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Interrelationship Between Serum Beta-Lysin, Lysozyme, and the Antibody-Complement System in Killing *Escherichia coli*

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The effects of different serum components alone and in conjunction with each other on *Escherichia coli* B were investigated. In general, the viability, turbidity, and electron microscope results were compatible with the following conclusions. The most efficient killing and destruction of *E. coli* B occurred when beta-lysin, lysozyme, and the antibody-complement system functioned in cooperation with each other at the serum concentration in isotonic solutions. The addition of sucrose protected the bacteria from the lethal and lytic action of these agents. Elimination of lysozyme from serum had the least effect on bactericidal activity, even though lysozyme treatment caused the cell wall to separate from the cytoplasmic membrane and caused clear areas to appear in the inner granular layer of the cell wall. Beta-lysin removal had an intermediate effect on the serum bactericidal activity. Beta-lysin treatment caused cell walls to collapse, allowed cytoplasmic contents to leak out of the cells, and stopped the separation of cell wall and cytoplasmic membrane, which normally takes place in 0.5 M sucrose solution. Inactivation of the complement eliminated the serum bactericidal activity against *E. coli* B. After treatment with antibody and complement, the cell walls became thick and indistinct, a portion of the cytoplasmic contents escaped, and patches of the middle layer of the cell wall appeared in freeze-etch preparations. Beta-lysin damaged the cytoplasmic membrane, lysozyme damaged the inner peptidoglycan layer of the cell wall, and the antibody-complement system damaged both the middle lipopolysaccharide layer of the cell wall and the cytoplasmic membrane.

The necessity of antibody and complement in the killing of gram-negative bacteria and beta-lysin in the killing of gram-positive bacteria by serum has been known for over 80 years. Lysozyme cooperates with the antibody-complement system in both the bacteriolysis and bactericidal action of serum on gram-negative organisms. Treatment of serum by bentonite removes lysozyme and inhibits both the lysis (1, 12, 19, 26) and killing (10, 11) of these organisms. The addition of lysozyme to serum or bentonite-treated serum will accelerate or partially restore the bactericidal activity. The observation of Glynn and Milne (11), that the addition of lysozyme at 10 times the serum concentration did not completely restore the bactericidal activity of bentonite-treated serum, caused them to postulate the existence of a bentonite-absorbable factor other than lysozyme, which is essential for serum bactericidal activity. Such a serum factor could be beta-lysin, even though purified beta-lysin is not bactericidal for gram-negative bacteria. In these experiments, *Escherichia coli* B was exposed to beta-lysin, lysozyme, and the complement-antibody system, alone or in combination with each other at physiological concentrations. Thin sections and freeze-etch preparations of the treated bacteria were examined with an electron microscope. The morphological changes were studied in conjunction with the viability and turbidity changes. The results helped clarify the interrelationships between the serum bactericidal components and determined the specific action sites of these different bactericidal agents. Since these experiments were carried out in both physiological saline solution (PSS) and 0.5 M sucrose solution, the significance of osmotic pressure in the killing of bacteria by serum was demonstrated.

MATERIALS AND METHODS

Organisms. The laboratory stock cultures of *E. coli* B were used in this study.

Beta-lysin, anti-beta-lysin, and lysozyme. Beta-lysin was purified and assayed as previously described

(5). In serum, beta-lysin can be stored at −70 C for months without loss of bactericidal activity. However, storage of the purified preparations in liquid form at any temperature, including −70 C, resulted in the loss of its bactericidal effect. In this study, freshly prepared beta-lysin was lyophilized. It maintained bactericidal activity for 1 year in this form.

Anti-beta-lysin was prepared in guinea pigs against rabbit beta-lysin as previously described (6).

Lysozyme was supplied by the Sigma Chemical Co., St. Louis, Mo. The egg white lysozyme had been crystallized three times, dialyzed, and lyophilized.

Determination of the percentage of lysis in E. coli B. All cultures were grown at 37 C, and the flasks were aerated by rotation. An overnight culture (2 ml) of *E. coli* B, grown in nutrient broth containing 0.1% glucose, was inoculated into 18 ml of nutrient broth and incubated for 2 h. The *E. coli* B was harvested and washed, and its concentration was adjusted in PSS so that a 1:10 dilution would have an optical density (OD) of 0.3 at 650 nm. The concentrated *E. coli* B suspension was then diluted 1:10 in a sample to be tested. All test samples containing bacteria were incubated at 37 C, and changes of optical density at 650 nm were recorded. The percentage of lysis was calculated by using the following formula:

$$\% \text{ lysis} = \frac{(\text{initial OD} - \text{final OD}) \times 100}{\text{initial OD of the bacterial suspension}}$$

Bactericidal tests. *E. coli* B was cultured and washed as described above. A 0.1-ml volume of the bacterial suspension, which contained approximately 5×10^7 cells, was added to 0.9 ml of the sample to be tested. The number of surviving organisms was determined after 10 min by preparing pour plates of various dilutions of the sample. The diluent was generally supplemented with 0.5 M sucrose to protect fragile cells.

Bentonite-treated rabbit serum (BRS). A 10-mg amount of bentonite was added per ml of rabbit serum, mixed throughly, and incubated at 37 C for 10 min. The mixture was then centrifuged to sediment the bentonite, and the supernatant fluid was collected and filtered through a 0.47-μm pore diameter Millipore membrane. This treatment removed both the beta-lysin and lysozyme, but did not alter the serum antibody or complement concentrations (11).

Thin-section electron microscopy. Bacterial cells were fixed for 2 h at 20 C with 2% electron microscope-grade gluteraldehyde. The cells were washed with 0.1 M sodium cacodylate buffer, pH 7.5, and then placed in 1% osmium tetraoxide at 0 C for 2 h. The specimens were washed again with buffer, suspended in 0.5% uranyl acetate, and allowed to stand overnight at room temperature. The cells were then washed with distilled water, dehydrated with ethyl alcohol and acetone, and embedded in Mollenhauer's (14) epoxy resin mixture number II. Thin sections were prepared using Sorvall Porter-Blum Mt-2 ultramicrotome. The sections were placed on Formvar-coated copper grids and poststained with lead citrate (23). The specimens were examined with a Hitachi HS-8 electron microscope with a 50-kV accelerating voltage.

Freeze-etch electron microscopy. The bacteria were sedimented by centrifugation. A drop of the residue was placed on a 3-mm gold disk; frozen specimens were placed on a −150 C table in a Balzers freeze-etching unit and freeze-etched as described by Moor and Mühlethaler (16, 17). After etching and shadowing, the specimens were warmed to room temperature and removed from the gold disks. The replicas were treated with chromic acid, washed with distilled water, and treated with Clorox to remove the organic material. The specimens were placed on uncoated copper grids and examined in a Hitachi HS-8 electron microscope with a 50-kV accelerating voltage.

RESULTS

Bactericidal tests for *E. coli* B were carried out to determine the effect of normal serum components on viability. Bactericidal tests were carried out with normal rabbit serum and heated rabbit serum, as well as BRS, lysozyme, and beta-lysin, alone and in combination with each other. All test solutions, including serum and its components, contained 0.5 M sucrose so that cell wall damage would not cause cytoplasmic membranes to rupture due to osmotic pressure. The results of these experiments are recorded in Table 1. Normal serum concentrations of lysozyme (1.2 μg/ml) and beta-lysin (32 U/ml) were used in these bactericidal tests. Normal serum from adult rabbits contained antibody against *E. coli* B in addition to complement, lysozyme, and beta-lysin. This serum was bactericidal for *E. coli* B and killed 99% of an inoculum of 10^7 *E. coli* B in 10 min at 37 C. The serum bactericidal activity was complement dependent, since heating at 56 C for 30 min eliminated the bactericidal activity without affecting the antibody, lysozyme, or beta-lysin concentrations of serum. BRS, which contained no beta-lysin or lysozyme but normal

TABLE 1. *Bactericidal action of rabbit serum and serum components in 0.5 M sucrose on E. coli B*

Preparation[a]	Survivors per ml	Survival (%)
0.5 M sucrose	4.5×10^7	100
Normal serum (Ab + C′ + beta-lysin + lysozyme)	4.7×10^5	1
Heated serum (Ab + beta-lysin + lysozyme)	4.3×10^7	95
BRS (Ab + C′)	6.7×10^7	100
BRS + beta-lysin + lysozyme	3.4×10^5	0.8
BRS + beta-lysin	3.2×10^5	0.7
BRS + lysozyme	3.8×10^7	85
Beta-lysin	6.3×10^7	100
Beta-lysin + lysozyme	6.5×10^7	100
Lysozyme	4.8×10^7	100

[a] Ab, Antibody; C′, complement.

levels of antibody and complement (11), was not bactericidal in sucrose as evidenced by 100% survival of the bacteria. The restoration of physiological levels of beta-lysin to BRS restored the bactericidal properties of the serum. In contrast, the addition of lysozyme to physiological concentrations did not restore the bactericidal activity of the BRS.

Some clarification of the action site of the antibody, complement, beta-lysin, and lysozyme system was derived by the study of electron micrographs of treated *E. coli* B in 0.5 M sucrose solution. Electron micrographs of the typical cells were compiled into Fig. 1, so that a comparison of these cells after the specific treatments could be made. There was always a slight separation between the cell wall and the cytoplasmic membrane when *E. coli* B had been in 0.5 M sucrose solution for 10 min. It was assumed that this periplasmic space was due to a loss of water because of the osmotic effects of the sucrose. When beta-lysin-treated cells (Fig. 1B) were compared with untreated cells, the most consistent difference was that the periplasmic space disappeared. This could be due to damage of the plasma membrane by beta-lysin with the loss of the semipermeable characteristics of the membrane. The most obvious lysozyme-induced alteration (Fig. 1C) was the extensive separation of the cytoplasmic membrane and the cell wall. The cytoplasmic membrane of these lysozyme-treated cells remained intact and enclosed the cytoplasmic contents. Cells treated with both beta-lysin and lysozyme (Fig. 1D) showed a marked separation of the cell wall from the cytoplasmic membrane and membrane damage. The release of cell contents from the cell was attributed to damage of the cytoplasmic membrane. Cells treated with BRS (Fig. 1E) lacked a clearly-defined periplasmic space. The cytoplasmic contents were less dense, and the cell walls were thicker and less distinct. These observations indicated that both the cytoplasmic membrane and the cell wall were damaged by the antibody-complement system.

The lysozyme-induced separation of the cell wall and the cytoplasmic membrane (Fig. 1C) disappeared or was greatly diminished when BRS was present with the lysozyme (Fig. 1F). The assumption that this was caused by antibody-complement action on the cytoplasmic membrane was in accord with the observation that the cytoplasm always appeared less dense when both agents were used. BRS-treated cells (Fig. 1E) were mainly rod-shaped, but became round when lysozyme was added (Fig. 1F).

The extent of morphological damage, which takes place when these bacteria are killed by the antibody-complement system working in concert with beta-lysin, is illustrated in Fig. 1G and 1H. The cell debris, which is seen when *E. coli* B is treated with normal rabbit serum (Fig. 1H), is comparable to that following treatment with beta-lysin in BRS (Fig. 1G). Although an occasional cell appeared normal, the vast majority of these cells appeared as ghosts.

Samples prepared for electron microscopy by the freeze-etch technique were unsatisfactory when bactericidal tests were performed in sucrose. The high sucrose concentration appeared to precipitate and mask structural details of the bacteria. This was not the case when bactericidal tests were done in the absence of sucrose. The results obtained when the various serum constituents were checked for bactericidal activity in PSS is shown in Table 2. The significance of osmotic shock in the bactericidal reaction becomes obvious when these results are compared with those obtained from bactericidal tests performed in 0.5 M sucrose solution (Table 1). The degree of killing was much greater in the absence of sucrose with the different serum constituents. Normal rabbit serum reduced the viable bacteria from 7.6×10^7 to 1.8×10^3/ml, for a bacterial survival of 0.002% in 10 min, when the bactericidal test was conducted in PSS. Serum bactericidal activity was completely eliminated when complement activity was destroyed by heat. The serum retained part of its bactericidal activity, killing 99.5% of the *E. coli* B, when the beta-lysin and lysozyme were removed by bentonite. When either lysozyme or beta-lysin were added to BRS at physiological concentrations, bactericidal activity was not enhanced. In fact, the addition of lysozyme to BRS consistently protected some of the bacteria from the bactericidal action of BRS. This result was unexpected since others (7, 8, 11, 19, 20, 24, 27) have shown that lysozyme, in concentrations 10 times those used in this study, increased bacteriolytic and bactericidal activity of BRS. The addition of both beta-lysin and lysozyme enhanced the bactericidal activity of BRS. The samples which contained either beta-lysin, lysozyme, or beta-lysin plus lysozyme were only slightly bactericidal.

To determine whether the neutralization of beta-lysin in rabbit serum would reduce the bactericidal activity against *E. coli* B, equal volumes of serum and heat-inactivated anti-beta-lysin were mixed. After incubation for 10 min at 37 C, the bacterial survival was determined by the standard plate count method. Normal serum reduced the number of viable *E. coli* B over 1,000-fold, whereas serum plus

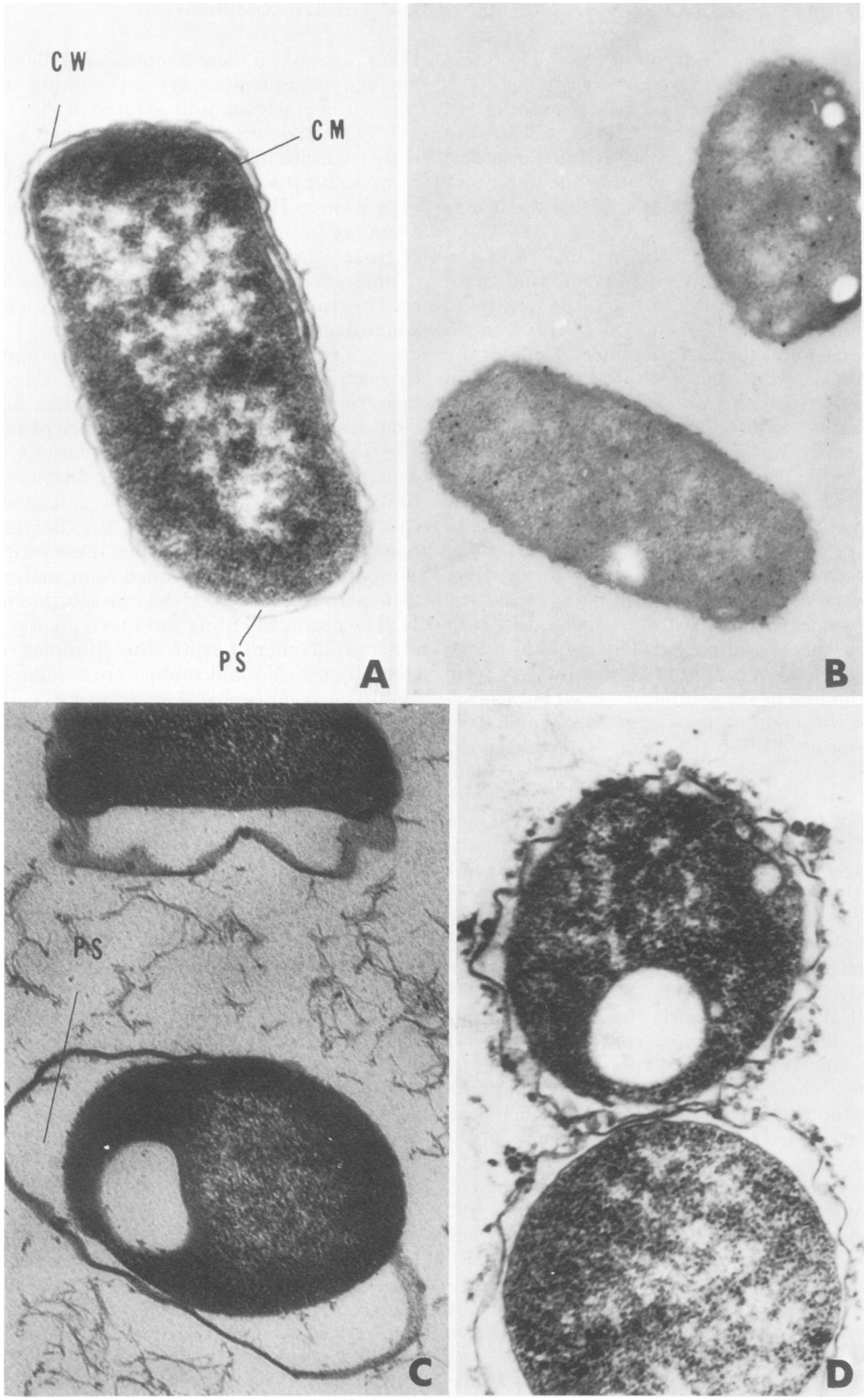

FIG. 1. *Effect of serum constituents alone or in combination on appearance of E. coli B as observed with the electron microscope thin-section technique. Treatments: (A) control (none), (B) beta-lysin, (C) lysozyme, (D) beta-lysin plus lysozyme, (E) BRS, (F) BRS plus lysozyme, (G) BRS plus beta-lysin and lysozyme, (H) rabbit serum. CW, Cell wall; CM, cell membrane; and PS, periplasmic space.* (×36,000)

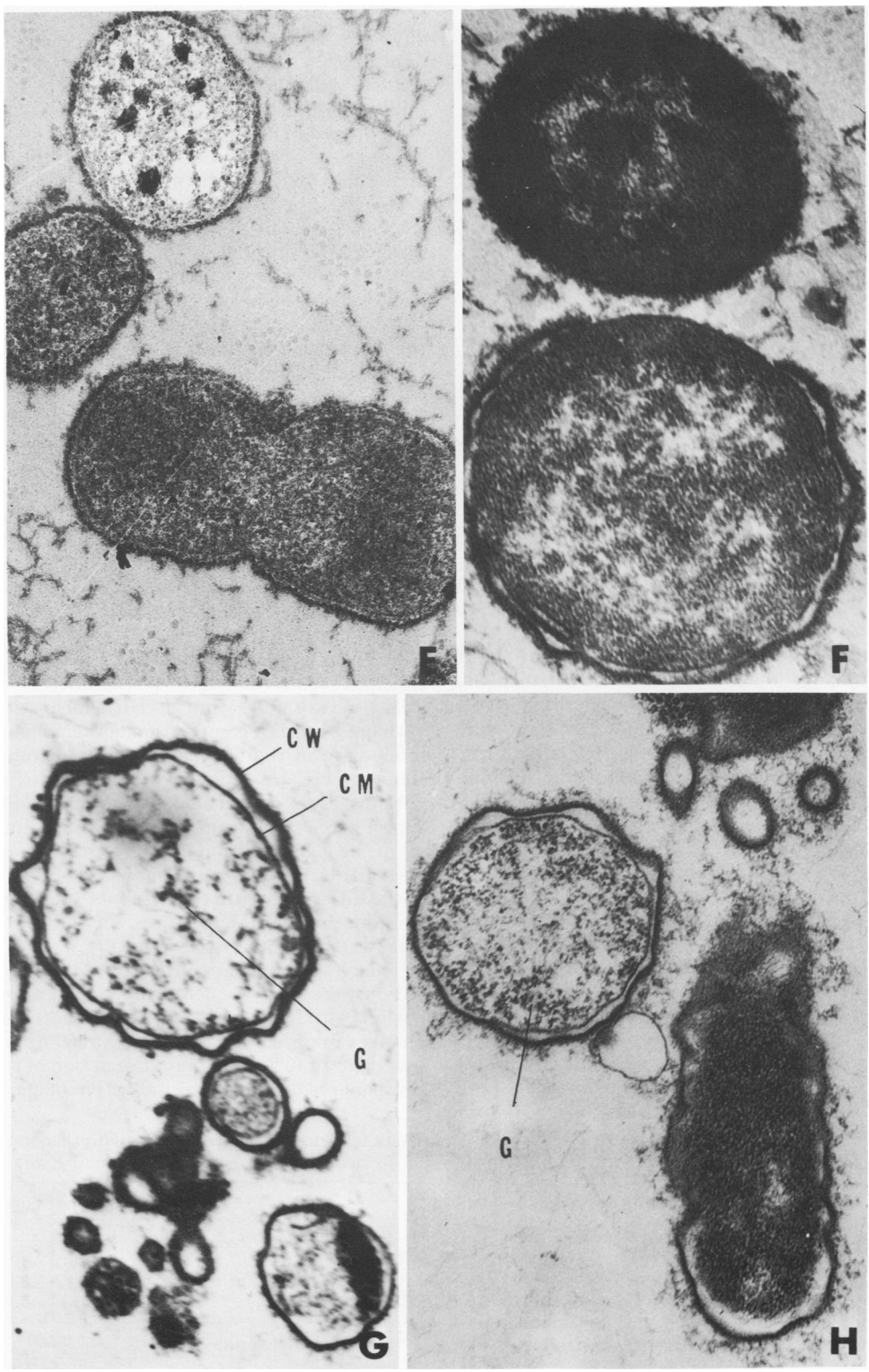

FIG. 1—*continued*

TABLE 2. *Bactericidal action of rabbit serum and serum components in PSS on E. coli B*

Preparation	Survivors per ml	Survival (%)
PSS	7.6×10^7	100
Normal serum (Ab + C′ + beta-lysin + lysozyme)	1.8×10^3	0.002
Heated serum (Ab + beta-lysin + lysozyme)	1.9×10^8	100
BRS (Ab + C′)	3.7×10^5	0.5
BRS + beta-lysin + lysozyme	2.7×10^4	0.04
BRS + beta-lysin	1.1×10^6	1.5
BRS + lysozyme	8.4×10^6	11
Beta-lysin	5.8×10^7	76
Beta-lysin + lysozyme	4.6×10^7	60
Lysozyme	4.6×10^7	60

anti-beta-lysin reduced the viable bacteria less than 10-fold (Table 3).

This freeze-etch electron microscopy study was undertaken with the expectation that further information would be acquired concerning the action sites of the bactericidal constituents of serum. Electron micrographs of cells exposed to different serum constituents in PSS for 10 min were compiled into Fig. 2. The freeze-etch technique revealed the surfaces of the different cell wall layers which were not distinguishable in thin-section studies. The control cell (Fig. 2A) had the three typical surfaces that were observed after freeze-etching. In general, the beta-lysin-treated *E. coli* B appeared normal, except that 48% of the cells exhibited a characteristic collapsing of either the cell wall or the cytoplasmic membrane (Fig. 2B). This indicated some loss of the cytoplasmic contents, which was consistent with the thin-section results. These beta-lysin-treated cells retained their typical rod shape. When *E. coli* B were treated with lysozyme (Fig. 2C), areas devoid of granules were observed above the cytoplasmic membrane. This was unexpected since others referred to this granular layer as the surface of the cytoplasmic membrane (2, 4, 21, 25). Most of these lysozyme-treated cells retained their typical rod shape. Treatment of *E. coli* B with BRS resulted in discrete patches on the second layer of the cell wall adhering to the inner peptidoglycan layer (Fig. 2D) in most of the cells. The cytoplasm of most of the BRS-treated cells was extensively damaged and pitted when the cells cross fractured. This abnormal cytoplasm could be the result of a loss of cell content or an increase in the water content of the cytoplasm. Although these cells appeared to swell, they still retained their rod shape. The presence of lysozyme in BRS during treatment of *E. coli* B resulted in a different type of cell damage (Fig. 2E). Most of the BRS-lysozyme-treated cells had patches composed of the middle layer of cell wall, but 60% of the patches were large and covered most of the cytoplasmic membrane. The remaining patches were small and could not be distinguished from those observed after treatment of *E. coli* B by BRS without lysozyme (Fig. 2D). More than 90% of these *E. coli* lost their characteristic rod shape and became round, due to the action of antibody, complement, and lysozyme. The pitting of the cytoplasm resembled that reported when the bacteria were treated with BRS without lysozyme. Whenever beta-lysin was present with the antibody-complement system, the damage to the bacteria was so extensive that very few surfaces were observed in the freeze-etch preparations. Practically all of these cells lost their rod shape and appeared as ghosts, consisting of empty cell walls (Fig. 2F), or appeared as a cross section of cells with pitted cytoplasm (Fig. 2G). This was the case when cells were exposed to either normal serum or BRS plus beta-lysin. The almost complete destruction of the bacteria, as seen with the electron microscope, is consistent with the bactericidal results that most of the bacteria were killed by normal serum or BRS reconstituted with beta-lysin. A compilation of the percentage of changes in the morphological appearance of freeze-etched *E. coli* B after treatment by various serum constituents is shown in Table 4. These percentages were obtained by examining all of the electron micrographs after the various treatments. These results show that the effects of the different serum constituents, alone and in combination with each other, on the bacterial cells were of a quantitative nature.

The lysis of a culture of *E. coli* B treated with various serum constituents, alone and in combination with each other, was calculated from changes in optical density read at 650 nm. This was done to further explain the action of serum components on these gram-negative bacteria. A

TABLE 3. *Neutralization of rabbit serum bactericidal activity on E. coli B by anti-beta-lysin*

Treatment	Survivors per ml	Survivors (%)
NRS[a]	1.0×10^5	0.077
NRS + anti-beta-lysin	4.3×10^7	33
PSS control	1.3×10^8	100

[a] NRS, Normal serum.

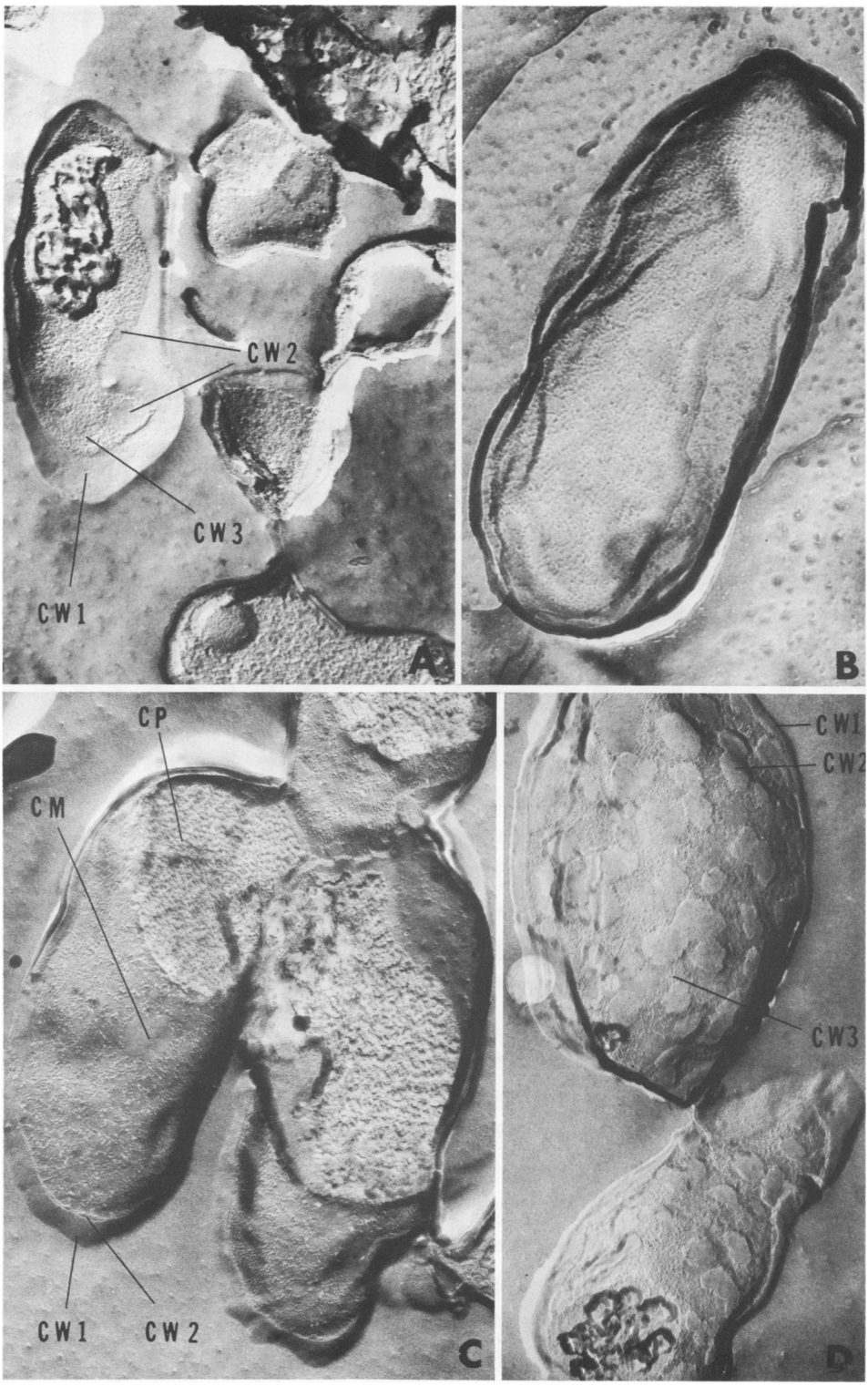

FIG. 2. *Effect of serum constituents alone or in combination on the appearance of E. coli B as observed with freeze-etch electron microscope technique. Treatments: (A) control (none), (B) beta-lysin, (C) lysozyme, (D) BRS, (E) BRS plus lysozyme, (F and G) BRS plus beta-lysin. CW1, Outer cell wall layer; CW2, middle cell wall layer; CW3, inner cell wall layer; CM, cell membrane; and CP, cytoplasm.* (×36,000)

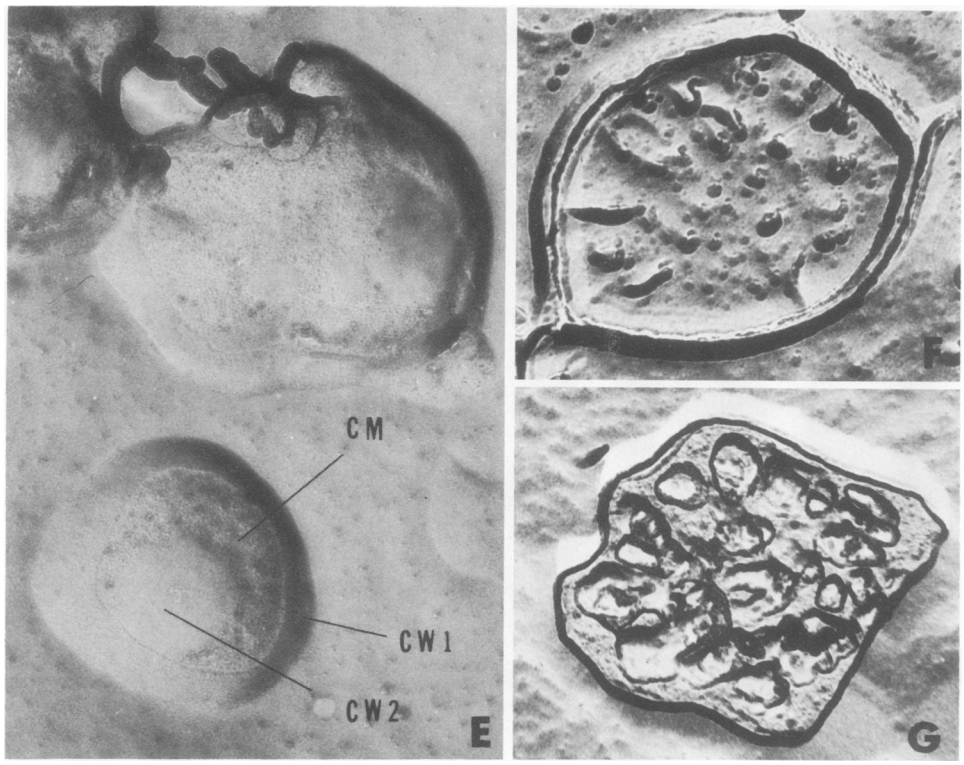

FIG. 2—*continued*

display of the data accumulated from these experiments is shown in Fig. 3. Normal serum and BRS, to which beta-lysin and lysozyme had been restored to normal levels, quickly caused the lysis of the bacteria, reaching at least 90%. BRS plus beta-lysin also lysed the bacteria, although this treatment was neither as rapid nor as effective as normal serum. Beta-lysin or lysozyme in PSS caused a slight nonspecific lysis of the bacteria. BRS plus lysozyme caused no lysis. The results of these experiments are consistent with the bactericidal data recorded in Table 2 and further demonstrate the requirement for all components of serum for the optimal activity against *E. coli* B.

DISCUSSION

It has been previously reported (24) that normal serum injures all of the peripheral structures of *E. coli*. This study confirmed the observation (1, 10, 12, 19, 26) that the antibody-complement system functions in conjunction with other serum constituents in the injury of these gram-negative bacteria. Even though beta-lysin, lysozyme, and the antibody-complement system augment each other, it is apparent that the antibody-complement system is of primary significance in the serum bactericidal activity for *E. coli* B. This was illustrated by the following observations. (i) Inactivation of complement by heat caused a complete loss of serum bactericidal activity. (ii) Beta-lysin and lysozyme, alone or in conjunction with each other, had little or no lethal effect on these gram-negative organisms. (iii) The antibody-complement system appeared to cause more widespread damage to peripheral structures than either beta-lysin or lysozyme. Alterations, similar to those reported on the lipopolysaccharide-phospholipid layer of the cell wall (7) and the cytoplasmic membrane (8), were observed after exposure to the antibody-complement system. In contrast, beta-lysin action appeared to be restricted to the cytoplasmic membrane, and lysozyme action restricted to the inner layer of the cell wall.

According to DePetris (3), the smoother outer layer of the *E. coli* cell wall is composed of lipoprotein, and the middle smooth layer of the

TABLE 4. *Freeze-etched E. coli B after treatment with different serum constituents*

Treatment	*E. coli* B (%) that appeared as:					
	Collapsed cells	Cells with distinct clear areas on cytoplasmic membrane	Cells with patches of the middle cell wall layer	Round cells	Cells with pitted cytoplasm	Cell wall ghosts
PSS control	0	0	4	0	2	0
Beta-lysin	48	17	5	0	5	2
Lysozyme	16	100	4	18	9	0
BRS	22	7	63	4	57	0
Beta-lysin + lysozyme	9	75	11	2	7	0
BRS + beta-lysin	[a]	[b]	[a]	94	92	39
BRS + lysozyme	[a]	[a]	89	94	84	2
BRS + lysozyme + beta-lysin	[a]	[a]	[a]	78	48	17
Normal rabbit serum	[a]	[a]	[a]	[a]	85	24

[a] Damage was so extensive that an accurate count for this alteration was not possible.
[b] The cell membrane was obstructed by the numerous patches of middle layer of the cell wall.

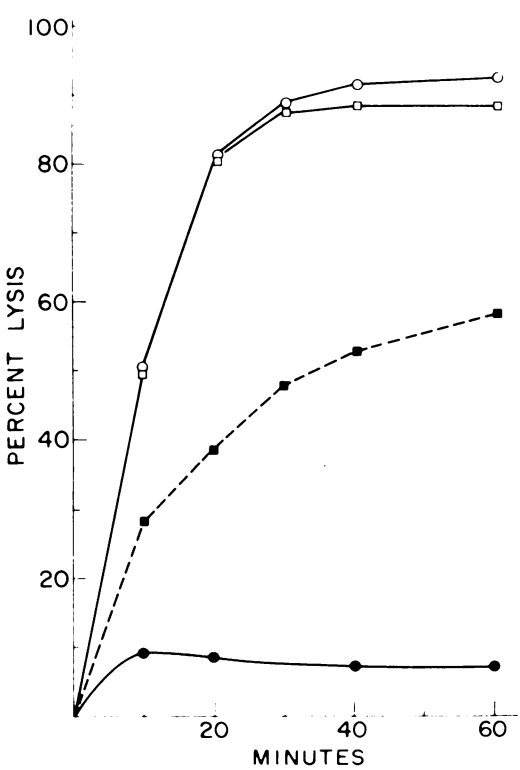

FIG. 3. *Percentage of lysis of E. coli B by rabbit serum and serum components in PSS. Where indicated, lysozyme and beta-lysin were used. Controls consisted of tests using beta-lysin, lysozyme, or PSS. Bentonite-treated serum plus lysozyme and heated serum caused no lysis, and, actually, turbidity increased. Symbols:* ○, *normal rabbit serum;* □, *BRS plus beta-lysin and lysozyme;* ■, *BRS plus beta-lysin;* ●, *controls.*

cell wall is composed of lipopolysaccharide. The inner granular surface is usually referred to as the cytoplasmic or protoplasmic membrane (2, 4, 21, 25). The particles on this inner layer are believed to be multienzyme complexes (15, 18) or transport sites (27). Our results indicate that these granules were part of the cell wall. It is our contention that this granular layer is the inner peptidoglycan layer of the cell wall or fragments of the peptidoglycan layer which are closely associated with the cytoplasmic membrane. This conclusion is based on the observation that 100% of the *E. coli* B treated with lysozyme developed obvious and distinct areas devoid of granules on the cytoplasmic membrane. The postulation that these granules are composed of peptidoglycan and are part of the innermost layer of the cell wall is strengthened by the previous finding that freeze-etched preparations of magnesium-starved *E. coli* have areas of cytoplasmic membranes devoid of granules (9). Magnesium is required for the synthesis of the peptidoglycan layer of the cell wall of *E. coli* (22). The close association between the innermost layer of the cell wall and the cytoplasmic membrane was confirmed by the thin-section studies. In these studies, lysozyme-treated cells developed a large space between the cell wall and the intact cytoplasmic membrane. Although Bayer and Remsen (2) observed areas in cytoplasmic membranes of untreated *E. coli* which were devoid of granules, such areas were much smaller and less extensive than those observed in this study following lysozyme treatment.

The patches of the middle layer of the cell wall observed on freeze-etched preparations

exposed to BRS in PSS were similar in appearance to those reported by Bayer and Remsen (2) when *E. coli* was frozen in the presence of 10% sucrose.

A comparison of the results of bactericidal tests, carried out in the presence or absence of 0.5 M sucrose solution, revealed that osmotic forces are of major significance in the killing of bacteria by the bactericidal constituents of serum. Larger numbers of bacteria survived when the serum, or its various components in different combinations, were tested for bactericidal activity in the presence of sucrose. These results are in accord with those of Feingold et al. (8), who showed that bentonite-absorbed serum had reduced bactericidal activity against *E. coli* in 0.6 M sucrose solutions.

Even though extensive morphological damage to either cell wall or cytoplasmic membranes can be caused by either the antibody-complement system, lysozyme, or beta-lysin, the *E. coli* often recover from this damage. This appears to be particularly significant when osmotic forces are reduced by the addition of sucrose to the test medium.

Beta-lysin has been shown to act on the cytoplasmic membrane of the highly susceptible gram-positive bacteria (13), but is not bactericidal for gram-negative bacteria. The results of this study demonstrated that beta-lysin also damaged the cytoplasmic membrane of gram-negative bacteria, but these organisms were still capable of surviving. This is consistent with the knowledge that the gram-stain differences are due to differences in the cell wall and not in the cytoplasmic membrane. It appears that the complex gram-negative cell wall is more effective than the gram-positive cell wall in enabling bacteria to survive cytoplasmic membrane damage.

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