

## THE COMPLEMENT-DEPENDENT BACTERIOLYTIC ACTIVITY OF NORMAL HUMAN SERUM

### I. THE EFFECT OF PH AND IONIC STRENGTH AND THE ROLE OF LYSOZYME

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It has been known for over 60 years that mammalian serum is capable of causing the dissolution, or lysis, of certain bacteria (1) and that complement may be involved in the process. Nevertheless, little experimental work has been done on the serum bacteriolytic reaction as such, no doubt because of the early introduction of the mammalian erythrocyte as the standard indicator for serum complement, and also because only a few types of bacteria are actually lysed by the complement system (2), compared with the many strains which are killed without obvious lysis.

The author's interest in the lysis of bacteria by complement stems from a more basic interest in determining (*a*) the nature of the complement substrate or receptor in cell membranes and (*b*) the biochemical nature of the lesion produced by complement at its substrate sites which leads to cell death and/or lysis. It is argued that the bacterial cell, being physically more robust than the erythrocyte or other mammalian cells, probably requires a more extensive and specific chemical change in the cell membrane before lysis can occur. Therefore, for studying the mode of lytic action of complement at the biochemical level, the bacterial cell may be the best choice. It is of interest in this context to recall the remark made by Muir (1931) in an article (3) on the hemolytic action of complement, "The effect of lytic action may be even more marked in the case of a bacteriolytic serum, almost complete solution of the bacteria being sometimes observed."

The studies reported here are concerned with determining the optimum conditions for, and the serum factors involved in, the lysis of a strain of *Escherichia coli* by normal human serum. This has led to certain deductions on the nature of the complement substrate and on the mechanism of bacteriolysis. An abstract of part of this work has previously been published (4).

#### *Materials and Methods*

*Bacterial Strain.*—The strain used throughout this work was an *Escherichia coli* designated "Lilly" which was obtained several years ago from Dr. W. S. Boniece, Eli Lilly Co., Indi-

anapolis. The culture gave rough colonies on McConkey's medium, a sedimented growth in nutrient broth and produced acid and gas from glucose and lactose, but not from sucrose. The strain was maintained on nutrient agar slants kept at 5°C. Cultures for lysis tests were usually grown for 18 hours at 37°C in beef infusion broth or in the synthetic medium of Davis and Mingioli (5). Nutrient agar was also used. Cells harvested from these media were washed twice in distilled water by centrifugation at room temperature, resuspended in either distilled water or 0.06 M NaCl, and held at 0°C.

*Serum.*—Blood was obtained from healthy human donors and allowed to clot 1 hour at room temperature. The serum was separated after a few hours of refrigeration and stored at -25°C. Sera were generally used within 1 to 2 months after collection, although no marked loss of hemolytic complement or of lytic activity towards *E. coli* was apparent after 6 months.

*Bacteriolysis Tests.*—Tests were made initially in 25 ml flat sided, screw cap bottles which fitted in the cell carrier of the Hilger spekker spectrophotometer. Later, 13 × 100 mm test tubes were used in a Coleman colorimeter. Readings of optical density were made using grey and red filters, respectively in the two instruments. In making the lysis tests, the vessels containing the requisite amount of buffer or other diluent were warmed to bath temperature (37°C) and serum added, usually to give a 1/10 or 1/20 dilution. The optical density was read immediately, after which bacterial suspension sufficient to give an optical density increment of 0.4 to 0.5 was added in a volume that was about 5 per cent of the total and the reading again taken (Time 0 reading). The vessels were then incubated in the water bath with periodic manual shaking and the optical density read at suitable intervals (Time *T* readings) to follow the course of lysis. The term lysis is used to denote a reduction in the optical density of the suspension. Per cent reduction in optical density (OD) was calculated by the expression:

$$\frac{\text{OD at time 0} - \text{OD at time } T}{\text{OD at time 0} - \text{OD of diluent} + \text{serum}} \times 100$$

Unless otherwise stated, lysis mixtures contained 0.04 M tris (2-hydroxymethyl-2-amino propane-1,3-diol) buffer, pH 8.4 and NaCl sufficient to make the total ionic strength, 0.06 (after the addition of serum).

*Assay of Complement.*—A procedure similar to that described by Mayer, Osler, Bier, and Heidelberger (6) was used.

*Assay of Lysozyme in Serum.*—The lysozyme content of serum was determined by a quantitative bacteriolysis test using crystalline egg-white lysozyme (Nutritional Biochemical Corp.) as standard and *Micrococcus lysodeikticus* American Type Culture Collection 4698 as test organism. The micrococcus was grown 2 days at 33°C on nutrient agar plates. The dilutions of serum and of standard lysozyme were made in 0.033 M phosphate buffer, pH 6.5, adjusted to ionic strength 0.1 by the addition of 0.045 M NaCl, and containing 0.05 per cent gelatin. A standard curve relating the per cent lysis to the lysozyme concentration was prepared each day using lysozyme levels from 0.025 to 0.25 μg/ml. Suitable dilutions of serum (e.g., 1/10 to 1/40) were then tested in parallel and the responses interpolated on the standard curve. It was assumed in interpreting the results that the lytic activity of normal human serum toward *M. lysodeikticus* was due to lysozyme and not to other serum factors.

*Absorption of Serum with Bentonite.*—Serum was depleted of lysozyme by absorption with bentonite, as suggested by Myrvik and Weiser (7). The bentonite was first washed by suspending 1 gm in 500 ml distilled water and giving repeated cycles of centrifugation and re-suspension in changes of distilled water until the pH of the suspension, initially close to 13.0, had fallen to 7.7. The washed bentonite was suspended to 100 ml and the larger particles which settled out during 15 minutes' standing were discarded. The fine material was then concentrated by centrifugation and the bentonite content determined by evaporating a portion to dryness at 110°C. The absorption of serum was done by centrifuging portions of bentonite

suspension, sufficient to give 5 mg dry wt per ml serum, discarding the supernatant, cooling to 0°C and adding serum at 0°C. The mixture of bentonite and serum was stirred for 15 minutes at 0°C, centrifuged 30 minutes at 10,000 RPM at 0°C and the supernatant decanted.

*Absorption of Serum with Bacteria.*—The bacterial suspensions used for the absorption of serum were prepared from cultures grown in liquid synthetic medium (5). The cells were collected by centrifugation, washed twice in water and suspended in water at 0°C. A portion of the suspension was dried to constant weight on a planchette to determine the concentration of bacteria in terms of mg dry wt per ml. Having done this, a portion of the suspension was centrifuged so as to provide a pellet containing 50 mg dry wt of bacteria for each millilitre of serum to be absorbed. The serum, cooled to 0°C, was added to the bacteria at 0°C and the

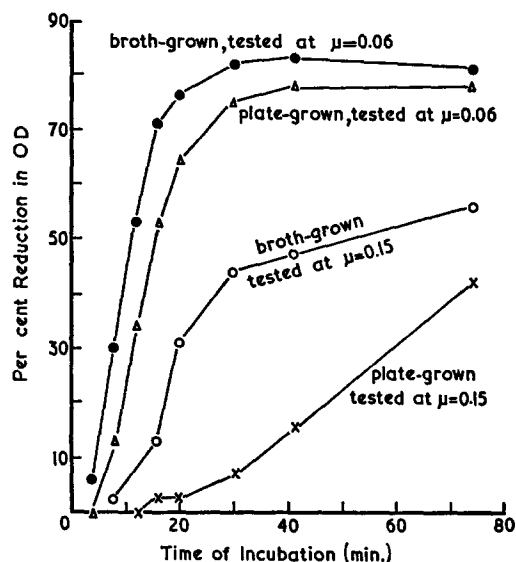


FIG. 1. Lysis of *E. coli* Lilly by human serum, showing the effect of using bacteria harvested from broth and from agar plates and the effect of conducting the tests in physiological saline ( $\mu = 0.15$ ), and in tris-buffered saline ( $\mu = 0.06$ ). The serum was used at  $\frac{1}{15}$  dilution.

mixture stirred 15 minutes at 0°C. Absorption was done at 0°C to prevent cell lysis and to minimize destruction of complement. The mixture was finally centrifuged 30 minutes at 12,000 RPM. at 0°C. The supernatant serum was then subjected to additional cycles of absorption, up to three altogether, using fresh portions of cells.

## RESULTS

### *General Observations*

It was found early in the investigation that both the initial rapidity and the final extent of lysis of *E. coli* Lilly by normal human serum was influenced by ionic strength and also by the type of culture medium from which the bacteria had been harvested. Thus, as shown in Fig. 1, lysis was rather slow and incomplete when the tests were made with serum diluted in physiological saline

(ionic strength 0.15), using cells harvested from beef infusion agar. In contrast, lysis was very rapid when the serum was diluted in tris-buffered saline of ionic strength 0.06, using cells harvested from beef infusion broth. Fig. 1 shows that under suitable conditions, lysis was detectable within 5 minutes and was complete at 20 minutes. Tests were made to ascertain whether the *coli*-lytic activity was a general property of human sera or whether only certain selected sera would show the effect. Table I shows results on the lytic activities of fourteen randomly chosen human sera tested in tris-buffered saline at ionic

TABLE I  
*Bacteriolytic Activity of Serum from Different Individuals*

Individual	Serum dilution	Reduction in OD after incubation	
		15 minutes	30 minutes
		<i>per cent</i>	<i>per cent</i>
B-h	1/10		62
B-l	1/20		85
B-m	1/15	58	75
D-d	1/15		75
F-t	1/15		75
K-r	1/20	73	83
K-y	1/15		79
M-n	1/20	50	80
M-y	1/10	81	
	1/20	77	
	1/40	37	
	1/80	18	
Mh-y	1/40		41
O-y	1/20		50
P-r	1/20		30
W-n	1/20		70
W-w	1/20		68

strength 0.06 using broth-grown cells. The tests were made over a period of more than one year and, therefore, the results with different sera are not exactly comparable. Nevertheless it is clear that each of the sera exhibited definite bacteriolytic activity at 1/10 to 1/20 dilution and, in the case of sample Mh-y, a 1/40 dilution was active. The sample P-r which appears in the table as the least active serum had been stored at  $-25^{\circ}\text{C}$  for several months, which may have resulted in some loss of activity.

The course of lysis was also followed by phase-contrast microscopy. The test mixtures consisted of 1/20 serum in tris-buffered saline as before, but the temperature was lowered to  $25-30^{\circ}\text{C}$  to slow the reaction. The bacteria, initially rod-shaped, were observed to swell, become spherical and then to lyse leaving remnants of the cell envelope in suspension. The organisms could be kept in

the spherical, pre-lysis condition (sphaeroplast form) by including 5 per cent or 10 per cent sucrose in the incubation medium. Another method of preparing sphaeroplasts was to use a higher concentration of cells in the usual lysis medium, incubate 2-3 mins. at 37° and then dilute 1/50 into 5 per cent sucrose. The sphaeroplasts so obtained could be collected by centrifugation and resuspended in either 5 per cent sucrose or in water. In the latter case, lysis occurred immediately, indicating the osmotic fragility of these forms. These observations recall those reported by Muschel, Carey and Baron (8) and Michael and Braun (9) on the formation of sphaeroplasts from *Salmonella typhi* and *Shigella dysenteriae*, respectively, by serum action.

#### *Ionic Strength, pH, and Temperature*

*Effect of Ionic Strength.*—To examine in more detail the effect of ionic strength on bacteriolysis, tests were made at a series of ionic strengths and in the presence of various buffers.

In a typical experiment with tris buffer, a 0.4 M stock solution pH 8.3 was used at a  $\frac{1}{10}$  dilution in each test mixture. It was calculated to contribute 0.02 to the total ionic strength. Serum was used at a final dilution of  $\frac{1}{20}$ , so that its contribution to ionic strength was 0.0075. The final ionic strength was then varied by adding different proportions of 0.15 or 1.0 M NaCl and distilled water. In actual practice the serum was added to the warmed mixtures of buffer, NaCl, and water, followed by the bacteria.

Fig. 2 shows the extent of bacteriolysis, as a function of ionic strength, after 15, 30, and 60 minutes' incubation. It is apparent that very low ionic strengths, in the region of 0.02 to 0.03 were not satisfactory for bacteriolysis and that a slightly higher value around 0.06 was optimum. Lysis was much less rapid at the physiological ionic strength 0.15 and was almost nil at 0.2.

Experiments were next made to confirm that the observed effects were due to ionic strength rather than to osmotic pressure or the action of specific ions. To do this, the amounts of tris buffer and serum were kept the same as before (total contribution to ionic strength = 0.0275) and a comparison was made of the effect of adding increasing molarities of NaCl,  $K_2SO_4$ , and glucose. The results in Fig. 3 show that in the series with  $K_2SO_4$ , optimum lysis was obtained with an added molarity of approximately 0.015. This gives a total ionic strength of 0.07, since a given molarity of  $K_2SO_4$  has three times the ionic strength of the same molarity of NaCl. This finding with  $K_2SO_4$  therefore supports the view that ionic strength rather than osmolarity or the presence of specific ions is the important variable. The results with glucose are in accord with this and are similar to other results (not shown) obtained with sucrose (a sugar not utilized by this organism).

To determine whether the lysis optimum at ionic strength 0.06 was dependent on using tris buffer and an alkaline pH, experiments at different ionic strengths were made using (a) serum adjusted to pH 6.6 without added buffer and b) Michaelis' veronal-acetate buffer pH 8.2. As shown in Fig. 4, the results were

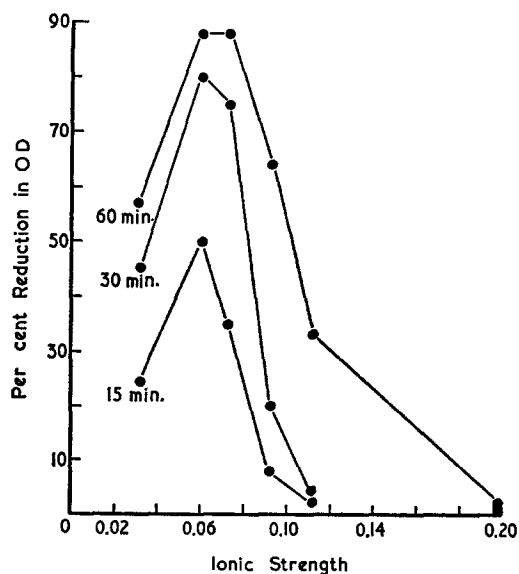


FIG. 2. The effect of ionic strength on the lysis of *E. coli* Lilly by human serum. Optical density (OD) readings were made after 15, 30, and 60 minutes of incubation.

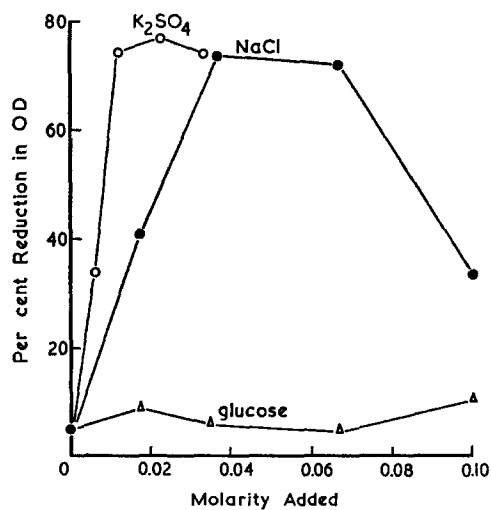


FIG. 3. The effect, on bacteriolytic activity, of adding different molarities of NaCl, K<sub>2</sub>SO<sub>4</sub> and glucose to a  $\frac{1}{20}$  dilution of serum in 0.04 M tris buffer. Incubation time was 20 minutes.

similar to those obtained with tris buffer and indicated that bacteriolysis proceeded optimally at ionic strength 0.06 independently of pH and type of buffer.

*The Effect of pH.*—The effect of pH on the serum bacteriolytic reaction was

studied both in the presence and absence of added buffers, all tests being made at ionic strength 0.06. In tests where no buffer was added, the serum was adjusted to the various pH values with NaOH or HCl.

The method of the experiment was first to titrate the serum to the desired pH value and then to calculate the amounts of 0.15 M NaCl and water needed to give a total ionic strength of 0.06. The bacteriolysis tests were then made by adding the requisite amounts of HCl or NaOH to the water plus NaCl, warming to bath temperature, and adding serum and bacteria successively in the usual way.

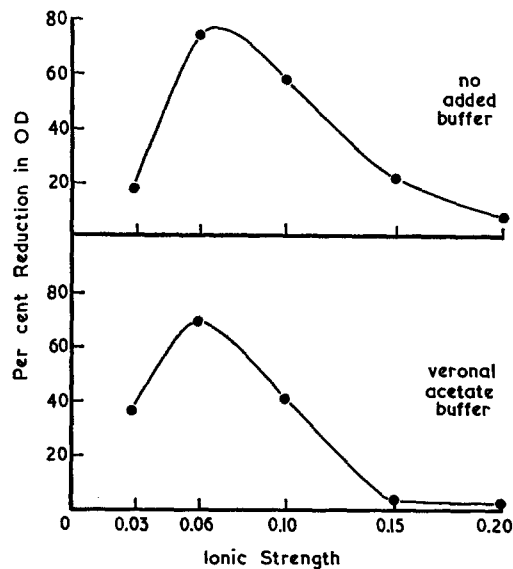


FIG. 4. Ionic strength and bacteriolytic activity of  $\frac{1}{20}$  serum: Comparison of unbuffered serum, pH 6.6, and serum in veronal-acetate buffer pH 8.3. Incubation time 20 minutes.

The results in Fig. 5 show that lysis was most rapid and complete close to pH 8.5 diminishing rapidly on the alkaline side of this value. The pH values plotted are those of the mixtures at the end of incubation but they did not differ significantly from the values predetermined in the blank run without bacteria.

The effect of pH on bacteriolysis was next studied using various buffers, notably tris, borate, and Michaelis' veronal-acetate.

Each buffer was used at a constant molarity and over a range of pH values, the total ionic strength being kept constant at 0.06 by calculating the ionic strength of the buffer from its acid-base titration curve and adding NaCl to the mixtures as required. Fig. 6 shows the pH *versus* bacteriolysis curves for serum in the presence of the three different buffers. With tris buffer (0.04 M) there was a definite optimum close to pH 8.3. With Clark and Lubs' borate buffer (0.025 M borate) there was a plateau in the region pH 7.5–8.5, but beyond 8.5 lysis was less rapid. With Michaelis' buffer (0.02 M veronal + 0.02 M acetate) most rapid lysis was at

pH 8.7. Additional experiments were made with this buffer in which the total molarity of veronal + acetate was varied between 0.005 and 0.05 M and, at each molarity, a range of pH values was prepared and adjusted to constant ionic strength 0.06.

It was found that the molarity of Michaelis' buffer did not influence the shape or position of the curve relating pH to bacteriolysis within the range examined.

*The Effect of Temperature.*—The effect of temperature on bacteriolytic activity was studied using serum at a 1/20 dilution in tris-buffered saline. The usual procedure of allowing the diluent to reach bath temperature before

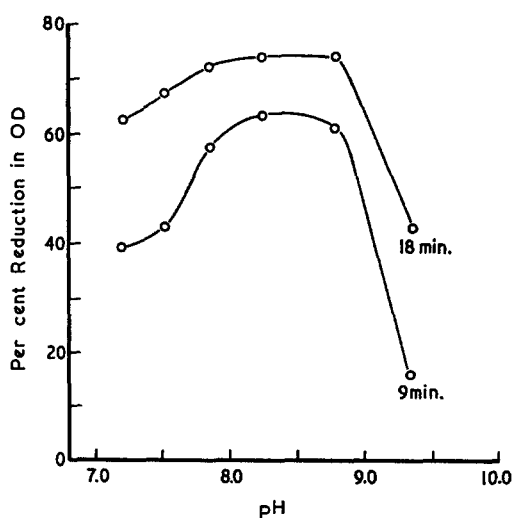


FIG. 5. Effect of pH on the bacteriolytic activity of serum without added buffer. The serum was used at  $\frac{1}{20}$  dilution and lysis readings taken at 9 and 18 minutes.

addition of serum was followed, and any temperature change produced by adding the 1/20 volume of serum, and the similar volume of bacteria to the 19 parts of diluent was neglected. Fig. 7 shows the extent of lysis as a function of temperature after 7, 15, and 30 minutes' incubation. It is apparent that at 7 minutes lysis was roughly proportional to temperature over the range 25–50°C. After 30 minutes, however, the lysis curve showed an optimum close to 37°C, suggesting that at the higher temperatures some inactivation of the bacteriolytic system in the serum was taking place. Temperatures below 20°C were not investigated apart from the finding that at 0°C lysis did not occur during 1 hour.

#### *Serum Factors Involved*

*Complement.*—An indication that complement was involved in this bacteriolytic system was obtained in tests on the effect of heating serum at 50°C and



56°C for 30 minutes. It was found (Table II) that whereas unheated serum gave appreciable lysis at a 1/50 dilution, the same serum which had been heated 30 minutes at 56°C was completely inactive at a 1/10 dilution. Serum heated 30 minutes at 50°C showed approximately two-thirds loss of bacteriolytic activity and this was found to parallel the loss in hemolytic complement.

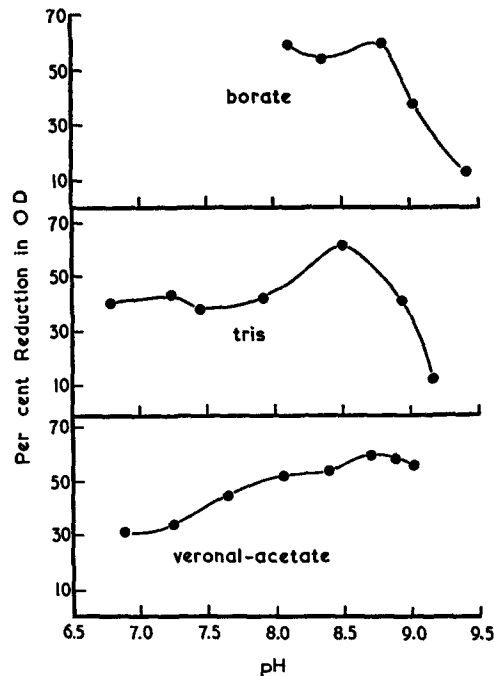


FIG. 6. The effect of pH on serum bacteriolytic activity in the presence of different buffers. The serum was used at  $\frac{1}{50}$  dilution and lysis readings were taken at 15 minutes. Ionic strength was constant at  $\mu = 0.06$  throughout. It should be noted that each buffer was tested on a different occasion and therefore the buffers can not be compared with each other as regards exact extent of lysis produced at 15 minutes.

A more specific demonstration of the participation of complement was made using serum which had been treated with an antigen-antibody system. The system used for this purpose was a suspension of washed sheep erythrocyte stromata sensitized with horse antibody. The amount used was just sufficient to inactivate 95 per cent of the hemolytic activity of the serum during 2 hours at 37°C. Such decomplemented serum was found to lack bacteriolytic activity (Table II).

In addition to the above, portions of serum were converted into the complement reagents R1, R2, R3, and R4. It was found (Table III) that these reagents showed only low bacteriolytic activity when tested individually, whereas mixtures such as R1 + R2 and R3 + R4 had substantial activity.

*Properdin*.—A sample of properdin-deficient serum (RPb) supplied by Dr. R. J. Wedgwood and found by him to have the characteristic bactericidal activity (10), in the presence and absence of added properdin, towards *Shigella dysenteriae* No. 377 was tested on *E. coli* Lilly. These tests showed the bacteriolytic activity of the RPb to be almost as great as that of the untreated serum.

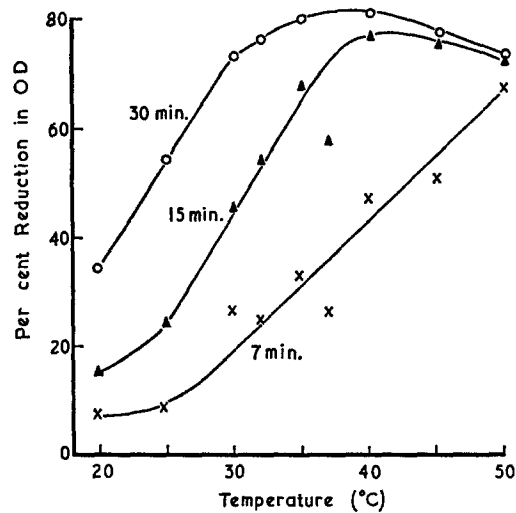


FIG. 7. Effect of incubation temperature on the rapidity of lysis. Optical density readings were taken at 7, 15, and 30 minutes.

TABLE II  
*Effect of Heat and of an Antigen-Antibody System on the Bacteriolytic Activity of Human Serum*

Treatment of serum	Dilution of serum in lysis test	Reduction in OD after 30 minutes
Nil	1/10	84
	1/20	85
	1/40	76
	1/80	30
50°C for 30 min.	1/10	80
	1/20	55
	1/40	9
56°C for 30 min.	1/10	1
Ag. Ab system	1/10	8

Addition of purified properdin to the RPb did not affect its bacteriolytic activity.

*Antibody.*—The participation of specific antibody was investigated by absorbing serum with viable, homologous cells at 0°C. The absorptions were

TABLE III  
*Bacteriolytic Activities of R1, R2, R3, and R4*

Reagent and dilution	Reduction in OD at 30 minutes
	<i>per cent</i>
Serum, 1/20	61
R1, 1/10	7
R2, 1/10	19
R3, 1/10	19
R4, 1/10	14
R1 + R2, 1/10 + 1/10	65
R3 + R4, 1/10 + 1/10	55

TABLE IV  
*Reduction of Serum Bacteriolytic Activity by Absorption with Homologous Cells and Restoration of Activity by Adding Back Heated Serum and Lysozyme*

Bacteriolytic system	Reduction in OD after 30 minutes
	<i>per cent</i>
Serum, 1/10.....	73
Once-absorbed serum, 1/10.....	52
Twice-absorbed serum, 1/10.....	23
Thrice-absorbed serum, 1/10.....	18
4×-absorbed serum, 1/10.....	14
Twice absorbed serum + 56°C 30 min. serum, 1/10 + 1/10.....	62
Twice absorbed serum + lysozyme, 1/10 + 2 µg/ml.....	69
56°C 30 min. serum, 1/10.....	0
Lysozyme, 2 µg/ml.....	4

repeated up to 4 times using 50 mg (dry wt) bacteria per milliliter of serum per absorption. It was found (Table IV) that two successive absorptions removed most of the bacteriolytic activity and it was noted also that hemolytic complement was not appreciably affected. On the assumption that loss of bacteriolytic activity by absorption was due to loss of antibody, the experiment of adding 56°C-heated serum to the absorbed serum was made, and a partial restoration of activity was found (Table IV). These findings were at first taken as evidence for the participation of antibody in the reaction until it was observed that

crystalline egg white lysozyme in trace amounts also was able to restore bacteriolytic activity to the absorbed serum (Table IV). This suggested that lysozyme rather than antibody was the important factor removed from the serum by the absorption. Assays for lysozyme were therefore made using *M. lysodeikticus*, and it was found that the initial level of lysozyme in this serum was 5.2  $\mu\text{g}/\text{ml}$ . After the first absorption it fell to 2.4  $\mu\text{g}/\text{ml}$ , and after the

TABLE V  
*Lysis of E. coli Lilly by Antimicrobial Agents*

Agent	Diluent	Reduction in OD after 60 minutes at 37°C
		<i>per cent</i>
Penicillin G, 4 mg/ml	Michaelis' buffer pH 8.3 $\mu = 0.06$ containing 20 per cent nutrient broth	5
Streptomycin sulfate, 1 mg/ml		5
Polymyxin B sulfate, 50 $\mu\text{g}/\text{ml}$		5
Chloramphenicol, 2 mg/ml		5
Na desoxycholate, 1 mg/ml	Michaelis' buffer pH 8.3, $\mu = 0.06$	5
Cetyltrimethyl ammonium bromide, 5 mg/ml		40*
Aerosol OT, 10 mg/ml		5
Lysolauryl ethanolamine, 0.5 mg/ml	Water	35
Lysolauryl lecithin, 0.5 mg/ml		5
Toluene, excess	Michaelis' buffer pH 8.3, $\mu = 0.06$	10
Lysozyme, 400 $\mu\text{g}/\text{ml}$	0.033 M tris buffer, pH 8.4, $\mu = 0.06$	5
Lysozyme 5 $\mu\text{g}/\text{ml}$ + EDTA 0.057 mM‡	0.017 M tris buffer, pH 8.4, $\mu = 0.06$	50

\* Superimposed agglutination.

‡ As described by Repaske (12, 13).

second absorption to 0.9  $\mu\text{g}/\text{ml}$ . Heating the serum 30 minutes at 56°C had no appreciable effect on the lysozyme activity. It should be noted that the level of egg white lysozyme which was effective in restoring bacteriolytic activity to absorbed serum was similar to that present in the serum initially and in heated serum. It was also observed that egg white lysozyme had a heat stability in the presence of serum proteins which was similar to that of the endogenous lysozyme of the serum. This was shown by an experiment in which 20  $\mu\text{g}/\text{ml}$  egg white lysozyme was added to a serum which had an initial activity corresponding to 4  $\mu\text{g}/\text{ml}$ , so as to give a mixture of activity 24  $\mu\text{g}/\text{ml}$ . After heating 30 minutes at 56°C, the total lysozyme activity was 20  $\mu\text{g}/\text{ml}$ , indicating that less than 20 per cent destruction of the over-all activity had occurred.

*Removal of Lysozyme with Bentonite.*—In place of bacteria as the absorbing agent, the aluminum silicate material, bentonite, which has a high affinity for lysozyme (11), was tested. It was found that a single absorption of serum with 5 mg (dry wt) washed bentonite per milliliter resulted in a reduction of the serum lysozyme level from 2.5 to less than 0.12  $\mu\text{g}/\text{ml}$ . The titer of hemolytic complement was not detectably reduced, *i.e.* less than 15 per cent. However, the *coli*-lytic activity was markedly diminished, and moreover, addition of lysozyme to 2  $\mu\text{g}/\text{ml}$  restored the activity to the original level. Lysozyme by itself at 5  $\mu\text{g}/\text{ml}$  had no bacteriolytic action on the coli.

#### *Lysis by Other Antibacterial Agents*

It was considered possible that *E. coli* Lilly might be an organism which autolysed readily when placed in an environment that was unfavourable for growth. Therefore the apparent bacteriolytic activity of serum would be attributable to the activation of an autolytic system in the organism rather than to any direct bacteriolytic effect of the serum itself. To explore this matter, the potential bacteriolytic activity of various antimicrobial agents was studied. The results in Table V show that apart from two cationic detergents, cetyltrimethyl ammonium bromide and lysolauryl ethanolamine, the various antibiotics and surface-active agents tested did not produce lysis under the conditions used. An interesting exception was lysozyme which had no lytic activity by itself at levels up to 400  $\mu\text{g}/\text{ml}$  but which was actively lytic at low levels (5  $\mu\text{g}/\text{ml}$ ) in the presence of ethylene diamine tetra-acetate (EDTA), as described by Repaske (12, 13). EDTA without lysozyme was not lytic.

#### DISCUSSION

Of the different physico-chemical variables studied, one of the most important in determining the rapidity and completeness of lysis of *E. coli* Lilly by human serum was the ionic strength of the medium. For although lysis occurred at the physiological ionic strength 0.15, it was much less than at ionic strength 0.06. The optimum at 0.06 was observed in serum without added buffer (*i.e.* diluted in water) and also in the presence of tris and veronal/acetate buffers. The effect did not appear to be due to specific ions, since  $\text{K}_2\text{SO}_4$  could be used in place of NaCl as the principal electrolyte. The organism itself was not osmotically fragile at ionic strength 0.06, for no lysis occurred when heat-inactivated serum was used. However, the observed optimum does not appear to correspond with any of the recognized activities of complement, although Levine, Wasserman, and Mills (14) have recently reported that complement fixation is promoted by ionic strengths below physiological. These authors suggested that the effect might be related to an accelerated activation of C'1 to C'1-esterase. Ionic strengths above physiological are known to inhibit complement fixation (14), and immune hemolysis (6), and also to stabilize C'1

in the pro-esterase form (15). In the present study, ionic strength 0.2 completely inhibited bacteriolytic activity.

The pH of the bacteriolytic system also influenced lysis and showed an optimum in the range pH 8.3-8.5. This was observed in the absence of added buffer and also in the presence of tris, veronal-acetate, and borate buffers. As with the ionic strength optimum at 0.06, so the pH optimum at 8.3-8.5 does not correspond with recognized activities of hemolytic complement. Nevertheless, it is known that complement is most active on the alkaline side of neutrality. For example, Mayer *et al.* (6) reported higher hemolytic activity at pH 7.6 than at pH 7.1. Becker (16) found eluted C'1-esterase (guinea pig) to be optimally active at pH 7.7, and Austen (17), working with human serum reported that the TAME-esterase activity of complement-treated antigen-antibody aggregates had an optimum at pH 8.4. However, in view of the participation of lysozyme in the present system, consideration should be given to the finding of Noller and Harstell (18, 19), extending earlier work by Nakamura (20), that high pH promotes the action of lysozyme on Gram-negative bacteria. If, therefore, the over-all bacteriolysis is due to the joint action of complement and lysozyme, then pH 8.4 may be the maximum value at which complement has adequate stability, yet is high enough to permit lysozyme to act.

A requirement for the four components of complement was established by experiments with R1, R2, R3, and R4, with heated serum, and with serum treated with an antigen-antibody system. An unexplained finding was that guinea pig serum, which has a higher titre of hemolytic complement than human serum, was less active in lysing *E. coli* Lilly. This did not appear to be due to lack of lysozyme, for the level of this agent was similar (2 to 5  $\mu\text{g}/\text{ml}$ ) to that present in human serum. Human serum heated at 56°C showed no enhancing effect when added to guinea pig serum. Normal pig serum had a bacteriolytic activity similar to human serum, but mouse serum was totally inactive, as might be expected from its deficiency of hemolytic (21) and bactericidal (22, 23) complement.

Properdin did not appear to be involved in the bacteriolysis of this organism, for a sample of RPb was as actively lytic as whole serum. It is likely, therefore, that *E. coli* Lilly belongs to the group of strains previously described (10) whose sensitivity to the properdin system, based on bactericidal tests, was classified as "unknown." It would, however, be desirable to obtain further data with other samples of serum and RPb.

Experiments on the absorption of serum with homologous cells at 0°C suggested initially that antibodies were involved in bacteriolysis, since a 56°C-heated serum restored activity to an absorbed serum in the classical manner. However, since egg white lysozyme had a similar activity in this system to heated serum, and since the serum lysozyme level was reduced by 80 per cent

by the absorption process, but was not affected by 56°C heating, it was concluded that lysozyme rather than antibody was the factor in the heated serum which restored bacteriolytic activity to the absorbed serum. (Muschel *et al.* (8) also have reported depletion of serum lysozyme by absorption of serum with Gram-negative bacteria.) The participation of the endogenous lysozyme of the serum in bacteriolysis was shown also by absorbing serum with bentonite, which is known to remove lysozyme. Serum treated in this manner had only a slight bacteriolytic activity against *E. coli* but became highly active when egg white lysozyme was added to it. It should be pointed out that this synergism of complement and lysozyme is not a new phenomenon and was described seven years ago by Amano *et al.* (24) and more recently by other investigators (8, 9).

For many years lysozyme was regarded as having little general importance in antibacterial immunity, since only the occasional organism appeared to be directly susceptible to its action. Recent work, however, has shown that lysozyme, in addition to its well known action on certain *Bacillus* and *Micrococcus* strains, is also active against most of the *Enterobacteriaceae* (18, 19). Gram-negative bacteria are not acted on directly by lysozyme but are, however, susceptible when certain co- and pretreatments with agents such as EDTA (12, 13), trypsin-butanol and high pH (18, 19), polymyxin (25), and alkyl-sulphate (26) are applied. The action of lysozyme on Gram-negative bacteria is probably a true enzymatic process for these organisms contain in their cell wall the mucopeptide substrate of lysozyme (27). Colobert (26) and Noller and Harstell (18, 19) suggested that the effect of the various co- or pretreatments with EDTA, etc., was to dissociate or disorganize a lipoprotein component of the Gram-negative bacterial cell wall, thereby allowing lysozyme to gain access to its substrate. In the present investigation, the bacteriolytic activity of normal human serum appeared to require the coparticipation of complement and lysozyme. In the light of the foregoing, it appears that complement may have basically the same action as agents such as EDTA, namely to prepare the way for lysozyme action by disorganizing or dissociating lipid or lipoprotein components of the cell wall. That is to say, the site of action of complement may be a cell wall lipid or lipoprotein. This is in accord with a previous suggestion (4) based on comparing the cell wall compositions of Gram-negative bacteria and of mammalian erythrocytes with those of Gram-positive bacteria. It was pointed out that the two former classes of cells have walls consisting mainly of lipoprotein whereas the Gram-positive bacteria, which as a group are generally considered to be resistant to destruction by complement, have walls relatively deficient in lipid and protein. The suggestion that complement may attack the phospholipid component of cell membranes has also been made by Ginsburg (28), following a study of the inhibitory action of phospholipids on the destruction of ascites tumor cells by antibody and complement.

The particular organism used in this investigation was chosen from a group of *E. coli* strains on the grounds of its high sensitivity to the bacteriolytic action of serum, a factor which should be borne in mind when considering the unexpected finding that neither antibody nor properdin appeared to be involved in bacteriolysis. The possibility of complement acting directly on bacteria was considered thirty years ago by Muri (29), "Accordingly, although the action of natural immune bodies concerned in bactericidal action has been demonstrated by the methods in a large number of instances, it appears scarcely justifiable to infer that this holds universally. The case is different from that of haemolysis, because complement is not fixed by red blood corpuscles unless they are sensitized by immune body, whereas, bacteria like various organic particles absorb complement directly and it may be that complement thus combined may sometimes have bactericidal effect." Further insight into this matter may be gained from a consideration of the surface chemistry of Gram-negative cells. Thus smooth Gram-negative organisms are less sensitive to serum bactericidal action than are rough strains. Smooth strains also differ from rough strains in their isoelectric point which, in turn is a reflection of differences in the chemistry of the cell surface, notably the predominance of O-antigen polysaccharide on the surface of the smooth form. Mudd, McCutcheon, and Lucké (30) have shown that one of the effects of antibody on a smooth Gram-negative bacterium is to raise the isoelectric point to a value close to that exhibited by a rough strain without antibody. Brown and Broom (31) suggested that a primary role of antibody in immune hemolysis was to raise the isoelectric point of the cell to a value such that electrostatic attraction of complement to the cell surface can take place. In the case of coli Lilly which is a very rough strain, analysis of isolated cell walls (32) has shown an extremely low content of lipopolysaccharide, or O-antigen. Moreover, this lipopolysaccharide is chemically primitive in that it contains glucose as its only non-amino sugar. The possibility is, therefore, that the cell wall of this organism may be suitable for direct complement action without the need for serum proteins such as antibody or properdin to combine with, and cover up polysaccharides on the cell surface.

With the increased knowledge of bacterial cell wall structure gained in recent years, it is of interest to consider what types of cell wall substances might form the substrate for the bacteriolytic reaction. For example, is it the lipopolysaccharide endotoxin as suggested by Rowley (33) and by Michael and Landy (34)? Possibly the concept of a single type of macromolecule acting as substrate may be inappropriate. Also the term "substrate" should at this stage be placed in quotation marks to indicate that the change produced in it by the serum factors may involve not the splitting of covalent bonds, as in an enzymatic reaction, but changes only in secondary valencies. In addition, it may be that different components of the cell surface mosaic act as "substrates" for



the different components of the bacteriolytic system. The evidence to date suggests that lipopolysaccharide is the "substrate" of antibody and of properdin (when involved), lipid B or lipid B protein complex is the "substrate" of complement, and cell wall mucopeptide the substrate of lysozyme. The over-all bacteriolytic reaction can then be regarded as involving the interaction of all these serum and cell wall components in a proper sequence, the result being a dislocation or dissolution of areas of the cell wall in a manner as yet unknown.

#### SUMMARY

The bacteriolytic activity of normal human serum on a rough strain of *E. coli* has been studied by a turbidimetric method.

Bacteriolysis was found to be markedly dependent on ionic strength and pH, with optima at  $\mu = 0.06$  and pH 8.3–8.5, respectively. The method of cultivating the cells also influenced the rapidity of lysis.

Lysis was temperature-dependent and was exhibited by all samples of human serum tested. Microscopically, organisms incubated with serum were observed to swell, lose their rod shape and eventually burst, leaving remnants of the cell membrane in suspension. Sphaeroplasts were obtained by brief exposure of cells to serum followed by dilution into 5 per cent sucrose.

The bacteriolytic reaction was shown to require complement. No definite requirement for properdin or specific antibody in this system could be demonstrated by the absorption of serum with zymosan and with homologous cells respectively. The latter procedure was found to reduce bacteriolytic activity by removal of serum lysozyme. Absorption of serum with bentonite also led to loss of bacteriolytic activity which could be restored with lysozyme. The organism was not lysed by lysozyme alone, but lysis occurred with lysozyme + EDTA in tris buffer.

The possibility of complement acting independently of antibody or properdin, in certain instances, is discussed in relation to bacterial cell wall structure. Data are presented supporting the hypothesis that the "substrate" of complement in cell membranes is a lipid or lipoprotein.

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