2	4	6	2
Heated at 70°C for 20 min., centrifuged						

To reconfirm the reliability of this method the recovery of varying quantities of RNA was tested. Varying quantities (2.0, 1.5, 1.0, 0.75, 0.5 ml.) of a 40 μ g per ml. solution of RNA were added to tubes (No. 1, 2, 3, 4, 5) containing 2.0 ml of 1:2 dilution of serum. A blank tube (No. 6) was prepared containing serum without RNA. A series of control tubes (No. 7, 8, 9, 10, 11) was also prepared. To these were added RNA without serum. Tube No. 12 served as the blank for these five tubes. To all tubes 0.5 ml. of 0.5 per cent solution of sodium desoxycholate were added. The total volume of each tube was adjusted to 5 ml. with Mg-saline as shown in Table 5. Aliquots (4.35 ml.) of each tube were treated with 0.15 ml. of 60 per cent perchloric acid and heated at 70°C for 20 minutes. The nucleic acid content was estimated in each centrifuged supernatant. The recovery of nucleic acid in the tubes after deproteinization was compared with that in the

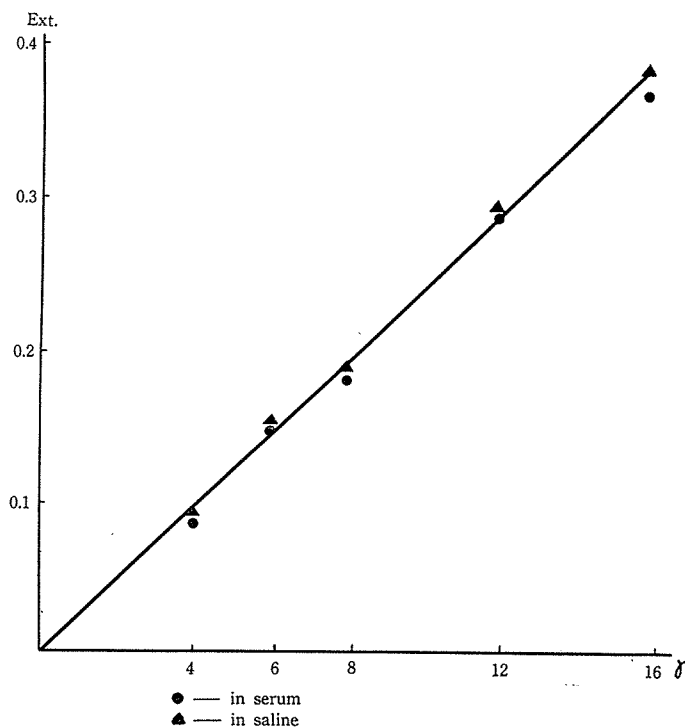


Fig. 3. Effect of deproteinization with perchloric acid on various concentration of RNA.

Table 5. Experimental conditions to test the effect of deproteinization by perchloric acid on the recovery of RNA

	1	2	3	4	5	6	7	8	9	10	11	12
Serum (1:2)	2.0	2.0	2.0	2.0	2.0	2.0	—	—	—	—	—	—
RNA solution	2.0	1.5	1.0	0.75	0.5	—	2.0	1.5	1.0	0.75	0.5	—
Mg-saline	0.5	1.0	1.5	1.75	2.0	2.5	2.5	3.0	3.5	3.75	4.0	4.5
Na-desoxycholate (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
Final concentration of RNA (γ /ml)	16	12	8	6	4	0	16	12	8	6	4	0
0.15 ml of 60% HClO_4 are added to 4.35 ml in each tube and the tubes are heated at 70°C for 20 min.												
Extinction (260 $m\mu$)	0.353	0.281	0.178	0.144	0.083	blank	0.375	0.290	0.183	0.153	0.093	blank

control tubes. Recovery was complete as shown in Figure 3.

iii) *Extraction of nucleic acid from serum spheroplasts*

With immune bacteriolysis of *E. coli in vitro*, serum spheroplasts formed were stable in ordinary medium. In this case, as shown later, nucleic acids were not liberated from the cells. Accordingly, for the estimation of nucleic acids in serum spheroplasts it was necessary to destroy these cells selectively without affecting normal cells. For this purpose several detergents were tested, *i. e.* Na-laurylsulfate, saponin, cetyltrimethyl ammonium bromide, cetylpyridinium chloride and Na-desoxycholate. Among them Na-desoxycholate was found to be the most suitable. Na-desoxycholate could destroy serum spheroplasts completely and did not affect the staining properties of normal cells at a final concentration of 1.0 to 0.025 per cent. Table 6 shows the extinction of nucleic acids from spheroplasts estimated

Table 6. Lytic effect of Na-desoxycholate on spheroplasts of *E. coli*

	1	2	3	4	5	6	7
Antiserum (1:100)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement (1:1)	0.5	—	—	—	0.5	0.5	0.5
Inactivated complement (1:1)	—	0.5	0.5	0.5	—	—	—
Mg-saline	2.5	2.5	2.5	2.5	3.5	3.5	3.5
Bacterial suspension (Ext. 0.4)	1.0	1.0	1.0	1.0	—	—	—
Incubated at 37°C 90 min.							
Microscopical findings	+++	—	—	—			
To these tubes were added:							
Na-desoxycholate (Each 0.5 ml of)	0.5%	10%	0.5%	5%	10%	0.5%	5%
Microscopical findings	*	—	—	—			
To 4.35 ml of each tube were added 0.15 ml of 60% perchloric acid and incubated at 70°C for 20 min. and centrifuged. Ultraviolet absorption in these tubes was measured in a Beckman spectrophotometer							
Extinction (260 $m\mu$)	0.452	0.033	0.007	0.008			

+++ : Almost all of bacteria changed to serum spheroplasts.

— : No bacteria changed.

* : Nearly all changed bacteria had disappeared.

in a Beckman spectrophotometer. Tubes 5, 6 and 7 are blanks for tubes 1, 2 and 3 respectively. 1.0 per cent final concentration of Na-desoxycholate could not liberate nucleic acid from bacterial cells without complement.

iv) *Summary of the experimental procedure*

The total process of this assay method is as follows. 0.5 ml. of antiserum, complement and 1.0 ml. of bacterial suspension are added to polyethylene tubes and the final volume is adjusted to 4.5 ml. with Mg-saline. Blanks are prepared containing the same constituents as the test series except that the bacterial suspension is replaced by Mg-saline. Each tube is stoppered and incubated at 37°C for the required period. After chilling, 0.5 ml. of 0.5 per cent Na-desoxycholate are added to the tubes. The tubes are agitated for a moment and then centrifuged at 8,000 r. p. m. for 10 minutes at 0°C. 4.35 ml. of each supernatant are pipetted into a stoppered tube containing 0.15 ml. of 60 per cent perchloric acid, agitated and heated at 70°C for 20 minutes. After chilling the tubes are centrifuged at 8,000 r. p. m. for 15 minutes. The nucleic acid content of each supernatant is estimated in a Beckman spectrophotometer at 260 m μ , subtracting the reading of the corresponding blank. The same experiment can also be performed as follows. The test series is set up as described above. In the blank series the same amount of bacterial suspension and antiserum are used and inactive complement is used in place of the active complement. Following procedures are the same. Though the data are not shown, the same result is obtained.

2) *Determination of 100 per cent bacteriolysis*

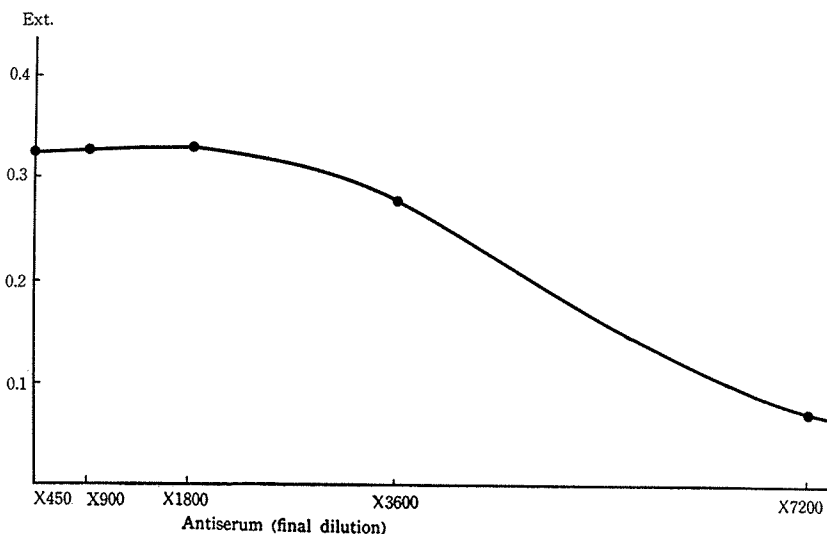


Fig. 4. Determination of excess amount of antiserum under our experimental condition.

	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'	11'	12'
Antiserum (1 : 100)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement (1 : 4)	(1 : 2)(1 : 2)											
	2.0	2.5	2.0	2.5	2.8	3.0	3.2	3.4	3.6	3.8	3.9	3.95
Mg-saline	2.0	2.5	2.0	2.5	2.8	3.0	3.2	3.4	3.6	3.8	3.9	3.95
Blanks of corresponding tubes												
	1	2	3	4	5	6	7	8	9	10	11	12
Antiserum (1 : 100)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement (1 : 4)	(1 : 2)(1 : 2)											
	2.0	1.5	2.0	1.5	1.2	1.0	0.8	0.6	0.4	0.2	0.1	0.05
Mg-saline	1.0	1.5	1.0	1.5	1.8	2.0	2.2	2.4	2.6	2.8	2.9	2.95
Bacterial suspension (Ext. 0.12)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Extinction (260 mμ)	0.325	0.331	0.327	0.312	0.233	0.283	0.278	0.225	0.163	0.062	0.053	0.010
%		100	98.4	94.5	72.6	85.0	83.7	67.9	49.3	18.7	16.1	3.0
Probit			7.062	6.598	5.54	6.04	5.98	5.46	4.98	4.11	4.01	3.12

ordinate, against the logarithms of the corresponding amounts of complement. A typical sigmoid curve was obtained. In addition, on plotting the per cent bacteriolysis as the ordinate of a probit scale, a linear relationship was obtained as shown in Figure 5. Thus the 50 per cent bacteriolytic unit of the complement could easily be calculated by the same method which was used for immune hemolysis.

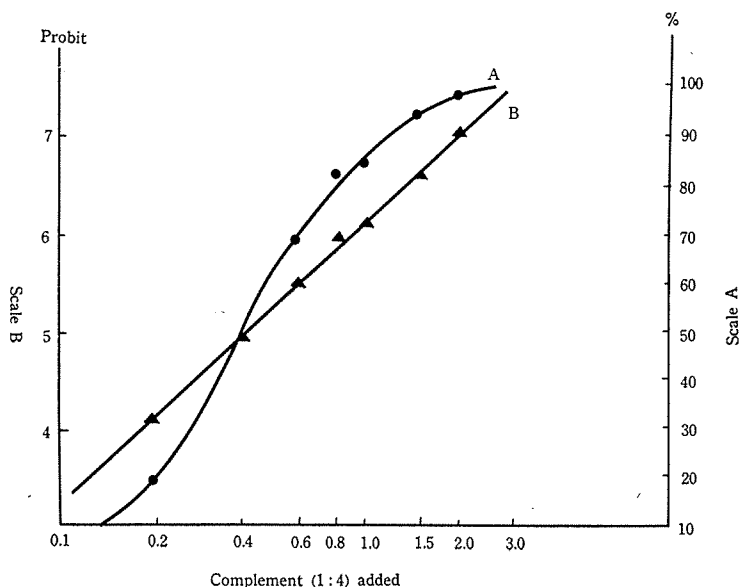


Fig. 5. Titration of complement with immune bacteriolysis,

4) Course of immune bacteriolysis

This experiment was conducted in the presence of excess antibody and complement. In this experiment surviving bacterial cells may synthesize nucleic acid utilizing the serum constituents as nutrient during the incubation period. In fact the nucleic acid content of cells incubated with inactivated complement increased. Therefore, if nucleic acid synthesis occurred in surviving cells it is too complex to analyse the reaction. All attempts to avoid this, by treating the cell suspension with ethanol, formalin or heat, were unsuccessful. Then we tried a method of Smollelis and Hartsell (1949) for estimating lysozyme activity, *i. e.* utilization of bacterial cells killed in ultraviolet light. By this method stopped the increase of nucleic acid in the cells during the incubation. A bacterial suspension (extinction 0.12) was exposed to ultraviolet light (19 watt Toshiba Germicidal lamp) at a distance of 10 cm. for 1 minute. The thickness of the bacterial suspension in the petri dish was less than 3 mm. By this treatment increase of nucleic acid during the incubation was prevented, and no disintegration of cells or bacteriolysis with inactivated complement was observed.

The course of immune bacteriolysis of intact cells was compared with that of cells irradiated by ultraviolet light (Table 8, Fig. 6). After about 20 minutes the amount of nucleic acid from intact cells increased markedly and both curves showed a plateau after about one hour. From the difference between the maximal extinctions of the curves it is shown that nucleic acids were synthesized during the course of immune bacteriolysis in intact cells. Such a phenomenon can easily be understood through our previous findings (Amano *et al.*, 1955), that bacterial cells could incorporate radioactive phosphoric acid during immune bacteriolysis.

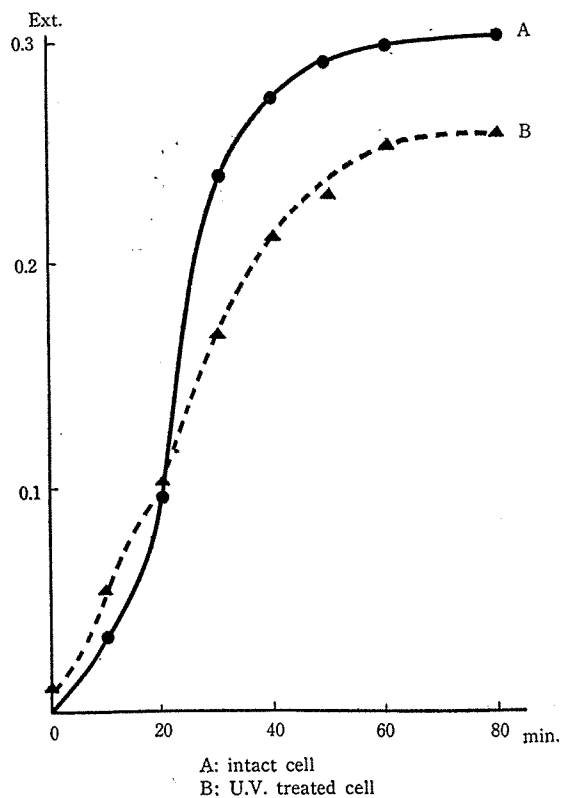


Fig. 6. Course of immune bacteriolysis of *E. coli B*.

DISCUSSION

Recently knowledge of the kinetics of immune hemolysis has progressed remarkably, and the precise mechanism of complement action has been studied mainly in immune hemolysis by Mayer and his colleagues (1958). Our method for the quantitative estimation of immune bacteriolysis is comparable in accuracy to the method of immune hemolysis. Furthermore in our method as in immune hemolysis, 0 per cent and 100 per cent lysis are the bases for the analysis of

Table 8.
Experimental conditions to study the course of immune bacteriolysis of *E. coli* B

	Intact cells								
	1	2	3	4	5	6	7	8	9
Antiserum (1:100)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement (1:4)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mg-saline	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	3.0
Bacterial suspension (Ext. 0.12)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—
Incubation time (min.)	0	10	20	30	40	50	60	80	
Extinction (260 m μ)	0.005	0.035	0.098	0.242	0.269	0.292	0.295	0.300	
%	1.7	11.7	32.7	80.7	89.7	97.3	98.3	100	
Microscopic finding	—	—	±	+	++	+++	++++	++++	
	U. V. irradiated cells								
	1'	2'	3'	4'	5'	6'	7'	8'	
Antiserum (1:100)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Complement (1:4)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
Mg-saline	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	
Bacterial suspension (Ext. 0.12)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
Incubation time (min.)	0	10	20	30	40	50	60	80	
Extinction (260 m μ)	0.004	0.58	0.108	0.169	0.213	0.229	0.240	0.246	
%	1	23.6	44.0	68.5	86.5	93.0	97.5	100	blank

++++: Almost all of bacteria changed to serum spheroplasts.

+++ : About three-fourths of bacteria changed to serum spheroplasts.

++ : About half changed to serum spheroplasts.

± : Most bacteria not changed.

— : No bacteria changed.

bacteriolysis. However in the plate count method or Muschel's method 100 per cent survival is the only basis. Utilizing our method it is possible to study the mechanism of complement action and the effect of other cellular substances on the one hand and surface structure of the microbial cells on the other.

It was difficult to completely remove bacteriolytic substances from fresh guinea pig serum, but it was achieved with zymosan. It is not yet clear what substances other than properdin are absorbed by zymosan. According to Inai *et al.* (1958), lysozyme, a bacteriolytic substance in human or guinea pig serum, can be absorbed by zymosan at low temperature. Therefore, it might be possible to find nonspecific bacteriolytic factors than lysozyme.

It was fortunate to discover that serum spheroplasts could be destroyed with Na-desoxycholate. Sodium desoxycholate does not absorb ultraviolet light at 260 m μ , nor interfere with deproteinization. However, normal cells of *Vibrio tyrogenus*, used previously in our experiments, were lysed by a low concentration of this agent.

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