INTERACTIONS OF THE COMPLEMENT SYSTEM WITH THE SURFACE AND ENDOTOXIC LIPOPOLYSACCHARIDE OF VEILLONELLA ALCALESCENS

By HOWARD A. BLADEN, Ph.D, HENRY GEWURZ, M.D., and
STEPHAN E. MERGENHAGEN, Ph.D.

*(From the Laboratory of Histology and Pathology and the Immunology Section of the
Laboratory of Microbiology, National Institute of Dental Research, National
Institutes of Health, Bethesda, Maryland)*

PLATES 93-98

The antibody-complement system required for immune hemolysis has been studied extensively and is being defined rapidly (1–5). The effect of antibody and complement (C') on the red blood cell recently assumed a dramatic morphological perspective when it was demonstrated that discrete holes or lesions were produced on the erythrocyte cell membrane during immune hemolysis (6).

Lysis and inhibition of growth of Gram-negative bacteria by mammalian serum can also be mediated by antibody and C' (7, 8). However, little is known about the mechanism of this reaction. In general, it is thought that the actual killing and lysis of the bacterial cell results from the effect(s) of C' and lysozyme (9, 10). It was suggested that C' action upon the lipoprotein layer of the cell membrane results in perforations of the cell surface, facilitating the access of lysozyme to its mucopeptide substrate. These events were postulated to culminate in lysis of the bacterial cell. Recently one step in this reaction, the presence of perforations or lesions after action of antibody and C', was demonstrated on spheroplasts of *Escherichia coli* (11). Mediation of these lesions by C' implied a possible similarity to the mechanism of action involved in the formation of lesions on erythrocytes. However, it was not established which membranes were affected by C', nor were the conditions necessary for this C'-mediated effect determined. In this report, attention is directed toward another Gram-negative genus, *Veillonella*, whose cell surface layers can be clearly differentiated by electron microscopy (12). The interaction of serum C' and the somatic endotoxic lipopolysaccharide of *Veillonella alcalescens* was investigated. The results clearly demonstrate that the lipopolysaccharide complex, as contained in the outer three-layer membrane of the cell (12), serves as a substrate for the action of serum C'. The results further suggest that in their reaction with lipopolysaccharide, the C' components go through a sequence of interactions similar to that required for immune hemolysis.

767

Materials and Methods

Materials

Culture.—Veillonella alcalescens, strain V5, was maintained in Rogosa's medium (13). This organism is an anaerobic, Gram-negative coccus originally isolated from human saliva and exhibits marked sensitivity to the bactericidal effects of fresh mammalian serum (14).

*Phenol-Water Extract ("LPS") of V. alcalescens.—*A biologically active lipopolysaccharide endotoxin was prepared from *V. alcalescens* by a minor modification of the phenol-water extraction procedure of Westphal and Lüderitz (15) as previously described (12). The material in the water-soluble phase was lyophilized and stored. Upon reconstitution, immediately prior to each experiment, it exhibited limited solubility in aqueous media and thus was used as a fine dispersion. This material is designated LPS throughout the text.[1]

*Rabbit Anti-Veillonella serum.[2]—*Antibody directed against *V. alcalescens* was prepared in rabbits as previously described (14).

*Sheep Erythrocytes.—*Sheep erythrocytes were obtained from the National Institutes of Health Animal Center, Farm Animal Unit, Poolesville, Md. 1 part of whole blood was aseptically drawn into 4 parts of Alsever's solution. The mixture was stored at 0–4°C for a minimum of 2 wk prior to use in hemolytic assays.

*Rabbit Anti-Sheep Erythrocyte Serum.—*The hemolysin was obtained from the Sylvana Chemical Company, Orange, N. J. (pool No. 0721651). Concentration of antibody for sensitization of the indicator erythrocytes was determined according to Mayer (1), and a 1:1000 dilution was used.

*Complement (C') Source.—*Fresh guinea pig serum was obtained in desiccated form from Baltimore Biological Laboratories, Baltimore, Md., and reconstituted prior to use with distilled water. In certain experiments fresh frozen guinea pig serum, obtained from Texas Biological Laboratories and maintained at − 70°C, was employed. Some preliminary experiments were performed with fresh human or precolostral calf serum (Colorado Serum Co., Denver, Colo.).

*Lysozyme.—*Crystalline egg white lysozyme was obtained from Worthington Biochemical Corp., Freehold, N. J. Dilutions of enzyme were made in distilled water for assay of lysozyme activity by a minor modification of the method of Hook, Carey, and Muschel (16). The enzyme was solubilized in a magnesium–saline solution or in Veronal-buffered saline when its effect upon the bactericidal or hemolytic activity of serum was tested.

*Bentonite-Absorbed serum.—*Bentonite was obtained from Fisher Chemical Co., Fair Lawn, N. J., and was washed twice in saline prior to use. 5 ml aliquots of serum were absorbed twice with 20 mg bentonite for 10 min at 0°C. Hemolytic C' activity of the absorbed serum was reduced 5–10% during this procedure, while detectable lysozyme activity was completely removed.

*Inhibition of C' activity.—*C'-depleted or C'-inhibited sera were prepared by (a) relatively selective inactivation of C' components by manipulations used to prepare "R-reagents" (1), carrageenin (17), and cobra venom factor C of Nelson (18, 19); (b) activation of the C' system with consequent depletion of hemolytic activity (1, 20, 21); and (c) inactivation of C' components and/or inhibition of intermediate reactions by various other compounds and reaction

---

[1] The following abbreviations are used throughout this paper: LPS, lipopolysaccharide endotoxin from *V. alcalescens*; PTA, phosphotungstic acid; GVB^{++}, Veronal-buffered saline supplemented with gelatin, calcium ions, and magnesium ions; EA, indicator sheep cells sensitized with rabbit antiserum; AHGG, aggregated human gamma globulins.

[2] Kindly prepared by Dr. Richard T. Evans.

conditions (1, 22–24).[8] The manipulations were performed and reagents prepared as indicated in Table I.

Methods

Electron Microscopic Procedures.—Initially whole cells of *V. alcalescens* were reacted with guinea pig and rabbit anti-*Veillonella* sera by a slight modification of the method of Evans,

TABLE I

Effect of Inhibition or Inactivation of Hemolytic C' Activity upon the Formation of Lesions on Veillonella Endotoxic LPS by Serum

Agent	Final concentration	Preincu-bation		Residual hemo-lytic C' titer	Lesions	Reference
		Time	Temp.			
		Min	*°C*			
1. Heat	—	20	56	<5	Negative	(1)
2. Hydrazine	0.075 M	90	37	<5	"	"
3. Zymosan*	1.35 mg/ml	90	37	30	"	"
4. "Midpiece" (euglobu-lin fraction)	—	—	—	<5	"	"
5. Aggregated human gamma globulins	3.2 mg/ml	60	37	<5	"	(20, 21)
6. Carrageenin	10 "	60	37	<5	"	(17)
7. Cobra venom factor C	1:1 (strength = 2½)	60	37	80	"	(18, 19)
8. Hypertonic NaCl	0.5 M	—	—	<5	"	(1)
9. Cold	—	480	0	10	"	(1, 22)
10. Congo red	0.5 mg/ml	—	—	<5	"	(23)
11. Hydrocortisone hemisuccinate	12 "	60	37	<5	"	(24)
12. Sodium gentisate	12 "	60	37	<5	"	(footnote 3)
13. Sodium salicylate	12 "	60	37	<5	"	"
14. Bentonite*	4 "	10	0	280	Strongly positive	(16)
No pretreatment (controls)	—	—	—	320	"	—

* Pretreated serum samples centrifuged for 10 min at 10,000 rpm and 0°C prior to assay.

Spaeth, and Mergenhagen (14). Certain subsequent experiments were performed using guinea pig serum without added antibody. Cells (0.3 ml) standardized to an OD of 1.0 at 660 mμ were reacted with 0.5 ml of 1:5 guinea pig serum and 1.2 ml of diluent (0.85% NaCl containing 0.063% MgCl₂·6H₂O). This reaction mixture was then incubated for 60 min at 37°C. The tubes were chilled and centrifuged; the resultant pellet was washed three times in diluent at pH 7.0 and resuspended. Control reaction mixtures were tested simultaneously. Microdrops were placed on Formvar-covered grids and negatively stained with 2% phosphotungstic acid

[3] Pickering, R. J., H. Gewurz, J. Gordon, and R. A. Good. Effects of sodium salicylate and related compounds on the complement system. In preparation.

(PTA) adjusted to pH 5.3 with 0.1 N NaOH. Surface membranes of *V. alcalescens* were visualized in sectioned material by methods previously described (12).

In order to demonstrate the effect of serum components on LPS, reaction mixtures containing 0.1 ml LPS (1 mg/ml), 0.5 ml guinea pig serum (1:5), and 0.4 ml diluent were incubated for 60 min at 37°C. After incubation, the material was sedimented and washed three times and the resultant pellet resuspended in 0.05 ml of diluent. A microdrop from the suspension was placed on a Formvar-covered grid and negatively stained with 2% PTA.

Ultrasonic fractionation of LPS (1 mg/ml) preparations were performed with a Branson Sonifier for 30 min at maximum output. The fractionated LPS was centrifuged at 105,000 g for 2 hr. The resultant sediment and supernate were lyophilized. Subsequent electron microscopic examination before and after reaction with fresh serum was performed as outlined above for negatively stained preparations.

In addition, untreated LPS was concentrated by centrifugation and the pellet was fixed with OsO_4, dehydrated through graded acetone, and embedded in Vestopal W. All preparations were examined in a Siemens Elmiskop I electron microscope at plate magnifications of 40,000 to 80,000 diameters.

Hemolytic C' Assays.—Two separate assays for hemolytic C' were used. In both, reactions were carried out in Veronal-buffered saline supplemented with gelatin, calcium ions, and magnesium ions (GVB^{++}) as described by Mayer (1). Indicator sheep cells were sensitized with the optimal concentration of rabbit antiserum in the presence of 0.01 M EDTA (15 min/30°C), washed, and resuspended to the appropriate cell concentration in GVB^{++}. This suspension is designated EA.

Correlation of Hemolytic C' Activity with Lesion-Inducing Capacity.—The effectiveness of various known inhibitors or inactivators of C' to destroy the capacity of fresh serum to produce lesions on LPS particles was determined by examination of negatively stained preparations. In parallel experiments, these pretreated sera were also assayed for their hemolytic capacity. An assay more sensitive than the conventional $C'H_{50}$ assay of Osler, Strauss, and Mayer (25) was used; smaller numbers of indicator cells were provided in smaller reaction volumes. In this assay, inhibitors or inactivators (1.0 ml) were reacted with serum dilutions (1.0 ml) for 60 min at 37°C. In some experiments, pretreated serum reagents (2.0 ml) were prepared. Serial dilutions of these test sera were made in 1.0 ml GVB^{++}; 0.5 ml EA (1 × 10^8/ml) and 1.0 ml GVB^{++} were added; and these final reaction mixtures were incubated for 60 min at 37°C. Buffer (5.0 ml) was added, the tubes were centrifuged, and cell lysis was determined on the basis of spectrophotometric determination of hemoglobin released into the supernatant.

Quantitation of the C'-Fixing Capacity of LPS.—A dilution of C' in 0.5 ml (containing 8–20 $C'H_{50}$) was incubated with 0.4 ml of GVB^{++} buffer or antiserum and 0.1 ml of the LPS suspension (10–3000 μg/ml) for 60 min at 37°C. An appropriate volume of GVB^{++} (usually 14.0 or 24.0 ml) was added and aliquots of this mixture were titrated for residual total C' activity ($C'H_{50}$/ml) according to the 7.5 ml assay of Osler et al. (25). C' component hemolytic activities were titrated by adaptations, previously described (19), of the methods of Mayer (1) and Nelson et al. (5). Guinea pig serum was the source of C'1 (5), C'2 (5), classical C'3 (C'-EDTA) (1), and the C' used in preparation of EAC'1,4. (1).

Bactericidal C' Activity against V. alcalescens.—Bactericidal C' activity was assayed by a modification of the "limited antibody" assay previously employed with anaerobic bacteria (14). *V. alcalescens* (0.5 ml of 6 × 10^7/ml) was incubated at 37°C with 1.0 ml dilutions of guinea pig serum, 0.5 ml optimal antiserum, and 0.5 ml magnesium-saline solution (with added lysozyme in some experiments). The reaction was performed in the bulb of a Thunberg tube, inside which the atmosphere was replaced by 95% N_2–5% CO_2, because of the anaerobic requirement of the organism employed. After 60 min the reaction mixture was added to 10 ml broth in the lower portion of the tube and incubation at 37°C continued. The growth of the

surviving bacteria was quantitated by hourly optical density determinations on a Beckman B spectrophotometer. Optimal antibody dilution for this assay was determined in preliminary experiments and was 1:500 for the antiserum used.

RESULTS AND DISCUSSION

Surface Defects on V. alcalescens after Reaction with Fresh Guinea Pig Serum. —Generally, after treatment with guinea pig serum, negatively stained cells of *V. alcalescens* appeared identical with untreated cells. Occasionally, however, cells with numerous, circular, dense spots were observed (Fig. 1). These areas, approximately 90 A in diameter, were considered to be "pits" or lesions in the cell surface and were morphologically identical with those previously shown on *E. coli* spheroplasts after treatment with specific antisera and C'. Globular extrusions were present on many cells and frequently contained numerous lesions identical with those seen on the surface of the whole cell (Fig. 2). Since lesions were observed on what was apparently the cell surface of *V. alcalescens*, and since these lesions were identical with those observed on *E. coli* (11) and erythrocytes (6) due to the action of C', further studies were undertaken to determine which membrane was acted upon and whether the lesions were C'-dependent.

Structural Relationship of Cell Envelopes of V. alcalescens and the LPS Fraction.—As reported for many Gram-negative bacteria, *V. alcalescens* has a cell envelope consisting of a three-layered outer membrane (OM), an inner solid membrane (SM), and a three-layered plasma membrane (PM) (Fig. 3). After phenol-water extraction to isolate the LPS fraction, the cells remained intact, but the original outer three-layered membrane was not evident. The solid membrane became the new outermost layer (compare Figs. 3 and 4). The morphological relationship of these structural entities on another strain of *Veillonella* has been described (12).

The isolated LPS, when negatively stained with PTA, were generally circular particles of widely varying sizes (Fig. 5). The LPS particles, when embedded and sectioned, appeared as somewhat circular structures with an outer membrane composed of two dense layers approximately 30 A wide separated by a less dense layer of 25 A (Fig. 6). The total width of this membrane, approximately 90 A, coincided closely with that of the outer three-layered membrane shown in Fig. 3. It appeared, therefore, that it was possible to remove selectively the single outer, unit-type membrane from *V. alcalescens*. After removal, this membrane was morphologically identifiable as particles which represented fragments of the three-layered outer membrane present on the whole cell.

If one assumes that in the bactericidal reaction with Gram-negative bacteria the primary step is some alteration of the outer membrane due to serum factors like C', then isolated portions of the membrane (LPS) should also interact with

serum factors. Further, morphological alterations similar to those seen on whole cells after action of C' should be observed.

Defects on LPS Particles after Reaction with Guinea Pig Serum.—Generally two types of structural entities were observed in LPS preparations after the addition of fresh guinea pig serum. One was the circular particle with discrete edges previously described (designated "A" particle) (Fig. 5). Many A particles appeared much larger after treatment with serum than did untreated LPS particles. Many appeared broken or "fractured" (Fig. 7), revealing an upper and lower side. Material which stained with PTA could frequently be observed at the open end of such particles. The second structure observed in LPS preparations after the addition of serum was a diffuse, amorphous material without regular structure (Fig. 8). The latter was seen only occasionally in untreated LPS preparations and will be referred to as "B" particles.

To determine whether the two types of particles observed in LPS preparations after reaction with serum were related, LPS preparations were submitted to ultrasonic fractionation, ultracentrifugation, and lyophilization. Reconstituted pellet material contained numerous particles which appeared identical with intact A particles although virtually all these particles were cracked or fractured. Reconstituted supernatant material contained large amounts of B particles and a few very small A particles. Since B particles were rarely seen in untreated LPS preparations and since cracked A particles were observed along with B particles after exposure to serum or ultrasonic treatment, it is likely that the B particles represent material released from A particles by the action of serum.

Electron microscopic examinations of negatively stained LPS after treatment with guinea pig serum revealed numerous lesions on the surface of both A and B particles (Figs. 9–12).[4] The defects on A particles (Figs. 9–10) were 85–90 A in diameter with a less dense zone of 20 A surrounding the dense central deposition of PTA. They were usually in a more concentrated array than the lesions on B particles but did not appear in any fixed pattern. Occasionally two or three lesions would form a chain or share an adjacent side; however, for the most part, the lesions were randomly spaced. The outer edge of A particles showing lesions were not continuous as in untreated LPS particles; rather they were serrated or indented and similar to the outer edge of erythrocyte membrane fragments containing lesions (6). It should be noted that not all A particles contained lesions and that increased incubation time did not increase the number of particles with lesions.

By contrast, the lesions on individual B particles were not as numerous and frequently were arranged in chains of up to twelve lesions (Fig. 11). While these chains usually appeared along the edge of the particle, many had single

---

[4] Similar lesions were seen after incubation of LPS with fresh human serum or precolostral calf serum (Colorado Serum Co.).

lesions widely spaced along their outer edges (Fig. 12). These lesions were morphologically identical with those observed on A particles.

Occasionally, incomplete particles which appeared to be fragments of A particles were observed (Fig. 13). These particles had numerous randomly spaced lesions present which served to differentiate them from B particles.

The individual lesions in both A and B particles were very similar to the individual "rings" or perforations observed in phospholipids, lecithin and cholesterol, after saponin treatment (26, 27). Though individual lesions on the LPS particles and phospholipids were similar, arrangement of lesions was different. A regular hexagonal array of pits was demonstrated in lecithin-cholesterol-saponin mixtures. A similar structural relationship was shown when saponin was reacted with certain mammalian cell and virus envelopes (28). When LPS and guinea pig serum were reacted, only a random arrangement of lesions on A particles was observed, suggesting a possible difference in location or availability of reactor sites on the membrane surface. The chains of lesions in B particles were somewhat similar, although longer and more discrete, to the chains of rings observed in cholesterol-saponin mixtures.

Attempts to show lesions on LPS after reaction with saponin failed and they suggest that the lipid moiety of the LPS macromolecule does not have appreciable amounts of lecithin or cholesterol. Whole cells of a number of Gram-negative bacteria do not contain either lecithin or cholesterol (29). In contrast, both lecithin and cholesterol are recognized as important constituents of many eucaryotic biological membranes.

C' Requirement for Lesion Formation on LPS Particles.—

To determine whether the appearance of lesions on the LPS particles after incubation with fresh guinea pig serum could be attributed to the action of the C' system, rather than alternate serum enzymes and/or other factors, a number of manipulations known to reduce or inhibit C' activity were performed with the serum prior to, as well as during, incubation with LPS. The ability of these pretreated sera to cause lesions on LPS as well as hemolysis of sheep cells was compared. The results of these experiments are summarized in Table I.

The procedures used in preparing so-called R-reagents (1), which are deficient in certain of the classical C' components and therefore unable to lyse sensitized erythrocytes, resulted in inability of the treated sera to form lesions on LPS. Similarly, serum reacted with either carrageenin or cobra venom factor C, stated to be specific inhibitors of C'1 and C'3c, respectively, lost its ability to induce lesions on LPS and had markedly diminished ability to hemolyze sensitized erythrocytes.

Reaction with aggregated human gamma globulins (AHGG) resulted in a loss of the capacity of the serum to hemolyze red blood cells and to induce lesions on LPS.

Agents such as hydrocortisone, salicylate compounds, and Congo red, all

known inhibitors of hemolytic C', also prevented lesion formation on LPS by serum.

Reaction conditions known to prevent or greatly retard hemolysis, e.g. hypertonic salt solutions and 0°C temperature, also prevented lesion formation on LPS. Experiments to determine the effect of chelation by EDTA upon lesion formation were not performed because LPS dissolved readily in the serum-EDTA mixture. By all parameters tested, loss of hemolytic C' activity correlated well with loss of capacity to induce lesions on LPS.
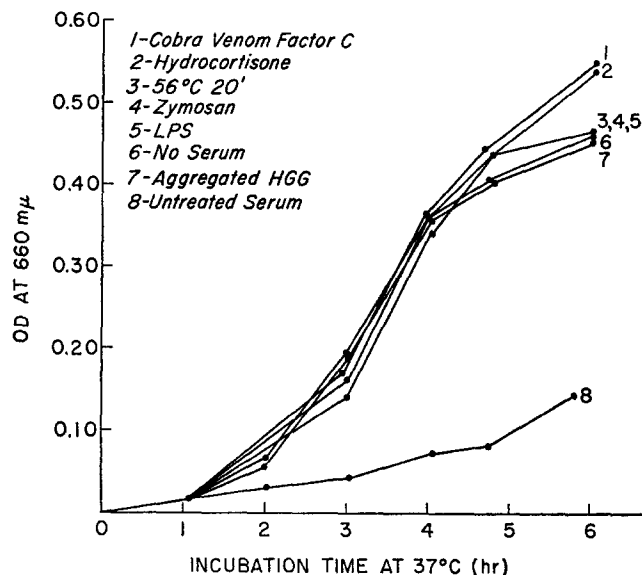
TEXT-FIG. 1. Inhibition of the serum bactericidal reaction against *V. alcalescens* by agents found to prevent lesion formation on LPS.

Effect of Inhibitors of Lesion Formation upon Serum Bactericidal Activity against V. alcalescens.—

It was determined whether agents and manipulations which inhibited lesion formation on the LPS would also inhibit the bactericidal effects of serum against the organism from which the LPS was isolated. Sera were pretreated with cobra venom factor C, hydrocortisone, heat, zymosan, *V. alcalescens* LPS itself, and AHGG.

The bactericidal potential of each serum so pretreated was lost (Text-fig. 1).

C' Fixation by LPS.—Since the results shown in Table I clearly demonstrated a C' dependence for lesion formation on LPS, it was considered that C' could be "fixed" during the process. Indeed, numerous investigators have found that various endotoxin preparations are "anticomplementary" (30–33).

Therefore, study of various parameters influencing the C' effect on *V. alcalescens* LPS was initiated.

LPS proved to be potent in its ability to "fix" C' (Text-fig. 2), e.g. 20 μg of LPS could fix 8 of 12 C'H_{50} of guinea pig C' during the 1 hr incubation period prior to hemolytic assay. No zone of antigen excess was observed. Exogenous antibody was not added to the normal guinea pig serum in these C' titrations. These results indicated that in our initial electron microscopic observations of lesions on the LPS, which involved reaction of 100 μg of LPS with approxi-
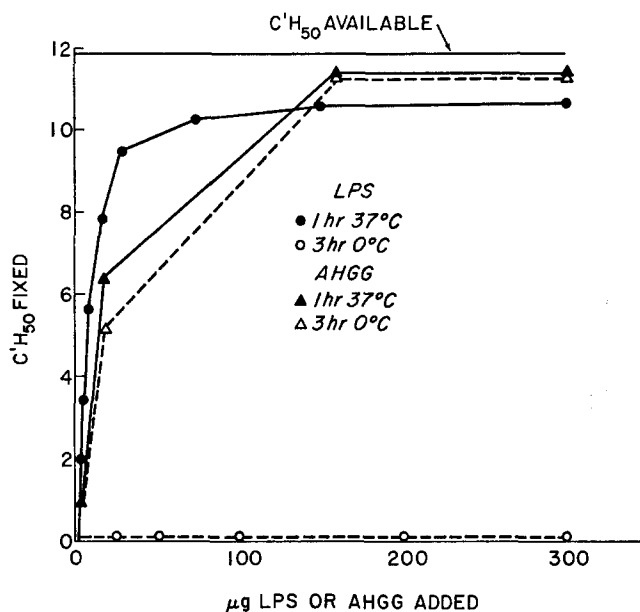
Text-Fig. 2. The effect of various amounts of LPS and AHGG on total hemolytic C' activity in guinea pig serum at 0°C and 37°C.

mately 20 C'H_{50} guinea pig C', the LPS substrate was greatly in excess of the least amount required for maximal C' fixation. This may provide a partial explanation for the absence of lesions on some LPS particles.

LPS was actively anticomplementary at 37°C in amounts as low as 4 μg, but not detectably so at 0°C in amounts as high as 300 μg (Text-fig. 2). Incubation of 10 μg LPS with guinea pig serum at 37°C led to progressive fixation until, for reasons not yet clear, a maximum fixation of C', always less than 100%, was reached after about 3 hr (Text-fig. 3). In contrast, no fixation of total C' occurred at 0°C. Indeed, reactions initiated at 37°C abruptly stopped fixing C' when the temperature was lowered to 0°C. Conversely, temperature elevation from 0°C to 37°C resulted in C' uptake in mixtures of

LPS and guinea pig serum. From these experiments, it was evident that C' fixation by LPS particles, like C'-dependent hemolysis of a sensitized erythrocyte (1, 22), was markedly dependent on time and temperature. Even though LPS had previously reacted with guinea pig serum for prolonged intervals at 0°C, was present when the sensitized erythrocytes were introduced, and had equal time for interaction at 37°C, it did not exhibit any ability to reduce the hemolytic activity of the guinea pig serum against the newly introduced cells. When LPS was preincubated at 37°C before addition of sensitized erythrocytes, hemolytic activity was reduced. This suggests that an interval of preincubation
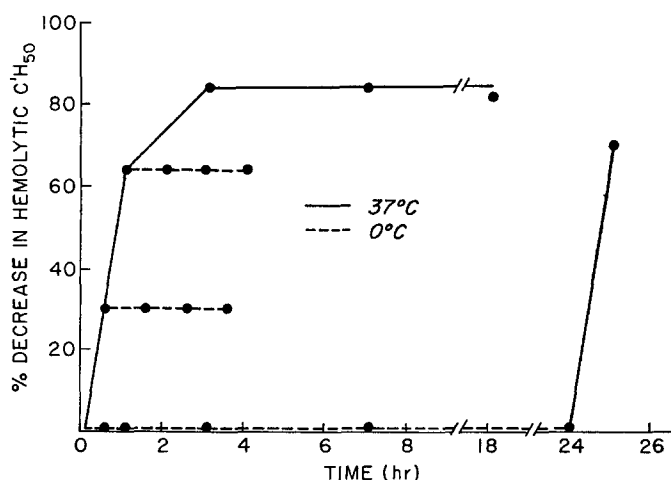
TEXT-FIG. 3. The effect of temperature on C'-fixation by 10 μg LPS during prolonged incubation. 12 $C'H_{50}$ were provided in the reaction mixture.

is necessary for LPS to divert C' activity otherwise available for other antigen-antibody systems.

Electron microscopy revealed that no defects were produced on LPS particles during incubation with guinea pig serum at 0°C. Temperature dependence for lesion formation on the erythrocyte membrane has been noted by others (34).

The ineffectiveness of microgram amounts of LPS to lower total C' hemolytic activity of serum at 0°C contrasts with the interaction of similar amounts of AHGG and serum. As indicated in Text-fig. 2, AHGG seemed equally anti-complementary at 37°C or 0°C. The inability of lipopolysaccharides and zymosan to fix significant amounts of C' at 0°C has been observed by others (30, 35)

The relative effects of increasing amounts of LPS and AHGG on the classical four C' component activities is shown in Table II. LPS fixed only small amounts of the early acting components ($C'1$, $C'4$, and $C'2$), but by compari-

son fixed large amounts of C'3. AHGG, on the other hand, fixed larger amounts of the early acting components but much less C'3. This relationship held when the amounts of total C' fixed by the two agents was similar (as with 10 μg LPS and 100 μg AHGG). These results suggest that antigens, as well as gamma globulins, may participate in C'-fixation by antigen-antibody complexes. Similar relative effects on C' component activities had been noted by Pillemer et al. (35) in comparing reactivity of human serum with zymosan and immune

TABLE II

Comparison of the Effects of V. alcalescens LPS and AHGG upon C' and C' Component Hemolytic Activities in Guinea Pig Serum

C' "fixation" expressed in 50% units*

| Material tested	Amount	C'	C'1	C'4	C'2	C'3
	μg					
LPS	10	6.2	<1000	<500	<300	61.5
LPS	25	10.0	<1000	<500	<300	76.9
LPS	100	>12.1	<1000	<500	<300	86.4
LPS	200	>12.1	<1000	<500	<300	88.4
AHGG	10	0.9	2500	2900	925	<10.0
AHGG	25	3.4	4150	4100	1225	<10.0
AHGG	100	6.7	5450	>4300	1525	31.0
AHGG	200	9.5	5830	>4300	1650	36.5
None	—	0	0	0	0	0
Activity available		14.0	6300	4500	2650	91.5

* V. alcalescens LPS and AHGG were incubated with 14.0 C'H$_{50}$ guinea pig C' in 1.0 ml buffered solution at 37°C for 1 hr. Residual C' component hemolytic activities were measured by assay with intermediates. In separate experiments, fixation of early-acting C' components by LPS, and formation of C'1a 4 sites on LPS were demonstrated.

complexes. This differential utilization of C' components may provide partial explanation of the differences observed in C'-fixation by LPS and AHGG at 0°C (Text-fig. 2).

The addition of rabbit anti-Veillonella serum increased the amount of C' fixed by LPS at 37°C, and further, this heterologous immune antibody allowed detectable C'-fixation to occur at 0°C. These experiments, summarized in Text-fig.4, defined the antibody concentration needed to potentiate the C'-fixing ability of LPS and to insure the experimental condition of "antibody excess." Whether the difference in the initiation of the C'-fixation by immune serum and factors normally present in serum (probably "natural" antibodies) was on a qualitative or quantitative basis we do not know. The interaction at

0°C, even in the presence of immune antibody, did not result in formation of electron microscopically visible defects on the LPS surface or in C'3 fixation.

The necessity of antibody for the lesion effect on LPS could not be critically tested since definitive exclusion of "natural" antibody in the C' source, directed against haptenic groups on the LPS macromolecule, was not possible. Attempts to absorb antibody with LPS resulted in partial solubilization of the LPS so that the total amount was no longer recoverable by centrifugation. This solubilized material was detectable in two ways: C' activity in absorbed serum was decreased during a 2 hr incubation period at 37°C, and C' activity in this serum was reduced selectively upon addition of rabbit anti-*Veillonella* serum during either 37°C or 0°C incubation. Therefore, all LPS or LPS-serum protein complexes had not been removed from the absorbed sera. Similarly, three successive absorptions with whole cells of *V. alcalescens* did
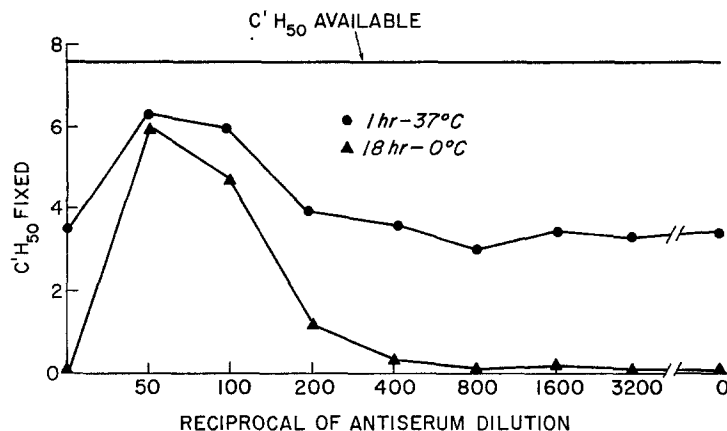
Text-Fig. 4. The effect of rabbit anti-*Veillonella* serum on C' fixation by 10 μg LPS at 0°C and 37°C

not reduced the reactivity of the test serum to LPS. Whether this resulted from similar solubilization of surface antigens or whether antigens on the LPS surface are not readily exposed on the surface of the intact bacterial cell is not yet clear. In any event, absorption emerged as a nonvalid criteria for determining the requirement for antibody in the LPS-C' interaction.

Effect of Lysozyme on Lesion Formation on LPS and on Bactericidal Activity against V. alcalescens.—

Previous investigators found that certain Gram-negative organisms were more susceptible to the bactericidal action of serum in the presence of lysozyme than in its absence. This led them to postulate a synergistic action of C' and lysozyme in the bactericidal reaction (9, 10).

To evaluate the role of lysozyme in the bactericidal reaction against *V. alcalescens*, experiments with lysozyme depletion and addition were performed. In the first set of experiments, bentonite-absorbed serum, which contained adequate hemolytic C' activity but was devoid of demonstrable lysozyme activity, caused lesion formation on the LPS particle. These lesions had the same appearance as had those with untreated serum. Further, addition of 20 μg egg white lysozyme did not alter the morphological appearance of these lesions.

In the next set of experiments, summarized in Text-fig. 5, the addition of lysozyme did not significantly augment the ability of guinea pig serum to inhibit bacterial growth. Serum absorbed with bentonite did exhibit a decreased bactericidal activity while hemolytic C' activity was only slightly decreased. The bactericidal capacity of this serum was not restored by readdition of lysozyme.

GENERAL CONSIDERATIONS

The intimate involvement of C' in the serum bactericidal reaction against certain Gram-negative bacteria is well documented (1, 3, 4, 8). In recent years, electron microscopic examination of cell-antibody-C' interactions has indicated
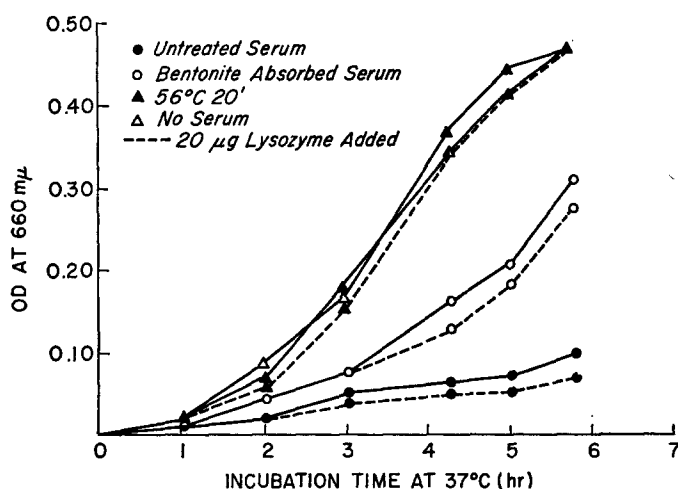
TEXT-FIG. 5. The effect of bentonite absorption and lysozyme supplementation on the serum bactericidal reaction against *V. alcalescens*.

that the C' enzymes initiate modifications of membranes of various mammalian (6, 37, 38) and bacterial (11) cells. Our recent observations with *E. coli* (11) demonstrated that C'-dependent lesions, perhaps complete perforations, were present on what was probably the surface of the spheroplast. In studies with *Veillonella* (12) it was possible to remove the single outer membrane selectively and to recover the lipopolysaccharide fraction.

In the present study it has been found that C' can induce electron microscopically visible lesions on the water-soluble, phenol-extractable LPS of *V. alcalescens*, i.e. upon "endotoxin" as defined by the extraction procedure of Westphal and Lüderitz (15), as well as upon the surface of the parent organism.

A role of C' in the formation of lesions on the LPS particles seems certain and probably is similar to its role in mediating lesions on the red blood cell. The diverse procedures of C' inhibition or inactivation used in this study, each

preventing lesion formation, together with uptake of hemolytic C' and C' components during the process of lesion formation, seem to establish this point.

Two attempts to describe the derivation of the LPS ("A" particle) from the intact cell as well as subsequent formation of "B" particles are presented in Text-fig. 6. In this scheme, the space between the outer membrane and the solid membrane of the parent organism contains material which becomes enclosed within the outer membrane as it folds and separates from the remainder
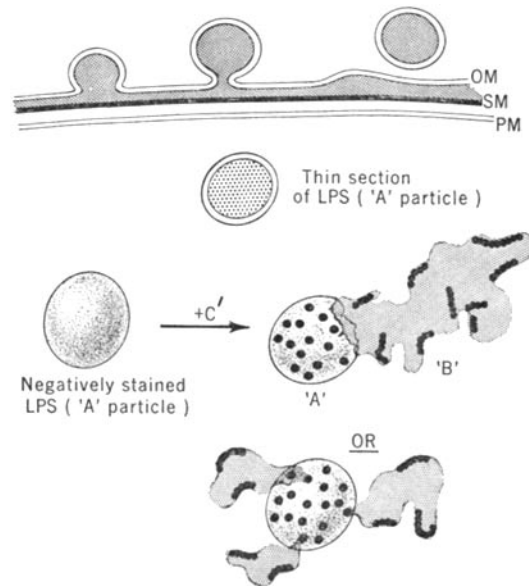
TEXT-FIG. 6. A schematic representation of possible derivation of LPS from the cell membrane of *V. alcalescens*, with subsequent formation of "A" and "B" particles by guinea pig serum. The three-layered outer membrane (OM), solid membrane (SM) and plasma membrane (PM) can be seen.

of the cell. The formation of LPS-containing globular structures or particles at the surface of the cell is similar to that illustrated by Knox, Vesk, and Work with *E. coli* (36). This intact particle is the described A particle, and contains B particle material. The latter could be liberated by the action of C' or by sonification. Numerous randomly spaced lesions on A particles might induce rupture and allow release of the B particle material. Alternatively, the lesions could be viewed as portals for leakage of the material through the membrane. For this case the lesions must extend completely through the three-layered membrane. As a third possibility, not illustrated in the figure, the B particles may represent ruptured and flattened A particles. The definite difference in the arrangement of the lesions present on B particles after C' action makes

this latter proposal seem unlikely. In any event, the presence of C'-mediated lesions on both types of particles indicates that both contain sites reactive with C'.

An observation which at present can only invite speculation is the presence of numerous lesions on certain single LPS particles while otherwise similar particles had none. If each lesion on a particle was the result of a single initiation of a C' sequence, one would expect single or multiple lesions on many particles rather than many lesions on single particles. On the other hand, if the formation of the initial lesion sensitized the adjacent membrane to a transferrable product in turn responsible for lesion formation (37), or if "transferring" antibodies or antibody–C' complexes were present in limiting amounts (39, 40), a "spraying" of hole formation would be seen. Rosse, Dourmashkin, and Humphrey (37) found that a single C' sequence is responsible for the formation of more than one lesion on the erythrocyte, and suggested that a single lesion may initiate lysis while others might be superfluous to this end. If an analogy can be made between *Veillonella* LPS and the mammalian erythrocyte, our results support these findings.

Attempts to determine whether antibody was necessary for lesion formation were unsuccessful. The solubilization of factors reactive with C' from LPS and intact organisms made absorption a noncritical test. Small amounts of bound or unbound antibody ("natural" antibody) may have remained in the serum along with the LPS material. An interaction of LPS and components of C' without initiation by antibody, as postulated by others (33, 41), could therefore not be excluded. Pillemer et al. (35) had claimed an interaction between C' and certain polysaccharides without an involvement of antibody, but an antibody requirement subsequently was shown (42, 43). While more data are needed, we feel it is not unlikely that the LPS-C' interaction studied here is also directed and initiated by antibody.

Several investigators (10, 44, 45) have suggested that LPS may serve as a substrate for the C' enzymes. The present investigation clearly shows that this is the case. The nature of an antigen, e.g. the presence of C' substrates and their proximity to haptenic sites, may well influence its reactivity with C'. Earlier it had seemed that the role of antigen in the C' reaction was primarily the modification or "aggregation" of antibody molecules (20, 21, 46). More recently, interactions between several C' components and the surface of cells have been demonstrated (3). Indeed the erythrocyte has been shown to accept numerous $\beta 1C$ (C'3c) molecules for each C'2 molecule activated on its surface (47). It is conceivable that endotoxic LPS is more reactive with C'3 than is AHGG (Table II) because of an ability to support more fully C' component interactions in the initiated C' sequence. This may contribute to certain biological properties of endotoxin.

The generalizing term most used in the past to describe the effects of endo-

toxin on C' in vivo (30, 32) and in vitro (30, 32, 33) has been "anticomplementary." The present studies have confirmed that LPS lowers C' titers in vitro, but have indicated that rather than by inhibition of C' (as suggested by the term anticomplementary), there seems to be a utilization of the entire C' sequence.

The role of lysozyme in the bactericidal reaction with *V. alcalescens* seems minimal. Serum absorbed with bentonite had no detectable amount of lysozyme, yet gave a positive bactericidal effect. Such serum still produced lesions on LPS. This was not surprising since lysozyme is active against β-1-4 linkages of mucopeptides (48) and not LPS. Further, electron microscopic studies on *E. coli* (49), *Spirillum serpens* (50), and *Veillonella parvula* (12) demonstrated that lysozyme acts upon the solid membrane; no effect of lysozyme was evident upon the outer LPS-containing membrane.

In the interaction between LPS and fresh mammalian serum, lesions dependent upon an intact C' system were formed on the LPS particles, concomitant with a loss of hemolytic activity ("fixation") in the reaction mixture. The C' system, by analogy with hemolytic C' (34), therefore exerts its terminal component effect of lesion induction upon the LPS. This sequence of events is known to include as by-products the promotion of phagocytosis and aggregation phenomena (3, 4, 51), the generation of anaphylatoxin (52, 53), and the production of factors chemotactic for neutrophils (54, 55). It has long been known that injection of bacterial endotoxins into higher vertebrates induces aggregation of platelets and polymorphonuclear leukocytes, along with neutrophil chemotaxis and alterations of vascular permeability (56, 57). Since C' interacts with LPS through points in the C' sequence that involve generation of biologically active factors, it may well be through the C' system, as suggested by others (30, 32, 58), that certain host reactivities to endotoxin are initiated or potentiated.

SUMMARY

Electron microscopic studies demonstrated that lesions were produced on the endotoxic lipopolysaccharide (LPS) as well as on the cell surface of *V. alcalescens* after reaction with fresh guinea pig serum. These lesions were approximately 90 A in diameter, and were seen on two characteristic structural entities derived from LPS preparations after incubation with serum. The use of numerous inhibitors, inactivators, and reaction conditions affecting hemolytic C' activity revealed that these lesions were mediated by the C' system. Concomitant with lesion formation, C' was fixed; the effect on classical C'3 activity was pronounced. It is concluded that endotoxic LPS, as contained in the outer three-layered membrane of the bacterial cell, is a substrate for the C' enzymes. It is suggested that certain biological activities of endotoxin may derive from its effects on the C' system.

BIBLIOGRAPHY

1. Mayer, M. M. 1961. Complement and complement fixation. *In* Experimental Immunochemistry. E. A. Kabat and M. M. Mayer, editors. Charles C. Thomas, Springfield, Illinois. 2nd edition. 133.
2. Mayer, M. M. 1964. Mechanism of haemolysis by complement. *Ciba Found. Symp. Complement.* 4.
3. Müller-Eberhard, H. J., U. R. Nilsson, A. P. Dalmasso, M. J. Polley, and M. A. Calcott. 1966. A molecular concept of immune cytolysis. *Arch. Pathol.* **82:**217.
4. Nelson, R. A., Jr. 1965. The role of complement in immune phenomena. *In* The Inflammatory Process. B. W. Zweifach, L. Grant, and R. T. McClusky, editors. Academic Press Inc., New York. 819.
5. Nelson, R. A., Jr., J. Jensen, I. Gigli, and N. Tamura. 1966. Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea pig serum. *Immunochemistry.* **3:**111.
6. Borsos, T., R. R. Dourmashkin, and J. H. Humphrey. 1964. Lesions in erythrocyte membranes caused by immune haemolysis. *Nature.* **202:**251.
7. Amano, T., K. Fujikawa, T. Morioka, A. Miyama, and S. Ichikawa. 1958. Quantitative studies on immune bacteriolysis. I. A new method of quantitive estimation. *Biken's J.* **1:**13.
8. Muschel, L. H., and H. P. Treffers. 1956. Quantitative studies on bactericidal actions of serum and complement. *J. Immunol.* **76:**1.
9. Amano, T., S. Inai, Y. Seki, S. Kashiba, K. Fujikawa, and S. Nishimura. 1954. Accelerating effect on the immune bacteriolysis by lysozyme-like substance of leucocytes and egg white lysozyme. *Med. J. Osaka Univ.* **4:**401.
10. Wardlaw, A. C. 1962. The complement-dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic strength and the role of lysozyme. *J. Exptl. Med.* **115:**1231.
11. Bladen, H. A., R. T. Evans, and S. E. Mergenhagen. 1966. Lesions in *Escherichia coli* membranes after action of antibody and complement. *J. Bacteriol.* **91:**2377.
12. Bladen, H. A., and S. E. Mergenhagen. 1964. Ultrastructure of Veillonella and morphological correlation of an outer membrane with particles associated with endotoxic activity. *J. Bacteriol.* **88:**1482.
13. Rogosa, M., R. J. Fitzgerald, M. E. MacKintosh, and A. J. Bemen. 1958. Improved medium for selective ioslation of Veillonella. *J. Bacteriol.* **76:**455.
14. Evans, R. T., S. Spaeth, and S. E. Mergenhagen. 1966. Bactericidal antibody in mammalian serum to obligatorily anaerobic gram-negative bacteria. *J. Immunol.* **97:**112.
15. Westphal, O., and O. Lüderitz. 1954. Chemische enforschung von lipopolysacchariden gram negativer bakterien. *Angew. Chem.* **66:**407.
16. Hook, W. A., W. F. Carey, and L. H. Muschel. 1960. Alterations in serum lysozyme and properdin titers of mice following X-irradiation or treatment with zymosan or endotoxin. *J. Immunol.* **84:**569.
17. Davies, G. E. 1965. Inhibition of complement by carrageenin: mode of action,

effect on allergic reactions and on complement of various species. *J. Immunol.* **8**:291.

18. Nelson, R. A., Jr. 1966. A new concept of immunosuppression in hypersensitivity reactions and in transplantation immunity. *Surv. Ophthamol.* **11**:498.

19. Gewurz, H., D. S. Clark, J. Finstad, W. D. Kelly, R. L. Varco, R. A. Good, and A. E. Gabrielsen. 1966. Role of the complement system in graft rejections in experimental animals and man. *Ann. N.Y. Acad. Sci.* **129**:673.

20. Ishizaka, T., and K. Ishizaka. 1959. Biological activities of aggregated gamma globulin. I. Skin reactive and complement-fixing properties of heat denatured gamma globulin. *Proc. Soc. Exptl. Biol. Med.* **101**:845.

21. Christian, C. L. 1960. Studies of aggregated γ-globulin. I. Sedimentation, electrophoretic and anticomplementary properties. *J. Immunol.* **84**:112.

22. McVickar, D. L., and G. R. Weinfeld. 1959. Immune hemolysis at 0°. I. Introduction: the role of amboceptor and complement. *J. Immunol.* **82**:290.

23. Gordon, J. 1930. The action of certain dyes on the bactericidal activity of normal serum and on haemolytic complement. *J. Pathol. Bacteriol.* **33**:47.

24. Gewurz, H., P. Wernick, P. G. Quie, and R. A. Good. 1965. Effects of hydrocortisone succinate on the complement system. *Nature.* **208**:755.

25. Osler, A. G., J. H. Strauss, and M. M. Mayer. 1952. Diagnostic complement fixation, I. A method. *Am. J. Syphilis, Gonorrhea, and Venereal Diseases.* **36**:140.

26. Bangham, A. D., and R. W. Horne. 1964. Negative staining of phospholipids and their structural modifications by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* **8**:660.

27. Lucy, J. A., and A. M. Glauert. 1964. Structure and assembly of macromolecular lipid complexes composed of globular micelles. *J. Mol. Biol.* **8**:727.

28. Dourmashkin, R. R., R. M. Dougherty, and R. J. C. Harris. 1962. Electron microscopic observations on Rous sarcoma virus and cell membranes. *Nature.* **194**:1116.

29. Scheuerbrandt, G., and I. Black. 1962. Unsaturated fatty acids in microorganisms. *J. Biol. Chem.* **237**:2064.

30. Gilbert, V. E., and A. I. Braude. 1962. Reduction of serum complement in rabbits after injection of endotoxin. *J. Exptl. Med.* **116**:477.

31. Kostka, J., and J. Sterzl. 1962. The action of endotoxin on complement. *Folia Microbiol. (Prague).* **7**:191.

32. Pearlman, D. S., J. B. Sauers, and D. W. Talmadge. 1963. The effect of adjuvant amounts of endotoxins on the serum haemolytic complement activity in rabbits. *J. Immunol.* **91**:748.

33. Muschel, L. H., K. Schmoker, and P. M. Webb. 1964. Anticomplementary action of endotoxin. *Proc. Soc. Exptl. Biol. Med.* **117**:639.

34. Hadding, U., H. J. Müller-Eberhard, and A. P. Dalmasso. 1966. Isolation of the terminal component of human complement. *Federation Proc.* **25**:485.

35. Pillemer, L., L. Blum, I. H. Lepow, D. A. Ross, E. W. Todd, and A. C. Wardlaw. 1954. The properdin system and immunity: I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science.* **120**:279.

36. Knox, K. W., M. Vesk, and E. Work. 1966. Relation between excreted lipopoly-

saccharide complexes and surface structures of a lysine limited culture of *Escherichia coli. J. Bacteriol.* **92**:1206.

37. Rosse, W. F., R. Dourmashkin, and J. H. Humphrey. 1966. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. III. The membrane defects caused by complement lysis. *J. Exptl. Med.* **123**:969.

38. Humphrey, J. H., and R. R. Dourmashkin. 1964. Electron microscope studies of immune cell lysis. *Ciba Found. Symp. Complement.* 175.

39. Willoughby, W. F., and M. M. Mayer. 1965. Antibody-complement complexes. *Science.* **150**:907.

40. Bowman, W. M., M. M. Mayer, and H. J. Rapp. 1951. Kinetic studies on immune hemolysis. II. The reversibility of red cell-antibody combination and the resultant transfer of antibody from cell to cell during hemolysis. *J. Exptl. Med.* **94**:87.

41. Skarnes, R. C. 1965. Nonspecific hemolysis of erythrocytes modified with bacterial endotoxins. *Ann. Inst. Pasteur.* **109**:66.

42. Nelson, R. A., Jr. 1958. An alternative mechanism for the properdin system. *J. Exptl. Med.* **108**:515.

43. Muschel, L. H. 1961. Antibody-complement system and properdin. *Vox Sanguinis.* **6**:385.

44. Rowley, D. 1956. Rapidly induced changes in the level of nonspecific immunity in laboratory animals. *Brit. J. Exptl. Pathol.* **33**:223.

45. Ginsburg, I. 1960. Action of phospholipids on the cytotoxic effect of rabbit antibodies against Ehrlich ascites tumor cells. *Brit. J. Exptl. Pathol.* **41**:648.

46. Osler, A. G. 1961. Functions of the complement system. *Advan. Immunol.* **1**:136.

47. Müller-Eberhard, H. J., A. P. Dalmasso, and M. A. Calcott. 1966. The reaction mechanism of B1C-globulin (C'3) in immune hemolysis. *J. Exptl. Med.* **123**:33.

48. Salton, M. R. J., and J. M. Ghuysen. 1959. The structure of *di*- and *tetra*-saccharides released from cell walls by lysozyme and *streptomyces* F_1 enzyme and the β (1 — > 4) N-acetyl-hexosaminidase activity of these enzymes. *Biochim. Biophys. Acta.* **36**:552.

49. Murray, R. G. E., P. Steed, and H. E. Elson. 1965. The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other gram negative bacteria. *Can. J. Microbiol.* **11**:547.

50. Murray, R. G. E. 1963. On the cell wall structure of *Spirillum serpens. Can. J. Microbiol.* **9**:381.

51. Gerlings-Petersen, B. T., and K. W. Pondman. 1962. Erythrophagocytosis: a study of the antigen-antibody-complement reaction. *Vox Sanguinis.* **7**:655.

52. Osler, A. G., H. G. Randall, B. M. Hill, and Z. Ovary. 1959. Studies of the mechanism of hypersensitivity. III. The participation of complement in the formation of anaphylatoxin. *J. Exptl. Med.* **110**:311.

53. Dias da Silva, W., and I. H. Lepow. 1965. Anaphylatoxin formation by purified human C'1 esterase. *J. Immunol.* **95**:1080.

54. Boyden, S. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J. Exptl. Med.* **115**:453.

55. Ward, P. A., C. G. Cochrane, and H. J. Müller-Eberhard. 1965 The role of serum complement in chemotaxis of leucocytes *in vitro. J. Exptl. Med.* **122**:327.
56. Thomas, L. 1954. The physiological disturbances produced by endotoxins. *Ann. Rev. Physiol.* **18**:467.
57. Stetson, C. 1965. Endotoxins. *In* Bacterial and Mycotic Infections of Man. R. J. Dubos and J. G. Hirsch, editors. J. B. Lippincott Co., Philadelphia. 4th edition. 304.
58. Spink, W. W., and J. Vick. 1961. A labile serum factor in experimental endotoxin shock: cross-transfusion studies in dogs. *J. Exptl. Med.* **114**:501.

EXPLANATION OF PLATES

PLATE 93

FIG. 1. A negatively stained whole cell of *V. alcalescens* after reaction with fresh guinea pig serum. Thin areas of the cell reveal numerous dense spots approximately 90 A in diameter surrounded by a less dense ring of 20 A. × 112,000.

FIG. 2. Extrusions from a cell reacted with guinea pig serum. Lesions identical with those observed on the surface of whole cells are seen. × 128,000.
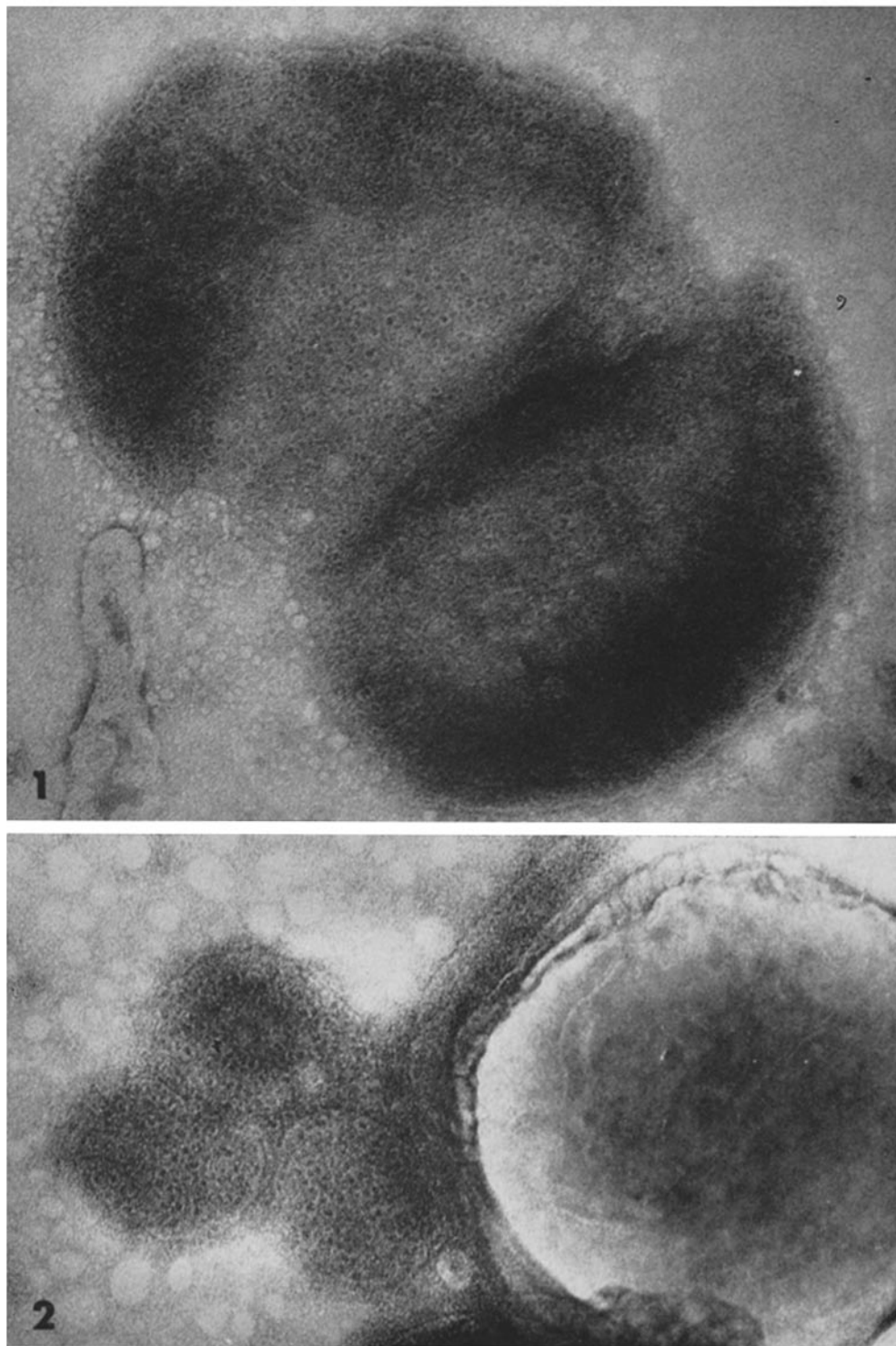
(Bladen et al.: Interactions of the complement system)

PLATE 94

FIG. 3. A thin section of *V. alcalescens*. The three-layered outer membrane (OM), solid membrane (SM), and plasma membrane (PM) can be seen. × 128,000.

FIG. 4. Appearance of *V. alcalescens* after phenol-water extraction. Note that the OM has disappeared and the outermost layer is the SM. × 160,000.

FIG. 5. Negatively stained LPS particles from *V. alcalescens*. × 200,000.

FIG. 6. In sections, LPS particles are predominantly circular with a three-layered membrane composed of two dense layers of 30 A separated by a less dense layer of 25 A. × 200,000.
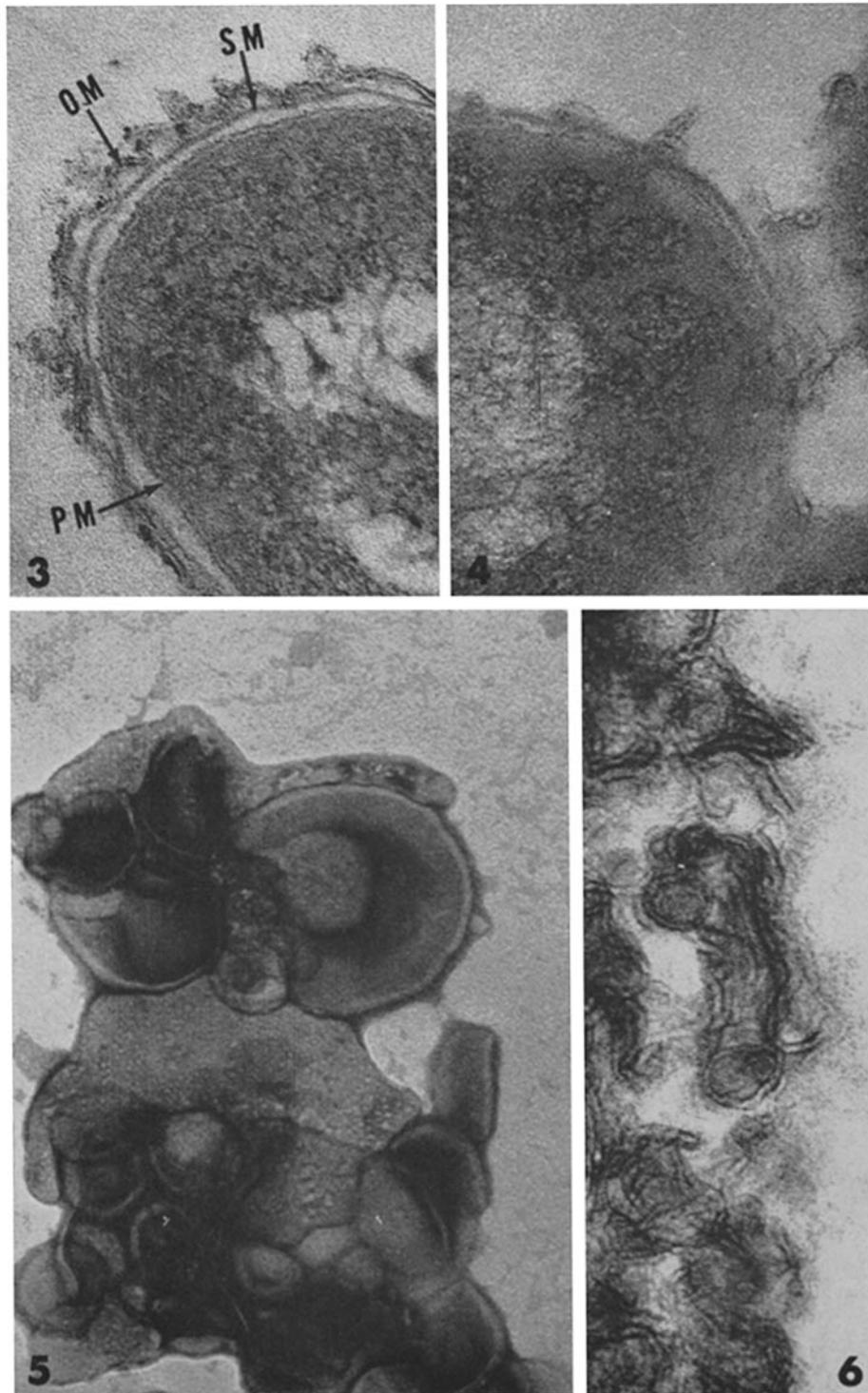
(Bladen et al.: Interactions of the complement system)

PLATE 95

FIG. 7. The appearance of negatively stained pelleted LPS particles after sonification and centrifugation. Many were ruptured and larger than those in untreated LPS preparations. Particles such as these, as well as the smaller ones, are considered "A" particles. × 136,000.

FIG. 8. Supernatant material after sonification and ultracentrifugation had numerous diffuse, amorphous particles. These are considered to be "B" particles. Negatively stained. × 136,000.
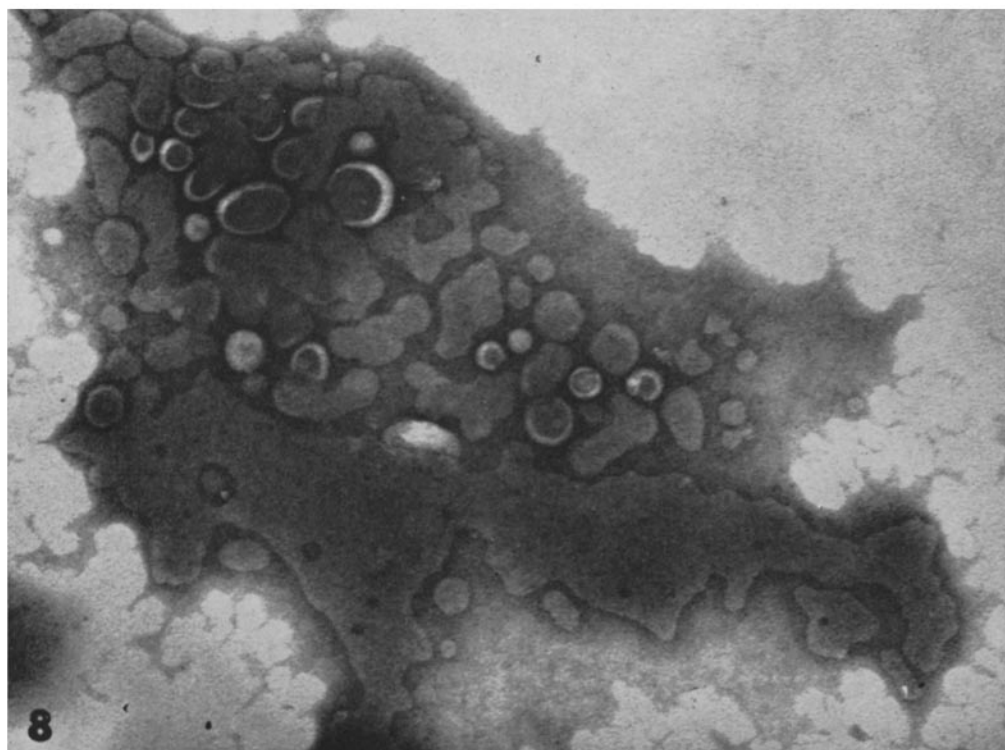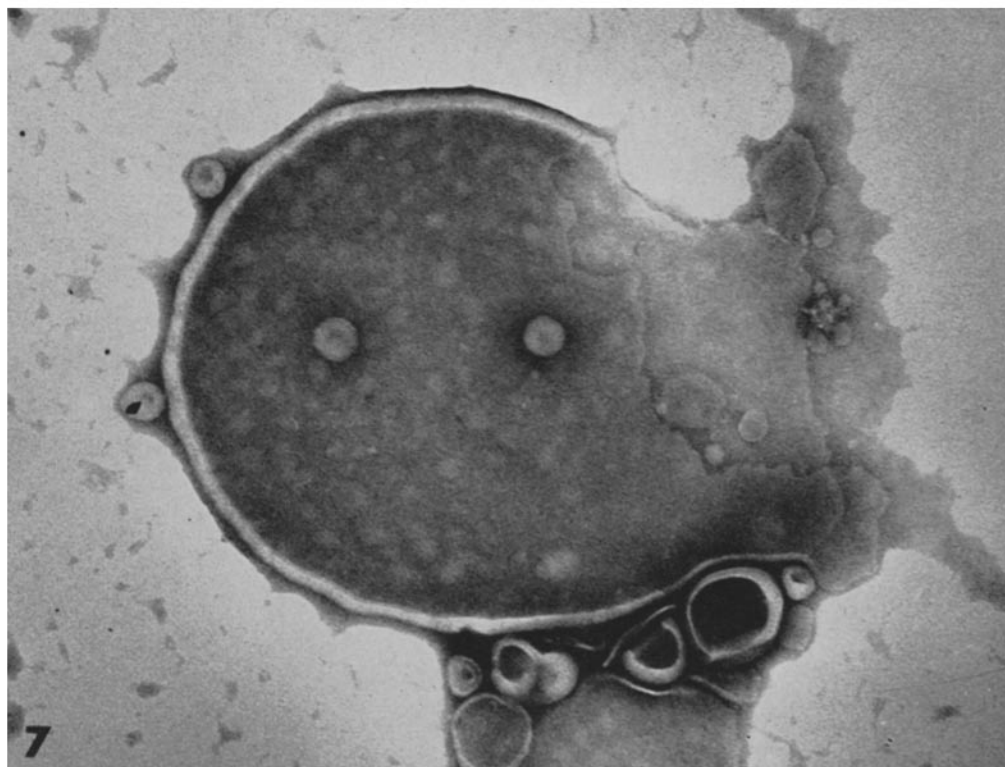
PLATE 96

FIGS. 9-10. Negatively stained "A" particles of LPS after treatment with guinea pig serum. Numerous 90 A diameter lesions containing PTA are randomly spaced over the surface of the particle. The edges of the particles are serrated. × 180,000, × 340,000.
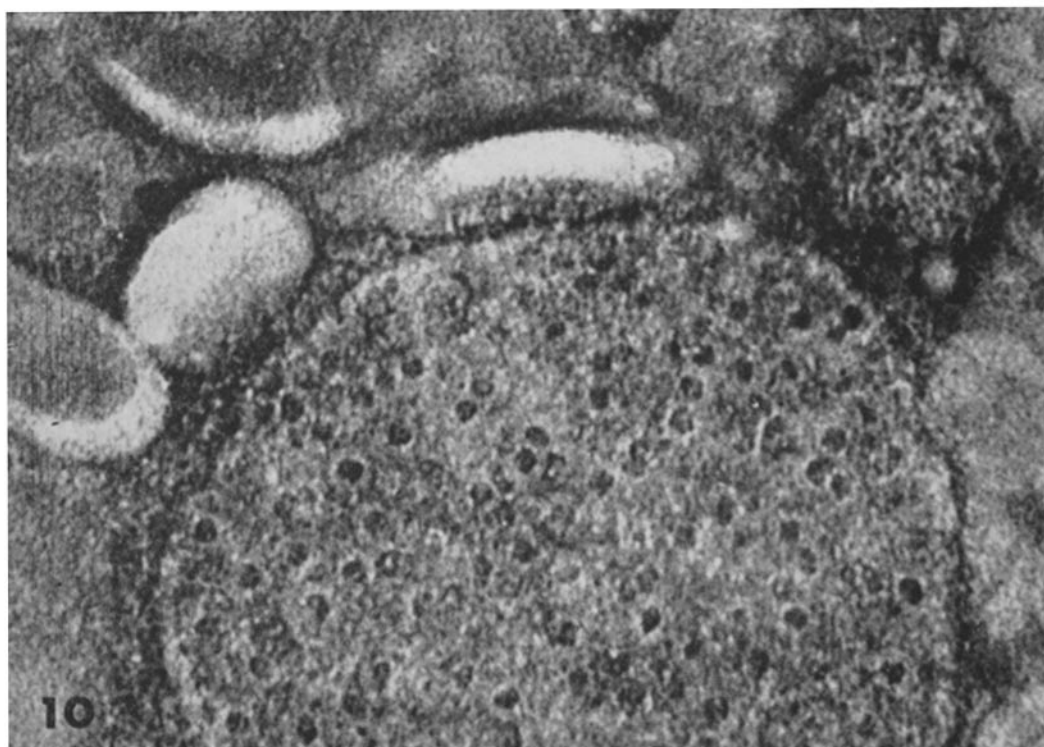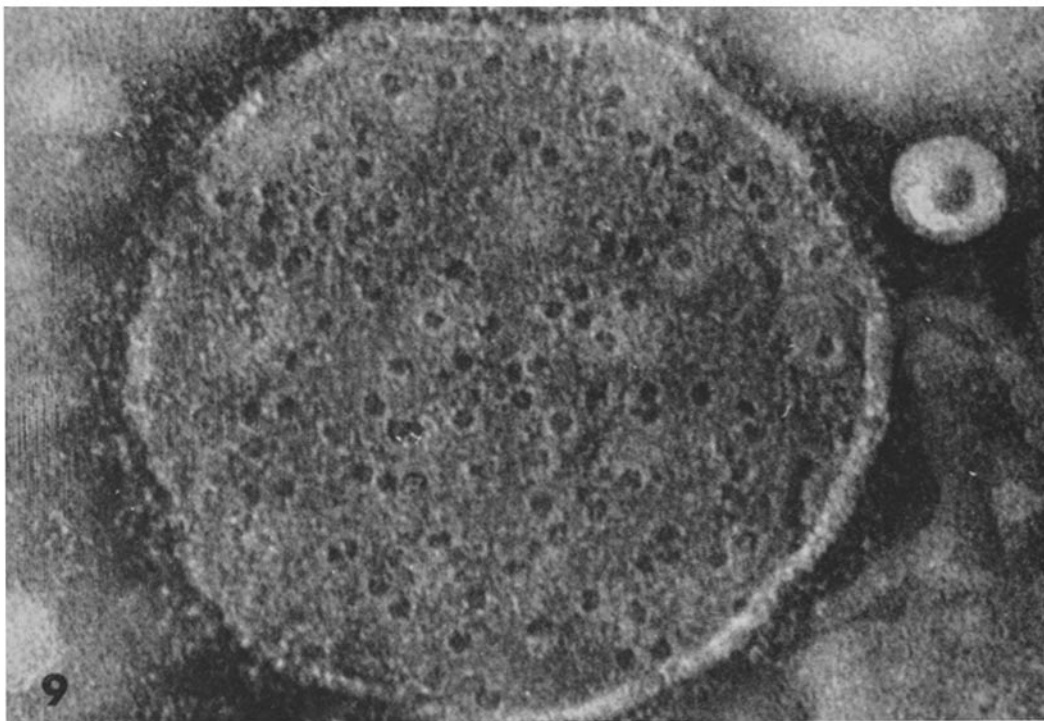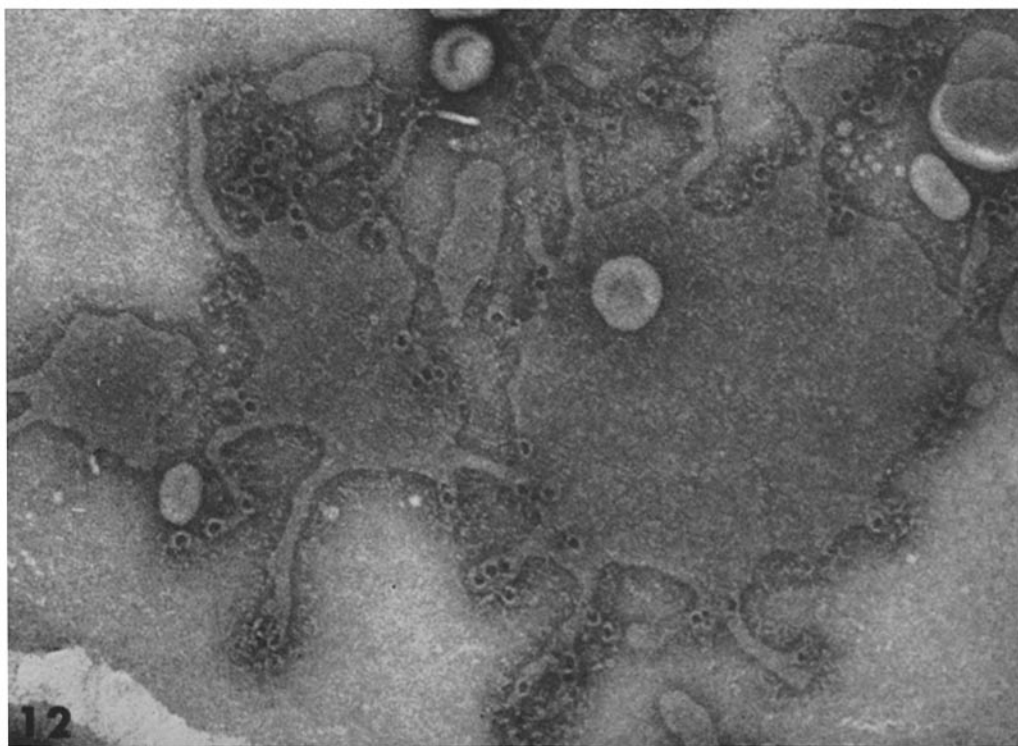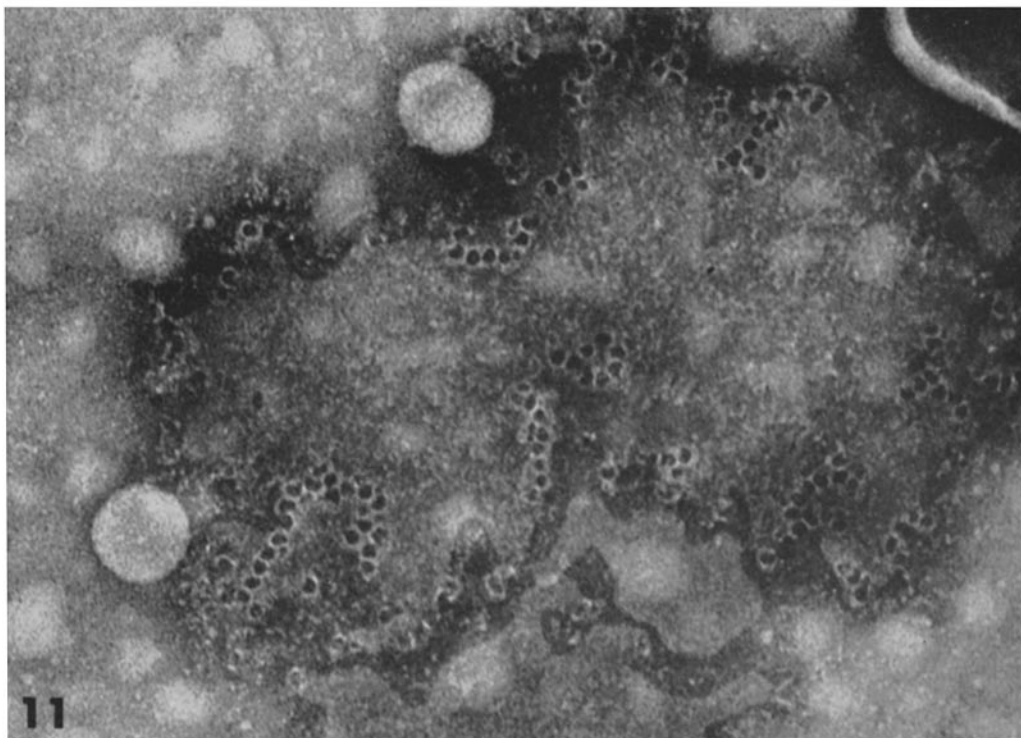
(Bladen et al.: Interactions of the complement system)

FIG. 11. In contrast to the randomness of lesions on "A" particles, those on "B" particles are usualy arranged in chains. Negatively stained. × 180,000.
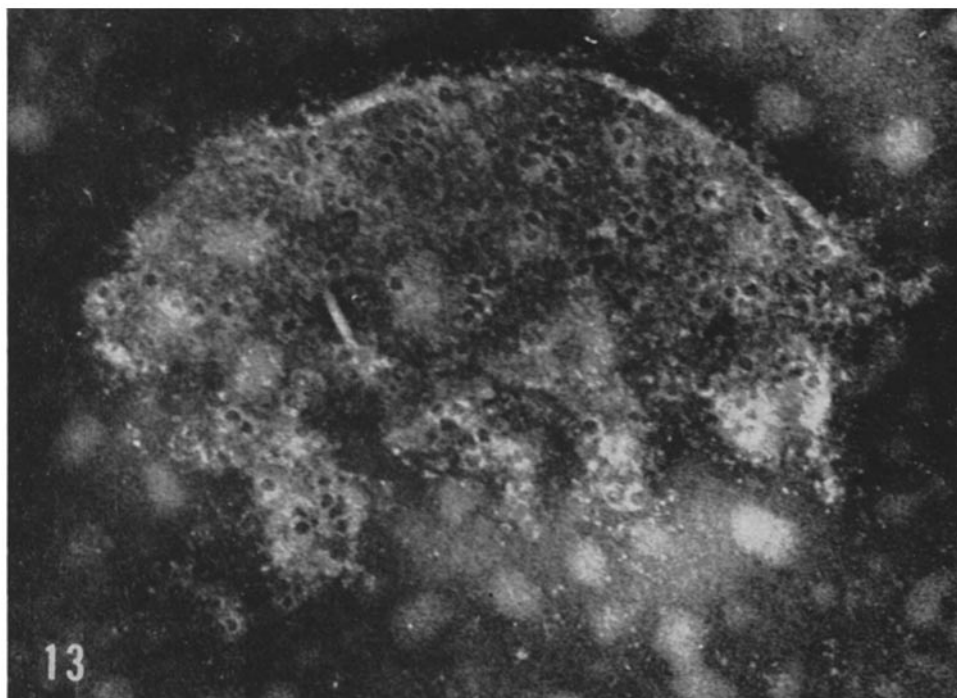
FIG. 12. Some "B" particles had single lesions present at various places along the periphery of the particle. × 148,000.

(Bladen et al.: Interactions of the complement system)

FIG. 13. Fragment of what was probably an "A" particle. Note the random distribution of the lesions as compared to the chains seen in Fig. 11 × 176,000.

(Bladen et al.: Interactions of the complement system)