THE SERUM BACTERICIDAL SYSTEM: ULTRASTRUCTURAL CHANGES IN NEISSERIA MENINGITIDIS EXPOSED TO NORMAL RAT SERUM

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Neisseria meningitidis is one of many Gram-negative organisms which are killed and lysed by mammalian sera (1). The serum bactericidal reaction depends upon combination of antibody with bacterial antigens and subsequent activation of the complement system (2). The reaction is similar to that which occurs during immune lysis of sheep erythrocytes (3) and Krebs' ascites tumor cells (4). In these latter systems, complement produces "holes" in the plasma membrane, leading to altered permeability and lysis.

Exposure of the Gram-negative bacteria *Escherichia coli* (5) and *Veillonella alcalescens* (6) to antibody and complement also results in the production of holes. However, in contrast to mammalian cells, most Gram-negative bacteria have a cytoplasmic membrane and a cell wall composed of a unit-type membrane and a layer of mucopeptide (7, 8). Production of holes in one of these layers would not be expected to result in the profound loss of osmotic control, seen in immune lysis of cells bounded solely by a plasma membrane. Thus, additional serum factors such as lysozyme seem important in immune lysis of Gram-negative bacteria (9).

We have studied the series of ultrastructural changes that occur in meningococci which are exposed to the bactericidal system of rat serum. Changes which are associated with killing are differentiated from those which occur as a consequence of, or subsequent to, the lethal injury. As an outgrowth of these observations some insight has been gained into the possible role of the cell wall laminae and the cytoplasmic membrane in maintaining osmotic integrity of the bacterium.

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Materials and Methods

Bacteria.—All strains of bacteria were obtained from the collection of the Department of Bacteriology, Walter Reed Army Institute of Research, Washington, D.C.

Two strains of *Neisseria meningitidis* were studied, A_1 (serotype A) and C_{11} (serotype C). These were isolated from the cerebrospinal fluid of human cases, subcultured once on "chocolate" agar (Mueller-Hinton Base, Difco), and stored in Greave's medium (10) at −70°C. Strain A_1 is susceptible to the bactericidal activity of normal rat serum, whereas strain C_{11} is not.

A strain of the genus *Herellea*, originally isolated as a laboratory contaminant, and a stock laboratory strain of *Neisseria catarrhalis* were also frozen in Greave's medium.

Preparation of Bacterial Suspensions.—Bacteria were cultured on Mueller-Hinton agar for 16 hr at 37°C in the presence of moisture and CO_2 (candle jar). The organisms were then subcultured for 5 hr on fresh Mueller-Hinton agar. After 5 hr incubation, the total particle count, as measured in a hemocytometer, approximated (± 10%] the viability (colony count).

Bacteria from 5 hr cultures were suspended in Dulbecco's phosphate buffered saline (PBS) pH 7.2 (Grand Island Biological Co., New York). The concentration was adjusted to an optical density of 0.20 at 650 mμ (16 × 125 mm Pyrex screw cap tube, Meteor Glass Co., Vineland, N.J.) in a spectrophotometer (Coleman Junior, Model 6A). This corresponded to a colony count of approximately 10^9 bacteria/ml.

Serum.—Adult male and female rats of the inbred Lewis strain (Microbiological Associates, Inc., Bethesda, Md.) were bled by cardiac puncture. The blood was pooled, allowed to clot at room temperature for 30 min, and refrigerated at 4°C for 3 hr. The serum was divided into aliquots and stored at −70°C. Immediately prior to use, the serum was thawed and passed through a 0.45 μ Millipore filter. All experiments were done with serum from a single pool. For the purposes of these experiments, serum obtained from rats which are not artificially immunized is referred to as normal rat serum (NRS).

Modified Serum Preparations.—Heat-inactivated rat serum (HRS): Normal rat serum was rendered complement deficient by heating at 56°C for 30 min.

Lysozyme-deficient rat serum (LDS): Bentonite was used to deplete normal rat serum of lysozyme (11). The bentonite (Fisher Scientific Co., Pittsburgh, Pa.) was washed three times in PBS and was added to normal rat serum in a final concentration of 5 mg (dry weight) per ml of serum. The serum was absorbed at 0°C for 120 min. Bentonite was removed by centrifugation (41,300 *g* for 15 min) at 0°C and the serum was filtered through a 0.45 μ Millipore membrane.

Absorption of rat serum with normal bacteria: Aliquots of normal rat serum were absorbed with 5 hr cultures of bacteria at 4°C for 2 hr, or at 37°C for 1 hr. The bacteria were washed once in PBS at 4°C, packed by centrifugation (45,900 *g* for 15 min), and added to serum at a final concentration of 10% (v/v). At the end of the absorption, the bacteria were removed by centrifugation at 4°C and the serum was passed through a 0.45 μ Millipore filter.

In one experiment, serum absorbed at 4°C was reabsorbed with fresh bacteria at 37°C for 1 hr.

Egg-white lysozyme: Three times crystallized egg white lysozyme (EWL, Grade A, Calbiochem) was added to normal rat serum and to bentonite-absorbed rat serum at a concentration of 100 μg EWL/ml serum. The lysozyme was kept in a desiccator at −20°C and was dissolved in PBS (10 mg/ml) immediately prior to use.

Complement Titers.—$C'H_{50}$ units/ml of normal and modified rat sera were determined by the method of Hook and Muschel (12). Amboceptor-coated sheep red blood cells were kindly provided by Mr. Earl Fife, Department of Serology, Walter Reed Army Institute of Research.

Lysozyme Determinations.—Lysozyme levels of normal and bentonite-absorbed rat sera were measured by the method of Wardlaw (11). An indicator strain of *Micrococcus lysodeikticus*

4698 was obtained from the American Type Culture Collection (courtesy of Mrs. Margaret Norman). Serum treated with bentonite failed to lyse *M. lysodeikticus*.

Penicillin-Treated Meningococci.—A 5 hr culture of *N. meningitidis* was transferred from Mueller-Hinton agar to broth culture (beef heart infusion + 0.3% yeast extract, Difco) and adjusted to an optical density of 0.02 at 650 mμ (25 × 130 mm sidearm flask, Bellco Glassware, Vineland, N.J.). The fluid culture was placed on a rotary shaker and agitated at 37C for 2 hr during which time the OD increased logarithmically to 0.02. Buffered potassium penicillin (Charles Pfizer, Inc., N. Y.) was added to a final concentration of 100 units/ml. The reaction was allowed to proceed at 37°C for 45 min. Aliquots were diluted 1:10 in PBS, NRS, and modified rat sera for further study.

Serum Bactericidal Reaction.—One part v/v normal or modified rat serum and one part v/v bacterial suspension (10^9 bacteria/ml) were added to eight parts v/v PBS (10^{-3} M Ca++, 5×10^{-4} M Mg++). The reaction mixture was incubated in a water bath at 37°C for varying time periods. The bactericidal reaction was stopped by diluting the reaction mixture 1:100 in PBS. Survivors were enumerated, after additional appropriate dilution, by plating on Mueller-Hinton agar. A nonbactericidal serum control, containing 10^8 bacteria in 1 ml of 10% HRS, was run simultaneously with the bactericidal reaction mixture.

Electron Microscopy.—Specimens were prepared for thin sectioning by standard procedures. The serum bactericidal reaction was stopped by adding one part v/v veronal-buffered 1% osmium tetroxide, pH 6.0 (13) directly to nine parts v/v of the bactericidal reaction mixture. The bacteria were recovered after centrifugation at 12,000 *g* for 10 min and the pellet was resuspended in fresh, undiluted fixative. After over-night fixation, the bacteria were washed in veronal buffer, pH 6.0, embedded in 3% agar, soaked in 1% uranyl acetate for 1–2 hr, dehydrated in graded alcohols, and embedded in Epon. Thin sections were stained with lead citrate (14) and were examined in a Siemens IA Elmiskop.

Specimens for negative straining were prepared by centrifuging the bacteria from the reaction mixture at 4°C, washing three times with distilled water, and mixing with equal amounts of 0.5 or 1% sodium phosphotungstate, pH 6.8, on grids which were covered with Parloidin films and stabilized with carbon.

RESULTS

Morphology of "Normal" Meningococci and Meningococci Exposed to Heat-Inactivated Rat Serum.—5 hr cultures of *N. meningitidis* were examined in thin sections after suspension for 5 min in either PBS (Fig. 3) or 10% heat-inactivated rat serum (HRS) (Figs. 4–6). The cells in the two preparations have identical morphology. The meningococcal cell wall is composed of two well-demarcated layers: outer membrane and dense line. The outer membrane of the wall and the cytoplasmic membrane are similar, each being 70 to 75 A thick and having a typical trilaminar, unit-membrane profile. These two membranes enclose the periplasmic space. A dense line, 15 to 25 A thick, divides the periplasmic space into inner and outer compartments. The relationship of these various layers and interposed spaces is shown in Fig. 6. The cytoplasm contains tightly packed ribosomes and a central, net-like nucleoid.

Effect of Normal Rat Serum on Morphology of N. meningitidis.—Meningococci were exposed to 10% normal rat serum (NRS) at 37°C for varying intervals after which samples were simultaneously taken for viability counts and fixation.

Table I records the rate of killing of meningococci (strain A_1) by NRS. At 30

TABLE I

*Bactericidal Activity of Normal and Modified Rat Sera**

Exp. No.	Organism	Sera		Incubation at 37°C	Survivors	Figures§
		Description‡	$C'H_{50}$			
			units/ml		%	
1	*Neisseria meningitidis* (A_1)	No serum	—	5 min	100	3, 31
2	" " "	HRS	<10	5 "	100	4
3	" " "	NRS	200	30 sec	100	7, 8
4	" " "	"	200	2 min	50	9, 10
5	" " "	"	200	5 "	<1	11, 12, 13, 24, 26
6	*Neisseria catarrhalis*	HRS	<10	5 "	100	14
7	" " "	NRS	200	5 "	<1	15
8	*Herellea sp.*	HRS	<10	5 "	100	16
9	" " "	NRS	200	5 "	<1	17
10	*Neisseria meningitidis* (A_1)	HLDS	<10	5 "	100	18
11	" " "	LDS	179	5 "	<1	19, 20
12	" " "	"	179	15 "	<1	21, 27
13	" " "	LDS + EWL	N.D.‖	5 "	<1	22, 23, 28
14	*Neisseria meningitidis* (C_{11})	NRS	200	15 "	100	30

* Bactericidal reaction mixture; 10^8 bacteria per ml of 10% rat serum in PBS.

‡ Abbreviations: HRS, heat-inactivated rat serum (56°C for 30 min); NRS, normal rat serum; LDS, lysozyme-deficient serum (bentonite-absorbed); HLDS, heat-inactivated LDS; EWL, egg white lysozyme (100 μg/ml).

§ Bacteria in Figs. 24, 26–31 incubated in appropriate sera for 15 min.

‖ Not determined.

sec (Experiment 3) there is no detectable killing; by 2 min (Experiment 4) 50% of the organisms are dead; and by 5 min (Experiment 5) the mortality exceeds 99% as compared to the controls (Experiments 1 and 2).

Bacteria exposed to NRS for 30 sec show only minimal morphological changes (Figs. 7 and 8). These consist of a sparse accumulation of amorphous, electron-dense material on the external surface of the outer membrane and foci of increased width in the outer periplasmic space.

At 2 min the majority of bacteria show distinct alterations in ultrastructure (Figs. 9 and 10). The outer membrane is less rugose and its trilaminar profile is less distinct than in controls. There is a considerable amount of electron-dense, fibrillar material on the exterior of the outer membrane. In many sites this material appears to protrude into (or from) the periplasmic space and to contact the dense line. The outer periplasmic space is slightly dilated, but this is partially obscured by the presence of the fibrillar material. The inner periplasmic space is greatly widened and is nearly devoid of electron-opaque material. No definite gaps are seen in the cytoplasmic membrane. Individual ribosomes appear more

distinct than usual due to increased separation of the particulate elements of the cytoplasm.

Organisms exposed to NRS for 5 min (Figs. 11–13) are greatly swollen and show marked accentuation of changes noted after 2 min. In addition, there are large gaps in the cytoplasmic membrane and herniation of ribosomes into the periplasmic space. The remainder of the ribosomes and the nucleoid material are widely separated to give the cytoplasm a "watery" appearance. Multiple, short discontinuous foci are present along the dense line. Fibrillar material appears to contact the remaining, solid portions of the middle dense line (Fig. 13).

Longer incubation of meningococci in NRS results in enhancement of the changes seen after 5 min. The cytoplasm and nucleoid are not identifiable after 30 min in serum. These gross alterations in ultrastructure are similar to those seen by Spitznagel and Wilson (15) in *E. coli*, which were incubated with guinea pig serum for 60 min.

Effect of Normal Rat Serum on Morphology of N. catarrhalis and Herellea sp.—The sequence of morphological changes which occurs in meningococci after exposure to NRS suggests that a series of events operate to produce death of the organisms. In order to determine whether all of these changes are required for killing Gram-negative bacteria or whether they are part of a spectrum of changes which occur following cell injury, two other serum-sensitive Gram-negative organisms, *Neisseria catarrhalis* and members of the genus *Herellea*, were studied.

The ultrastructure of cells from a 5 hr culture of *N. catarrhalis* (Fig. 14) is similar to that of meningococci. However, the middle dense line is thicker (35 to 45 A) in *N. catarrhalis* than in *N. meningitidis* (15 to 25 A). Treatment of *N. catarrhalis* with NRS for 5 min kills more than 99% of the organisms (Table I, Experiment 11). The morphology of such NRS-treated cells is shown in Fig. 15. As in the case of the meningococcus, there is fibrillar material on the outer membrane, focal disruption of the cytopasmic membrane, and periplasmic and intracytoplasmic edema. Unlike *N. meningitidis*, the dense line in NRS-treated *N. catarrhalis* is intact.

The salient features of the structure of normal *Herellea sp.* (Fig. 16) include extended fimbriae on the cell surface, a markedly rugose outer membrane, and closely apposed cell wall laminae. Incubation of *Herellea sp.* in NRS for 5 min kills more than 99% of the cells (Table I, Experiment 13) and results in morphological changes shown in Fig. 17. The morphological alterations resemble those seen in meningococci after 2 min exposure to NRS (Figs. 9 and 10). There is accumulation of fibrillar material on the cell surface, loss of rugosity of the outer membrane, separation of cell wall layers by edema of the periplasmic space, and moderate edema of the cytoplasm. In contrast to NRS-treated meningococci, serum killed *Herellea sp.* have no breaks in continuity of either cytoplasmic membrane or dense line.

Components of the Bactericidal System of Normal Rat Serum.—It has been established in several mammalian sera that the bactericidal activity against various Gram-negative bacteria is due to the combined action of antibody and complement (1). Properdin has been incriminated in some of these systems (16). The antibody can be present in the sera of nonimmunized animals ("natural antibody") (17) or in hyperimmune sera (18). Both IgG and IgM antibodies have been shown to be active in the serum bactericidal system (19).

In the present experiments the components of the bactericidal system of normal rat serum were defined mechanistically. Rat serum was absorbed at 0°C and/or 37°C with *N. meningitidis* (A_1) or *Herellea sp.*, and residual bactericidal activity was measured.

Results in Table II indicate that the bactericidal system of rat serum is composed of a specific recognition mechanism (presumably antibody) and a common effector mechanism (presumably complement). Absorption of serum at 0°C with either *N. meningitidis* (A) (Experiment 3) or *Herellea sp.* (Experiment 4) removes specific antibodies, as shown by a selective decrease in bactericidal titer against the homologous cells. The retention of bactericidal activity against heterologous organisms demonstrates that complement is not removed by absorption at 0°C. Further, results in Table II indicate that fixation of complement in the bactericidal system depends upon prior attachment of antibody. At 37°C both *N. meningitidis* and *Herellea sp.* fix complement (Experiments 5 and 6), but in serum previously absorbed with these organisms at 0°C complement fixation no longer occurs (Experiments 7 and 8). When serum devoid of antibody (absorption at 0°C) is added to serum devoid of complement (heterologous absorption at 37°C), the complete bactericidal system is reconstituted (Experiments 9 and 10).

Role of Lysozyme in Bactericidal Reaction of Rat Serum against N. meningitidis.—Serum lysozyme (muramidase) is known to play a role in the complement-dependent bactericidal system against Gram-negative bacteria (11). Hydrolysis of cell wall mucopeptide by lysozyme (20) is the principle factor that brings about lysis of many serum-sensitive organisms (21). Although it may increase the efficiency of the bactericidal process, there is less certainty that lysozyme is necessary for killing of Gram-negative bacteria (22, 33). In order to assess the contribution of lysozyme in killing of meningococci by serum, the following experiments were performed. Meningococci (A_1) were exposed to rat serum from which lysozyme had been removed (LDS) by absorption with bentonite. Also, the bacteria were treated with NRS and LDS to which 100 μg/ml egg white lysozyme (EWL) was added. The maximum concentration of bacteria (10^8/ml) killed by 10% NRS was used to assure detection of either a significant change in rate or a decrease in number of organisms killed by the modified serum preparations.

Results in Fig. 1 show that removal of lysozyme from rat serum by absorption with bentonite has little effect on the efficiency of killing of meningococci.

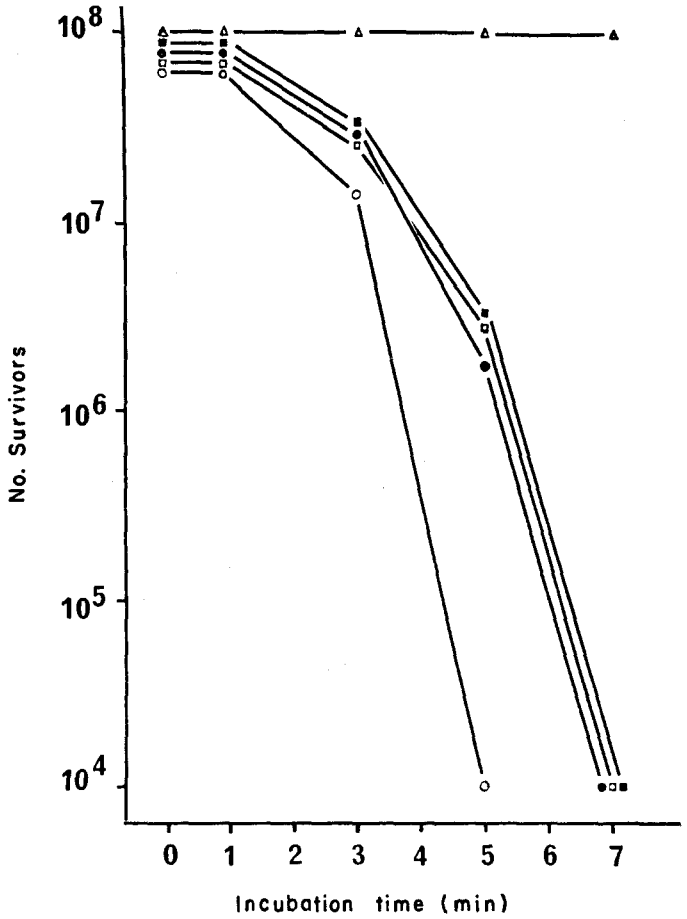

FIG. 1. Effect of bentonite and egg white lysozyme (EWL) on bactericidal activity of normal rat serum against *N. meningitidis* (A_1). □——□, normal serum; ■——■, bentonite-absorbed serum; ○——○, normal serum + EWL (100 μg/ml); ●——●, bentonite-absorbed serum + EWL (100 μg/ml); △——△, controls: heated serum (56°C × 30 min), bentonite-absorbed heated serum, and heated serum + lysozyme (100 μg/ml). Colony count for all points at 0 and 1 min 10^8 bacteria/ml.

More than 99% of the bacteria are killed after 5 min exposure to either NRS or lysozyme-deficient serum (LDS). Also, there is no increase in rate of killing upon addition of 100 μg EWL to 1 ml of LDS, and only a slight increase in bactericidal activity after addition to EWL to NRS. Control preparations show that neither lysozyme nor bentonite are, in themselves, toxic to meningococci. Thus, there is no killing by PBS + EWL, HRS + EWL or bentonite-adsorbed HRS.

While lysozyme appears to play a minor role, if any, in the actual killing of

meningococci by NRS, morphological alterations following exposure to LDS and NRS are strikingly different. In contrast to NRS-treated cells (Figs. 11–13), meningococci which are exposed to LDS for 5 min (Figs. 19 and 20) have only slightly dilated periplasmic spaces, little or no intracytoplasmic edema, and intact cytoplasmic membranes and dense lines. Preferential dilatation of the inner periplasmic space, seen in NRS-treated organisms, is not present in meningococci exposed to LDS. In fact, dilatation of the outer periplasmic space seems to predominate in the latter organisms. Incubation of meningococci in LDS for 15 min (Fig. 21) does not modify the extent or types of alterations seen after 5 min. The only morphological points of similarity between NRS- and LDS-killed meningococci is the presence of fibrillar material on the cell surface.

Addition of EWL to lysozyme-deficient serum has no discernible augmentative effect on the rate of killing meningococci (Fig. 1). The ultrastructural appearance of meningococci exposed to LDS + EWL for 5 min (Fig. 22 and 23) is quite different, however, from that of organisms killed by LDS alone. Addition of EWL to LDS is associated with disappearance of the dense line and marked periplasmic and intracytoplasma edema. Nevertheless, LDS + EWL does not fully reproduce the spectrum of morphological alterations seen in NRS-treated meningococci. Thus, there are no breaks in continuity of the cytoplasmic membrane of LDS + EWL treated cells, despite the fact that edema of the cytoplasm exceeds that seen in NRS-treated bacteria. In common with both NRS- and LDS-killed meningococci, fibrillar material is present on the outer membrane of cells treated with LDS + EWL, and is seen to extend into (or from) the periplasmic space.

Wardlaw (11) has suggested that *E. coli* can be lysed by lysozyme in the presence of complement alone. This does not appear to be the case with meningococci. As seen in Table II (Experiment 3), the bactericidal activity of normal rat serum to *N. meningitidis* (A_1), but not to *Herellea sp.*, can be removed by absorption with meningococci at 0°C. Addition of 100 μg/ml EWL does not restore the ability of such absorbed serum to kill meningococci. The results indicate that both antibody and complement are required before lysozyme can affect the meningococcus.

Appearance of "Holes" in the Wall of N. meningitidis.—Serum-associated holes: Holes have been described in the membranes of mammalian (3, 4) and bacterial cells (5, 6) after exposure to antibody and complement. Similar holes are seen in meningococci treated with normal rat serum. In the present experiments, meningococci (A_1) were incubated in 10% NRS for 15 min, washed in distilled water, and negatively-stained with phosphotungstate (PTA). Numerous elliptical to spherical collections of PTA are distributed over the entire surface of organisms treated in this manner (Fig. 24). These serum-associated holes or "pits" vary from 90 to 130 A in greatest diameter (mean = 110 A), and

TABLE II

Bactericidal Activity of Rat Serum Against N. meningitidis and Herellea Sp.

Exp. No.	Treatment of normal rat serum	Bactericidal titer*		$C'H_{50}$
		N. meningitidis (A_1)	*Herellea sp.*	
				units/m
1	Untreated	64	128	200
2	Incubated 37°C for 60 min	64	128	194
3	Absorbed with *N. meningitidis* (A_1)‡ 0°C for 120 min	<4	128	185
4	Absorbed with *Herellea sp.*‡ 0°C for 120 min	64	<4	189
5	Absorbed with *N. meningitidis* (A_1)‡ 37°C for 60 min	<4	<4	<20
6	Absorbed with *Herellea sp.*‡ 37°C for 60 min	<4	<4	<20
7	Absorbed with *N. meningitidis* (A_1) at 0°C for 120 min followed by absorption with fresh meningococci at 37°C for 60 min	<4	128	177
8	Absorbed with *Herellea sp.* at 0°C for 120 min followed by absorption with fresh *Herellea sp.* at 37°C for 60 min	64	<4	181
9	Equal volumes of serum-preparations 3 and 6 mixed	32	64	95
10	Equal volumes of serum-preparations 4 and 5 mixed	32	32	91

* Reciprocal of highest twofold serum dilution killing more than 50% of bacteria after 30 min incubation at 37°C.

‡ One part v/v packed bacteria added to nine parts v/v normal rat serum.

are surrounded by a rim of diminished electron density. The lesions appear to be randomly distributed.

The electron microscopic image of a negatively stained bacterium records features of several superimposed laminae of the cell wall, which may be penetrated to varying depths by the negative stain. For this reason, it is impossible to localize holes to one layer of the wall by simple examination of the whole negatively-stained bacterium. To determine the location of the lesions more precisely, we studied the appearance of serum-induced holes in organisms whose walls were modified either by penicillin or egg white lysozyme.

Penicillin pretreatment of meningococci was used to exclude both the cytoplasmic membrane and the middle dense line as locations of serum-induced holes. The surface of log phase meningococci exhibits blebs after treatment with penicillin (Fig. 25). The blebs appear to be composed only of the outer membrane of the cell wall. Both the cytoplasmic membrane and the dense line course along the base of the bleb. Therefore, demonstration of holes in penicillin-induced blebs after NRS-exposure (Fig. 26) rules out both the cytoplasmic membrane and the dense line as the layers in which the holes are located.

Mucopeptide cannot be excluded as the site of hole production in the preceding experiment because it is not clear whether the mucopeptide layer is identical to the electron-dense line of the cell wall or merely associated (but not visible) with the dense line. The possibility that mucopeptide is the substrate for serum-induced holes is ruled out by an experiment in which meningococci (A_1) are exposed to serum plus egg white lysozyme (100 μg/ml). Because lysozyme digests mucopeptide (22) holes located in the layer would be expected to disappear after such treatment. This is not the case. Holes are similar in size, shape, number and distribution in cells exposed to LDS (Fig. 27) or to LDS + EWL (Fig. 28). Thus, mucopeptide along with cytoplasmic membrane and dense line,

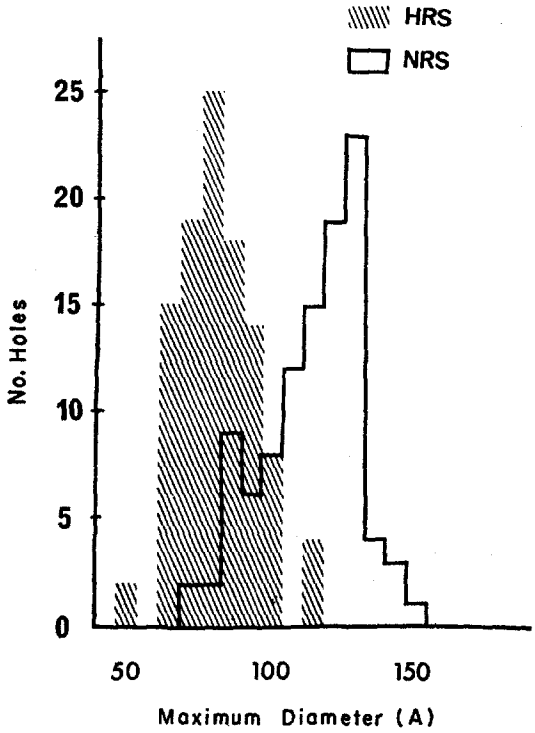

FIG. 2. The size-frequency distribution is shown for the "holes" which were present in one normal rat serum-treated meningococcus (A_1) and two similar organisms that were exposed to heated rat serum. Micrographs of the specimens were obtained with identical electron microscopic and photographic enlargement factors. The foci of negative stain accumulation or holes on the resulting prints were measured to the nearest 0.1 mm with a micrometer containing enlarging eyepiece, and the maximum diameters of the holes was plotted as shown in the figure. The mean value for holes in HRS-treated organisms (shaded area) is 82 $\pm$ 1.8 A, whereas the mean maximum diameter for holes in the NRS-treated meningococcus is 110 $\pm$ 3 A. The difference between these two means is significant ($P < 0.01$) and establishes that two populations of holes are present.

described above, are excluded as the sites of formation of serum-induced holes. The holes must be located in the only lamina which has not been excluded, the outer membrane of the cell wall.

Naturally occurring "holes": In addition to serum-associated lesions, holes are also seen in the walls of meningococci which have *not* been exposed to antibody of complement. We first noted these defects in meningococci (A_1) which were incubated with heat-inactivated (decomplemented) rat serum. Similar holes are also seen in serum-resistant meningococci (C_{11}) after exposure to NRS (Fig. 30). These naturally occurring holes (Fig. 29) are smaller (mean diameter = 82 A) and less numerous than those seen in serum-killed (NRS-treated) cells of the same strain (Fig. 24).

It could be argued that the naturally-occurring holes are artifactitious, resulting from exposure of the bacterium to repeated water washing; or that the small size and number of holes reflects incomplete action of rat serum. To exclude both of these possibilities, a drop from a 24 hr broth culture of *N. meningitidis* (A_1) was placed directly on a coated grid and stained with PTA (Fig. 31). Typical 82 A diameter holes are present in the walls of such organisms.

The relation of serum-induced holes to naturally occurring holes in the walls of meningococci is not clear. Fig. 2 is a size distribution plot of the two types of lesions. Analyses of the two curves reveals that, despite some overlapping, the serum-induced and naturally occurring "holes" represent two distinct populations.

DISCUSSION

Exposure of susceptible meningococci to suitable concentrations of normal rat serum initiates a series of alterations in cellular homeostasis which eventuate in death and lysis of the organism. Ultrastructural changes which occur in affected cells during the bactericidal process include the following: accumulation of fibrillar, electron-dense material on and in the cell wall, development of holes or pits in the outer membrane, partial dissolution of the dense line, enlargement of the periplasmic and cytoplasmic spaces, and appearance of large gaps in the cytoplasmic membrane with resultant outpouring of cytoplasmic contents beyond their usual confines.

All of the alterations seen in organisms exposed to the complete bactericidal system are not invariably associated with killing, per se. Bentonite-absorbed serum (lysozyme deficient) efficiently kills meningococci but does not produce all of the above mentioned changes in morphology. Such meningococci have holes in the outer membrane, accretion of fibrillar material on the surface, and evidence of modest fluid accumulation in the periplasmic space. They do not show disruption of the dense line, marked intracytoplasmic edema, rupture of the cytoplasmic membrane, or spillage of cytoplasmic contents. These latter changes appear to be either postmortem alterations or reflections of antemortem

processes not intimately associated with death of the meningococcus. *N. catarrhalis* does not show changes in the dense line and *Herellea sp.* exhibits no disruption of either the dense line or the cytoplasmic membrane after exposure to normal rat serum, although both of these organisms are killed.

Changes in the two inner laminae (dense line and cytoplasmic membrane) may be factors which determine whether or not serum-treated cells can repair the induced injury. Muschel (23) and Davis et al. (32) have demonstrated that some serum injured *E. coli* can be rescued by addition of excess magnesium to the medium. Dienes showed that "serum-killed" *Salmonella typhosa* can be recovered as L-forms if they are transferred to an environment with high osmolality soon after exposure to serum (24). It is possible that manipulation of the external environment can rescue organisms in which holes in the outer membrane and periplasmic edema are present. It is less likely that recovery of viability can be effected by any means once rupture of the cytoplasmic membrane and the middle dense line occur.

The initial insult to the gram negative bacterium by the serum bactericidal system is presumed to result in holes in the cell wall. The creation of holes by antibody and complement in antigenic membranes has been demonstrated by negative staining in several types of mammalian cells (3, 4) as well as in *E. coli* (5) and *V. alcalescens* (6). The lesions are known to reside in the plasma membrane of mammalian cells. It is not entirely clear from previous morphological studies whether the lesions are located in the plasma membrane (5), the outer membrane (6) or the intermediate layer of the Gram-negative bacterial wall. We have demonstrated that the 110 A diameter accumulations of negative stain (holes) are located in the outer membrane of intact, whole meningococci which have been killed by rat serum. Complement-dependent holes are present in penicillin-induced blebs, which are composed entirely of outer membrane; and similar holes persist in serum-treated organisms whose mucopeptide is removed by egg white lysozyme. The location of lesions in the outer membrane of meningococci correlates with the production of holes by antibody and complement in purified endotoxin (25).

We have also demonstrated that meningococci which have not been killed by antibody and complement have holes in their cell walls. Small accumulations of negative stain (82 A in mean diameter) are seen in the walls of serum-susceptible cells which (*a*) have not been exposed to any serum component, or (*b*) have been exposed to heated, uncomplemented rat serum. Furthermore, 82 A diameter holes are present in walls of serum-resistant (C_{II}) meningococci before and after treatment with normal rat serum.

The small, naturally occurring holes, when carefully measured, are readily differentiated from the larger, complement-dependent holes. It is clear, therefore, that the presence of focal accumulations of negative stain on bacterial membranes does not necessarily signal the bactericidal reaction of complement. The present experiments do not provide information about the physiological role of holes in walls of normal meningococci or possible relationships of naturally occurring and complement-induced holes.

One of the well documented actions of complement is alteration of the permeability of cell membranes (26). This alteration is presumably related to the production of defects, which are thought to be either full thickness holes or superficial pits, in the outer membrane of Gram-negative bacteria. The first ultrastructurally defined consequence of such altered permeability in the serum-treated menigococcal cell wall is widening of the periplasmic space. This finding is suggestive of local fluid accumulation or edema. The pattern of periplasmic edema seems to depend on the presence of absence of a serum factor, probably lysozyme, which is removed by bentonite. In meningococci exposed to normal rat serum, edema occurs chiefly in the inner periplasmic space, between the middle dense line and cytoplasmic membrane. This contrasts with the pattern seen in cells reacted with bentonite-adsorbed serum. In the latter organisms, widening of the outer periplasmic space predominates. The dense line which separates the inner and outer periplasmic compartments is most likely mucopeptide (27) or globular protein associated with mucopeptide (28), as shown in the present experiments by removal of this lamina by egg white lysozyme. It would appear, therefore, that the difference of fluid accumulation in the two systems depends on permeability characteristics of mucopeptide. Such periplasmic edema, if associated with ion shifts similar to those described between serum-injured mammalian cells and the external medium (26), might disrupt the major oxidative processes associated with the cytoplasmic membrane of the bacterium (29).

Edema of the periplasmic space in NRS treated meningococci is followed by intracytoplasmic edema, disruption in continuity of the cytoplasmic membrane, and escape of cytoplasmic contents from their usual confines. The occurrence of periplasmic edema before intracytoplasmic edema suggests that the cytoplasmic membrane is a permeability barrier which, in conjunction with the outer membrane and mucopeptide, functions to regulate the fluid and ion environment of the organism's internal *milieu*.

Intracytoplasmic edema is not necessarily accompanied by loss of continuity of the cytoplasmic membrane. In meningococci exposed to bentonite absorbed serum and egg white lysozyme there is marked periplasmic and intracytoplasmic edema but no visible breaks in the cytoplasmic membrane. The lack of correlation between cytoplasmic edema and rupture of the cytoplasmic membrane suggests that the cytoplasmic membrane does not undergo simple osmotic rupture. An alternative explanation is that the gaps in the cytoplasmic membrane result from the action of an intracellular product, perhaps autolytic enzyme, which operates following initial injury to the cell. Such a mechanism might be involved in the case of *N. meningitidis*, which is extremely autolytic (30). *N. catarrhalis*, on the other hand, is not autolytic (30) but shows the same breakdown of cytoplasmic membrane after treatment with normal rat serum that is seen in meningococci. Therefore, it is unlikely that an autolytic type of mechanism alone accounts for dissolution of the cytoplasmic membrane.

It is possible that gaps develop in the cytoplasmic membrane due to the action of a serum factor which, in addition to lysozyme, is removed by bentonite. The existence of such a substance has been postulated by Glynn and Milne (22). These authors found full restitution of lytic activity against *E. coli* if egg white lysozyme was added to human serum which had been rendered lysozyme deficient by anti-lysozyme antibody. However, egg white lysozyme could not restore the full lytic capability to serum which was absorbed with bentonite. The present experiments suggest that such

a bentonite-absorbable factor could bring about lysis by acting on the cytoplasmic membrane to effect its dissolution. It should be noted, however, that rupture of cytoplasmic membrane is only one of several mechanisms by which lysis, as defined classically by a decrease in turbidity of a bacterial suspension, can conceivably occur.

One of the most striking morphological changes associated with the serum bactericidal process is the progressive accumulation of electron dense, fibrillar material on the cell wall outer membrane. This material appears to extend through the outer membrane and contact the middle dense line.

The present experiments indicate that fibrillar material accumulates only on membranes which have been damaged by the antibody-complement system. There is no fibrillar material on the surface of meningococci which are exposed to heat-inactivated rat serum or to sera from which either antibody or complement has been selectively removed.[1] Nor is fibrillar material present on the surface of serum-resistant meningococci (C_{II}) which are incubated with normal rat serum.[1]

It cannot be determined from the electron micrographs whether fibrillar material arises within the bacterial cell and is extruded through a damaged, "porous" outer membrane or whether the material is derived from serum and extends into the periplasmic space. The two possibilities are not mutually exclusive.

Spitznagel has described the loss of ^{32}P-labeled macromolecules from radioactively-labeled *E. coli* following exposure to normal guinea pig serum (31). Loss of such material was associated with gross disruption of the bacterial cell (15). In the present experiments, it seems unlikely that macromolecules from cytoplasm contribute significantly to the formation of fibrillar material. Both NRS-treated meningococci, which show marked architectural disorganization, and LDS-treated meningococci, which show only minimal alterations, have similar amounts of fibrillar material on their surfaces. It is possible, however, that fibrillar material arises from the cell wall itself.

Both antibody and complement are bound to the surface of serum-sensitive meningococci during the bactericidal process. It is reasonable to speculate that these specifically reacting substances contribute to the formation of fibrillar material. Alternatively, fibrillar material may be composed of serum constituents which adhere nonspecifically to the damaged outer membrane. The distinctive fibrillar character and radial orientation of the material and its presence in the outer periplasmic space argue against this latter interpretation.

SUMMARY

Exposure of meningococci to the bactericidal system of normal rat serum initiates a series of ultrastructural changes that accompany death of the organ-

[1] Unpublished observations.

ism. These morphological alterations consist of complement-dependent holes in the cell wall outer membrane, edema of the periplasmic space and cytoplasm, and accumulation of fibrillar material in and on the cell wall. Dissolution of the dense line and rupture of the cytoplasmic membrane also occur in meningococci exposed to normal rate serum. These latter two changes, however, are not seen in meningococci that are killed by bentonite-absorbed (lysozyme-deficient) serum, nor are they invariably present when other serum-susceptible organisms (*N. catarrhalis* and *Herellea sp.*) are treated with normal rat serum.

The pattern of development of edema in serum-treated meningococci suggests that the cell wall outer membrane, mucopeptide layer, and cytoplasmic membrane act synergistically to maintain osmotic equilibrium of the bacterium. Death of the bacterium seems related to alteration of permeability following injury to the outer membrane.

Holes are demonstrable, by negative straining, in the outer membrane of the meningococcal cell wall after exposure to the bactericidal effects of rat serum. The lesions are 110 A average greatest diameter and depend on the presence of antibody and complement for their formation. In addition, 82 A diameter holes are present in the walls of normal, untreated meningococci. The relation of complement-dependent holes to the smaller, naturally occurring holes is not known.

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tide in sections of the cell wall of *Escherichia coli* and other gram negative bacteria. *Can. J. Microbiol.* **11**:547.

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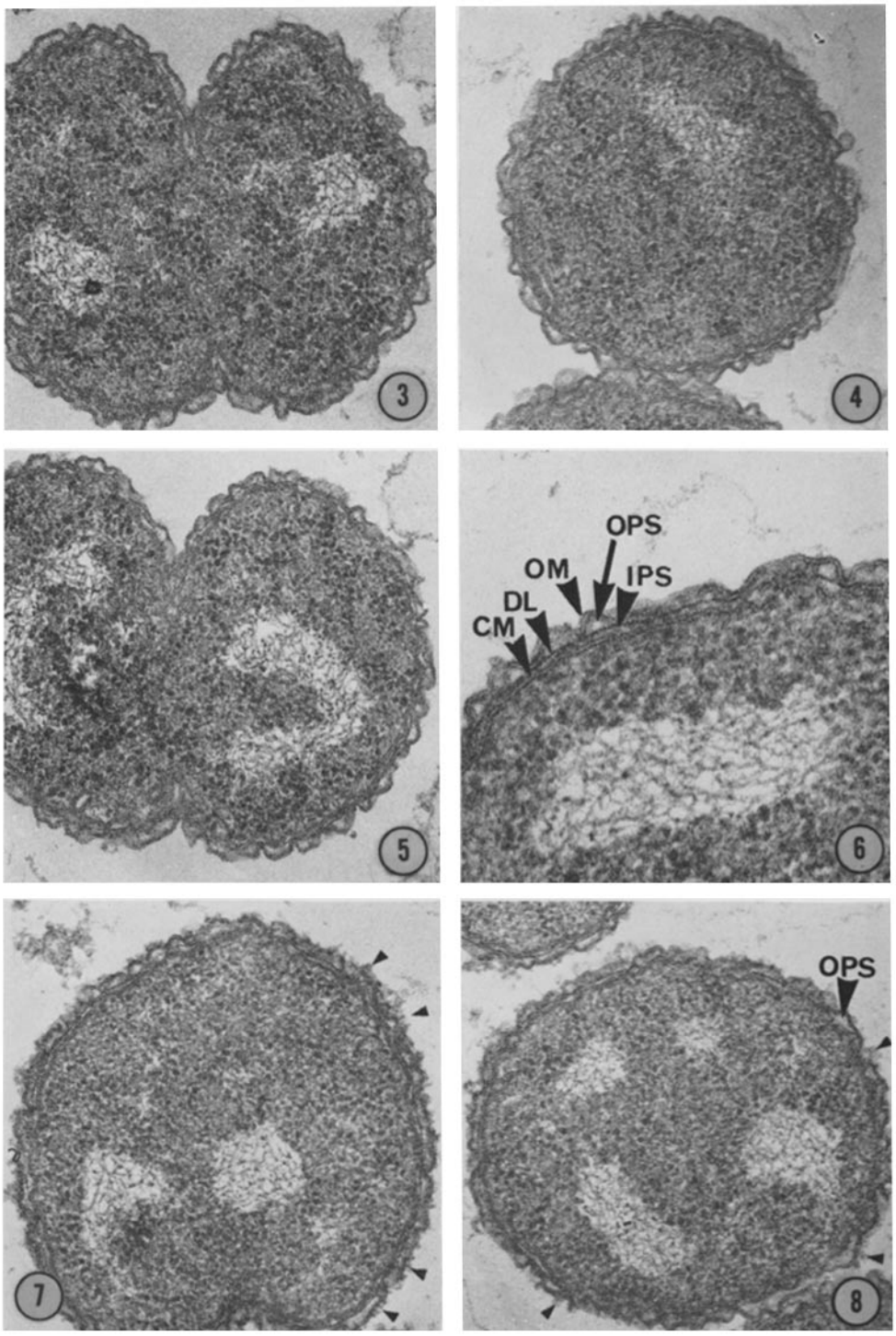
FIG. 3. Untreated, incompletely divided form of *Neisseria meningitidis* from 5 hr culture on Mueller-Hinton agar. The cell wall is composed of two layers (see Fig. 6) and has a rugose surface. No fibrillar material is present on the outer membrane. The cytoplasmic contents are compact, except for the net-like nucleoid. × 70,000.

FIGS. 4 and 5. *N. meningitidis* (A_1) exposed to heat-inactivated rat serum (56°C × 30 min) for 5 min. There are no alterations in wall or cytoplasmic ultrastructure as compared to untreated controls (Fig. 3). × 70,000.

FIG. 6. Detail of wall of meningococcus (A_1) exposed to heat-inactivated rat serum for 5 min. The outer membrane (*OM*) and dense line (*DL*) of the cell wall, and the cytoplasmic membrane (*CM*) enclose the outer periplasmic space (*OPS*) and the inner periplasmic space (*IPS*). × 140,000.

FIGS. 7 and 8. *N. meningitidis* (A_1) exposed to normal rat serum (NRS) at 37°C. 30 sec after exposure to NRS there is a small amount of amorphous, electron-dense material (arrows) on the outer membrane and possible focal widening of the outer periplasmic space. × 70,000.



FIGS. 9 and 10. *N. meningitidis* (A_1) exposed to normal rat serum (NRS) at 37°C. After 2 min exposure to NRS there is abundant electron-dense material on the cell surface. The material has a distinctly fibrillar appearance, is radially oriented, and seems continuous with similar material in the outer periplasmic space. The inner periplasmic space is markedly widened and appears "empty." The cytoplasmic membrane is intact and the over-all density of the cytoplasm is only slightly decreased. × 70,000.

FIGS. 11 and 12. *N. meningitidis* (A_1) exposed to NRS at 37°C. By 5 min, NRS-treated meningococci show marked morphological alterations. Large gaps in the cytoplasmic membrane (*CM*) are associated with spillage of ribosomes (*R*) beyond the usual confines of the cytoplasm. There are many small foci of discontinuity in the middle dense line (see Fig. 13). Individual ribosomes and strands of nucleoid are separated to give the cytoplasm a "watery" appearance. × 70,000

FIG. 13. Higher magnification of *N. meningitidis* exposed to NRS for 5 min at 37°C. There are numerous gaps in the dense line (see rectangular enclosure). Radially oriented fibrillar material in the outer periplasmic space seems to extend through the outer membrane and to contact the remaining segments of dense line. × 140,000.

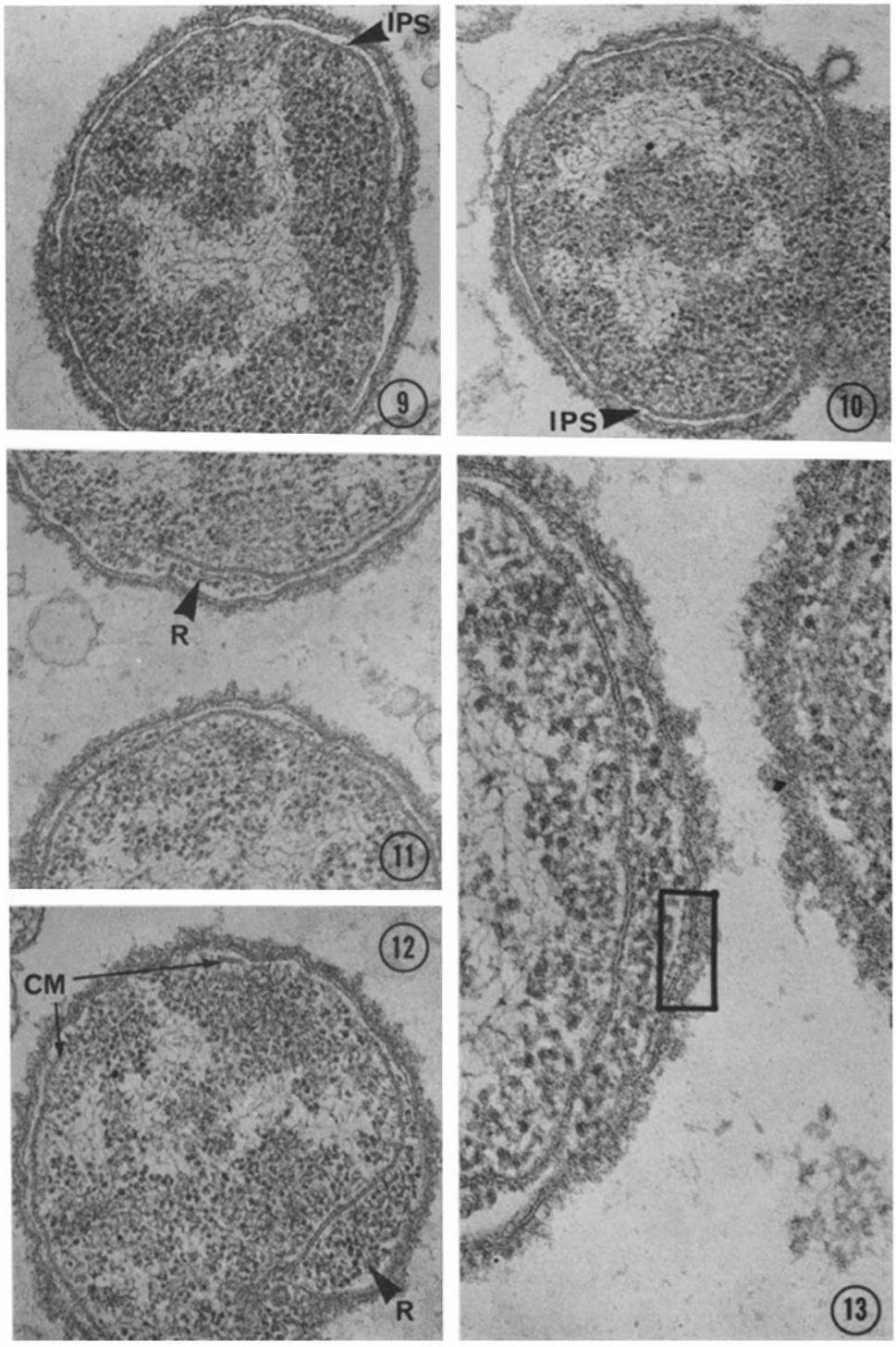


FIG. 14. *Neisseria catarrhalis* has essentially the same morphlogy after exposure to heat-inactivated rat serum as *N. meningitidis* (see Figs. 3–5). The dense line is thicker (35–45 A) than that in the meningococcus. × 70,000.

FIG. 15. Exposure of *N. catarrhalis* to NRS for 15 min results in morphological alterations similar to those seen in meningococci at 5 min (Figs. 11 and 12). However, in *N. catarrhalis* the dense line (*DL*) remains intact. There are large gaps in the cytoplasmic membrane (*CM*) and marked intracytoplasmic edema. Large amounts of fibrillar material are present on the outer membrane and in the outer periplasmic space. × 70,000.

FIG. 16. *Herellae sp.* exposed to heat-inactivated rat serum for 15 min. The bacterium has closely apposed cell wall laminae, extended fimbriae, and a compact cytoplasm. × 70,000.

FIG. 17. Incubation of *Herellea sp.* with NRS for 5 min produces morphological alterations similar to those seen in meningococci at 2 min (Figs. 9 and 10). The fimbriae appear curved and admixed with electron-dense, fibrillar material. Edema is prominent in the inner periplasmic space and cytoplasm. The cytoplasmic membrane and dense line are intact. × 70,000.

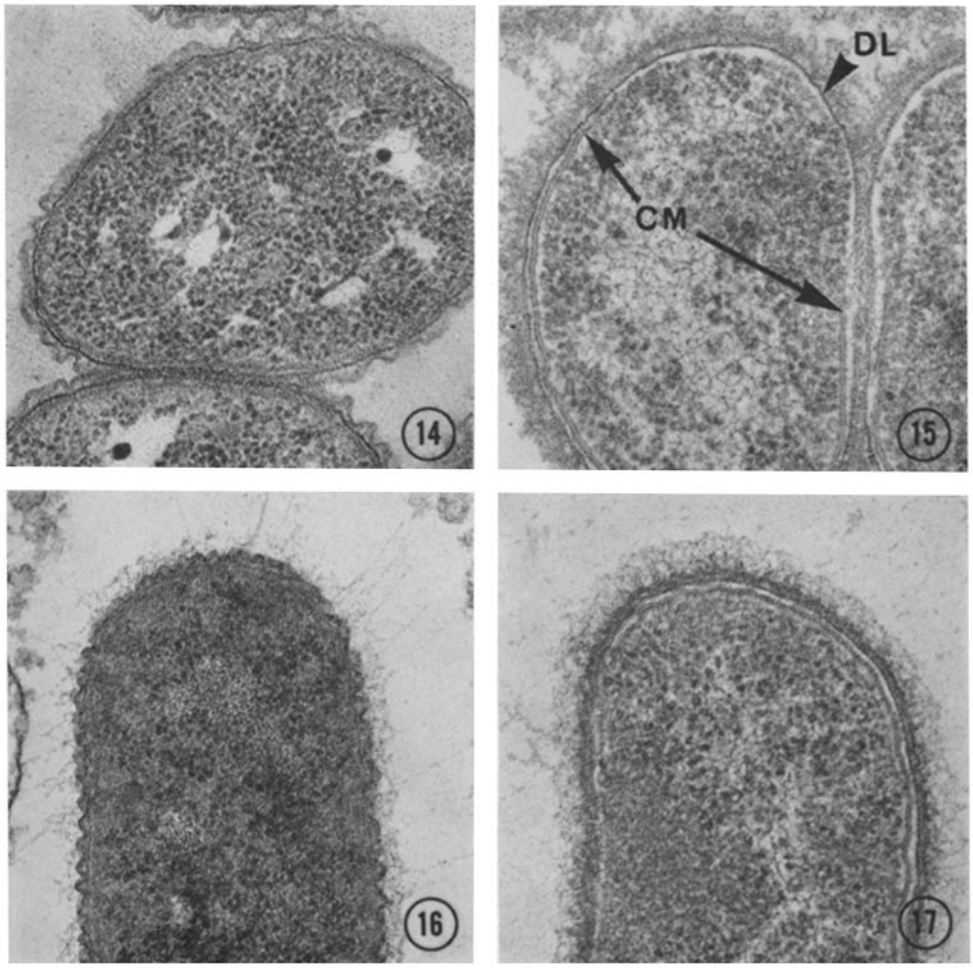
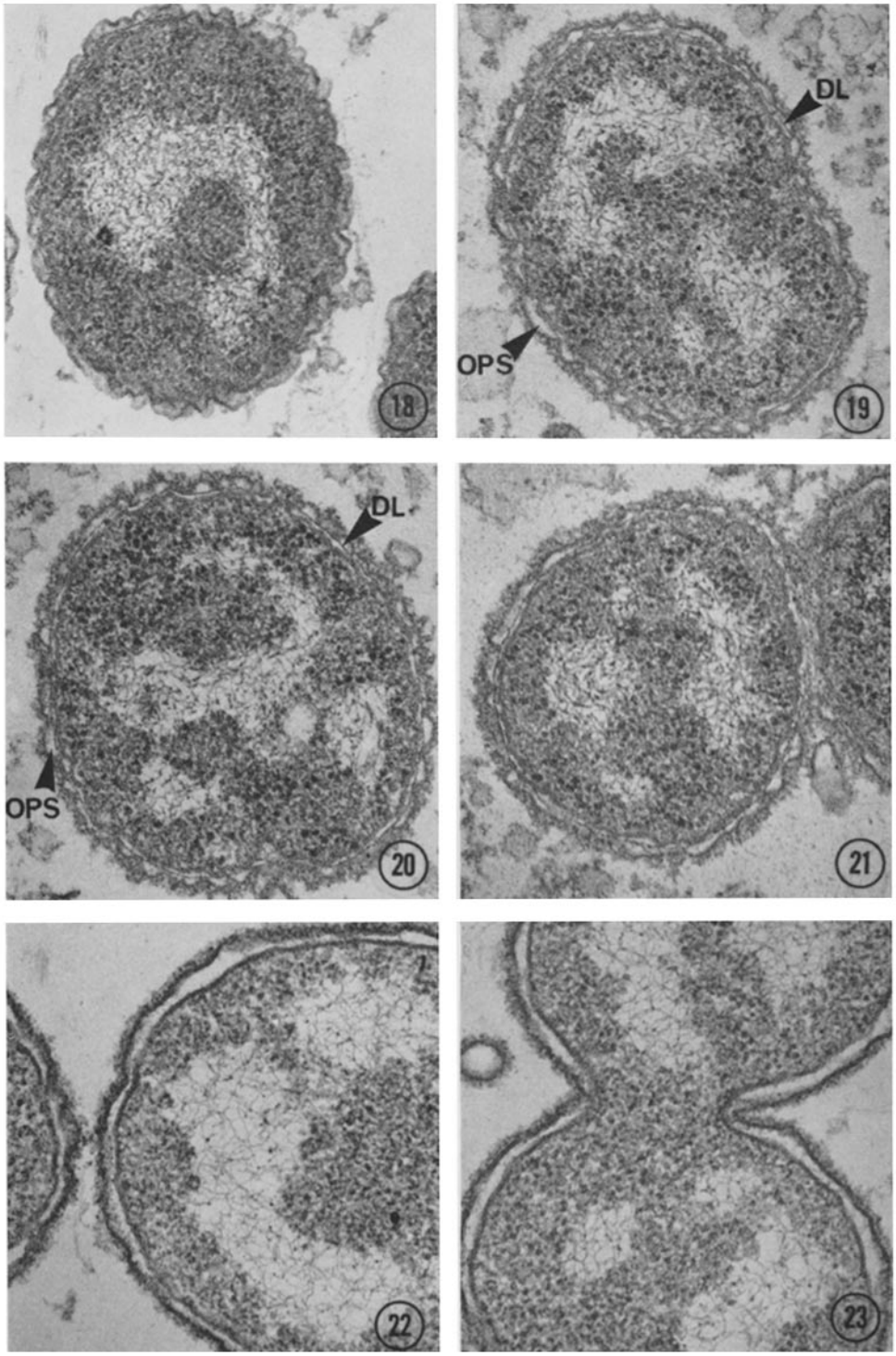


FIG. 18. *N. meningitidis* (A_1) exposed to heat-inactivated, lysozyme-deficient (bentonite-absorbed) rat serum for 5 min at 37°C. No ultrastructural changes are seen. × 70,000.

FIGS. 19 and 20. *N. meningtidis* (A_1) treated with lysozyme-deficient serum for 5 min. There is accumulation of fibrillar material on the surface, dilatation of the periplasmic space, and slight intracytoplasmic edema. Edema of the outer periplasmic space (*OPS*) predominates over that in the inner periplasmic space. The dense line (*DL*) is intact. × 70,000.

FIG. 21. *N. meningitidis* (A_1) exposed to lysozyme-deficient serum for 15 min. There is no progression of ultrastructural changes seen at 5 min (Fig. 20.) × 70,000.

FIGS. 22 and 23. Addition of 100 μg/ml egg white lysozyme to lysozyme-deficient serum alters the appearance of meingococci seen in Figs. 19–21. The dense line is absent and the periplasmic space is markedly dilated. There is marked edema of the cytoplasm. The cytoplasmic membrane is intact. × 70,000.



FIGS. 24–30. All organisms (except Fig. 25) were treated with serum, washed three times in distilled water, and negatively stained with sodium phosphotungstate.

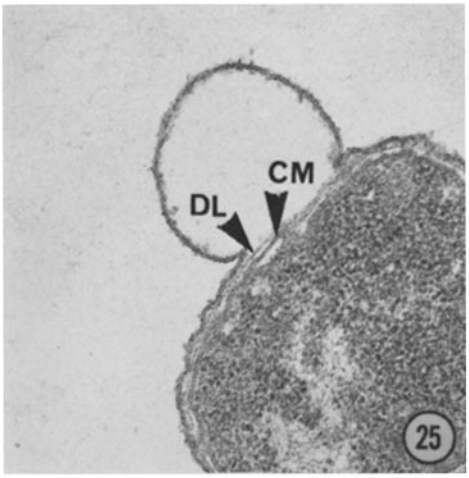
FIG. 24. *N. meningitidis* (A_1) exposed to NRS for 15 min at 37°C. Discrete foci of accumulated phosphotungstate are scattered over the entire surface of the bacterium. These foci, which presumably represent holes or pits, are 110 A in mean greatest diameter and are surrounded by halos of diminished electron opacity. × 100,000.

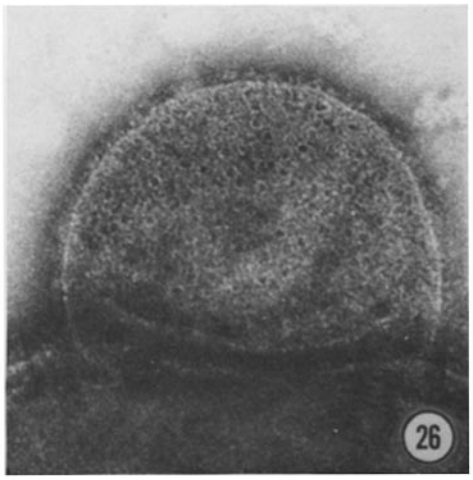
FIG. 25. *N. meningtidis* (A_1) treated with penicillin (100 units/ml) for 45 min at 37 C. A typical bleb, composed entirely of outer membrane, is present. The interior of the bleb is devoid of electron-opaque material and contains neither dense line (*DL*) nor cytoplasmic membrane (*CM*), which are seen at the base. × 70,000.

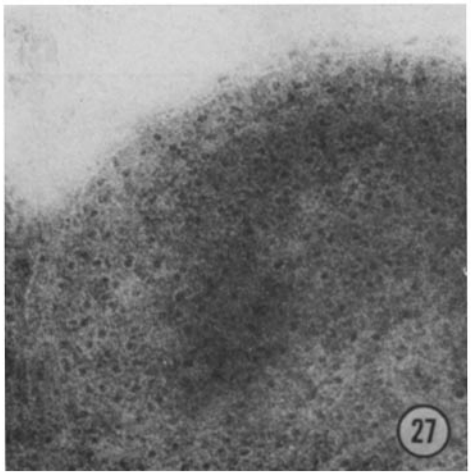
FIG. 26. Penicillin-induced outer membrane bleb on the surface of a meningococci (A_1); 15 min exposure to NRS. Numerous holes, similar to those seen in Fig. 24, are present on the surface of the bleb. × 100,000.

FIG. 27. Holes are present on the surface of this meningococcus (A_1) which was incubated for 15 min with lysozyme-deficient (bentonite-absorbed) serum. × 100,000.

FIG. 28. Typical 110 A holes are present in *N. meningtidis* (A_1) after treatment for 15 min with lysozyme-deficient serum plus 100 μg/ml egg white lysozyme. × 100,000.







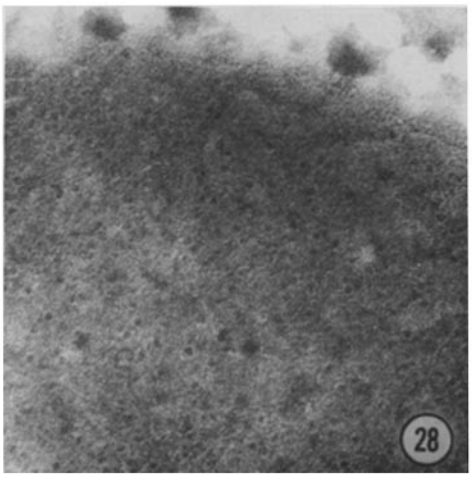


FIG. 29. *N. meningitidis* (A_1) exposed to heat-inactivated rat serum for 15 min. Local accumulations of phosphotungstate are present. These foci (circled) are smaller (82 A mean greatest diameter) and less numerous than holes seen in serum-killed meningococci (Fig. 24). Each hole is surrounded by a halo of decreased electron opacity. × 100,000.

FIG. 30. Strain C_{11} of *N. meningitidis* is not killed by normal rat serum. 82 A diameter holes are present on the surface of these bacteria before (not shown) and after (shown) exposure to NRS fr 15 min. × 100,000.

FIG. 31. Direct negative staining (no intermediate washing step) of untreated meningococcus (A_1) taken from a 24 hr Mueller-Hinton broth culture. Numerous 82 A diameter holes are present. The holes have a halo of decreased electron density and are similar to those seen in Figs. 29 and 30. × 100,000.