EFFECTS OF HUMAN NEUTROPHIL GRANULE EXTRACT
ON NEISSERIA GONORRHOEAE

by

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Neisseria gonorrhoeae, grown to log-phase in vitro, was treated with human neutrophil granule extract. Bacterial viability, macromolecular synthesis, and membrane permeability were then evaluated. Killing was time and concentration dependent, with greater than 90% loss of viability at 60 minutes with 750 μg/ml granule extract. Increasing Mg++ concentrations progressively inhibited killing by granule extract. Granule extract bactericidal activity was due to oxygen-independent antimicrobial systems of neutrophils.

Killing of N. gonorrhoeae by granule extract was not due to the inhibition of RNA or protein synthesis. Granule extract treated bacteria, which had lost 80-90% viability, incorporated the same amount of radiolabelled precursor into trichloroacetic acid precipitable material as did untreated bacteria. Ribonucleic acid synthesis was slightly more resistant than protein synthesis to the effects of granule extract.

Bacterial membrane permeability was increased in the presence of granule extract. Actinomycin D was found to enter cells and inhibit RNA and protein synthesis in granule extract treated bacteria but not in untreated bacteria. Disruption of membranes by detergent or granule extract enhanced the association of ³H-actinomycin D with N. gonorrhoeae.
Granule extract and actinomycin D showed a synergistic bactericidal effect. Chloramphenicol did not enhance the bactericidal activity of granule extract.
CHAPTER I

INTRODUCTION

Polymorphonuclear leukocytes (PMN or neutrophils) constitute an important part of the defense mechanisms a human possesses in the fight against microbial infections. Their function is to move toward, phagocytose, and eventually kill and degrade any microorganism that has gained entrance into a host organism. Without normally functioning PMN, a host is much more susceptible to the invasive and virulent factors of many bacteria.

Much research has been devoted to the phagocytic powers of neutrophils. Most of the work centers on two groups of factors: (1) those present in the host which facilitate phagocytosis by neutrophils (e.g., immunoglobins and complement; see Klebanoff and Clark, 1978, Ch. 4, for an extensive review), and (2) factors possessed by bacteria which impede or inhibit phagocytosis (e.g., capsules, Verbrugh et al., 1979; Wilkinson et al., 1979; and staphylococcal protein A, Peterson et al., 1977). These and other studies indicate that phagocytosis is a dynamic bacteria-PMN interaction, which is dependent upon many factors, and that the kinetics of phagocytosis vary with the
species and even strains of bacteria studied (Klebanoff and Clark, 1978, Ch. 4).

Polymorphonuclear Leukocyte Bactericidal Activity

The microbicidal or killing activity of PMN also has been documented extensively (for review, see Klebanoff and Clark, 1978, Ch. 7). These studies have focused on three aspects of the bactericidal activity of PMN: bactericidal activity associated with metabolic changes, bactericidal characteristics of phagolysosomes and the contents of granules, and bacterial properties which alter bactericidal activity.

Bactericidal Activity Associated with Metabolic Changes

During phagocytosis, or attempted phagocytosis, neutrophils are stimulated and exhibit an increase in oxygen uptake and utilization of glucose through the hexosemonophosphate shunt (HMPS) (Sbarra and Karnovsky, 1959; DeChatelet, 1978). A product of the HMPS is an increased level of intracellular NAD(P)H^+, which is oxidized by an NAD(P)H^+ oxidase, forming superoxide (•O_2^-) (Karnovsky, 1968). Subsequent to the formation of •O_2^-, other oxygen derivatives increase, such as hydrogen peroxide (H_2O_2), singlet oxygen (^1O_2), and hydroxyl radical (OH·). As possible bactericidal agents, these oxygen derivatives constitute the oxygen dependent antimicrobial system. The oxygen derivatives may
be bactericidal themselves, or they may be part of other bactericidal systems, such as $H_2O_2$ in the myeloperoxidase (MPO)-halide-$H_2O_2$ antimicrobial system (for review, see DeChatelet, 1975).

Hydrogen peroxide in relatively high concentrations is microbicidal by itself. It may be important in the overall microbicidal activity of PMN against bacteria which cannot degrade $H_2O_2$, such as the catalase negative bacteria. The concentration of $H_2O_2$ within normal phagocytosing neutrophils varies over time, due to its use by the MPO mediated antimicrobial system and its conversion to other oxygen derivatives. However, since release of $H_2O_2$ into the extracellular medium has been shown to occur (Root et al., 1975), the development of a bactericidal concentration surrounding an ingested bacterium is questionable. Thus, the major role of $H_2O_2$ in the neutrophil antibacterial systems is believed to be in conjunction with MPO and a halide, most likely chloride.

Superoxide, as an independent antimicrobial agent within PMN, is thought to play a minor role. Aerobic or aerotolerant bacteria produce large quantities of superoxide dismutase to combat the toxic effects of superoxide. Since most pathogenic bacteria are aerobic or aerotolerant, they are relatively resistant to superoxide. Ismail, Sawyer, and Wegener (1977) found that of $H_2O_2$ and $O_2^-$ produced in vitro in the purine-xanthine oxidase reaction, the $H_2O_2$ was
bactericidal for N. gonorrhoeae while \( \cdot O_2^- \) was not. Since \( H_2O_2 \) is formed directly from superoxide, the major role of superoxide in the bactericidal process appears to be in the formation of \( H_2O_2 \).

Although singlet oxygen has yet to be definitively demonstrated in intact PMN, its presence is suggested by the demonstration of light emission from intact bacteria during phagocytosis (Allen, Stjernholm, and Steele, 1972). Singlet oxygen as a bactericidal agent in neutrophils has been suggested by the work of Krinsky (1974). He found that bacteria containing singlet oxygen quenchers were killed less readily than bacteria of the same species which lacked quenchers. Although evidence on the presence and role of singlet oxygen is circumstantial, it must be considered as a potential bactericidal agent.

The myeloperoxidase-halide-\( H_2O_2 \) antimicrobial system is dependent upon the formation of \( H_2O_2 \). Thus, it is an oxygen dependent antimicrobial system (Klebanoff and Clark, 1978, p. 410). This extensively researched system is believed by many to be the most potent bactericidal system of human neutrophils. Although the kinetics of killing of various bacteria by MPO-halide-\( H_2O_2 \) have been delineated, the molecular mechanisms of killing are still unclear. Recent work by Thomas (1979a, 1979b) suggests that killing may be due to oxidizing agents formed by the MPO-halide-\( H_2O_2 \) system, where subsequent oxidation of bacterial membrane
components may be involved in bacterial death. Shohet et al. (1974) found that neutrophil induced lipid peroxidation and release of unsaturated fatty acids from phagocytosed pneumococci coincided with death. Although not confirming any specific bactericidal mechanisms, these findings indicate that oxidation of bacterial membrane components may play an important role in the killing of bacteria by PMN.

In neutrophils that do not exhibit stimulated metabolic activity, as found in patients with chronic granulomatous disease (CGD), there is decreased bactericidal capacity. The CGD neutrophils show a reduced ability to form $\cdot O_2^-$ and $H_2O_2$, which decreases the potency of the oxygen dependent antimicrobial systems. The finding that CGD patients are highly susceptible to bacterial infections emphasizes the importance of normally functioning neutrophils in fighting bacterial infections. For optimal bactericidal activity to occur, neutrophils must increase oxygen consumption and convert the oxygen to forms that are bactericidal or to ones that may be used to create other bactericidal agents.

Bactericidal Characteristics of Phagolysosomes and the Contents of Granules

When a bacterium is phagocytosed, it is surrounded by a bilayer membrane derived from the neutrophil cytoplasmic membrane. This structure, termed a phagosome, fuses with cytoplasmic granules to form a phagolysosome. Bactericidal activity occurs within this phagolysosome
(Densen and Mandell, 1978). Sequestration of bacteria in this manner, away from the nutrient-rich plasma and interstitial fluids, could lead to bacteriostatic and perhaps bactericidal conditions for some organisms. The confinement of bacteria within a phagolysosomal structure maintains a close association with the bactericidal agents.

The phagolysosomal pH has been shown to drop to 6.0-6.5 within a few minutes after its formation (Mandell, 1970; Jacques and Bainton, 1978). Acidic conditions are believed to facilitate the bactericidal activity in several ways. Many bacteria, such as the pneumococcus, are sensitive to acidic conditions, and the intraphagolysosomal pH may be directly bactericidal to certain bacteria. The toxicity of the acidic pH must be viewed as selective, since many bacteria, such as the lactobacilli, grow well at an acidic pH. A more important aspect of the acidic phagolysosome may be the activation of granule enzymes. The MPO-halide-H₂O₂ antibacterial system has an acidic pH optimum (McRipley and Sbarra, 1967). While not believed to be directly bactericidal, various degradative enzymes have greatest activity at an acidic pH.

Neutrophil granules contain a minimum of 30 different enzymatic activities. Only a few are believed to be bactericidal. Upon fusion of granules with phagosomes, these proteins are in a position to exert their activities on bacteria. The activities of all these proteins, except
myeloperoxidase, are independent of oxygen or its derivatives. They are referred to as oxygen-independent antibacterial systems. Four proteins have been implicated in the bactericidal or bacteriostatic action of neutrophils.

**Myeloperoxidase.** In patients with an hereditary MPO deficiency, neutrophils, which otherwise appear to be normal, kill bacteria at an abnormally slow rate (Lehrer, Hanifin, and Cline, 1969). This finding indicates the importance of the bactericidal activity of the MPO-halide-$\text{H}_2\text{O}_2$ system within neutrophils. Other bactericidal aspects of myeloperoxidase have been discussed previously in relation to its dependence on the formation of $\text{H}_2\text{O}_2$.

**Lysozyme.** The enzymatic activity of lysozyme cleaves the N-acetyl-glucosamine-N-acetyl-muramic acid linkages of peptidoglycan in bacterial cell walls (Strominger and Tipper, 1974, pp. 164-184). Bacteria vary greatly in their susceptibility to lysozyme, due to variations in the chemical composition of their cell walls. When bacteria are incubated with lysozyme and agents that decrease bacterial cell membrane stability, the lytic action of lysozyme is enhanced (Leive, 1974). Thus, the bactericidal activity of lysozyme within PMN is probably dependent on the type of bacteria ingested and other granule components which may enhance its activity.
Lactoferrin. Apolactoferrin, the iron unsaturated form of lactoferrin, is bactericidal for *Vibrio cholerae* and *Streptococcus mutans* but not for *E. coli* in *in vitro* assays (Arnold, Ide, and McGhee, 1977). The bactericidal effect was found to be dependent on the iron chelating properties of apolactoferrin. Rabbit PMN that had phagocytosed antibody-ferritin complexes, which saturated intracellular lactoferrin with iron, exhibited a decreased bactericidal capacity for *Pseudomonas aeruginosa* (Bullen and Wallis, 1977). This finding indicates that lactoferrin may play a role in intracellular killing. Since analogous experiments have not been performed with human PMN, a more precise role for lactoferrin cannot be stated.

Cationic Proteins. The bactericidal cationic proteins are divided into two major groups based on their physical and chemical properties. The chymotrypsin-like cationic proteins have molecular weights of 25,000 to 29,000 daltons (Olsson and Venge, 1974). They are bactericidal toward both gram-positive and gram-negative bacteria, although gram-positive are most susceptible (Odeberg and Olsson, 1975). When purified, the chymotrypsin-like cationic proteins are extremely effective bactericidal agents. Concentrations of $10^{-6}$ M and $2.5 \times 10^{-6}$ M kill more than 90% of $2 \times 10^6$ *S. aureus* and *E. coli*, respectively, within 30 minutes. Because the bactericidal activity was
not found to be dependent on the chymotrypsin-like protease activity, the bactericidal activity was probably due to their cationic nature (Odeberg and Olsson, 1975).

The bactericidal/permeability increasing (B/PI) cationic protein has a molecular weight of approximately 59,000 daltons and has no detectable protease, peroxidase, or lysozyme activity. Therefore, it may be a non-catalytic bactericidal protein (Weiss et al., 1978). Gram-negative bacteria are very susceptible to the bactericidal properties of this protein when purified, but gram-positive bacteria appear to be totally resistant. The cationic proteins will be discussed in more detail with respect to the specific alterations they induce in bacteria.

The phagolysosome as a bactericidal compartment is an extremely complex milieu of granule proteins, bacteria, and any extracellular molecules that may have been ingested with the bacteria. Although individual granule components are bactericidal, the killing within phagolysosomes is probably due to the synergistic action of many different granule proteins. Odeberg and Olsson (1976b), using in vitro bactericidal assays, found a synergistic bactericidal action between granulocyte elastase, which is not bactericidal alone, and both the MPO-\textsubscript{H$_2$O$_2$}-halide and chymotrypsin-like cationic protein bactericidal systems. Elastase potentiates the bactericidal activity of lysozyme (Thorne, Oliver, and Barrett, 1976) and the B/PI protein (Olsson et al, 1978,
The non-bactericidal PMN acid hydrolases also potentiate the lytic action of lysozyme (Neeman, Lahav, and Ginsburg, 1974). Thus, the bactericidal action of neutrophils is due to the contents of the granules and oxygen derivatives working in a synergistic fashion, and it is augmented by the acidic pH of the phagolysosome.

Bacterial Properties that Alter Bactericidal Activity

Physiological variation among bacterial species results in altered killing and degradation by PMN. In general, the gram-negative bacterial with their protective lipopolysaccharide (LPS) layer are more resistant than the gram-positive which lack LPS (Cohn, 1963a, 1963b). The LPS creates an impermeable barrier to some bactericidal agents, such as lysozyme, which cannot reach their substrates (Leive, 1974). The relationship between LPS and sensitivity to neutrophil granule extracts has been shown by Rest, Cooney, and Spitznagel (1977, 1978) and Modrzakowski and Spitznagel (1979). These studies showed that LPS deficient mutants of E. coli, S. typhimurium, and S. minnesota were much more sensitive to the non-oxidative bactericidal mechanisms of whole or fractionated granule extracts than were their parent strains that contained the full component of LPS. As the carbohydrate content of the mutants decreased, the bacteria became more susceptible to the bactericidal activity of the granule extracts. Conversely, Rest and Spitznagel
(1978) found that these mutants were killed by the isolated MPO-Cl⁻-H₂O₂ bactericidal system less readily than were their normal parental strains.

Staphylococcal protein A has been found to reduce the susceptibility of *S. aureus* to the antimicrobial activity of neutrophils (Suen and Allen, 1980). The effect of the protein A was mediated via the immunoglobulins of normal human sera. Strains of *S. aureus* lacking the proteins, and non-specifically binding less immunoglobulins, were much less resistant to killing by neutrophils. These results indicate that even slight bacterial variations will alter their response to neutrophil bactericidal mechanisms.

Of the literature reviewed, no bacteria have been found to be totally resistant to the bactericidal mechanisms of human neutrophils. However, *Mycobacterium microti* and *M. lepraemurium* were totally resistant to killing by mouse peritoneal PMN (Smith, Barr, and Alexander, 1979), suggesting that these organisms may be resistant to human PMN. Some bacteria, such as some of the obligate intracellular parasites, resist killing by inhibiting phagolysosome formation. Other bacteria resist the initial phagocytic process. There are several other mechanisms by which bacteria resist killing, but for most bacteria, once they have been ingested and phagolysosome formation has taken place, they eventually are killed.
Specific Bacterial Alterations
Associated with Killing and Degradation

Rabbit PMN

Much of the research investigating the effects of neutrophils on bacteria has employed rabbit PMN. In vitro studies with intact rabbit neutrophils have demonstrated that with phagocytosis and killing of the gram-positive organisms, Bacillus subtilis and Micrococcus lysodeikticus, there is an immediate cessation of bacterial protein synthesis. However, in the gram-negative bacterium, E. coli, protein synthesis continues for a time after ingestion and killing (Elsbach et al., 1973). E. coli subjected to bactericidal concentrations of disrupted rabbit granulocytes can regulate macromolecular synthesis 30 minutes after a 95% loss of viability (Elsbach et al., 1974). Fractionation of rabbit neutrophil granules produces several bactericidal components with cationic properties that (1) have an associated phospholipase A₂ activity and hydrolyze bacterial phospholipids (Elsbach et al., 1979), (2) increase the permeability of E. coli without gross ultrastructural alterations in the bacterial envelope (Van Houte et al., 1977), and (3) inhibit staphylococcal cell membrane enzymatic functions (Walton and Gladstone, 1975; Walton, 1978). The above findings indicate that the different effects of rabbit granulocytes on bacteria are due to the different fates of bacterial envelopes. The greatest alterations possibly occur at membranes. If the bacterial envelope
retains sufficient structural integrity, then cytoplasmic biochemical functions may be retained after loss of ability to divide.

Rabbit neutrophils differ from human neutrophils in several ways. The concentrations of cationic proteins are lower in human than in rabbit neutrophils. The cationic proteins of rabbits have lower molecular weights and a higher content of basic amino acids than those in humans (Olsson and Venge, 1972). Also, pH optimums and susceptibility to inhibitors differ in human and rabbit cationic proteins (Tsung, Kegeles, and Becker, 1977), and there is an associated phospholipase $A_2$ activity in rabbit but not human cationic proteins (Weiss and Elsbach, 1977). Due to these differences, only human neutrophils and their contents will be considered further.

Human PMN

Alteration of bacteria by human PMN is a continuous process once ingestion has occurred. The alterations that have been detected can be divided into two general areas: (1) those which occur or can be correlated with bacterial loss of viability, and (2) alterations which are part of a degradative process of the bacteria. There is no finite point at which one ends and the other begins; however, a division is based on the objectives of the literature cited.
Alterations Which Are Correlated with Bacterial Loss of Viability. Oxidation (Thomas, 1979a, 1979b) and decarboxylation (Selvaraj et al., 1974) of bacterial components by the isolated MPO-$\text{H}_2\text{O}_2$-$\text{Cl}^-$ antimicrobial system have been observed to correlate with bacterial loss of viability. Thomas (1979a) found that MPO catalyzed the oxidation of chloride to hypochlorous acid (HOCl), and about 30 to 50% of the oxidizing equivalents of HOCl were detected as nitrogen-chloride derivatives of peptides released from the bacteria. The nitrogen-chloride derivatives could further oxidize other bacterial components (Thomas, 1979b). Oxidation of bacterial sulfhydryl groups also closely correlates with the bactericidal action of the system. Lactobacillus acidophilus lysine transport is inhibited by the MPO-mediated antimicrobial system (Klebanoff and Clark, 1978, pp. 429-430), indicating that oxidation and/or decarboxylation of essential membrane proteins could lead to bacterial death.

Peroxidation of unsaturated bacterial lipids by neutrophils also has been correlated with bacterial death (Shohet et al., 1974). Using normal and CGD neutrophils, Shohet et al. (1974) demonstrated that the release of radio-labelled unsaturated lipids, but not saturated lipids, was dependent on oxidative mechanisms. The release of lipids was attributed to peroxidation by $\text{H}_2\text{O}_2$, and lipid release correlated with bacterial death.
Bacterial alterations by the non-oxidative anti-microbial systems have been shown to occur (Shayegani, 1968; Odeberg and Olsson, 1976a; Weiss et al., 1978). Extensive damage to staphylococcal membranes by granule lysates lead to the formation of staphylococcal L-forms (Shayegani, 1968). When preopsonized \textit{S. aureus} was incubated with lysates plus non-immune sera, L-forms (on hypertonic solid media) were obtained. The greater survival of \textit{S. aureus} on hypertonic media than on isotonic media indicates that the granule lysate had a damaging effect on the cell surface, which may have consisted of exposure of areas of the cytoplasmic membrane to osmotic shock. Although viability on the hypertonic media was not 100%, survival indicated that extensive cell surface damage may occur without loss of intracellular functions.

Chymotrypsin-like cationic proteins exhibit more effects on the functions of gram-positive than gram-negative bacteria and also are more bactericidal toward gram-positive than gram-negative organisms (Odeberg and Olsson, 1976a). These proteins inhibit (1) protein, RNA, and DNA synthesis in both \textit{E. coli} and \textit{S. aureus}; (2) oxygen consumption by \textit{S. aureus}; and (3) the energy driven $^{86}\text{Rb}^+$ influx into \textit{S. aureus}. The efflux of $^{86}\text{Rb}^+$ from \textit{E. coli} was enhanced by the cationic proteins whereas efflux from \textit{S. aureus} was unaltered. All of these effects on bacterial functions and viability were found to be dose-dependent.
Binding of the B/PI cationic protein to susceptible gram-negative bacteria shows a direct correlation with its permeability increasing and bactericidal activities (Weiss et al., 1978). Leucine incorporation into TCA precipitable material was only slightly inhibited when the loss of viability was greater than 90%. When *E. coli* was subjected to bactericidal concentrations of this cationic protein, there was a slight activation of bacterial phospholipases. However, this activation was not necessary for an increase in permeability. The change in permeability was believed to be at the outer membrane, which is the permeability barrier for actinomycin D. Actinomycin D is permeable in the presence, but not in the absence of, the B/PI cationic protein (Olsson et al., 1978).

**Alterations Which Are Part of the Degradation of Bacteria.** Extensive degradation of bacteria generally occurs after the majority of bacteria have lost the ability to divide (Cohn, 1963a; Neeman et al., 1976; and for a recent review, see Elsbach, 1980). Research concerning the degradation of bacteria by human PMN has used the release of radio-labels from bacteria and electron microscopic observation as indicators of bacterial degradation. By incubating pre-labelled bacteria with granulocytes, Cohn (1963a) found that small molecular weight molecules were degraded and released first, after which acid insoluble materials of both
gram-negative and gram-positive bacteria were degraded. This indicated that lysis had occurred. The release of acid soluble counts was found to be greater from gram-positive than gram-negative bacteria. A greater release of radio-labels from gram-positive bacteria than gram-negative was also observed when bacteria were incubated for 16-18 hours in granule lysates (Lahav and associates, 1974, 1975). With gram-positive bacteria, the cell membranes were extensively degraded over 18 hours, as seen by the release of glucosamine, muramic acid, glutamic acid, and ribitol (Lahav et al., 1974).

Electron microscopic observation of phagocytized Neisseria meningitidis indicated that the sequence of events of degradation began with the formation of an electron lucent cytoplasm, subsequent ballooning of the outer trilaminar membrane, and finally the formation of bacterial membrane vesicles (DeVoe, Gilchrist, and Storm, 1973). These events occur over 6 hours, and although many bacteria appeared degraded, others appeared relatively intact. Degradation of N. gonorrhoeae began within 30 to 60 minutes after phagocytosis, indicating a difference in susceptibility to the degradative action of PMN (Ward, Glynn, and Watt, 1972).

**Purpose of the Present Study**

The exudate from urethral infections with N. gonorrhoeae (GC) is characterized by many neutrophils and GC.
The GC are either free or associated with the neutrophils. Two proposed mechanisms of GC resistance to the neutrophil bactericidal action are (1) resistance to phagocytosis and (2) resistance to intracellular killing. Virulent colony type GC resist phagocytosis whereas avirulent colony type GC are readily ingested by neutrophils (Roberts, 1977). The exact mechanism(s) of this resistance has not been demonstrated. Witt, Veale, and Smith (1976) and Veale, Finch, and Smith (1976) have presented evidence that virulent colony type GC remain viable within neutrophils longer than do avirulent colony type GC. Others, however, have presented evidence that virulent and avirulent GC are killed once they are ingested (Watt, 1970; Ward et al., 1972; Gibbs and Roberts, 1975). Virulent and avirulent GC colony types are killed equally by neutrophil granule extract (Rest, 1979) and H₂O₂ and ·O₂⁻ (Ismail et al., 1977) indicating that virulence may not be due to intracellular survival. Thus, the interaction of GC with neutrophils is very important in the understanding of gonococcal infections.

The lethal changes in bacteria due to the bactericidal agents of human neutrophils are not known. The following work presents observation of the effects of granule extract on macromolecular synthesis and membrane permeability in *N. gonorrhoeae*. By studying these important bacterial functions, further insight is gained into how the gonococcus, and bacteria in general, are killed by neutrophils.
CHAPTER II

MATERIALS AND METHODS

Neutrophil Granule Extract

Neutrophil granule extract was prepared as described by Rest (1979). Neutrophils were obtained from healthy adult human subjects by leukapheresis and were sedimented through hydroxy-ethyl starch or Plasmagel (HTI Corp., Buffalo, N. Y.) to separate the neutrophils from red blood cells. Ficoll-Hypaque was employed to separate the neutrophils from other white blood cells. Final neutrophil suspensions were > 92% pure. Neutrophils were homogenized to 90% breakage in 0.34 M sucrose, with the granules being separated from cell debris by differential centrifugation (250 xg, 15 minutes). The granules were pelleted (20,000 xg, 20 minutes), and the contents were extracted overnight at 3°C with 0.2 M sodium acetate buffer (pH 4.0) with 10 mM CaCl₂. After centrifugation to remove granule membranes, the supernatant was dialyzed against phosphate buffered saline (PBS), pH 7.4, in dialysis tubing with an average molecular weight cutoff of 3,500 d. Protein concentration of the dialyzed granule extract was determined by the method of Lowry et al. (1951) using egg white lysozyme as standard. The PBS dialyzed granule extract in this form was used in
all assays and hereafter will be referred to as granule extract (GE).

**Bacteria**

*Neisseria gonorrhoeae* (GC) strain F62 was obtained from P. Fred Sparling, University of North Carolina, Chapel Hill. Cultures were maintained on GC medium base agar (Difco Corporation, Detroit, MI) supplemented with 200 ng/ml cocarboxylase, 4 mg/ml dextrose, 100 µg/ml glutamine, and 1.25 µg/ml ferric nitrate. Cultures were selectively passaged daily. Only colony type 4, described by Kellogg and associates (1963, 1968) was used in these studies.

Broth grown bacteria, which were used in assays, were started from plate-grown bacteria that had been incubated for 15-22 hours at 36°C in a humidified incubator containing 5 to 8% CO₂ in air. Bacteria were removed from plates with a sterile loop and suspended in gonococcal broth media (GCB) to an optical density at 550 nm (OD₅₅₀) of 0.35 to 0.40 in a Spectronic 20 spectrophotometer (Bausch and Lomb Inc., Rochester, N. Y.). Five milliliters of this suspension were added to 16 ml of GCB in a side arm flask. This GC suspension yielded a turbidity reading of 10-15 Klett units (Klett-Summerson Photoelectric Colorimeter, Klett Manufacturing Co., N. Y.). The only differences between the GCB and solid media were that the iron was deleted from the GCB and 400 µg/ml of NaHCO₃ were added to
replace the $\text{CO}_2$ in air. Broth cultures were incubated in side arm flasks at $37^\circ\text{C}$ in a water bath shaker (Gyrotory Water Bath Shaker, Model G76, New Brunswick Scientific Co., Edison, N. J.) rotating at 164 cycles per minute. Mid-log phase GC (grown 3-3.5 hr. to a turbidity of 120-150 Klett units) were diluted in fresh GCB to an OD$_{550}$ of $0.30 \pm 0.01$. A 1:1 dilution of this GC suspension was used in all assays and yielded $1.0-2.0 \times 10^8$ colony forming units (CFU) per ml of GCB.

**Bactericidal/Incorporation Assays**

Biosynthetic activity of GC in the presence of GE was determined by incorporation of radiolabelled precursors into TCA (trichloroacetic acid) precipitable material. Assays to determine bacterial viability and incorporation in the presence of GE were performed in sterile disposable 13 x 100 borosilicate glass tubes. Incubations were done under the same conditions as for the growth of bacteria in broth. The total volumes of the assay mixtures varied with different experiments and were determined by the number of data points desired for individual experiments. The assay mixtures consisted of the following: (1) GC suspended in GCB, which comprised approximately one-half the assay volume; (2) GE, whose volume varied in order to achieve the desired protein concentration, and (3) GCB or PBS in varying volumes to achieve the desired total assay mixture volume.
Control assays were set up in the same manner, except that PBS replaced GE.

Bacterial viability was determined by the streak plate method. At time zero and the times indicated in the results section, 20 \( \mu l \) of assay mixture were removed and diluted in GCB. Fifty microliters of the appropriate dilution were spread on fresh GC agar plates in duplicate, incubated for 24 to 36 hours, and bacterial colonies counted. For all experiments, the viability at the different time points was compared to the initial (time zero) viability. Viability was defined as the ability of GC to form colonies on solid media. The viability results were expressed as the number of colony forming units (CFU) per ml of assay mixture.

**Radiolabels and Trichloroacetic Acid Precipitation**

To determine incorporation over time in the presence of granule extract, radiolabelled precursors were added at time zero. For pulse label experiments, precursors were added at the times indicated in the results section. All radiolabels were obtained from Amersham Corp., Arlington Heights, IL. Prior to addition to the assays, the radiolabels were diluted in GCB or PBS. The final precursor concentration was set by adding unlabelled precursor. To study nucleic acid synthesis, 5-\(^3\)H-uridine (6 mM, 1.67 \( \mu Ci/\mu mole \)) and 6-\(^3\)H-uracil (2 mM, 10 \( \mu Ci/\mu mole \)) were used. To determine protein synthesis, a \(^3\)H-amino acid mix
(concentration and specific activity varied with individual amino acids) and 4,5-³H-L-leucine (2 mM, 10 µCi/µmole) were used.

At times indicated in the results section, 100 µl of assay mixture was removed in triplicate and placed in 2 ml of ice cold 10% (w/v) TCA in 13 x 100 mm tubes. The tubes were immediately vortexted and placed on ice for 1 hour. At one hour the TCA containing aliquots were filtered through 25 mm, 0.45 µm pore cellulose filters (Amicon, Lexington, MA). The tubes and filters were rinsed first with 10 ml of 5% (w/v) ice cold TCA, and then the filters had a rinse of 4 ml ice cold 95% ethanol. The filters were dried under an infrared heat lamp and placed in minivials (West Chemical Products, San Diego, CA) containing 4 ml of Formula 963 scintillation fluid (New England Nuclear, Boston, MA). Counting was done in a Tracor Analytic Liquid Scintillation System, Model G892, scintillation counter. Only the counts of the triplicate samples that had sample channels ratio numbers within 5% of each other were used in the results.

To control for nonspecific radiolabel attachment to bacteria and/or filters, two controls were run with each experiment. Two assay tubes were placed one each in a 60°C water bath or on ice for thirty minutes. Label was added for 10 minutes, and three 100 µl samples were removed and processed as described above. The counts per minute (CPM)
were averaged, and the average was subtracted from the CPM of experimental results.

**Bacterial Permeability**

Actinomycin D (200 ng/ml) and chloramphenicol (100 ng/ml) were used to study permeability changes that might occur in the GC when exposed to GE. These inhibitors of ribonucleic acid and protein synthesis, respectively, were added to assays with the labelled precursors to observe their intracellular presence by means of their inhibitory activity. Experimental procedures were the same as described above except that actinomycin D or chloramphenicol was added with the label.

$^3$H-Actinomycin D (0.5 µCi/ml, 0.2 nM) uptake by GC during a 10 minute pulse was also used as an indicator of bacterial permeability. The label was added to bacteria that had been incubated with GE and at the indicated times, 100 µl were removed, filtered, and rinsed with 5 ml of room temperature PBS. Filters were dried and counted as described previously.

**Materials**

Phosphate buffered saline used in dialysis and controls contained per liter of distilled, deionized water: NaCl, 8 gm; KCl, 0.2 gm; NaHPO$_4$, 1.15 gm; and KH$_2$PO$_4$, 0.2 gm. Egg white lysozyme, Ficoll, L-leucine, uracil, uridine, actinomycin D, and chloramphenicol were purchased
from Sigma Chemical Co., St. Louis, MO. Hypaque (50% sodium) was obtained from Winthrop Laboratories, Menlo Park, CA. All chemicals were of analytical reagent grade if available, and other materials were of the highest quality commercially available.
CHAPTER III

RESULTS

Bactericidal Activity of Granule Extract

Prior experimentation had determined that a minimum of $1.0 \times 10^8$ CFU/ml of GC was necessary to obtain reproducible counts for incorporated radiolabels. To obtain 99+% killing of $1.0 \times 10^8$ CFU/ml by 60 to 120 minutes, 750 $\mu$g/ml of GE was used. Killing at this concentration was exponential over 120 minutes with 50% viability occurring at approximately 20 minutes (Fig. 1). Seven different GE preparations were used throughout this study, and only small variations in bactericidal activity existed between the preparations. These variations were not significant enough to be a factor in the incorporation experiments. Control GC remained viable as indicated by an increase in CFU (Fig. 1), with roughly three bacterial doublings occurring over 120 minutes. Variations in the assay volumes did not affect the bactericidal activity of the GE.

The bactericidal activity of GE was dose dependent (Fig. 2). Concentrations up to about 75 $\mu$g/ml reduced bacterial growth or were bacteriostatic. Greater concentrations were logarithmically bactericidal up to 750 $\mu$g/ml.
Fig. 1. Bactericidal Activity of 750 μg/ml Granule Extract -- Viability at indicated times is that percent of viable bacteria at zero time. Data are expressed as the mean ± S.E.M.
Fig. 2. Effect of Different Concentrations of Granule Extract on Gonococcal Viability and $^3$H-Uridine Incorporation into Acid-Precipitable Material -- Incubation time was 60 min. Incorporation is that number of CPM in a 10 min pulse at 60 min as compared to a zero time control pulse. Results are expressed as the mean ± S.E.M.
Killing of GC by 750 μg/ml GE was inhibited by 1.0 and 20 mM Mg\textsuperscript{++} in a dose dependent manner (Table 1). Magnesium at these concentrations did not affect bacterial viability in PBS controls.

Table 1. Effect of Mg\textsuperscript{++} on the Bactericidal Activity of 750 μg/ml Granule Extract

<table>
<thead>
<tr>
<th>Assay Contents</th>
<th>% Viability at 30 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>174.7</td>
</tr>
<tr>
<td>PBS + 20 mM Mg\textsuperscript{++}</td>
<td>166.4</td>
</tr>
<tr>
<td>GE</td>
<td>11.7</td>
</tr>
<tr>
<td>GE + 1.0 mM Mg\textsuperscript{++}</td>
<td>36.0</td>
</tr>
<tr>
<td>GE + 20 mM Mg\textsuperscript{++}</td>
<td>112.0</td>
</tr>
</tbody>
</table>

Viability is expressed as the per cent of initial bacterial inoculum.

Effect of Granule Extract on Macromolecular Synthesis

When radiolabelled precursor molecules were added at zero time with GE, incorporation into TCA precipitable material continued at a reduced rate for the initial 60 minutes as compared to controls (Fig. 3b). Incorporation of \textsuperscript{3}H-uridine and \textsuperscript{3}H-L-leucine was 59 and 32% of their respective controls at 60 minutes. Viability during the first 60 minutes decreased to 2 to 13% of zero time viability (Fig. 3a). At 120 minutes, the TCA precipitable counts had decreased from the 60 minute counts for
Fig. 3. Effect of Granule Extract on Nucleic Acid and Protein Synthesis -- A—A, uridine control; •—•, uridine + GE; Δ—Δ L-leucine control; o—o, L-leucine + GE. Granule extract and label were added at zero time. Representative experiment of three.
3\textsuperscript{H}-uridine and 3\textsuperscript{H}-uracil incorporation. Results are not shown for 3\textsuperscript{H}-uracil incorporation; however, they were almost identical to uridine in per cent incorporation. This decrease suggested degradation of radiolabelled nucleic acids after about 60 minutes. Incorporation of 3\textsuperscript{H}-L-leucine, in contrast, increased over the entire 120-minute test period. Incorporation of 3\textsuperscript{H}-uridine and 3\textsuperscript{H}-L-leucine into TCA precipitable material in control GC was almost linear over time, corresponding to an increase in CFU. Thus, nucleic acid and protein synthesis continue at a reduced rate in GC that are being rendered non-viable by GE, in comparison to GC that are viable and continually dividing.

Pulse Labelling of N. gonorrhoeae in the Presence of Granule Extract

To define better the relationship between GE induced loss of viability and macromolecular synthesis, GC were pulse labelled after incubation with granule extract. At the indicated times, GC were pulsed for 10 minutes, and incorporation was compared to zero time PBS control incorporation. This procedure allowed for the comparison of macromolecular synthesis in a certain number of viable, dividing, control GC versus the same number of non-dividing GC in the presence of GE. Thus, a better idea of the ability of GC to synthesize macromolecules at any given time could be attained.
Nucleic acid synthesis, as indicated by the incorporation of $^3$H-uridine into TCA precipitable material, was found to remain functional after a greater than 80% loss of viability (Fig. 4a). As the viability of GC decreased, $^3$H-uridine incorporation remained the same as or greater than controls for the first 45 minutes. This incorporation of $^3$H-uridine into TCA precipitable material was found to be independent of the concentration of GE between 50 and 1000 $\mu$g/ml GE within 60 min (Fig. 2).

In contrast, $^3$H-uracil incorporation decreased with a loss of viability (Fig. 4b). Ten-minute $^3$H-uracil pulse incorporations at incubation times greater than 60 minutes decreased to less than 10% by 120 minutes. Controls exhibited increased incorporation of radiolabels concomitant with an increase in CFU.

Differentiation of $^3$H-uridine and $^3$H-uracil incorporation into DNA and/or RNA was attempted using the method of Schmidt and Thannhauser (1945). Bacteria incubated in only GCB were pulse labelled for 10 min with $^3$H-uridine or $^3$H-uracil. Aliquots were removed, TCA precipitated, and then hydrolyzed with either acid or base and processed as for the assay samples. Acid and base hydrolysis of $^3$H-uridine labelled GC showed that 18% of the total TCA precipitable material was acid resistant (pH 1.5 at 100°C for 30 min), and no label was found to be incorporated into base resistant material (pH 12 at 37°C for
Fig. 4. Pulse Labelling of Gonococcal Nucleic Acids in the Presence of Granule Extract -- Per cent viability is the number of viable bacteria at indicated times compared to the initial viable inoculum. Per cent incorporation is the number of CPM at indicated times compared to a time zero PBS control pulse. Results expressed as the mean ± S.E.M. Numbers above bars indicate actual per cent incorporation.
20 hr). Hydrolysis of $^3$H-uracil labelled macromolecules showed that 17% of the label was in acid resistant material, and 1% was in base resistant material. The rest of the counts passed through the filters and were considered to be both in acid and base sensitive material. Therefore, the incorporation of $^3$H-uridine into TCA precipitable material was indicative of RNA synthesis, and $^3$H-uracil incorporation indicated both DNA and RNA synthesis.

Pulse labelling of protein with $^3$H-L-leucine showed that the protein synthetic capabilities of GC remained intact despite a greater than 90% loss of viability (Fig. 5). As GC steadily lost viability over 45 minutes due to GE, protein synthesis during a 10 minute pulse was the same as or greater than controls. Incubation of GC with GE for greater than 60 minutes inhibited incorporation to about 10% of controls by 120 minutes. Control assays showed increasing incorporation over time with an increase in the number of CFU.

With an increase in GC membrane permeability due to the GE, one might expect the intracellular pools of radiolabels to increase as compared to control GC. Incorporated counts in the GE-treated GC might then be inflated compared to the amount of synthesis that actually occurred. To account for this possible variation in radiolabel pools, the following experiment was performed. Bacteria were incubated with GE or PBS for 30 minutes and
Fig. 5. Pulse Labelling of Gonococcal Proteins in the Presence of Granule Extract -- Data are expressed as in Fig. 4.
then pulsed with $^3$H-uridine. The assay tubes were then centrifuged to pellet the GC, and the supernatant was removed. The pellet was resuspended in PBS, and 100 µl aliquots were placed directly into scintillation fluid and counted. GE and PBS incubated GC had $1.65 \times 10^5$ CPM/ml and $1.98 \times 10^5$ CPM/ml associated with them, respectively. These similar total cell associated counts indicated that the intracellular pools of $^3$H-uridine were more than likely the same. These results reduced the possibility of RNA synthesis in GE-treated GC appearing normal due to increased labelling of RNA.

To evaluate bacterial viability and incorporation of $^3$H-uridine into RNA after removal of GE, the following experiment was performed. Bacteria were incubated with GE or PBS for increasing time periods, and viability was determined. The GC were then washed once to remove GE and incubated for 60 minutes in GCB. Viability was again determined, and the GC were pulse labelled for 10 minutes with $^3$H-uridine. After washing and during the GCB incubation, the GC continued to lose viability (Fig. 6). This finding indicated that the GE was not completely removed by the wash and that some GE components were possibly adhering to the GC. When the GC were centrifuged during the wash, there was a greater than 10-fold decrease in volume after removal of the supernatant. Since the pelleted GC were then resuspended to the same volume as before centrifugation,
Fig. 6. RNA Synthesis and Viability in Gonococci After Removal of Granule Extract — ■—■, viability at indicated times; •—•, viability after wash and 60 min incubation as described in text. Numbers above bars are actual per cent incorporation.
this process would reduce the GE concentration from 750 μg/ml to less than 75 μg/ml. And, as indicated in Fig. 2, GE at 75 μg/ml or less is not bactericidal.

The ability of GE-treated GC to incorporate \(^{3}\)H-uridine after washing and incubation had also decreased in parallel with viability. An eventual decline in RNA synthesis would be expected in GC killed by any mechanism. Adherent GE components would also continue disrupting the GC and thus inhibit RNA synthesis to a greater extent. Control assays showed an increase in viability and incorporation after the wash and 60-min GCB incubation.

**Effect of Granule Extract on the Membrane Permeability of N. gonorrhoeae**

Many different gram-negative bacteria are normally impermeable to actinomycin D (Act D). When the outer membrane of these bacteria is disrupted, Act D can enter the cell, and its intracellular presence is indicated by inhibition of RNA and protein synthesis. This fact was used to study permeability changes that might occur in GC due to GE. Both Act D and chloramphenicol (CAP) were used as indicators of membrane permeability.

The GC were found to be relatively sensitive to both Act D and CAP. Subinhibitory concentrations of 200 ng/ml for Act D and 100 ng/ml for CAP were used in the assays.
In these experiments, GC were incubated with 750 μg/ml GE for 30 minutes and viability determined. The GC were then pulse labelled for 10 minutes with radiolabels plus Act D or CAP, and viability was again determined after the pulse.

In the presence of GE, Act D inhibited both protein and RNA synthesis (Table 2). There was roughly a three-fold inhibition of protein synthesis over GE-treated GC and a total inhibition of RNA synthesis. Actinomycin D plus GE exhibited a 17 to 20 times greater bactericidal effect on GC than did GE alone. Thus, GE at 750 μg/ml increases the permeability of GC membranes, at least to Act D. Control assays with PBS and Act D were essentially the same as straight PBS controls with respect to both incorporation and viability (Table 2). Equivalent results were found with an incubation time of 60 minutes. Experiments similar to these, except that GC were preincubated with Act D and then exposed to GE and labelled precursors, gave almost identical results. In contrast, CAP did not inhibit protein synthesis or enhance the loss of viability of GC in the presence of GE (Table 2). Control assays with CAP and PBS were the same as straight PBS controls.

The association of ³H-Act D with GC was found to be greater in the presence of GE than in its absence (Fig. 7). When GC were incubated with 0.1% Triton X-100, there was a greater association of ³H-Act D than when GC were incubated
Table 2. Actinomycin D and Chloramphenicol Inhibition of Protein and RNA Synthesis

<table>
<thead>
<tr>
<th></th>
<th>Protein Synthesis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RNA Synthesis&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Incorporation 30 min % Viable 30 min</td>
<td>% Incorporation 40 min % Viable 40 min</td>
</tr>
<tr>
<td>Act D + GE</td>
<td>14.0 ± 2.0 26.8 ± 6.8</td>
<td>0.26 ± 0.01 4.5 ± 1.5</td>
</tr>
<tr>
<td>GE</td>
<td>47.0 ± 6.0</td>
<td>4.95 ± 16.5</td>
</tr>
<tr>
<td>Act D + PBS</td>
<td>96.0 ± 3.0 165.0 ± 9.2</td>
<td>180.0 ± 21.0 141.0 ± 3.5</td>
</tr>
<tr>
<td>PBS</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CAP + GE</td>
<td>46.0 ± 9.0 26.5 ± 6.1</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>GE</td>
<td>37.5 ± 8.5</td>
<td>7.0 ± 1.8</td>
</tr>
<tr>
<td>CAP + PBS</td>
<td>97.5 ± 10.5 141.0 ± 6.9</td>
<td>160.0 ± 15.0 150.0 ± 21.0</td>
</tr>
<tr>
<td>PBS</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>H-A.A. mix and 4,5-<sup>3</sup>H-L-leucine as precursors.

<sup>b</sup>5-<sup>3</sup>H-uridine as precursor.

<sup>c</sup>Results expressed as the per cent of the mean ± S.E.M. of the respective times PBS control.
Fig. 7. Association of $^3$H-Actinomycin D with N. gonorrhoeae -- GC were incubated with the indicated agents for 30 min and then pulsed with $^3$H-Act-D (200 ng/ml) for 10 min. At indicated times aliquots were filtered, rinsed and counted to determine association of label with GC. Results expressed as the mean ± S.E.M. ■—■, Triton X-100; ●—●, GE; ▲—▲, PBS control.
with GE. Table 3 shows the spectrophotometrically determined absorbance of these three preparations. The absorbance of the Triton X-100 assay decreased with a corresponding decrease in viability, which is indicative of lysis. The PBS and GE assays, however, showed increasing absorbance over time. The increasing absorbance of the PBS assay was due to an increase in CFU, whereas the increase in absorbance with GE is not at present explainable. Extensive lysis of GC by the GE would have been masked by the increasing absorbance, but this is unlikely when one considers the decrease observed with Triton X-100. Thus, the greater the disruption of GC membranes, the greater the association of $^3$H-Act D with GC.

Table 3. Absorbance of $^3$H-Actinomycin D Uptake Assays

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>PBS Control</th>
<th>0.1% Triton X-100$^a$</th>
<th>Granule Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.000 (100)</td>
<td>0.000 (100)</td>
<td>0.375 (100)</td>
</tr>
<tr>
<td>30</td>
<td>0.146 (174)</td>
<td>-0.174 (0.44)</td>
<td>0.476 (16.1)</td>
</tr>
<tr>
<td>60</td>
<td>0.340 (242)</td>
<td>-0.179 (0.05)</td>
<td>0.580 (0.02)</td>
</tr>
</tbody>
</table>

At zero time the spectrophotometer was blanked at 550 nm to the PBS control containing GC, and was used as the blank for the other assays. At indicated times the absorbance and viability were determined. Numbers in parentheses indicate per cent viability.

$^a$Absorbance readings on spectrophotometer for Triton X-100 assay were 9.826 at 30 minutes and 9.821 at 60, indicating a decrease from 0.000.
CHAPTER IV

DISCUSSION

Human neutrophil GE and some of its specific purified components are bactericidal for a wide range of bacterial species. Most of the research in the area has dealt with the mechanisms of action of GE and its components as bactericidal agents. Few investigations of bacterial changes in response to GE have been done. In the experiments reported here, GE was found to (1) have a bactericidal effect on GC, (2) "kill" GC without inhibiting macromolecular synthesis, and (3) increase the membrane permeability of GC. The following discussion will be divided into these three areas.

**Killing of N. gonorrhoeae by Granule Extract**

The bactericidal activity of GE used in this research was due to the non-oxidative bactericidal systems. Very few, if any, of the enzymatic activities necessary for oxygen derivative formation and oxygen-dependent killing are present in the GE. Although MPO is an important antibacterial agent and is present in the GE, it probably does not play a role in the killing of GC for four reasons. First, the MPO-Cl⁻-H₂O₂ antimicrobial system has been found
to be inhibited by normal strength bacteriological media (Rest, 1979). Second, Rest (1979) also found that addition of catalase to GE bactericidal assays does not inhibit killing. Third, hydrogen peroxide was not added to the bactericidal assays in my experimentation. Finally, any $H_2O_2$ that might be produced by the components of the assay system or GC itself would most likely be degraded by the active GC catalase. Thus, the primary bactericidal activities of the GE may be due to the cationic proteins and lysozyme. Other granule components, such as elastase and collagenase, may add a potentiating effect.

Only two studies have examined the bactericidal activity of granule contents on GC (Watt, 1970; Rest, 1979). The results presented here parallel those of Rest (1979), where the killing of GC by GE was found to be both time and concentration dependent. By varying the concentration of GE and/or bacteria, the kinetics of killing were altered. Watt (1970) found that 90% of $6 \cdot 10^5$ GC/ml were killed within 5 min by 50 $\mu$g/ml of granule cationic proteins. Since concentrations of the cationic proteins or bacteria were not varied by Watt (1970), a comparison of the kinetics of killing with my results is not possible. In comparing the bactericidal activity of granule contents on GC, the conditions and phase of growth must be considered (Rest, 1979). In this study, and the above cited studies, GC that
were grown to log-phase in broth culture were highly sensitive to the bactericidal agents of GE.

Several results presented here indicate that the cationic proteins have an important role in the bactericidal activity of GE. As found with GE (Table 1), the bactericidal activity of the chymotrypsin-like cationic proteins (Odeberg and Olsson, 1976a) and the B/PI cationic protein (Weiss et al., 1978) is inhibited by Mg\(^{++}\). The bactericidal activity of 70 \(\mu g/ml\) of chymotrypsin-like cationic proteins is inhibited by 20 \(mm\) Mg\(^{++}\), whereas 2.5 \(mm\) Mg\(^{++}\) inhibits 4 \(\mu g/ml\) of the B/PI cationic protein. An ionic concentration of 0.15 \(M\) NaCl inhibits the chymotrypsin-like cationic proteins (Odeberg and Olsson, 1975). In the Mg\(^{++}\) inhibition experiments of Weiss et al. (1978) and Odeberg and Olsson (1976a), the ionic concentration was held constant by decreasing the NaCl concentration before the addition of Mg\(^{++}\). In my experimental procedure, however, the ionic concentration was not lowered to account for the Mg\(^{++}\). The final ionic concentration equalled 0.19 \(M\) NaCl, and the bactericidal activity of the chymotrypsin-like cationic proteins was probably inhibited. A NaCl concentration of 0.15 \(M\) does not inhibit the bactericidal activity of the B/PI cationic protein (Odeberg and Olsson, 1975). Partial inhibition of the chymotrypsin-like cationic proteins may have occurred during extract dialysis, since the PBS used to dialyze the
acetate extract contained 0.14 M NaCl. Thus, the bactericidal activity of the GE may be reduced to the B/PI cationic protein and lysozyme.

The continuing loss of viability of GE-treated GC, which had been washed and incubated in broth (Fig. 6), is indicative of cationic protein bactericidal activity. The chymotrypsin-like cationic proteins are preferentially adsorbed out of unfractionated GE by various microorganisms (Drazin and Lehrer, 1977), and their bactericidal activity is dependent on binding to microbial surfaces (Odeberg and Olsson, 1976a). The bactericidal activity of the B/PI cationic protein is also dependent on binding to bacterial surfaces (Weiss, Beckerdite-Quagliata, and Elsbach, 1980). Release of these cationic proteins is dependent on increasing Mg$^{++}$ and/or ionic concentrations (Odeberg and Olsson, 1976a). Since the Mg$^{++}$ or ionic concentration was not altered during the wash in my experimental procedure, the cationic proteins probably remained adherent to the GC and continued their bactericidal activity.

The loss of viability in GC due to GE may be enhanced by the activation of autolysins. The autolysins have amidase, phospholipase A, and lysophospholipase enzymatic activities and are associated with GC membranes. Upon activation, the autolysins hydrolyze their substrates. Subsequent lysis of the cells may occur (Morse, 1979). Cacciapuoti, Wegener, and Morse (1978) observed that
hydrolysis of peptidoglycan and phospholipids can occur when GC are in a buffer. This hydrolysis may cause a loss of viability and a loss of integrity of the cell envelope. However, they found that neither hydrolytic activity accounted for autolysis of the GC. Thus, autolysin activation may occur without lysis. Mechanisms of autolysin activation include depletion of glucose in the media and cessation of bacterial growth (Morse and Bartenstein, 1974). Depletion of glucose is probably not a factor in these studies since the PBS control and GE assays contained the same amount of glucose, and no lysis was observed in the controls (Table 3). Granule extract, however, does cause cessation of bacterial growth, and subsequent activation of the autolysins may occur. The purified B/PI cationic protein induces the activation of phospholipase A in E. coli (Weiss et al., 1978). However, this activation is not necessary for the bactericidal effects of the phospholipase A activity. Although no studies have directly compared autolysis in GC and E. coli, evidence does indicate that GC are much more autolytic than E. coli and other gram-negative bacteria (Morse, 1979). This suggests that if phospholipase A activation does occur in GC, then autolysin activation may play a role in the killing of GC by GE. The above evidence indicates autolysin activation is one aspect of GC metabolism that must be considered in relationship to GE killing.
Macromolecular Synthesis

The continuation of macromolecular synthesis with a concomitant loss in viability occurs in E. coli treated with the chymotrypsin-like cationic proteins (Odeberg and Olsson, 1976a) and the B/PI cationic protein (Weiss et al., 1978). E. coli treated with the B/PI cationic protein do not show a decrease in protein or nucleic acid synthesis over 30 min, even though greater than 99% killing has occurred (Weiss et al., 1978). Nucleic acid and protein synthesis in E. coli treated with the chymotrypsin-like cationic proteins continue at a rate of about 60% of untreated E. coli for the first 30 to 60 min (Odeberg and Olsson, 1976a). This reduced rate of nucleic acid synthesis was also observed when GC were treated with GE at zero time and incorporation measured at 60 and 120 min (Fig. 3). However, protein synthesis in GE-treated GC was only 30% of untreated GC. Odeberg and Olsson (1976a) interpret the decrease in E. coli macromolecular synthesis as a direct inhibition of biosynthetic activity by the cationic proteins. My interpretation is that cationic protein or GE-treated bacteria which are being rendered non-viable cannot be expected to continue macromolecular synthesis at the same rate as untreated, dividing bacteria.

If a comparison of control bacteria with GE or cationic protein-treated bacteria is to be made, then one must compare the synthesis of untreated control bacteria
with an equal number of treated bacteria. This comparison was made by pulse labelling GC that had been treated with GE (Figs. 4 and 5). The results showed that as GC lost viability, their ability to synthesize macromolecules was the same as controls. Therefore, GE does not kill GC by inhibiting macromolecular synthesis. Some other lethal change must have occurred prior to the eventual cessation of macromolecular synthesis. Perhaps if the chymotrypsin-like cationic protein-treated E. coli had been pulse labelled, the same amount of synthetic activity might have been observed as compared to untreated bacteria. With my interpretation, the decrease in macromolecular synthesis is a result of other changes that are induced in bacteria by GE or the chymotrypsin-like cationic proteins.

Further support of the finding that GE does not kill GC by inhibiting macromolecular synthesis comes from the synergistic bactericidal activity of GE and Act D (Table 2). A synergistic bactericidal action suggests that the bactericidal mechanisms of the two agents are different. The bactericidal action of Act D is through the inhibition of RNA and protein synthesis, whereas the bactericidal action of GE is not known. If GE killed GC by inhibiting macromolecular synthesis, then the combined bactericidal activities of GE and Act D would be more additive than synergistic.
Conclusions about the effect of GE on DNA synthesis in GC cannot be made. The amount of radiolabel incorporation into DNA during a 10 min pulse is too low to quantitate adequately DNA synthesis. Deoxyribonucleic acid synthesis in GE-treated GC might be important since bacterial DNA is associated with the cytoplasmic membrane. The association of bacterial DNA with the cytoplasmic membrane is believed to be at or near the origin of replication (Moat, 1979). Since GE and its components alter bacterial membranes, a direct effect on DNA synthesis may occur.

**Membrane Permeability**

Resistance to Act D in gram-negative bacteria is due to an impermeable outer membrane (Leive, 1974). The outer membrane, which is composed of LPS and associated lipids and proteins, is permeable only to small molecular weight molecules. When the outer membrane is disrupted by ethylenediaminetetraacetic acid (EDTA), larger molecules such as Act D may pass through (Leive, 1974). The bacteria are then sensitive to Act D inhibition of RNA synthesis.

This same process is seen with the B/PI cationic protein of neutrophil granules. Weiss et al. (1978) found that Act D resistant *E. coli* could be made sensitive by treatment with this cationic protein. Granule extract treated GC also showed enhanced sensitivity to Act D. The B/PI cationic protein and GE thus exert similar effects on
bacterial membranes. Possibly, the B/PI cationic protein in the GE increased the permeability of GC. In the experiments of Weiss et al. (1978), the B/PI cationic protein was in a purified state. Therefore, any changes observed in bacteria could be attributed to this protein. Since GE contains many different proteins, the increase in permeability seen in GC cannot be attributed solely to the B/PI cationic protein.

In comparison to wild type *E. coli*, the outer membrane of GC is more permeable (Wolf-Watz et al., 1976). *Escherichia coli* strain S15 is resistant to 27.5 μg/ml of Act D (Weiss et al., 1978), whereas GC were found to be resistant to concentrations less than 200 ng/ml of Act D. This difference appears to lie in the chemical composition of the LPS. The LPS of GC contains fewer outer membrane proteins than *E. coli*, and the carbohydrate content is lower (Wolf-Watz et al., 1976). The outer membrane proteins of GC are also more hydrophilic than those of *E. coli*, and Wolf-Watz et al. (1976) suggest this may be the reason for the greater sensitivity of GC to antibiotics. The passage of charged antibiotics may be facilitated by the presence of these hydrophilic proteins.

The permeability increasing activity of the B/PI cationic protein is dependent on binding to bacterial surfaces (Weiss et al., 1980). Gram-negative, but not gram-positive bacteria, bind this cationic protein (Weiss et al., 1978), indicating that binding is to the outer
membrane. Different strains of *E. coli* and *S. typhimurium* possess different lengths of LPS core and O antigen polysaccharides. Smooth strains with long chain polysaccharides are more resistant to various bactericidal agents than rough strains with short chain polysaccharides. Rough strains are more sensitive to GE (Rest et al., 1977, 1978), various fractions of GE (Modrzakowski et al., 1979), and the B/PI cationic protein (Weiss et al., 1980).

*Neisseria gonorrhoeae* has an intermediate rough LPS with a core polysaccharide and little or no O antigen (Wiseman and Caird, 1977). According to Rest (1979), GC are killed to about the same degree as intermediate rough *E. coli* and *S. typhimurium*. Thus, when comparing GC to other bacteria, an increase in permeability due to GE may be expected, since GC have most of the characteristics of the more sensitive bacteria.

The lack of synergism observed between CAP and GE in the inhibition of GC protein synthesis is not readily explicable. Neu and Winshell (1970) also found a lack of synergy between the bactericidal activities of CAP and EDTA-treated *E. coli*. The strain of *E. coli* they used also had a genetically determined resistance to CAP. The GC used in this study were not totally resistant to CAP. I can only speculate that perhaps the GE altered the CAP in some way to inhibit its action on bacterial ribosomes; or, an increase in permeability does not enhance the action of CAP.
**E. coli** treated with either EDTA (Leive, 1965a, 1965b) or the B/PI cationic protein (Weiss et al., 1978) lose their ability to divide, retain macromolecular synthetic capability, and become more permeable to Act D. Treatment with Mg$$^{++}$$, however, uncovers a difference in mechanisms. Magnesium reverses the inability of EDTA-treated cells to divide, whereas it has no effect on inhibition of cell division by the B/PI cationic protein. After a 30 min GE treatment of GC, viability was 27% (Table 2). The same table shows that upon the addition of Act D at 30 min, there was a total inhibition of RNA synthesis. This finding indicates that the membranes of the remaining viable bacteria had been altered to become more permeable. The difference in the ability of **E. coli** to divide when treated with Mg$$^{++}$$ and EDTA or the B/PI cationic protein, and the increase in permeability found in GE-treated GC indicate that the B/PI cationic protein and GE may not kill solely by increasing bacterial permeability. The increase in permeability may coincide with, or precede, some other change in the bacteria. More research into the specific changes that occur in bacterial membranes in response to the granule bactericidal agents needs to be done to clarify these and other questions.
Conclusions

The conclusions of this research are summarized as follows.

1. The cationic proteins may have an important role in the bactericidal activity of GE on GC. Observations showed that Mg$^{++}$ inhibits the bactericidal action of GE and that the bactericidal components adhere to GC. Weiss et al. (1980) have suggested that the most important non-oxidative antimicrobial system of neutrophils is the B/PI cationic protein.

2. Neisseria gonorrhoeae is not killed by GE due to the inhibition of protein or RNA synthesis. Protein and RNA synthesis, as shown by pulse labelling, continue in GC despite the loss of viability due to GE. No conclusions concerning DNA synthesis can be made.

3. Some structural integrity of GC is probably retained after the loss of viability. The findings that no lysis occurred due to GE and that protein and RNA synthesis continued after loss of viability suggest the retention of structural integrity. Electron microscopic observation of GE-treated GC needs to be done to clarify this point.

4. Granule extract increases membrane permeability of GC. An increase in permeability was shown by Act D inhibition of protein and RNA synthesis in GE-treated GC. Whether this is a lethal change or not cannot
be concluded now. Results presented indicate that an increase in permeability may not be lethal but may precede or occur simultaneously with a lethal event. As indicated by the results presented here, and the results of others, the lethal change that is induced in bacteria by granule components is probably in the bacterial membrane. A more in-depth study of membrane structural and functional changes in response to GE is needed in order to answer the question of how GE kills bacteria.
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