THE CELL ENVELOPE OF *NEISSERIA GONORRHOEAE*

by

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ABBREVIATIONS

Å	angstrom
Ci	curie (2.22×10^{12} disintegrations per minute)
CPM	counts per minute
CuP	cupric di(1,10-phenanthroline)
DMA	dimethyl adipimidate
DMS	dimethyl suberimidate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DO	dissolved oxygen
dpm	disintegrations per minute
DTBP	dithiobispropionimidate
DTSP	dithiobis(succinimidyl propionate)
EDTA	ethylenediaminetetraacetic acid
FFDNB	1,5-difluoro-2,4-dinitrobenzene
FFDS	4,4'-difluoro-3,3'-dinitrodiphenyl sulfone
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA	human leukocyte antigen
KDO	2-keto-3-deoxyoctonate
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
β-ME	β-mercaptoethanol
MOMP	major outer membrane protein
MS	manuscript
MW	molecular weight

N.	*Neisseria*
NEM	N-ethylmaleimide
P	protein
PAGE	polyacrylamide gel electrophoresis
PG	peptidoglycan
POMP	principal outer membrane protein
R-type	"rough"-type
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulfate
S-type	"smooth"-type
T1	colony type 1
T2	colony type 2
T3	colony type 3
T4	colony type 4
TCA	trichloroacetic acid
TDA	tartryldiazide
TDCA	tartryldi(ε-aminocaproylazide)
TDGA	tartryldi(glycylazide)
TEA	triethanolamine
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Outer membrane proteins of Neisseria gonorrhoeae have recently been reported to be associated with colonial coloration (154), interaction with polymorphonuclear leukocytes (154), resistance or sensitivity to antibiotics (44), and resistance to normal human serum and complement (58). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis is a powerful tool for examining membrane proteins. Since the outer membrane of gram-negative bacteria, such as N. gonorrhoeae, is the structure that interfaces with the host environment, information on its components will lead to a further understanding of the organism and the diseases it causes.

This study examined outer membrane proteins of N. gonorrhoeae and sought to accomplish the following:

1) To chemically cross-link gonococcal outer membrane proteins to determine whether stable protein complexes exist in the native outer membrane.

2) To compare the cross-linking patterns of outer membrane proteins from different strains and colonial types of N. gonorrhoeae.

3) To investigate the role of the gonococcal outer membrane in autolysis.

4) To determine the effect of environmental parameters (i.e. level of dissolved oxygen) on gonococcal outer membrane protein composition. The pertinent literature pertaining to these studies is reviewed in the following section.

II. LITERATURE REVIEW

Gonorrhea is the most prevalent bacterial disease in the world today, yet much remains to be discovered about the pathogenesis and virulence of the causative organism, Neisseria gonorrhoeae. Sexual contact is the primary route of transmission with infection being most prevalent in the more sexually active age groups. The organism usually infects the genito-urinary tract, pharynx, and rectum, where gonococci appear to attach to and penetrate mucosal surfaces, with columnar rather than squamous epithelial cells being invaded (4, 169). The invading bacteria appear to enter the cells directly rather than passing between cell junctions (169). By three or four days post-infection capillaries become dilated with infiltration of leukocytes. Soon granulocytes accumulate in subepithelial spaces, and the almost diagnostic purulent exudate forms (4, 169).

Gonococci secrete an IgA protease (6, 123, 124), but the role of this enzyme in gonococcal infections is unknown. N. gonorrhoeae is not known to secrete any other extracellular factors, so it is likely that the interaction of bacteria with host is mediated by cell surface components. Any progress toward prevention of this prevalent venereal disease of man will undoubtedly be assisted by further investigation of the gonococcal cell surface.

A. Colony types

Kellogg et al. (76) reported the existence of four morphologically distinct colony types, designated types 1, 2, 3, and 4 (T1, T2,

T3, T4). Jephcott and Reyn (69) identified a fifth colony type (T5). Evidence obtained from human studies indicated that T1 and T2 colony types predominate in clinical isolates (76, 77, 79, 145). Colony types 1 and 2 are considered virulent and must be selectively subcultured daily to prevent conversion to types 3 and 4, which are considered avirulent. T1 and T2 gonococci have pili on their surface while T3 and T4 cells lack these appendages.

In addition to classification by colony type, recent observations have indicated that light- and dark-colored variants of N. gonorrhoeae exist. Diena et al. (24) reported that light variants of T1 and T2 colonies revert to T4 colonial type, whereas dark variants of T1 and T2 colonies give rise to T3 colonies. Swanson (153, 154) has observed that "twitching motility" is present in piliated light colonies, absent in nonpiliated colonies (light or dark), and is only rarely seen in piliated dark colonies. Dark colonies tend to have many more zones of adhesion than light colonies, and dark colonies are more trypsin sensitive than light colonies. Swanson (155) later used the terms "opaque" and "transparent" in place of "dark" and "light" to describe these gonococcal variants. Upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, outer membranes of dark colonies contain two bands (26,000 and 28,000 daltons) not found in light colonies. Further knowledge of differences and similarities among gonococcal colonies may aid in attaining the goal of eradication of this disease.

B. Cell envelope of gram-negative bacteria

Gram-positive and gram-negative bacteria have distinctly different cell envelopes. A gram-positive bacteria has a cytoplasmic membrane, a thick peptidoglycan layer, and polysaccharide and teichoic acid components in its cell wall. A gram-negative bacteria, in contrast, has a cytoplasmic membrane, a thin peptidoglycan layer, and a lipopolysaccharide-containing outer membrane. The complexity of the gram-negative cell envelope results in difficulty in resolving its component parts.

1. Cytoplasmic membrane

N. gonorrhoeae is a gram-negative diplococcus which possesses a "typical" gram-negative cell envelope: that is, an inner cytoplasmic membrane, a peptidoglycan layer, and an outer membrane. The chemical composition of the cytoplasmic membrane has been shown to be similar to other biological membranes (97), consisting of a phospholipid bilayer containing both intrinsic and extrinsic proteins. The inner membrane can be obtained by ethylenediaminetetraacetic acid (EDTA)-lysozyme treatment of the cells, followed by density gradient centrifugation (101, 115). This membrane contains many cytochromes, other electron transport and oxidative phosphorylation proteins, enzymes for complex lipid biosynthesis, and enzymes involved in later stages of cell wall biosynthesis. Also, the cytoplasmic membrane acts as a physical barrier to retain metabolites and exclude external compounds. This barrier contains specific

transport systems which allow cells to accumulate necessary substrates without loss of intermediary metabolites and cofactors.

2. Peptidoglycan

The peptidoglycan component of the cell envelope imparts cell rigidity, maintains cellular shape, and assists the cytoplasmic membrane in maintaining osmotic stability in hypotonic, isotonic, and metabolically unfavorable environments. The layer of peptidoglycan is situated between the cytoplasmic and outer membrane in an area called the periplasmic space. Pressure inside the bacterial cell probably forces the cytoplasmic membrane against this rigid layer (132), even though electron micrographs of fixed and sectioned cells usually show an electron transparent layer between the cytoplasmic membrane and the peptidoglycan. The procedures used to prepare cells and/or thin sections for electron microscopy most likely produce this phenomenon. These two structures may be joined in the living cell by nascent peptidoglycan or sites of adhesion (131).

Certain enteric bacteria contain a lipoprotein (9, 10) which anchors to the peptidoglycan and extends into the outer membrane. This lipoprotein is one of the most abundant proteins in Escherichia coli, existing in a free form in the outer membrane as well as in a bound form that covalently links peptidoglycan to the outer layer of the cell envelope (63). Thus, in organisms which possess this lipoprotein, a layer of protein can be envisioned between the outer membrane and the peptidoglycan. No lipoprotein molecule has been demonstrated in Neisseria gonorrhoeae (175).

Recently, Hebeler et al. (50, 51) described a peptidoglycan-associated protein(s) in cells grown at pH 6.0. This phenomenon was not observed when cells were grown at pH 8.0 or 7.2, and did not appear to be strain specific. However, it has not been determined whether the increase in amino acid content of the peptidoglycan was due to production of more than one protein, nor has the physiologic role of this protein component been elucidated.

The peptidoglycan of gram-negative bacteria is a rather open framework, due to a minimal amount of peptide cross-linking (131). The chemical composition of the peptidoglycan of N. gonorrhoeae consists of muramic acid, glutamic acid, alanine, meso-diaminopimelic acid, and glucosamine in approximate molar ratios of 1:1:2:1:1 (48, 175). The gonococcus is similar in this respect to all gram-negative bacteria thus far examined (38, 98, 139). Peptidoglycan is composed of amino sugars and amino acids and does not contain phospholipid, which accounts for the absence of freeze-fracture cleavage planes (59) and double track membranes in electron micrographs. The open structure of this layer produces a barrier which is probably effective only against large molecules (16).

3. Periplasmic space

The periplasmic space of gram-negative bacteria contains various binding proteins and periplasmic enzymes. If the outer membrane is disrupted, these proteins are released into the environment (57). It is not known whether periplasmic enzymes are compartmentalized in specific areas of the periplasmic space (171) or are evenly

distributed and possibly associated with lipopolysaccharide or other structural components of the cell envelope (15). In either case, the periplasmic space with its rigid peptidoglycan framework provides an enriched and conditioned environment at the exterior of the bacterial cytoplasmic membrane.

4. Outer membrane

The outer membrane is similar to the cytoplasmic membrane in that it contains phospholipid and protein and exhibits a double track morphology when thin sections are viewed in the electron microscope (115). In earlier literature this layer has been referred to as the L-layer since it alone contains lipopolysaccharide (LPS) (115, 144). The outer membrane is unique to gram-negative bacteria and confers special properties upon organisms which have it (25). This layer, unlike the cytoplasmic membrane, does not carry out active transport, but instead acts as a molecular sieve (100).

The phospholipid content of the outer membrane of gram-negative bacteria is quantitatively similar to, but qualitatively different from, that of the cytoplasmic membrane (172). The phospholipids appear to be in a hexagonal configuration in the bilayer (31, 32), and produce cleavage planes typical of biological membranes upon freeze fracturing (32). The inner layer of the outer membrane has been found to contain more phospholipids than the outer layer (144). When unsaturated fatty acids are substituted for saturated fatty acids in the membrane cellular permeability increases, allowing lysozyme and other agents to penetrate (133).

Sud and Feingold (151) examined the phospholipid and fatty acid composition of N. gonorrhoeae. The phospholipids, comprising 8% of the dry weight of the cells, consisted of phosphatidylethanolamine (70%) and phosphatidylglycerol (20%), with small amounts of phosphatidylcholine and cardiolipin also present. The most prevalent fatty acids of the phospholipids were palmitic (16:0) and a C16:1, with myristic (14:0) and a C18:1 acid present in smaller amounts. The gonococcus is one of the few gram-negative bacteria that contains phosphatidylcholine, and differs from other gram-negative bacteria in that no cyclopropane fatty acids or acids with more than 18 carbon atoms were detected (2).

Lipopolysaccharide (LPS, endotoxin) consists of two moieties with different physiochemical characteristics, a heteropolysaccharide and a covalently bound lipid, lipid A (91). The polysaccharide component is composed of the core oligosaccharide and an O-specific side chain (91). The O-specific side chain carries the antigenic determinants for serological specificity, acts as a receptor for phage (87), and is species and/or strain specific. The core oligosaccharide is covalently bound to lipid A and is group specific (91). Lipid A has a glucosamine-phosphate backbone to which are attached long chain fatty acids (90). An unusual sugar, 2-keto-3-deoxyoctonate (KDO) is associated with the core oligosaccharide portion of LPS (47, 113) and thus can be used as an LPS marker. Investigations have shown that neither the polysaccharide portion (78) nor KDO (129)

is responsible for LPS toxicity, but rather that this toxic property of LPS is due to lipid A (35, 36).

The hydrophobic lipid A portion of lipopolysaccharide is thought to be situated in the hydrophobic zone of the outer membrane (15, 114). Osborn (114) has suggested that the hydrophobic portion facilitates transfer of LPS from the inner membrane, the site of its synthesis, to the outer surface of the envelope. Since polar parts of LPS protrude from the outer membrane, the resulting configuration has been called a "picket fence" (16). Deletion of a distal part of the LPS molecule would shorten the effective barrier thickness, while an intact "picket fence" might protect organisms by keeping reactions with antibody and complement a safe distance away.

Mutants have been isolated which synthesize LPS lacking the O-specific side chain or various parts of the core oligosaccharide (88, 110, 138, 150). These mutants are commonly designated "rough strains", whereas cells producing complete LPS are designated "smooth strains" (111). Rough mutants exhibit increased permeability to antibiotics and lysozyme (138) and show a marked increase in release of periplasmic enzymes during growth (88). These data suggest the LPS as well as the phospholipid continuum is essential to the barrier function of the outer membrane.

Ions are important in the retention of LPS in the membrane bilayer. Repaske (127) found that ethylenediaminetetraacetic acid (EDTA), a chelator of divalent cations, permitted lysozyme to act on several gram-negative bacteria. Spheroplasts formed by the action of

EDTA and lysozyme are sensitive to actinomycin, which does not affect intact E. coli (94). Leive (80, 81) subsequently found that EDTA treatment alone makes cells sensitive to actinomycin. This permeability increase is general, and is consistent with a partial breakdown of a passive permeability barrier. Prolonged exposure to EDTA is toxic to bacteria (85), resulting in RNA damage and cell death, presumably due to chelation of divalent cations necessary for ribosomal integrity and enzyme function (85). Shorter exposure periods indicate that EDTA is affecting primarily the outer rather than the cytoplasmic membrane (84).

Mg^{2+} and Ca^{2+}, the predominant ions in the cell envelope, are chelated by EDTA. Leive (82, 86) has demonstrated that EDTA treatment of E. coli released LPS (33-50%) plus some phosphatidylethanolamine (5-20%) and a little protein (1-2%). This would explain the observation that EDTA-treated E. coli cells, when diluted into medium containing excess Mg^{2+} and Ca^{2+}, require approximately half a generation of growth to restore the permeability barrier (83). The release of LPS was very rapid and paralleled the increase in permeability (86). Leive (84) has suggested that LPS released by EDTA is in equilibrium with LPS that is in a non-releasable form [LPS$\cdots Mg^{2+}$ (or Ca^{2+})$\rightleftharpoons$LPS$\cdots$X] to explain the observation that only one third to one half of the cellular LPS is released in the presence of EDTA. This non-releasable form of LPS is hypothesized to result from binding through hydrophobic and ionic interactions with other molecules in the membrane. Altering the divalent cation concentration in the

growth medium alters the equilibrium of LPS, and in turn modifies the fraction of LPS released by EDTA (84).

Much controversy exists in the literature concerning the composition and characterization of the LPS of N. gonorrhoeae. Stead et al. (147) extracted LPS from 5 strains of gonococci, and finding that the oligosaccharides contained glucose, galactose, glucosamine, heptose, KDO, and phosphate, concluded that O-side chains were absent. All strains also contained the same fatty acids - β-OH-10:0, β-OH-12:0, β-OH-14:0, 12:0, 14:0, 16:0, 16:1, 18:0, 18:1. No differences were found when virulent and avirulent or penicillin-sensitive and -resistant strains were compared.

In a similar study Perry et al. (119) isolated LPS from T1 and T4 colony types. The R-type LPS extracted from all T4 organisms appeared to be essentially identical. However, LPS from isogenic T1 colony types yielded S-type LPS, sometimes in association with R-type LPS. Later it was reported (120, 121) that yields of S-type LPS were inconsistent and frequently did not appear at all. Upon reinvestigation it was suggested that the suspected O-type polysaccharides detected in their preparations could be artifact (121).

Gonococcal LPS has been shown to be the receptor for pyocins (137) and the antigen to which bactericidal antibody in normal human serum is directed (41). Presently, work is in progress which uses these criteria to investigate the nature of gonococcal LPS.

Outer membrane proteins have been studied most extensively in E. coli. These proteins are found in small variety but in large

quantity, making them easy to isolate and characterize. In 1970, Schnaitman (141) described a protein band in SDS-polyacrylamide gels of the E. coli outer membrane which accounted for up to 70% of the total outer membrane protein. Inouye and Yee (62, 64) later showed that this single band actually contained several distinct proteins. It is now known that the "major outer membrane protein" of Schnaitman consists of at least 4 different proteins. The designations of these proteins are shown in Table 1. Another major outer membrane protein in E. coli is the previously mentioned lipoprotein, described by Braun (9).

The resolution and migration of the major outer membrane proteins depends on the SDS-gel system used, and can vary with acrylamide concentration, extent of cross-linking, pH and ionic strength of the buffer, voltage and current, and the method of sample solubilization. In addition, variation in outer membrane protein profiles can vary with culture conditions and bacterial strain (92, 142). With these factors in mind, the major outer membrane proteins of E. coli have been identified and described.

Matrix proteins (proteins Ia and Ib) exist in a tight, noncovalent association with the peptidoglycan (PG). These proteins can be isolated as a complex with peptidoglycan and bound lipoprotein, or in a form dissociated from peptidoglycan (134). Rosenbusch (134) reported that protein Ia of E. coli strain BE had a molecular weight of 36,500 and appeared to be a single peptide containing 334 amino acid residues and no nonprotein components. Proteins Ia and Ib

Table 1. Nomenclature of the major proteins of the outer membrane of E. coli K-12 as described by various investigators

DiRienzo et al. (25)	Bragg (7)	Schnaitman (3)	Henning (140)	Lugtenberg (92)
Matrix protein				
Ia	A_1, A_2	1a	1a	b
Ib		1b	1b	c
TolG protein	B	3a	II*	d
Lipoprotein	F		IV	

appear to be very similar and may differ only by modification of amino acid residue(s) (3, 140).

The relative amounts of Ia and Ib vary with growth conditions (3, 92). Both contain a high content of β-structure (134), in contrast to many other "intrinsic" membrane proteins which show a high content of α-helical structure. Proteins Ia and Ib migrate in a monomeric form on SDS-gels only if heated above 70° C in the presence of SDS (134). These same conditions release the matrix proteins from the peptidoglycan, as does exposure to 0.5 M NaCl, indicating that the association with peptidoglycan is probably due to ionic interactions (25). LPS stimulates the binding of matrix proteins to peptidoglycan (177). Inactivation of bacteriophage by purified matrix proteins is enhanced by the presence of LPS, suggesting an interaction of LPS and matrix proteins in vivo (19).

Electron microscopy reveals that matrix protein molecules are arranged in a hexagonal lattice (134, 149). The hexagonal arrangement is observed in both the presence and absence of peptidoglycan (149), indicating that protein-protein interactions are a major force in maintaining the lattice. Triplets of indentations (2 nm diameter) seen with negatively-stained preparations have been hypothesized to represent transmembrane pores (149). Nakae (106, 107) showed that incorporation of matrix proteins of Salmonella typhimurium and E. coli B into artificial LPS-phospholipid vesicles enhanced their permeability to sucrose. Cytoplasmic proteins are ineffective in reforming membrane vesicles (109). Vesicles reconstituted with matrix

proteins exhibit molecular sieving properties very similar to those of intact outer membranes, in that oligosaccharides of molecular weight greater than 900 are excluded (108). Lipoprotein-containing vesicles did not exhibit the same exclusion properties (107). This further suggests that matrix proteins form passive diffusion pores through the outer membrane (25).

The tolG protein is distinguished from the matrix proteins by its trypsin- and pronase-sensitivity (62) and its heat-modifiable behavior on SDS-gels (7, 64, 142). The apparent molecular weight of this protein increases when the membrane is solubilized in SDS-buffer at temperatures above 50° C (7, 64, 142). The tolG protein is a receptor for certain bacteriophage (19, 95, 163) and has a function in F-pilus-mediated conjugation (95). Mutants lacking tolG are unable to be recipients during conjugation, due to lack of pair formation (143). Also, purified tolG protein will inhibit conjugation and inactivate phages (56). This activity is enhanced with the addition of LPS, suggesting the interaction of tolG and LPS *in vivo* (163).

Lipoprotein (molecular weight 7,000), described by Braun and Reyn (9), contains 58 amino acid residues and links to every tenth to twelfth *meso*-diaminopimelic acid residue of the peptidoglycan. The free form of the lipoprotein is chemically identical to the bound form, except it does not contain any peptidoglycan components (65). There are twice as many molecules of the free form (4.8×10^5 per cell) (63) as of the bound form (2.4×10^5 per cell) (9, 10).

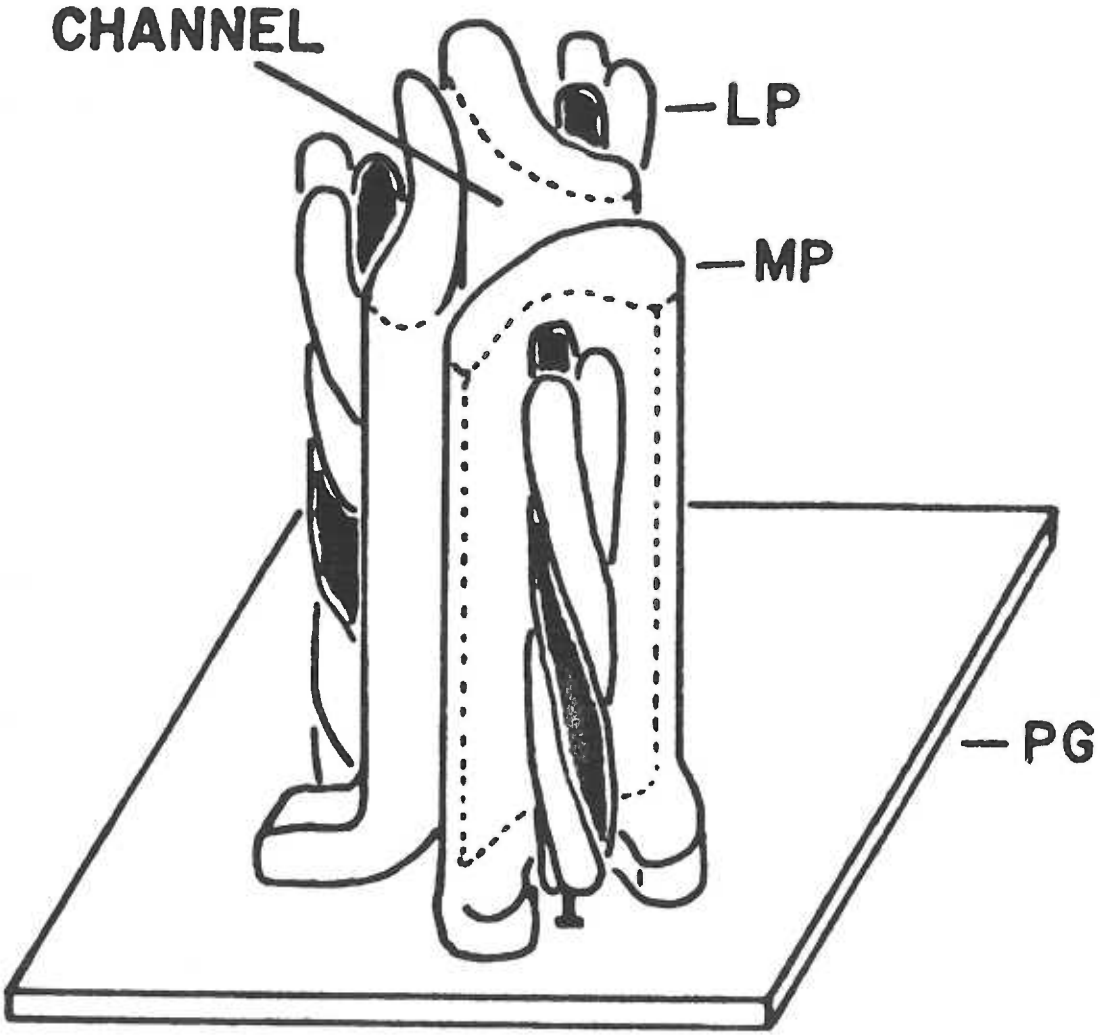
This protein has a very high α-helical content (8, 11). Inouye (61) and Braun (11) have proposed different models for the assembly of the lipoprotein, but this question is still unanswered. It has not yet been determined whether the lipoprotein is exposed on the outer surface of the membrane.

Minor proteins exist in the outer membrane, and may number between 10 and 20. Many are receptors for phage and colicins, and some are necessary for uptake of nutritional substrates (25).

LPS seems to be exclusively localized in the outer layer of the bilayer (105), while phospholipids are located in the inner layer (144). Evidence indicates that most of the outer membrane proteins are exposed to the external environment (74). The protein composition of the outer membrane is variable, but the total amount of protein remains relatively constant (92, 142). Mutants of E. coli have been isolated which lack matrix proteins and tolG (56). No apparent defects in structural integrity were observed in these mutants. As yet, no mutants have been isolated which lack matrix proteins, tolG, and lipoprotein.

DiRienzo, Nakamura, and Inouye (31) have proposed an assembly model of matrix protein and lipoprotein (Fig. 1). Three molecules of matrix protein, mainly in β-structures, form a hydrophilic diffusion pore 1.5-2.0 nm in diameter. Each matrix protein molecule is stabilized by a triple coiled-coil of lipoprotein molecules containing 2 molecules of free form and 1 molecule of bound form lipoprotein. The lipoprotein molecules may fit into the identations seen on electron micrographs. This would be compatible with evidence that the

Figure 1. Postulated assembly model of the matrix protein and lipoprotein. Abbreviations: MP, matrix protein; LP, lipoprotein; and PG, peptidoglycan. From DiRienzo et al. (25).


CHANNEL
LP
MP
PG

lipoprotein is evenly distributed over the cell surface in greater molecular numbers than the matrix protein. Further experiments are necessary to test this model.

C. Outer membrane of Neisseria gonorrhoeae

The outer membrane of N. gonorrhoeae is an undulating membrane 7.5-8.5 nm thick (104). When viewed in the electron microscope the outer membrane appears more loosely attached to the peptidoglycan than the outer membrane of E. coli, possibly due to the lack of a lipoprotein anchoring molecule (175). No significant difference in outer membrane density exists between E. coli and N. gonorrhoeae (174, 175).

Devoe and Gilchrist (23) observed cell wall blebs in log-phase cultures of N. meningitidis. These blebs, produced by budding of the outer membrane, contain outer membrane components, including LPS. Such blebs have also been described in N. gonorrhoeae (42, 99, 102), and are of a size similar to those found in N. meningitidis.

Russell (136) reported that the envelope of N. sicca contained proteins which exhibited different electrophoretic mobilities depending upon whether the samples were solubilized at 37° C or 100° C. This phenomenon was later observed in strains of N. gonorrhoeae (52, 165).

Wolf-Watz et al. (176) compared the outer membrane of E. coli and N. gonorrhoeae and found that, in their hands, the E. coli outer membrane possessed 10-12 polypeptides, with the predominant protein having a molecular weight of 44,000 daltons. N. gonorrhoeae

subjected to the same treatment yielded about 8 proteins, the predominate one having an apparent molecular weight of 35,000 daltons. The outer membrane proteins of the gonococcus were easily extracted in Tris-saline, which released no proteins from E. coli. The E. coli outer membrane could only be extracted with detergents, suggesting that the outer membrane proteins of N. gonorrhoeae may be more hydrophilic than those of E. coli.

Johnston and Gotschlich (72) isolated the outer membrane of N. gonorrhoeae strain 2686 (T4) and subjected it to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). They found a relatively simple protein profile with proteins of 34,500, 22,000, and 11,500 molecular weight (MW) accounting for the majority of the protein. The major outer membrane protein (MOMP) (MW 34,500) accounted for 66% of the total protein of the outer membrane, and had an isoelectric point (pI) of 4.6. The other outer membrane proteins had a pI of 4.0. The five outer membrane proteins visualized represent those proteins not solubilized by the EDTA used in isolating the outer membrane. Tris-saline extraction of purified outer membrane yielded an aggregate containing the 34,500, 22,000, and 11,500 molecular weight proteins.

Frasch and Gotschlich (33) developed a relatively gentle method for the extraction and isolation of outer membrane proteins from group B meningococci utilizing 0.2 M LiCl at 45° C for 2 hr. Extraction was carried out on a gyratory shaker in the presence of glass beads. A high molecular weight protein-LPS complex could be

isolated from the supernatant of the extraction suspension by gel filtration or ultracentrifugation. This complex, located on the surface of the outer membrane, was called the serotype antigen, and proved to be an effective immunogen in rabbits.

Johnston, Holmes, and Gotschlich (73) pursued the possibility of using the gonococcal major outer membrane protein as a serotyping antigen. They found this protein to be an integral part of a high molecular weight complex containing 2 proteins and LPS. The major protein could not be resolved into more than one peptide utilizing various gel systems. Tris-saline-extracted gonococcal outer membranes produced 9 bands upon SDS-PAGE. When 14 strains were compared, the most notable finding was the variance in subunit molecular weight of the different major outer membrane proteins, ranging in molecular weight from 38,000 to 32,000 daltons. Fifty-seven strains of gonococci from New York City were divided into seven classes according to the subunit molecular weight of their major outer membrane protein. When other strains from New York City and strains from foreign countries were examined, the number of serotype classes increased to 16. A second protein of lower subunit molecular weight covaried with the MOMP. Colony types T1, T3, and T4 possessed identical MOMP's; serospecificity was therefore retained through subculture. Contact pairs of infected individuals appeared to be infected with the same serotype of N. gonorrhoeae.

The gonococcus is polyantigenic (LPS, outer membrane proteins, pili). Therefore, immunological studies and biochemical

characterization require the preparation of isolated components of the outer membrane. Heckels (52) described a procedure for isolating the two major outer membrane proteins and LPS of the gonococcus. Lithium acetate-extracted outer membrane yielded 5 bands on SDS-PAGE. When the outer membrane from cells labeled with dansyl chloride-cycloheptaamylose was extracted, only the two major proteins and pili were fluorescently labeled. Treatment of the outer membrane with 6 M urea removed some interfering minor proteins. Subsequent incubation of the membrane preparation in alkaline buffer containing 5 mM EDTA and 1% cholate yielded a soluble fraction enriched in protein II (24,000 MW), whereas the insoluble fraction was enriched in protein I (36,500 MW). The soluble fraction was concentrated and applied to a Sephadex G-75 column, with resultant separation of protein II and LPS. Protein II was further purified by passage over a G-200 column, which removed pure protein II from protein II found in association with other outer membrane proteins. The insoluble residue was extracted with 6 M guanidine thiocyanate, and the supernatant was dialyzed against 6 M urea. After concentration, the solution was applied to a Sephadex G-200 column to obtain a peak of purified protein I. Protein II, when present, is the protein that shows anomalous electrophoretic mobility with different temperatures of solubilization. From these experiments it would appear that both protein I and protein II are tightly associated with the membrane and with each other.

Heckels (54) reported that the parent strain of one of his laboratory strains, P9, contained a 60,000 MW protein (protein III) not

seen after repeated subculture of the strain. This protein was readily extracted by 6 M urea, which had been used in previous studies as a preliminary step to remove minor proteins. Later, Heckels (53) used ^{125}I-lactoperoxidase to label whole cells, and found that protein I and protein II, when present, were labeled heavily, but protein III was labeled to a much lesser degree. Other bands visible on autoradiography were probably minor outer membrane proteins and/or cytoplasmic protein contamination. When isolated membranes were ^{125}I-labeled, protein III was readily labeled, indicating that only small amounts of protein III are expressed at the outer surface of the intact gonococcal cell.

Swanson (154) reported a 28,000-29,000 MW outer membrane protein which appeared to be involved in the association of gonococci with leukocytes, and another protein (26,000-28,000 MW) whose presence determines trypsin-sensitivity and coloration and aggregation characteristics of colony forms. This latter protein may be the same as Heckels' protein II.

Evidence of an altered outer membrane protein in dark-colored variants of both piliated and non-piliated gonococci was presented by Walstad et al. (165). A 28,000 MW outer membrane protein exhibited variable behavior on SDS-PAGE which was dependent on the presence of urea and boiling. This may be the same protein as that observed by Swanson (154) and Heckels (52).

Frasch and Mocca (34) observed that two predominant outer membrane proteins in a strain of N. meningitidis group B demonstrated

heat-modifiable behavior. The predominant outer membrane protein, protein b (41,000 MW), migrated as a high molecular weight aggregate when heated at 56° C for 1 min in the presence of SDS and urea. It was proposed that protein b existed in the outer membrane as trimers or tetramers. The other heat-modifiable outer membrane protein, protein e (28,000 MW), migrated with an apparent molecular weight of 21,000 daltons (protein e*) after heating at 56° C for 1 min. Protein e shifted its apparent molecular weight to 28,000 daltons only after heating at 100° C for 2 min. This is similar to the 28,000 MW protein of the dark-colored gonococcal colonies (165). When gonococcal outer membranes were heated at 100° C in the presence of urea, only a 28,000 MW band was observed. In the absence of urea and boiling, the predominant protein had a molecular weight of 20,000 daltons. Urea without boiling or boiling without urea produced a major band at 28,000 MW and a minor band at 20,000 MW.

Hildebrandt et al. (58) transformed a serum-sensitive gonococcal strain to serum resistance with DNA from 3 different disseminated strains (serum resistant). This transformation was accompanied by structural and antigenic changes in the MOMP. Upon transformation, the subunit molecular weight of the MOMP changed from that of the recipient (MW 39,000) to that of the donor (MW 36,500). The shift in MW was accompanied by a concomitant change in antigenic type. The authors suggested that a MOMP of a characteristic subunit molecular weight may be related to serum resistance.

Pili have received much attention as possible immunoprophylactic agents. These surface appendages are approximately 80 Å in diameter and extend up to 2 microns in length from the cell (70, 156). Pili are composed of protein with an aggregate molecular weight of $1\text{-}20\text{x}10^6$ daltons (13). The monomeric protein has a MW of 17,000-21,500 and is subject to strain variation (12, 130).

Novotny and Turner (112) reported that type 3 and 4 gonococci have pili antigens in their envelope although they do not possess pili. Pili have been implicated as virulence factors, as mediators of attachment to eucaryotic cells (14, 66, 67, 96, 125, 152, 157, 159, 168), and as the cellular component responsible for increased resistance to phagocytosis by some colony forms (40, 125, 158, 162). Some trials have been initiated using pili as an immunizing agent; results of these experiments are still being analyzed.

Several groups of investigators (21, 55, 68, 128) have reported the presence of capsules on N. gonorrhoeae. Capsules were demonstrated with the wet India ink technique of Duguid (26), with the best encapsulation found on freshly isolated strains. The putative gonococcal capsule was suggested to be polysaccharide (68). The polysaccharide capsule of N. meningitidis has antiphagocytic properties. Whether the gonococcus does or does not have such a capsule remains a matter of controversy.

D. Autolysis

When a bacterial cell grows and divides, the cell envelope of that bacteria must also enlarge. Lytic enzymes have been described

(39) which cleave components of the envelope to permit addition of new material. Glycosidases, amidases, and endopeptidases are classes of such lytic enzymes (39). Any uncontrolled or unbalanced activity of these enzymes can lead to autolysis of the bacterial cell. The autolytic nature of N. gonorrhoeae has been the subject of several recent investigations.

Morse and Bartenstein (103) observed that glucose-limited cultures of N. gonorrhoeae were autolytic, whereas nitrogen-limitation or the addition of chloramphenicol or rifampicin did not induce autolysis. Hebeler and Young (48) further examined the autolytic behavior of the gonococcus. They found that the optimum pH for autolysis was 9.0 and the optimum temperature was 40° C. Autolysis was not dependent on the growth phase of the organism, but potassium ions (0.1 M) did increase the rate of autolysis. Data from the hydrolysis of radioactively labeled peptidoglycan suggested that autolytic enzymes were active over a large part of the cell surface.

Elmros et al. (28) observed that a low environmental pH and divalent cations stabilized nongrowing cells. On the other hand chelating agents, such as EDTA, promoted autolysis. Buffer had an effect on autolysis rate, with cells suspended in Tris(hydroxymethyl)-aminomethane (Tris) buffer exhibiting a higher rate than cells suspended in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), phosphate, or Tris-maleate buffers. Sucrose, spermine, and polyvinylpyrrolidone also prevented autolysis. It was suggested that the outer membrane plays an important role in mechanical stability, since

divalent cations, low pH, and low temperature had a protective effect. The stability imparted by divalent cations may be a result of cross-linking of envelope components via ionic bonding (1).

Mechanical stability was not found to correlate with cellular viability. Elmros et al. (29) showed that viability was less at pH 5 and 6, where the cells were stabilized, than at pH 7.2, the optimal pH for survival. However, low temperatures which decreased lysis also preserved viability. Divalent cations, with the exception of Cu^{++} which is toxic, both stabilized cells and enhanced their viability in buffer.

Hebeler and Young (49) attempted to purify the major autolysin of N. gonorrhoeae with little success. This enzyme was found to be an N-acetylmuramyl-L-alanine amidase (E.C. 3.5.1.28), which had a pH optimum of 5.5 to 8.5. The amidase was tightly bound to the cell envelope, since Triton X-100 and NaCl were needed to effectively remove it. Neither substance alone would liberate the autolytic enzyme, and problems in purification arose from difficulty in removing Triton X-100.

Gonococci suspended in buffer undergo peptidoglycan hydrolysis. The rate of peptidoglycan hydrolysis in HEPES buffer was maximal at pH 8.5, and was similar in the presence or absence of Mg^{2+} (170). Therefore, the stabilization of gonococci by divalent cations is not due to an effect on peptidoglycan hydrolysis. Cells stabilized at pH 6.0 exhibited a rate of peptidoglycan hydrolysis which was 50% of maximum. Incubation of cells with chloramphenicol or rifampicin

before suspension in HEPES buffer (pH 8.5) reduced the rate of autolysis (170). This would suggest a slow turnover of the peptidoglycan hydrolase and would eliminate the possibility that a regulatory protein is involved in the sequence of events leading to autolysis.

Recently, Rosenthal (135) found evidence that gonococci possess hexaminidase (glycan-splitting) activity in addition to amidase activity. The role of this hexaminidase may be more important in turnover of gonococcal peptidolgycan than previously thought, since data presented suggested that this enzyme might account for up to 80% of the total soluble peptidoglycan generated from turnover.

Peptidoglycan hydrolysis is probably necessary for autolysis of N. gonorrhoeae. However, available data would also suggest a role for the outer and/or cytoplasmic membrane in this phenomenon. Guymon and Sparling (43) examined wild-type and antibiotic-resistant and -hypersensitive isogenic strains of N. gonorrhoeae for crystal violet uptake, rates of autolysis, and response to lysozyme. Mutation to nonspecific resistance (ery locus) led to relative impermeability of crystal violet, as compared to wild-type. This penetration barrier could be overcome with the addition of 5 mM EDTA. This mutation also resulted in lower rates of autolysis and decreased sensitivity to lysozyme in the absence of divalent cations. On the contrary, a mutation at the nonspecific hypersensitivity locus env resulted in increased crystal violet uptake, increased rates of autolysis, and increased sensitivity to lysozyme in the presence of Mg^{2+}. This suggested that permeability and autolytic rate are dependent

upon the composition, configuration, and integrity of the outer membrane, which can be altered by mutation and/or environmental factors.

These antibiotic-resistant and -sensitive isogenic strains were analyzed to discover whether cell envelope alterations simultaneously occurred (44). No differences were detected in phospholipid or lipopolysaccharide composition. However, mutation to nonspecific resistance (mtr-2) was accompanied by a seven-fold increase in the amount of a minor 52,000 MW outer membrane protein and a 32% increase in the extent of peptidoglycan cross-linking. Addition of nonspecific hypersensitivity loci (env-1 or env-2) to a strain carrying mtr-2 resulted in phenotypic reversal of resistance due to mtr-2, a reduction in amount of the 52,000 MW protein, and a reduction in the extent of peptidoglycan cross-linking. Addition of another locus, pen B2, which increases resistance to penicillin and tetracycline, resulted in disappearance of the wild-type MOMP (36,900 MW) and appearance of a new species of MOMP (39,400 MW). Yet another locus, env-3, which imparts nonspecific hypersensitivity, had no effect on outer membrane proteins and caused only a slight decrease in peptidoglycan cross-linking. It remains to be determined whether the altered characteristics seen in the transformants are primary or secondary effects of other, undetected cell envelope changes.

E. Extraction of outer membrane

The classic procedure used for isolation of the outer membrane of gram-negative bacteria involves treatment of the bacterial cell with EDTA and lysozyme (101, 115). EDTA increases the permeability

of the outer membrane so that lysozyme, normally excluded by the intact outer membrane, can pass through and degrade the peptidoglycan layer (127). Once the peptidoglycan is degraded, the organism is osmotically fragile. Therefore, this procedure is performed in the presence of 20% sucrose, which stabilizes the spheroplasts thus formed. Transfer of spheroplasts to a hypotonic environment results in breakage of the membranes and release of cytoplasmic components. The membranes can reanneal and form closed membrane vesicles. The membrane suspension is separated into cytoplasmic and outer membrane fractions by isopycnic sucrose density centrifugation (115). The resultant bands are identified by chemical and enzymatic analyses. Electron transport components are used to determine the cytoplasmic membrane band, while carbohydrate, LPS, and hexosamine are located primarily in the outer membrane band (72, 115).

When this procedure is applied to the gonococcus, three bands appear in the gradient (72). The first band ($p°=1.141$ g/cm^3) contains cytoplasmic membrane, the second ($p°=1.176$ g/cm^3) contains both cytoplasmic and outer membrane material, and the third ($p°=1.219$ gm/cm^3) contains outer membrane. Proteins can then be extracted from this outer membrane preparation using detergents (Triton X-100, sodium deoxycholate) or 150 mM NaCl in 10 mM Tris-HCl buffer, pH 7.4, for 2 hr at 37° C.

One problem with applying this method to gonococci is that EDTA alone can cause extensive lysis of cells (28). This has also been observed in Pseudomonas aeruginosa (27). Such sensitivity to EDTA

may result in fragmentation or loss of outer membrane components, and may also result in decreased yields of outer membrane material.

Zollinger et al. (178) used N. meningitidis outer membrane blebs isolated from culture supernatants as a source of outer membrane. An alternative procedure called for incubation of cells in buffer containing 0.01 M EDTA, followed by two passages of the cell suspension through a hypodermic needle. This theoretically sheared off outer membrane which was then isolated by differential centrifugations.

Hatefi and Hanstein (46) utilized chaotropic agents as a means of extracting membrane proteins. Kauzmann (75) hypothesized that apolar groups form hydrophobic bonds mainly because of their thermodynamically unfavorable interaction with water, rather than as a result of attraction for each other. Chaotropic agents are defined as those inorganic anions which favor the transfer of apolar groups to water. By making water more disordered and lipophilic, the hydrophobic bonds of membranes should be weakened, with a resultant increase in the water solubility of particulate proteins. These agents, used at concentrations of 2 M or less, have the added advantage that, unlike detergents, they do not denature proteins or lead to loss of loosely bound cofactors. In some instances, weak chaotropic agents (Br^-, NO_3^-, Cl^-) are more effective than potent chaotropes (SCN^-, ClO_4^-) in membrane resolution, perhaps due to the contribution of electrostatic forces as well as hydrophobic ones.

Hook (60) showed by electron microscopy that when whole cells of N. gonorrhoeae are extracted with 1 M NaSCN the outer membrane peels

away from the cell. The treated cells remained intact, but were no longer viable and appeared to contain aggregated cytoplasmic material. LiCl and NaSCN were compared as extracting agents. LiCl extraction was found to be a gentler method, releasing less cellular nucleic acid into the final membrane preparation. LiCl treatment did not inactivate succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH), which can be used to assay cytoplasmic membrane contamination of the outer membrane preparation. Activities of SDH and LDH in the final outer membrane preparation were 1.5% and 0.3%, respectively, of those found in the total envelope preparation. When various chaotropic agents were compared, 1 M LiCl yielded the highest ratio of protein to phospholipid. These data suggest that extraction with 1 M LiCl is a gentle procedure which does not denature protein or remove significant amounts of protein from the membrane matrix.

F. Chemical cross-linking

Chemical cross-linking can provide useful information on quaternary structure of oligomeric proteins and large molecular aggregates (122). This technique can be applied to either soluble proteins or protein components of a membrane. The most versatile cross-linking method utilizes a bifunctional reagent; that is, one with two reactive groups capable of bridging side chains of amino acids in a protein(s). Application of a bifunctional reagent to a protein suspension can yield 1) intramolecular cross-links, 2) intermolecular cross-links (homopolymers), and/or 3) intermolecular cross-links

between protein complexes composed of different proteins (heteropolymers) (173).

Variables that should be considered when selecting reaction conditions for cross-linking are protein concentration, protein-to-cross-linker concentration ratio, pH, and ionic strength (173). Upon completion of the reaction, excess reagent must be eliminated, then the derivatives obtained are characterized.

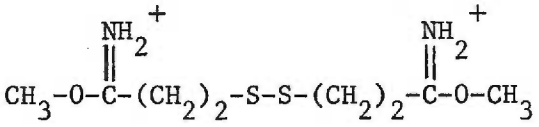
Chemical cross-linkers fall into many classes. Reagents can be homobifunctional or heterobifunctional, water soluble or insoluble, cleavable or non-cleavable, flexible or non-flexible (18, 71, 122, 173). The reactive end groups may have differing degrees of specificity in their reactions with proteins. Some cross-linking reagents only react upon photochemical activation. Thus, a reagent can be selected which will have optimal reactivity for a given protein-containing system.

Certain assumptions are made about modification of proteins by cross-linking reagents: 1) not all of the protein functional groups are modified, 2) within each molecular weight category a variety of species exist with different numbers of intra- and interchain cross-links, but only the first cross-link provides information, and 3) the molecular weight contribution of the added cross-linker is negligible in comparison to the molecular weight of the protein chains (122).

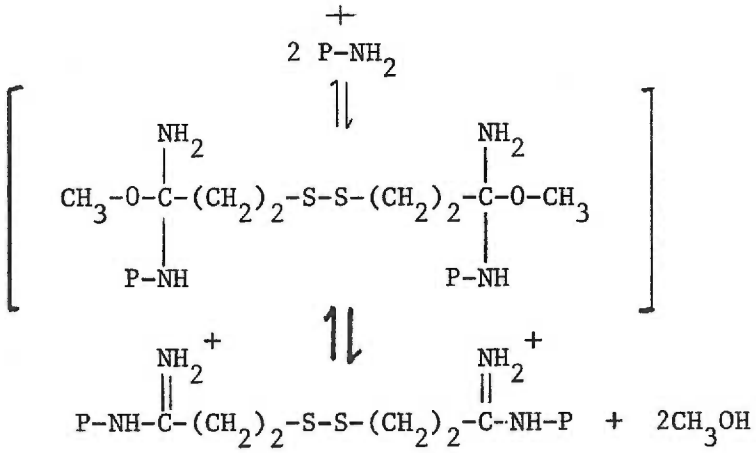
A variety of cross-linking reagents (structure and specifics of reaction) have been discussed in recent reviews (18, 71, 122, 173).

Tables 2 and 3 list the cross-linking reagents mentioned in this literature review. Attention shall be focused on one of the more utilized classes of cross-linking reagents, the alkyl imidates or imidoesters.

Reaction of imidates with protein appears to be confined exclusively to primary amino groups (122). The product of this reaction is an amidine, which retains the positive charge of the original amino group and results in little alteration of physical properties or biologic activity. The cross-linking process is illustrated by the following reaction (71, 122):



Dimethyl-3,3'-dithiobispropionimidate (DTBP)



This schematic reaction would result in the formation of a homopolymer of two units of $P\text{-}NH_2$, joined by a chemical bridge 12 Å in length. If two different species of protein were present ($P^1\text{-}NH_2$, $P^2\text{-}NH_2$) and were separated by 12 Å, the reaction could yield

Table 2. Properties of selected amino-specific non-cleavable cross-linking reagents

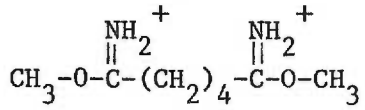
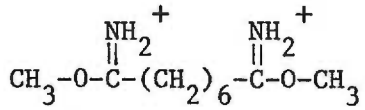
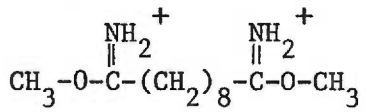
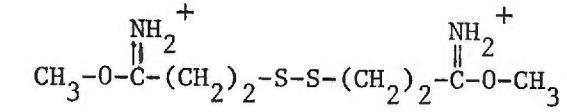
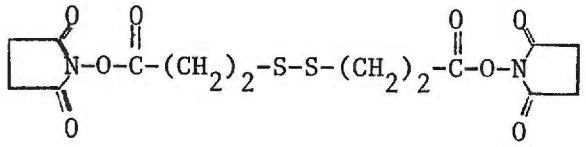
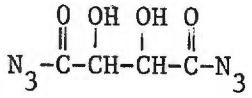
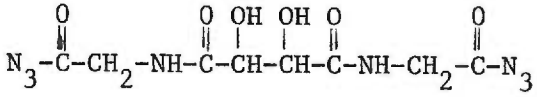
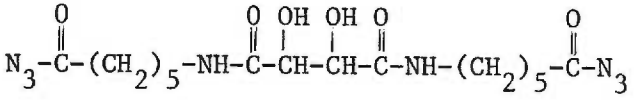
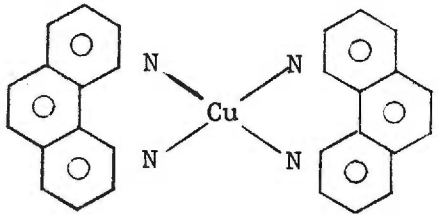
Name	Formula	Abbreviation	Approximate maximum linkage dimension
Dimethyl adipimidate		DMA	9 Å
Dimethyl suberimidate		DMS	11 Å
Dimethyl sebacimidate			

Table 3. Properties of selected cleavable cross-linking reagents

Name	Formula	Abbreviation	Approximate maximum linkage dimension
Amino specific			
Dimethyl 3,3'-dithio-bispropionimidate		DTBP	12 Å
Dithiobis(succinimidyl-propionate)		DTSP	11 Å
Tartryldiazide		TDA	6 Å
Tartryldi(glycylazide)		TDGA	13 Å
Tartryldi(ε-amino-caproylazide)		TDCA	23 Å
Sulfhydryl specific			
Cupric di(1,10-phenan-throline)		CuP	0 Å

P^1-NH-(R)-S-S-(R)-NH-P^2, a heteropolymer. These are desired reactions; however, the imidate can undergo many other reactions which render it useless as a cross-linker. The reaction path of the imidate is determined by the pH of the reaction mixture, which therefore is a critical parameter in this system. For maximum formation of amidine with a minimum number of side products, the reaction should be carried out at or above pH 10. Although many proteins cannot tolerate such alkaline conditions, even for short periods of time, one or two minutes at this pH produces a substantial amount of amidine.

If the reaction must be carried out at or below pH 8, lower yields of amidine will be produced and undesirable side reactions may occur (122). Large excess of cross-linker will only produce more unwanted side reactions, so sequential addition of small amounts of reagent is preferred.

Synthesis of cleavable protein cross-linkers has allowed investigators to reduce cross-linked complexes into their component parts. The cross-linking reagent mentioned previously, dithiobispropionimidate (DTBP), contains a disulfide bond which can be easily cleaved with a reducing agent (dithiothreitol, β-mercaptoethanol). One disadvantage to the disulfide cross-linkers is that they may undergo disulfide interchange. When used in conjunction with two-dimensional polyacrylamide gel electrophoresis, however, the advantages of this type of cleavable cross-linker far outweigh the disadvantages.

Preparations of cross-linked material are electrophoresed on a one-dimensional gel in the absence of β-mercaptoethanol. Upon completion of electrophoresis, the gel is heated in buffer containing reducing agent. The gel is then placed upon a second-dimensional slab and electrophoresed at right angles to the first dimension (166). Cleavage of cross-linked complexes results in off-diagonal spots which migrate faster in the second dimension than in the first, due to liberation of lower molecular weight components. If a heteropolymer is cleaved, its components should lie on a straight line, and thus the components can be identified as members of the same cross-linked complex. Intrapeptide disulfide bonds, either naturally occurring or produced by the cross-linking reaction, may produce spots on the opposite side of the diagonal as a result of alteration of the effective Stokes radius (122). Also, multiple complexes of varying Stokes radii may produce smearing of bands in SDS-polyacrylamide gels, with concomitant difficulties in identification (122). The demonstrated existence of a certain cross-linked complex can be interpreted with some confidence. The absence of a complex, however, may or may not be significant.

Chemical cross-linkers have been used to study ribosomes (5, 93), erythrocytes (148, 166, 167), histones (160, 161), oligomeric enzymes (20), and bacterial membranes (22, 30, 45, 116-118, 126). Wang and Richards (166) treated isolated human erythrocyte membranes with dimethyl dithiobispropionimidate (DTBP) and the o-phenanthroline cupric ion complex (CuP), which oxidizes intrinsic sulfhydryl groups.

They found during sample preparation for electrophoresis that disulfide-containing cross-links were very labile to heat (100° C for 3 min will destroy the cross-links). If different acrylamide concentrations were used in the first and second dimension, a skewed but reproducible and usable diagonal line resulted. Glycoproteins migrated off-diagonal in the presence or absence of reducing agent when gel concentrations in the two dimensions were different, due to properties of glycoproteins per se. To aid in identification of off-diagonal spots, the authors advised slight overloading of the first dimensional gel so that streaking would result in a line in the second dimension connecting all off-diagonal spots derived from the same band. This procedure also aided in the detection of minor spots.

Wang and Richards (166) classified their off-diagonal spots into three categories: 1) off-diagonal spots with their centers on a vertical line, 2) off-diagonal spots whose centers are not on a vertical line with any other spot, and no part of a given spot overlaps with any other spot, and 3) off-diagonal spots whose centers are not in a vertical line, but parts of the spots overlap with each other. The first type of spots, representing heteropolymers, were rarely observed with their cross-linked erythrocyte membranes. The second type of spots, seen at a low degree of cross-linking, were the easiest to interpret and represented homopolymers or heteropolymers with a "missing" partner. With higher degrees of cross-linking, type 3 spots were most prevalent. It could not be determined whether this resulted from heterogeneity of the same component or from one

component being part of two different cross-linked complexes of similar, high molecular weight. Spots of the second type, assumed to represent dimer, trimer, and higher oligomers, often have molecular weights less than those expected for integral multiples of the monomer. The branched structure of a cross-linked complex may lead to anomalous electrophoretic mobility when compared to mobility-molecular weight relations for single linear polypeptides.

Very high molecular weight complexes of erythrocyte membrane proteins were observed which barely entered the first dimensional gel (166). After cleavage of disulfide bonds, these complexes produced a large number of monomer bands in a vertical line below the first dimension origin. It was further shown that these bands resulted from cross-linking, not from trapping of a portion of the total protein applied (166).

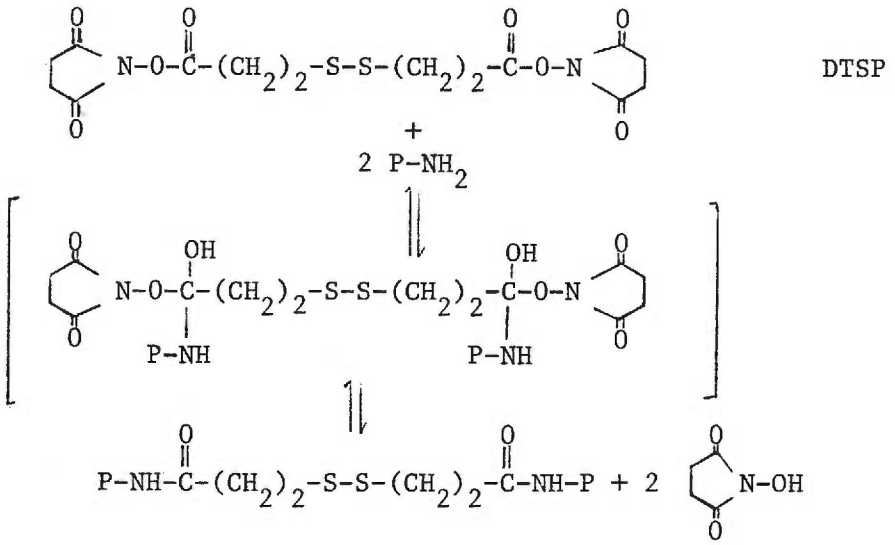
Cross-linking was demonstrated with both DTBP and CuP. The patterns obtained were different, but yet surprisingly similar. CuP requires the close proximity of two -SH groups to form a disulfide bond. These neighboring -SH groups must be accessible to the CuP. In addition, it was demonstrated that N-ethylmaleimide (NEM) inhibited cross-linking by CuP, but had no effect on DTBP cross-linking. NEM-treatment, therefore, did not alter contact between peptide chains. The data also suggest that most, if not all, DTBP cross-linking is due to reaction with amino groups, rather than as a result of disulfide exchange.

The majority of off-diagonal spots detected after two-dimensional SDS-PAGE of cross-linked erythrocyte membranes were tentatively identified as homopolymers, which is in agreement with the findings of Steck (148), who suggested oligomeric proteins may be a general feature of membrane architecture. However, are these observed cross-linked complexes the result of accidental collisions of peptides freely floating in the membrane bilayer, or do they in actuality represent stable oligomeric complexes in the membrane? Given a large number of different proteins found in the whole membrane, the number of accidental complexes that might form would be very large, with no complexes occurring more frequently than any other. The resolution of the detecting system would not pick up any of these numerous complexes. The visualization of off-diagonal spots would only result if the monomers were part of a stable, bonded complex or if the monomers had restricted mobility in the membrane matrix.

Cross-linking of intact erythrocytes yielded the same pattern as cross-linked isolated membranes (166). No difference was observed in cross-linking patterns when the reaction was done at 0° C rather than at room temperature.

Lomant and Fairbanks (89) reported the synthesis and properties of a new cleavable protein cross-linker, [^{35}S] dithiobis(succinimidyl propionate) (DTSP). This reagent quantitatively acylates free primary and secondary aliphatic amino groups in either organic or aqueous media within two minutes at 23° C. Hydrolysis of the active N-hydroxysuccinimide ester termini in buffer at pH 7 is relatively

slow, so low concentrations of reagent yield optimal cross-linking. DTSP is not directly water soluble, but the reagent can be dissolved first in a small volume of polar organic solvent (dimethylformamide, dimethyl sulfoxide, or acetone), which can then be added to 50-200 volumes of buffer. The reaction of DTSP with protein is schematically represented (37, 71):



The authors claim DTSP to be a superior reagent to DTBP since it will react at pH 7, has a long solution half-life, does not require large reagent excess, and in general reacts under conditions less favorable to disulfide interchange.

A series of bifunctional reagents were reacted with skeletal muscle myosin (17). It was calculated that dimethylimidoesters of adipic, suberic, and sebacic acid reacted with 0.5% of the free amino groups, DTBP reacted with 1.5%, and DTSP reacted with 10% of the free amino groups. The half-time of DTSP was found to be 90 min; in

contrast, the half-time in solution of DTBP was 15 min. For this system, the success of cross-linking was dependent on the bifunctional reagent chosen. The solubility of myosin was altered by acylation of the amino groups (DTSP), while DTBP left the net positive charge unchanged. Introduction of cross-links did not appear to alter the α-helical structure of the rods, but did change the overall symmetry.

DTSP (also called Lomant's reagent) has also been applied to purified human histocompatibility antigens (HLA) (146). A heteropolymer (54,000 MW) was detected which contained components of 44,000 MW (heavy chains) and 12,000 MW (β_2-microglobulin), but no dimers of the 44,000 or 12,000 MW proteins or higher oligomers were observed.

Lutter et al. (93) used a series of acyl azides to cross-link E. coli ribosomes. These reagents contain a cis-glycol which is cleaved by periodate treatment. However, periodate may also cleave sugar residues in the system, and the product aldehydes formed are known to be intrinsically reactive (122).

Haller and Henning (45) treated whole cells of E. coli with a variety of non-cleavable imidoesters prior to ghost purification. This cross-linking treatment produced ghosts that were insoluble in boiling 1% SDS. In contrast, ghosts prepared from non-cross-linked cells were soluble in 1% SDS at room temperature. Ghost purification involved a trypsin step. Trypsinized ghosts, when treated with imidoesters, did not become resistant to boiling in 1% SDS. In the absence of trypsin exposure, ghosts could be cross-linked so as to become resistant to boiling in SDS. It was suggested that extensive

protein-protein interactions must exist in the E. coli outer membrane.

Whole cells of E. coli were treated with acyl azides and cleavable diimidoesters by Palva and Randall (116). The outer membrane was isolated and subjected to two-dimensional SDS-PAGE. No cross-linking was detected with tartryldiazide (TDA; 6 Å bridge length). Tartryldi(glycylazide) (TDGA; 13 Å) produced an off-diagonal spot from a complex of 73,000 MW, which was identified as protein I (37,000 MW). Two less intense spots of the 37,000-dalton protein were seen originating from complexes of higher molecular weight, and were concluded to represent trimers and tetramers of protein I.

Further investigations were undertaken by these researchers (117). Whole cells, isolated cell walls, and protein-peptidoglycan complexes were cross-linked. The cleavable acyl azide, TDGA, produced dimers and trimers of protein I. Proteins treated with the non-cleavable imidoester, dimethylsuberimidate (DMS), were resolved on one-dimensional gels. The resultant gel pattern showed a continuous series of multimers, with every third one more abundant than the two immediate lower ones when protein-peptidoglycan complexes were cross-linked. When purified protein I was treated with DMS, only dimers and trimers were observed, with no evidence found for formation of higher multimers. This would agree with the hypothesis of Rosenbusch (134) that protein I is arranged in a lattice structure with a basic repeating unit of three protein I molecules. Higher

multimers can only be visualized if the protein remains associated with peptidoglycan or the membrane matrix.

Protein I is known to consist of protein Ia and protein Ib. Protein Ia but not Ib is involved in nucleotide permeation through the outer membrane. Palva and Randall (118) cross-linked a strain of E. coli containing both Ia and Ib, then used high-resolution SDS-gel electrophoresis to determine what cross-linked complexes were formed. A low concentration of TDGA was used so as to produce only dimers. The resultant gel patterns indicated that dimers of Ia-Ia and Ib-Ib occurred, but there was no evidence for formation of a Ia-Ib complex. Thus, the two forms of protein I tend to be complexed only to similar molecules. Although small amounts of heteropolymer formation cannot be ruled out, the multimers formed have been suggested to represent two different species of pores in the outer membrane, as has been hypothesized by van Alphen et al. (164).

Reithmeier and Bragg (126) used DTSP to cross-link protein I-peptidoglycan complexes, isolated outer membrane, and cell wall (outer membrane and peptidoglycan) preparations. Dimers, trimers, and higher multimers of protein I were found, but no cross-linking of protein I to peptidoglycan was detected. Heat-modifiable protein II* formed high molecular weight oligomers. No cross-linking of protein I to protein II was detected. Protein II and the free form of the lipoprotein could be linked to the peptidoglycan. The free form of the lipoprotein could be linked to itself and to protein II.

Chemical cross-linking, while still a relatively new technique, seems to have unlimited applicability in the fields of microbiology, biochemistry, and immunology. When applied to membrane systems, this procedure can be a powerful tool in elucidating the architecture of membrane proteins.

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Manuscript 1

Cross-linking analysis of outer membrane proteins of *Neisseria gonorrhoeae*

ABSTRACT

The arrangement of proteins in the outer membrane of Neisseria gonorrhoeae was investigated through the use of chemical cross-linkers and two-dimensional sodium dodecyl sulfate - polyacrylamide gel electrophoresis. Cross-linked isolated outer membranes yielded dimers and trimers of the major outer membrane protein. Also, data were obtained suggesting a stable interaction between the major protein and protein II, the second most prevalent protein in the gonococcal outer membrane.

INTRODUCTION

The outer membrane of Neisseria gonorrhoeae appears to be typical of gram-negative bacteria. In contrast to the cytoplasmic membrane, it contains relatively few proteins, with one or two proteins predominating (8, 9). The major outer membrane protein (MOMP) (9) has also been referred to as the principal outer membrane protein (POMP) (2) or protein I (6). The apparent molecular weight of this protein is strain specific (9), and variations in molecular weight have been correlated with antigenic differences (7, 9). The other predominant protein has a lower apparent molecular weight and has been termed the covariant protein (9) or protein II (6); in some gonococcal strains this protein exhibits heat-modifiable behavior (6, 22).

Much work has been done on the qualitative and quantitative analysis of gonococcal outer membrane proteins, but as yet no one has investigated the architecture of these proteins in the membrane matrix. Introduction of chemical cross-linking for nearest-neighbor analysis has facilitated exploration of outer membrane protein arrangement. Cleavable cross-linking reagents, used in conjunction with two-dimensional sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), have provided much information on the arrangement of proteins in the outer membrane of Escherichia coli (3, 13-16). The predominant outer membrane protein of E. coli appears to be preferentially cross-linked to itself, with formation of dimers, trimers, and low yields of higher multimers (13-16). Accumulating evidence supports the hypothesis that protein I occurs as a trimer in the membrane of E. coli (14, 16, 17, 19).

The technique of chemical cross-linking was applied to isolated outer membranes of different strains and colony types of N. gonorrhoeae. The data obtained show both similarities and differences in the arrangement of outer membrane proteins of N. gonorrhoeae and E. coli.

MATERIALS AND METHODS

Organisms. N. gonorrhoeae strains F62 and 1362 were used in these studies. Strain F62 was obtained from the Neisseria Reference Laboratory, Public Health Service Hospital, Seattle, Wash. strain 1362 was a clinical isolate obtained from the Multnomah County

Health Department, Portland, Ore. The identity of both organisms was confirmed by cell morphology in gram-stained smears, oxidase reaction, and the production of acid from glucose, but not from maltose, sucrose, or fructose. Stock cultures were suspended in skim milk, frozen in a dry ice-acetone mixture, and stored at -70° C. Colony types (10) were selected, subcultured, and maintained in a 4% CO_2 incubator on GC agar (Difco) plates.

Medium and growth conditions. The basal medium contained the following per liter of distilled water: proteose peptone no. 3 (Difco), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; and soluble starch, 1 g. The final pH of the medium was 7.2. A growth factor supplement similar to IsoVitaleX (BBL) but lacking glucose, $NaHCO_3$ (420 mg/liter), and glucose (5 g/liter) were added after autoclaving. In some cases, 0.5% yeast extract was used in place of the defined growth factor supplement. For growth of large quantities of piliated colony types, 20 mM glutamate was added to the basal medium.

Inocula were prepared by suspending growth from a GC agar plate in 25 ml of basal medium. This suspension was incubated at 37° C in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) for 6-8 h. The entire suspension was then added to 1300 ml of prewarmed medium and incubated at 37° C in a gyratory shaker overnight.

Chemicals. Sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, β-mercaptoethanol, and Coomassie Blue R-250 were purchased from Bio-Rad Laboratories, Richmond, Calif. Dimethyl

adipimidate (DMA; 9 Å), dimethyl suberimidate (DMS; 11 Å), dimethyl 3,3'-dithiobispropionimidate (DTBP; 12 Å), dithiobis (succinimidyl propionate) (Lomant's reagent, DTSP; 11 Å), 1,5-difluoro-2,4-dinitrobenzene (FFDNB; 5 Å), and 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone (FFDS; 9 Å) were purchased from Pierce Chemical Co., Rockford, Ill. All other chemicals were of reagent grade.

Outer membrane preparation. Outer membranes were prepared by a modification of the method of Frasch and Gotschlich (5). Cultures (7.8 liters) were harvested by centrifugation at 4° C (10,000 x g, 10 min). The overnight cultures were also analyzed for colony type composition at this time by inoculation of a GC agar plate. Cultures of piliated colony types consisted of greater than 75% T1 or T2 colony types. The pellet obtained by centrifugation was resuspended in 0.15 M NaCl and placed in tared centrifuge tubes. After centrifugation (12,000 x g, 10 min), the cell pellet was resuspended in 1 M LiCl (20 ml/g wet weight of cells) and extracted by shaking with glass beads for 2 h at room temperature on a reciprocal shaker. The extracted cell suspension was decanted and centrifuged at 12,000 x g for 10 min at 4° C. The resulting supernatant was centrifuged at 30,000 x g for 45 min at 4° C. The 30,000 x g supernatant was then centrifuged at 85,000 x g for 2.5 h at 4° C in a Beckman L5-65 ultracentrifuge (type 30 rotor). The resulting pellet was washed twice with 0.15 M NaCl and centrifuged (12,000 x g, 5 min). The supernatant was carefully removed and stored at 4° C.

Cross-linking of isolated outer membranes. DMA, DMS, and DTBP were dissolved in buffer [0.5 M triethanolamine (TEA), pH 8.5, containing 0.05 M $MgCl_2$] at a concentration of 8 mg/ml. Outer membrane preparations (1-2 mg protein) were diluted in the same buffer to a volume of 0.5 ml. The solutions were kept on ice and the cross-linking reaction was initiated with addition of 0.5 ml cross-linker solution to the membrane suspension. For cross-linking with FFDNB, FFDS, and DTSP, the membranes (1-2 mg protein) were diluted to a volume of 0.99 ml in cold 0.2 M TEA, pH 8.5, containing 0.02 M $MgCl_2$. FFDNB and FFDS were dissolved in acetone at a concentration of 200 mg/ml. DTSP was dissolved in acetone, dioxane, or dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml. Cross-linking reactions were initiated by the addition of 10 µl cross-linker.

Cross-linking reactions were allowed to proceed for 15 min at 0° C, with occasional shaking of the solution. The reaction mixture was then diluted with 20-30 volumes 0.15 M NaCl (pH<7.0) and centrifuged at 85,000 x g for 2.5 h at 4° C (type 30 rotor). The pellet was resuspended in a small volume of 0.15 M NaCl and stored at 4° C.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE of gonococcal outer membrane preparations was done according to the method of Laemmli (11). Samples (80-200 µg protein) were prepared in buffer [10% glycerol, 3% (w/v) SDS, 62.5 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 6.8] and heated at 100° C for 45 seconds prior to electrophoresis. In some experiments, non-cross-linked samples were heated in the same buffer in the presence of 5% (v/v)

β-mercaptoethanol (β-ME) for 5 min at 100° C. One microliter tracking dye (0.05% bromophenol blue) was added to the sample before electrophoresis.

One dimensional cylindrical gels (ca. 70 mm in length) contained 8.75% or 10% acrylamide. Stacking and separating gels were formulated according to Laemmli (11) except 0.1% (v/v) TEMED was used for polymerization of the stacking gel. Samples were electrophoresed at 1 mA/gel through the stacking gel, then at 2 mA/gel through the separating gel. Running time through the cylindrical gels was approximately 3 h. After electrophoresis of preparations treated with cleavable cross-linking reagents, the gels were removed and sliced in half lengthwise. One half was heated at 100° C for 30 min in buffer [62.5 mM Tris-HCl, pH 6.8, containing 3% (w/v) SDS and 5% (v/v) β-ME]. The other half was stained in 0.25% (w/v) Coomassie blue in methanol:acetic acid:H_2O (45:9:45), as were whole gels of preparations treated with non-cleavable cross-linkers.

The slab gel apparatus used was purchased from Pharmacia Inc., Piscataway, N.J. The gel slabs were 3 mm thick, 73 mm wide, and approximately 65 mm in length. A 5-10 mm stacking gel was added before electrophoresis. The separating gels were made as above, except 0.04% (v/v) β-ME was added. The stacking gel was either made as above or consisted of 1% (w/v) agarose (Calbiochem, San Diego, Calif.) and 1% (v/v) β-ME in 0.125 M Tris-HCl (pH 6.8) containing 0.1% (w/v) SDS. The half-cylindrical gels which had been exposed to β-ME were rinsed several times with water and placed on top of the slab gels.

The rods were covered with the agarose-β-ME buffer solution, and a small amount of tracking dye was placed on the bottom end of the rod with a tuberculin syringe. Samples were electrophoresed at 10 mA/slab through the stacking gel and at 20 mA/slab through the separating gel. Running time was 3-3.5 h. The slabs were removed and sometimes were soaked overnight in a prestain solution consisting of isopropanol: acetic acid:H_2O (25:10:65). The slabs were stained as described above. Both slabs and rods were destained in acetic acid:methanol:H_2O (7.5:5.0:87.5) at 60° C in the presence of BioRex RG501-X8 resin (Bio-Rad Laboratories). Destained gels were stored in a 0.02% (w/v) solution of sodium azide. Some slab gels were subsequently dried.

Protein determination. Protein concentration was determined by the method of Bradford (1), modified by using a commercial reagent (Bio-Rad Laboratories). Bovine plasma gamma globulin (Bio-Rad Laboratories) or bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as the standard.

RESULTS

Cross-linking with non-cleavable reagents. Outer membrane preparations from N. gonorrhoeae strain F62 colony type 4 were cross-linked with various reagents and analyzed on one-dimensional SDS-polyacrylamide gels. Figure 1 shows that FFDS-treated membranes appear identical to the control F62 (T4) membranes. DMA- and DMS-treated membranes seem to yield very similar gel patterns;

however, both are different from the F62 (T4) control. The band corresponding to protein II (32,000 daltons) in the non-cross-linked control is greatly decreased in DMA- and DMS-treated membranes. Cross-linked preparations exhibited new bands corresponding to molecular weights of 63,000, 107,000, 114,000, and 148,000. In addition, a heavily stained area is seen at the top of both gels.

DMS and DTBP have the same reactive end groups and similar bridge lengths, yet DTBP-treated membrane preparations show dissimilar SDS-PAGE patterns. New bands with apparent molecular weights of 63,000, 93,000, 142,000, and 147,000 appear when outer membranes are treated with DTBP. Less high molecular weight material was observed at the top of the gel.

No differences in SDS-PAGE bands were observed when DTBP- and DTSP-treated outer membranes were compared. However, treatment of outer membranes with FFDNB resulted in a smeared gel pattern suggestive of cross-linking (high molecular weight material at the top of the gel and a darkened area at ca. 60,000 daltons). Cross-linking apparently occurred with several of the reagents tested, but a definitive determination of the components of the new high molecular weight complexes cannot be made from analysis of one-dimensional gels only.

Two-dimensional analysis of DTSP-treated F62 outer membranes. *N. gonorrhoeae* strain F62 (T4) outer membranes were cross-linked with DTBP and DTSP and analyzed by two-dimensional SDS-PAGE as described. Similar two-dimensional gel patterns were observed with

both reagents (data not shown). DTSP can be dissolved in a variety of polar organic solvents. This reagent was dissolved in three different solvents to compare their effects on cross-linking of gonococcal outer membranes. Figure 2 shows the results obtained from such an experiment. DTSP dissolved in acetone produced off-diagonal spots corresponding to dimer and trimer complexes of protein I (MOMP). An off-diagonal spot corresponding to protein II was also observed. However, it cannot be determined on this particular gel if this spot arose from a complex of protein I-protein II or protein II-protein II. Acetone- and dioxane-dissolved DTSP produced similar patterns, except for the indication of multimers of protein I above the level of trimer with acetone-dissolved DTSP. DTSP dissolved in DMSO produced no off-diagonal spots, and the corresponding one-dimensional gel suggested that some proteins had been lost in the presence of this solvent (61,000 and 37,500 dalton proteins) when compared to one-dimensional patterns produced with acetone or dioxane. Acetone was selected as the solvent of choice for further experiments.

The finding that protein II may be cross-linked to protein I was surprising, since cross-linking data from E. coli only gave evidence of homopolymers of the principal outer membrane protein (13-16). To determine whether this phenomenon was a general property of gonococcal outer membranes, both virulent and avirulent colony types of two different strains were examined.

N. gonorrhoeae strain F62 (T2) outer membranes were treated with DTSP. The results were identical to those seen with outer

membranes isolated from T4 cells (Fig. 3). Multimers of protein I were observed at the level of dimer and trimer, and protein II appeared to form dimers either of protein I-protein II or protein II-protein II. These results suggest that the observed cross-linking pattern does not differ among colony types.

Two-dimensional analysis of DTSP-treated strain 1362 outer membranes. Cross-linked outer membranes from N. gonorrhoeae strain 1362 (T4) exhibited an identical pattern in two-dimensional SDS-PAGE as cross-linked membranes from strain F62 (Fig. 4). However, with strain 1362 the off-diagonal spots at the level of dimer are quite distinct and appear to be aligned vertically, whereas with cross-linked F62 outer membranes the spots were not as definitely aligned. This observation suggests that protein II is linked to a subunit of protein I, instead of forming a homopolymer of protein II. In both strains the off-diagonal spot at the level of dimer corresponding to protein I was darker than the spot corresponding to protein II, suggesting that protein I may be linked to either protein II or to more protein I.

Cross-linked outer membranes from type 1 cells of strain 1362 resembled those from type 4 cells except for the appearance of off-diagonal spots of lower molecular weight (Fig. 5). These spots were not seen on the control slab, and seemed to arise from cross-linking of components in the 14-19,000 molecular weight range.

DISCUSSION

Isolated gonococcal outer membranes can be effectively cross-linked with a variety of bifunctional reagents. It was interesting that FFDS did not appear to cross-link proteins in the outer membrane while FFDNB, a cross-linking reagent with a shorter bridge length, did. These reagents react preferentially with epsilon amino groups of lysine residues and/or phenolic groups of tyrosine residues, but also react with sulfhydryl and imidazole groups (24). Perhaps the conformation or availability of these outer membrane protein residues was such that FFDS could not react. Since FFDNB produced smeared rather than distinct stained bands, it could not be determined whether this reagent cross-linked proteins more frequently than it modified them by monofunctional substitution or intramolecular cross-linking (23).

DMA- and DMS-treatment produced similar gel patterns, even though the bridge lengths of these reagents are different (9 vs. 11 Å). In contrast, DMS and DTBP have more similar bridge lengths (11 and 12 Å), yet show more differences in SDS-PAGE cross-linking patterns than DMA and DMS. All three chemicals have the same reactive end groups; however, DTBP differs from DMA and DMS in that it contains a cleavable disulfide bond. One possible explanation of the differences observed is disulfide interchange. However, Wang and Richards (23) found no detectable differences in DTBP cross-linking of N-ethylmaleimide-treated or -untreated erythrocyte ghosts,

suggesting that most if not all cross-linking was due to reaction of DTBP with free amino groups of proteins.

In strain F62, protein I has an apparent molecular weight of 34,000 daltons and protein II one of 32,000 daltons. Dimers of I - I and II - II would only have an apparent molecular weight difference of 4,000 (68,000 vs 64,000 daltons), with a mixed dimer of I - II having an intermediate molecular weight. It would be difficult to determine the components of new high molecular weight complexes solely on the basis of calculated molecular weights. The apparent molecular weights of the new bands appearing after cross-linking do not seem to be integral multiples of the molecular weights of protein I or protein II. It has been suggested, however, that cross-linked complexes may not fit the molecular weight-mobility relationship determined from non-cross-linked proteins (23), perhaps due to an alteration of the Stokes radius or a change in SDS-binding to modified polypeptides (4, 18). Such problems demonstrate why cleavable cross-linking reagents used in conjunction with two-dimensional SDS-PAGE analysis can be used for resolving the association of membrane proteins.

Although DTBP- and DTSP-treated gonococcal outer membranes produced similar gel patterns, DTSP was chosen for further study because of its availability and because it produced clearer off-diagonal spots. Multimers of protein I (MOMP) were observed at the level of dimer and trimer. This is in agreement with cross-linking data obtained with E. coli. Palva and Randall (14) and Reithmeier and Bragg (16) found dimers and trimers of protein I in E. coli when cell walls, isolated

outer membranes, or protein-peptidoglycan complexes were treated with a variety of bifunctional reagents. Reithmeier and Bragg (16) also demonstrated cross-linking of protein B (protein II) and protein F (free form of the lipoprotein). Protein II could be cross-linked into very high molecular weight complexes, while protein F was cross-linked to a dimer and possibly a trimer form. Both of these proteins appeared to be situated such that they could be cross-linked to peptidoglycan. Endermann and Henning (3) also found that protein II and lipoprotein were cross-linked to the peptidoglycan layer of E. coli. Palva and Randall (14) and Endermann and Henning (3), however, showed no evidence of cross-linking of protein II into higher multimers.

A surprising finding was the indication that protein I was cross-linked to protein II in gonococcal outer membranes. This association appears to exist only at the level of dimer and not at the level of trimer. After mercaptan cleavage, analysis of the second-dimensional slabs suggests that the off-diagonal spots corresponding to protein I and protein II at the level of dimer are on a vertical line. Reithmeier and Bragg (16) presented evidence that dimers of protein I and dimers of protein II exhibit a definite diagonal skewing of off-diagonal spots. In some of our slabs the off-diagonal spots are somewhat elongated from left to right, making interpretation a bit more difficult, but we believe that these spots arise from a cross-linked complex containing one molecule of protein I and one molecule of protein II. The off-diagonal spot at the level of dimer corresponding to protein II is always less intense than

that corresponding to protein I. This would allow one to hypothesize interaction of protein I with either molecules of protein I or molecules of protein II. No off-diagonal spots corresponding to protein II are seen at the level of trimer. Perhaps the quantity of protein II linked at this level is below detectable limits, or possibly cross-linking of protein I to the trimer level favors the close association of three identical molecules, with the exclusion of protein II from such a complex.

N. gonorrhoeae does not possess a lipoprotein similar to that found in E. coli (25). Therefore, it should not be surprising to find a different arrangement of outer membrane proteins. Another remarkable result was the evidence of cross-linking of lower molecular weight components in strain 1362 (T1). The arrangement of off-diagonal spots would indicate that a heteropolymer had been formed from two lower molecular weight proteins. More strains will have to be examined to determine if this is a strain-related phenomenon.

Rosenbusch (17) and Steven et al. (19) postulated that protein I of E. coli was arranged in a hexagonal lattice, with each morphological unit containing three molecules of protein I (19). Cross-linking data obtained with E. coli provided further evidence for this arrangement (14, 16). Some investigators have shown a hexagonal arrangement of proteins in the outer membrane of N. gonorrhoeae by electron microscopy (12, 20, 21). Data obtained with cross-linked gonococcal outer membranes are compatible with a similar arrangement since protein I could be cross-linked to a trimer. The arrangement

of gonococcal outer membrane proteins appears to differ from that of E. coli in that protein I was also found in association with protein II. Further studies may show the hexagonal arrangement of the major outer membrane protein to be a general characteristic of all gram-negative bacteria.

The observation that protein I can be cross-linked to protein II in N. gonorrhoeae indicates that, at least in certain regions of the outer membrane, protein I and protein II are found in close association. This is compatible with the findings that 1) upon extraction of the serotyping antigen both proteins are removed (9), and 2) protein I and protein II are separated from each other during purification attempts only with fairly harsh treatments (6 M guanidine thiocyanate) (6). Heckels (6) showed via fluorescent labeling of intact gonococci that these two major proteins are located at the cell surface. Gonococcal outer membrane proteins have been mentioned as possible immunoprophylactic agents, and since protein I and protein II are exposed at the surface of the gonococcus, the association of these proteins may be important in antigenic presentation and interaction with host.

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Figure 1. One-dimensional SDS-PAGE analysis of N. gonorrhoeae strain F62 (T4) outer membranes treated with various cross-linking reagents. A, control; B, DMA; C, DMS; D, DTBP; E, DTSP; F, FFDNB; and G, FFDS.

A B C D E F G

Figure 2. Effect of solvent on cross-linking of N. gonorrhoeae strain F62 (T4) outer membranes. A. Control; B. acetone; C. dioxane; D. dimethyl sulfoxide.

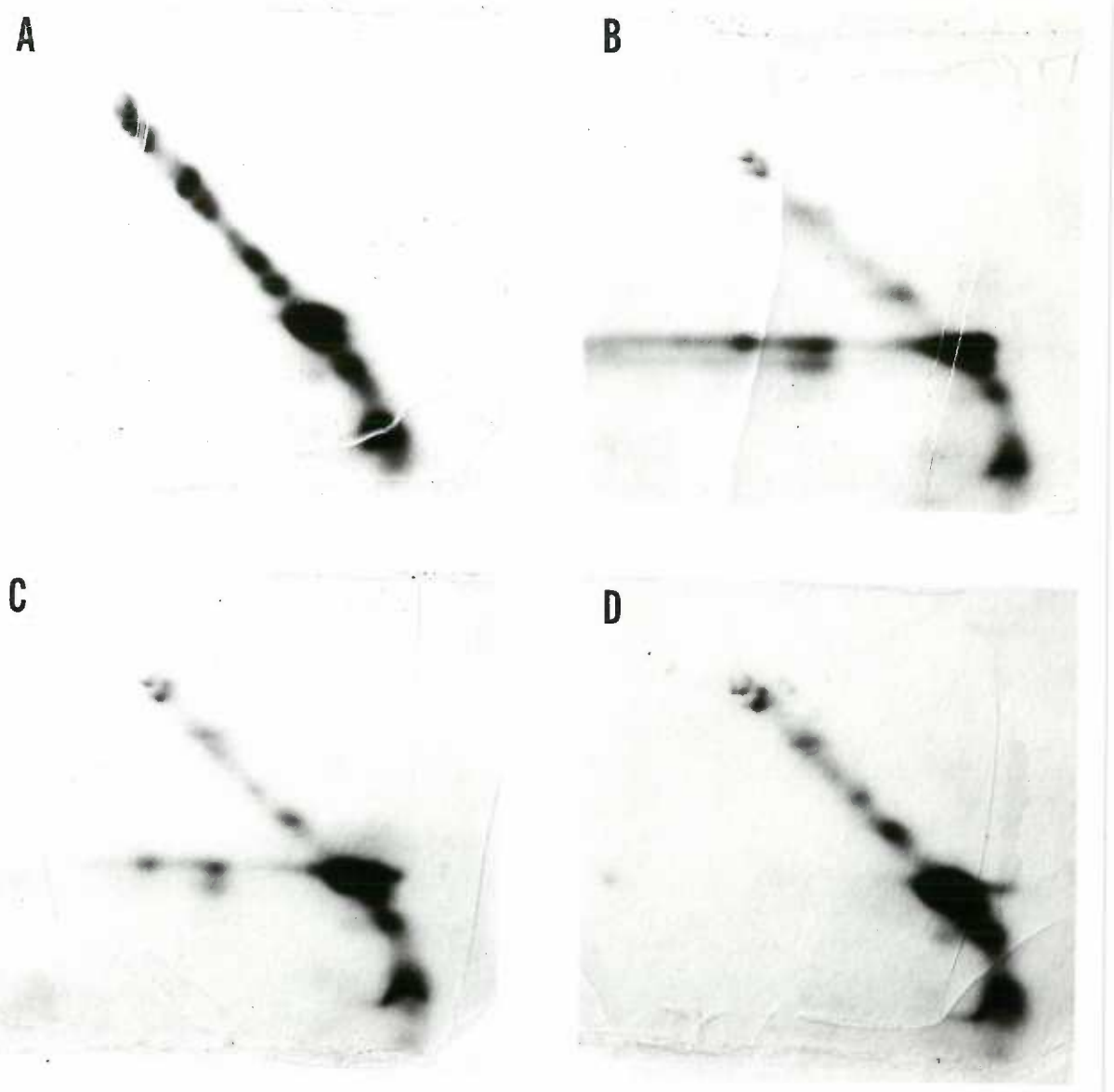

A
B
C
D

Figure 3. DTSP-treated outer membranes of N. gonorrhoeae strain F62 (T2).

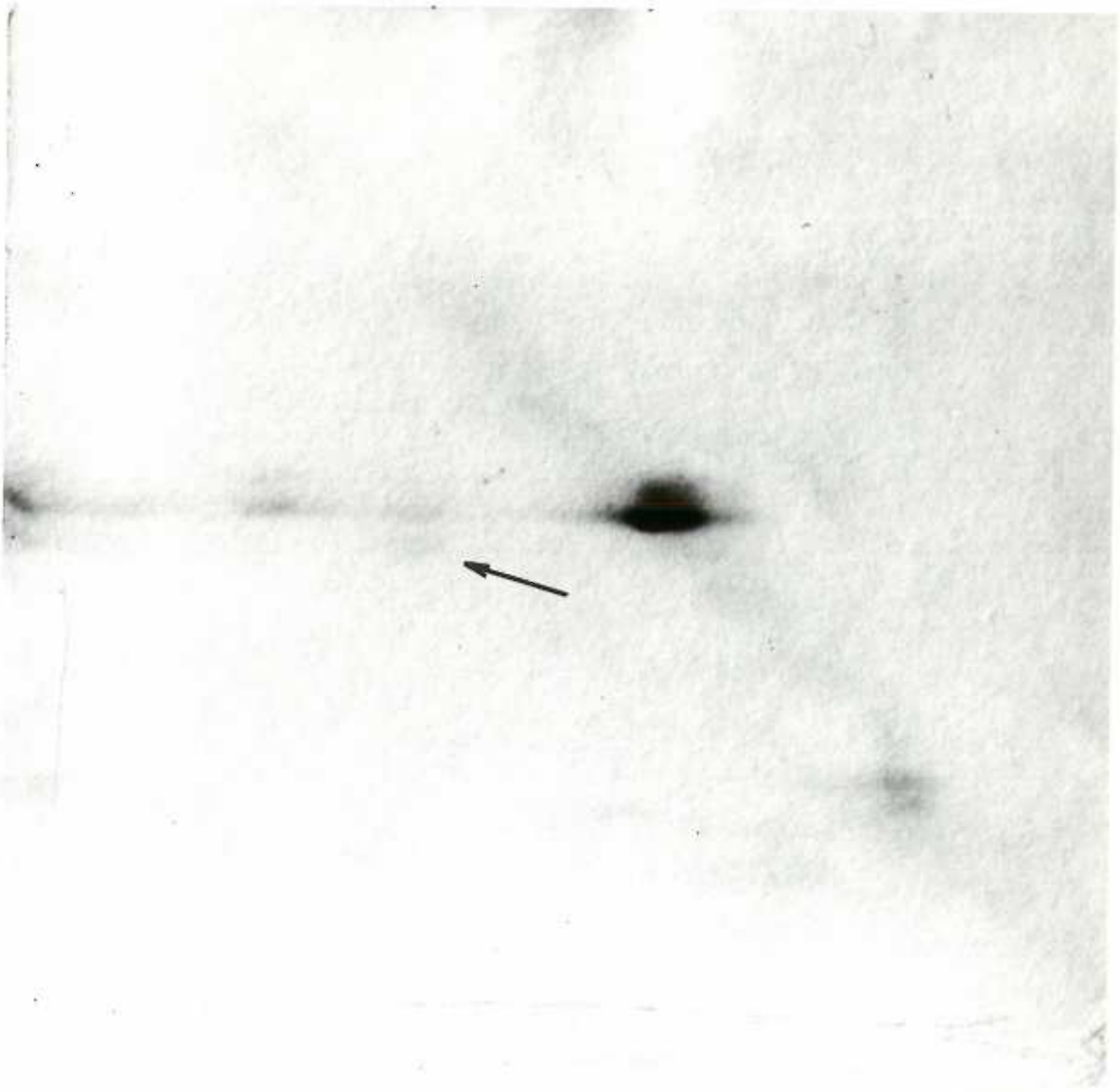
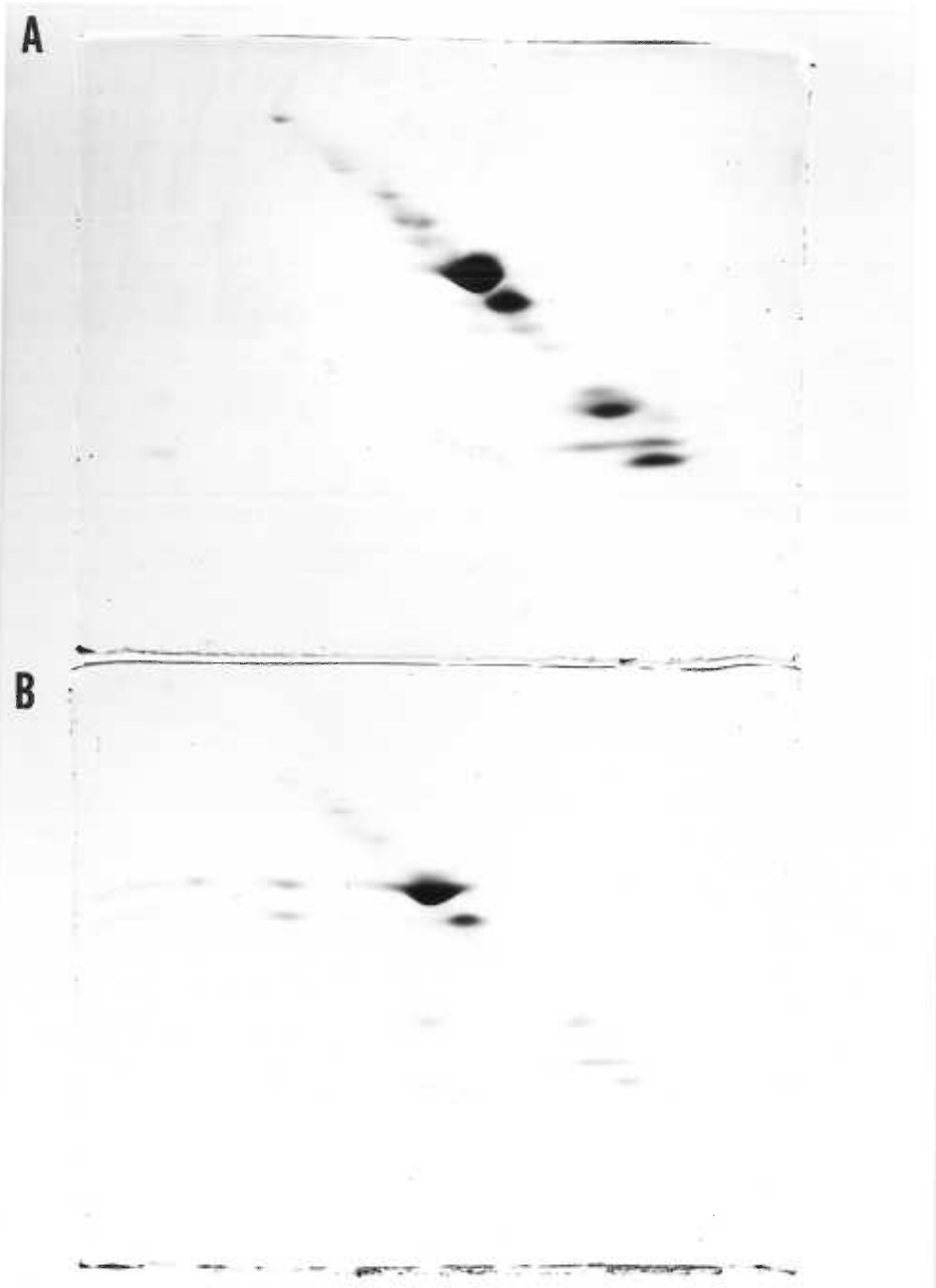


Figure 4. Control (A) and DTSP-treated (B) outer membranes of N. gonorrhoeae strain 1362 (T4).


A
B

Figure 5. Control (A) and DTSP-treated (B) outer membranes of N. gonorrhoeae strain 1362 (T1).


A
B

Manuscript 2

Cell envelope of *Neisseria gonorrhoeae*: Role of divalent cations in stabilization against autolysis

ABSTRACT

Neisseria gonorrhoeae strain JW-31 exhibits a high rate of autolysis when suspended in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer at pH 8.5. Autolysis was suppressed at pH 6.0 or by the addition of divalent cations (Mg^{2+}, Ca^{2+}). Peptidoglycan hydrolysis and viability were similar in the presence or absence of Mg^{2+}. Cells were labeled during growth in medium containing either [8-^{3}H]-adenine or [2,3-^{3}H]-arginine. At pH 8.5, strain JW-31 released a considerable amount of ^{3}H-adenine-labeled material. Divalent cations or an acid pH abrogated this release. Similarly, divalent cations or an acid pH reduced the release of both ^{3}H-adenine- and ^{3}H-arginine-labeled material from strain JW-31 when suspended in HEPES buffer containing 20% sucrose, as compared to material released at pH 8.5 in the presence of sucrose. Coelectrophoresis of outer membranes labeled during growth with [U-^{14}C]-lysine and [4,5-^{3}H(N)]-lysine-labeled material released by cells suspended in buffer indicated that at an acid pH or in the presence of divalent cations much of the material released was outer membrane and/or periplasmic protein. This evidence suggests that at pH 6.0 or in the presence of divalent cations autolysis is prevented by stabilization of the cytoplasmic membrane.

INTRODUCTION

Neisseria gonorrhoeae autolyzes rapidly when suspended in buffer at an alkaline pH (10). Previous studies on this phenomenon determined that divalent cations (4, 5, 19), acid pH (4, 19), low temperature (4, 5), and hypertonic environments (4, 5) protected nongrowing gonococci. Wegener et al. (19) observed that there was no correlation between peptidoglycan (PG) hydrolysis and the rate of autolysis in N. gonorrhoeae. Later, Cacciapuoti et al. (3) showed that phospholipid hydrolysis occurred in the absence of cellular lysis in both an autolytic and non-autolytic strain of N. gonorrhoeae. Divalent cations, which inhibit autolysis, enhanced hydrolysis of endogenous phospholipids. Thus, differences in PG and phospholipid hydrolysis cannot explain the protective effect of divalent cations.

Investigations into the lytic behavior of gonococci suspended in buffer have thus far examined changes in culture turbidity. To better understand the mechanism(s) of stabilization by divalent cations and an acid pH, we radiolabeled cellular components and determined their fate when cells were suspended in a stabilizing or autolytic environment. The data obtained suggest that divalent cations and an acid pH stabilize the cytoplasmic membrane of N. gonorrhoeae.

MATERIALS AND METHODS

Organisms. N. gonorrhoeae strain JW-31 was used in these studies. The properties and maintenance of this strain have been previously described (12, 13).

Medium and growth conditions. The basal medium contained the following per liter of distilled water: proteose peptone no. 3 (Difco), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; and soluble starch, 1 g. The final pH of the medium was 7.2. A growth factor supplement similar to IsoVitaleX (BBL) but lacking glucose, $NaHCO_3$ (420 mg/liter), and glucose (5 g/liter) were added after autoclaving. For some experiments a chemically defined medium (14) containing 10 µg/ml lysine was used.

Inocula were prepared by suspending the growth from GC agar (Difco) plates in basal medium and inoculating 300 ml of fresh basal medium as previously described (11). Inocula for experiments utilizing defined medium were prepared by suspending the growth from chemically defined medium-agar plates in a solution of the medium salts, and 300 ml of defined medium [0.5% (w/v) inoculum] were inoculated to an initial turbidity of 35-40 Klett units. Cultures were incubated at 37° C in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Turbidity was measured by Klett-Summerson colorimetry, filter no. 54 (540 nm).

Chemicals and radioisotopes. All chemicals were of reagent grade. The following isotopes were obtained from New England Nuclear

Corp., Boston,Mass.: [8-^{3}H]-adenine (specific activity, 18.9 Ci/mmol); [2,3-^{3}H]-L-arginine (specific activity, 21.5 Ci/mmol); [^{14}C(U)]-L-lysine (specific activity, 285 mCi/mmol); [4,5-^{3}H(N)]-L-lysine (specific activity, 72.13 Ci/mmol).

Release of ^{3}H-adenine-labeled material from cell suspensions. N. gonorrhoeae strain JW-31 was grown for 2 generations in basal medium containing 2 µCi [8-^{3}H]-adenine per ml of medium. Cells were harvested, resuspended in fresh medium lacking [8-^{3}H]-adenine, and incubated for 20 min at 37° C. Cells were then harvested and resuspended in 50 mM HEPES buffer (pH 8.5 or 6.0), with or without 20 mM $MgCl_2$. The cell suspensions were incubated at 37° C and at intervals 5 ml aliquots were removed, centrifuged (4100 x g, 5 min), and the supernatants placed at 4° C. Radioactivity was determined from 0.2 ml aliquots of the supernatants. To determine the activity associated with cellular nucleic acid material, triplicate 1 ml aliquots of the supernatants were added to tubes containing 1 ml 20% trichloroacetic acid, incubated at 4° C for 30-60 min, and filtered onto Whatman GF/C filter discs. Filters were processed and radioactivity determined as previously described (19).

Release of material from cells suspended in 20% sucrose. Cultures were grown for 2 generations in basal medium containing [8-^{3}H]-adenine (4 µCi/ml of medium) or [2,3-^{3}H]-arginine (2 µCi/ml of medium). The cells were harvested and resuspended in 50 mM HEPES buffer (pH 8.5 or 6.0), containing 20% sucrose and additions as

indicated. At zero time and after incubation for 60 min, 2 ml aliquots of the cell suspension were centrifuged and the release of ^{3}H into the supernatant was determined. The data are expressed as the difference between the percent ^{3}H released at 0 and at 60 min.

Coelectrophoresis of ^{14}C-lysine-labeled outer membrane proteins and ^{3}H-lysine-labeled released material. Strain JW-31 was grown to late exponential phase (140-160 Klett units) in a chemically defined medium containing either [$^{14}C(U)$]-lysine (0.1 μCi/ml of medium) or [4,5-$^{3}H(N)$]-lysine (1.3 μCi/ml of medium). The ^{3}H-labeled culture was centrifuged (4100 x g, 5 min) and resuspended to one-half its original volume in 50 mM HEPES buffer containing 20% sucrose (pH 8.5) with or without 20 mM $MgCl_2$ or $CaCl_2$, or in 50 mM HEPES buffer containing 20% sucrose (pH 6.0). The suspensions were incubated at 37° C for 1 hr, then centrifuged (4100 x g, 5 min). The supernatants were decanted, dialyzed against distilled water, and lyophilized. The lyophilized material was weighed and resuspended in a small volume (500-750 μl) of 0.15 M NaCl. The ^{14}C-labeled culture was centrifuged at 4° C (10,000 x g, 10 min) and washed once in 0.15 M NaCl. The washed cell suspension was centrifuged (12,000 x g, 10 min, 4° C) in tared centrifuge tubes, and the pellets resuspended in 1 M LiCl (20 ml/g wet weight of cells). Glass beads (5 mm diameter, 120-150 per 1-liter flask) were added and the suspension was shaken on a reciprocal shaker at room temperature for 2 hr. The suspension was then decanted and centrifuged (12,000 x g, 10 min at 4° C). The resulting supernatant was centrifuged at 31,000 x g for 45 min at 4° C. The

supernatant was decanted and subjected to ultracentrifugation (85,000 x g, 2.5 hr, 4° C). The 85,000 x g pellet was washed twice with 0.15 M NaCl and resuspended in a small volume of 0.15 M saline. After centrifugation (3,000 x g, 10 min, 4° C) the supernatant was carefully removed and stored at 4° C.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Weber and Osborn (18). Preparations of outer membrane or material released in buffer in the presence of 20% sucrose were diluted in 0.01 M sodium phosphate buffer (pH 7.0), containing 3% (w/v) SDS and 2% (v/v) β-ME. Equal volumes of radiolabeled preparations were used in sample preparation for double-label gels. Samples were heated at 100° C for 5 min and 2 µl 0.05% bromophenol blue and 2 drops of glycerol were added. The polyacrylamide gels had a final concentration of 7.5% acrylamide. On some gels a stacking gel was applied, which consisted of 5 ml 0.07 M sodium phosphate buffer (pH 6.0), containing 0.2% (w/v) SDS, 4.5 ml of a stock solution of acrylamide to a final concentration of 3.3%, 0.5 ml ammonium persulfate (3 mg/ml), and 7-10 µl N,N,N',N'-tetramethylethylenediamine (TEMED). Samples were layered on the gels and carefully overlaid with gel buffer (0.1 M sodium phosphate, pH 7.2). Gels were electrophoresed at 8 mA/gel. Radiolabeled gels were frozen at -20° C before slicing. Slices (1 mm) were placed in scintillation vials and solubilized in 0.15 ml 30% H_2O_2 at 50° C. After cooling, 5 ml of Aquasol (New England Nuclear) were added and the vials counted

in a Beckman L5-345 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.).

Estimation of ^{3}H and ^{14}C counts in slices of double-labeled gels was done according to Freifelder (6). The blank contained 0.15 ml H_2O_2 and one slice of non-radiolabeled gel in 5.0 ml Aquasol. The ^{14}C and ^{3}H standards contained one slice of non-radiolabeled gel, 0.15 ml H_2O_2, and 100 µl ^{14}C-toluene (4.15×10^5 dpm/ml) or ^{3}H-toluene (2.20×10^6 dpm/ml), respectively, in 5.0 ml Aquasol. The counting efficiencies for ^{14}C and ^{3}H were 66% and 25%, respectively. The overlap of ^{3}H counts into the ^{14}C channel was less than 0.1%. The overlap of ^{14}C counts into the ^{3}H channel was 40%.

Gels containing unlabeled material were stained for 2 h with Coomassie blue (18) and destained in H_2O:methanol:acetic acid (87.5:5:7.5) at 60° C in the presence of BioRex RG501-X8 resin (Bio-Rad Laboratories, Richmond, Calif.). The destained gels were stored in a solution of 0.02% (w/v) sodium azide.

Miscellaneous determinations. Protein concentrations were determined by the method of Bradford (2), modified by using a commercial reagent (Bio-Rad Laboratories). Bovine plasma gamma globulin (Bio-Rad Laboratories) was used as the standard. Total lipid phosphorus was measured by the procedure of Ames and Dubin (1). 2-Keto-3-deoxyoctonate (KDO) was determined by a modification (16) of the thiobarbituric acid method of Weissbach and Hurwitz (20).

RESULTS

Effect of pH and Mg^{2+} on autolysis. Wegener et al. (19) have shown that incubation in buffer at an acid pH (pH 6.0) or in the presence of divalent cations suppressed autolysis of N. gonorrhoeae strain JW-31, while incubation in buffer at an alkaline pH (pH 8.5) resulted in high rates of autolysis. Table 1 compares the rates of autolysis, peptidoglycan (PG) hydrolysis, and viability of strain JW-31 when suspended in 50 mM HEPES buffer at pH 6.0 or 8.5, with or without added Mg^{2+}. Peptidoglycan hydrolysis was similar and viability was greatly reduced under all conditions. There was no apparent correlation with rate of autolysis.

Strain JW-31 was grown in liquid medium containing [8-^{3}H]-adenine in order to label the nucleic acids. Previous studies (14) ascertained that ca. 90% of the incorporated ^{3}H-adenine was associated with the nucleic acid-containing cell fraction, whereas the remainder was associated with soluble cell pool. Labeled exponential phase cells were harvested and resuspended under the conditions listed in Table 1. Samples were removed at 30 min intervals over a 90 min period and were assayed for release of total label and labeled trichloroacetic (TCA)-precipitable material. The results (Fig. 1A) show that autolyzing cells (HEPES buffer, pH 8.5) release 71% of their initial activity over a 90 minute period. Most of the material released was of large molecular weight and could be precipitated with TCA. The 71% decrease in cell-associated ^{3}H-activity is comparable to the 62% decrease in turbidity observed when cells were suspended under similar

conditions. Cells incubated in buffer at pH 6.0 or at pH 8.5 in the presence of Mg^{2+} did not release a significant amount of labeled adenine (Fig. 1B) and did not decrease in turbidity, suggesting that the cytoplasmic membrane was intact.

Release and characterization of material from cells incubated in 20% sucrose. As previously observed, conditions which reduce the rate of autolysis do not maintain viability. Thus, in the absence of cell lysis, some processes must be occurring which lead to cell death. To further investigate this phenomenon, gonococci were labeled with either ^{3}H-adenine or ^{3}H-arginine. The radiolabeled gonococci were suspended in HEPES buffer containing 20% sucrose to simulate the hypertonic *in vivo* environment.

Table 2 shows that cells incubated in 20% sucrose at pH 8.5 release a considerable amount of both cellular nucleic acid and protein. The addition of divalent cations prevented the loss of significant quantities of cellular nucleic acids. However, the presence of divalent cations did not prevent release of significant amounts of cellular protein, since *ca*. 20% of incorporated ^{3}H-arginine was released. Incubation in 20% sucrose at pH 6.0 prevented release of both protein and cellular nucleic acid.

The material released under the conditions described above was characterized as described in Materials and Methods. Table 3 shows that 90% of the cell mass was released during incubation in HEPES buffer (pH 8.5) containing 20% sucrose. In the presence of 20 mM Mg^{2+} or Ca^{2+}, cells released 18% and 17% of their original dry

weight, respectively. Only 14% of the original dry weight of cells was released during incubation in HEPES buffer (pH 6.0).

The released material was assayed for protein, 2-keto-3-deoxy-octonate (KDO), and total phosphate. The material released during incubation in HEPES buffer (pH 8.5) containing 20% sucrose, contained more than 15 times the amount of protein and 10 times the amount of KDO and phosphate as were released by cells incubated in the presence of divalent cations or at pH 6.0 (Table 3). The quantities of protein, KDO, and phosphate released by cells suspended at pH 6.0 or at pH 8.5 in the presence of Mg^{2+} or Ca^{2+} were similar. However, twice as much protein was released in the presence of Ca^{2+} as was released by cells at pH 6.0 or at pH 8.5 in the presence of Mg^{2+}.

SDS-PAGE of a mixture of ^{14}C-lysine-labeled outer membrane proteins and ^{3}H-lysine-labeled material released from strain JW-31. To determine whether proteins released by strain JW-31 suspended in HEPES buffer containing sucrose were similar to those present in the outer membrane, cells were labeled during growth with ^{3}H-lysine and suspended as described in Materials and Methods. The material released into the supernatant was dialyzed, lyophilized, resuspended in a small volume, and co-electrophoresed on SDS-polyacrylamide gels with a ^{14}C-lysine-labeled outer membrane preparation. The results are shown in Figure 2. Note that the scale for ^{3}H activity released at pH 8.5 is 20 times greater than the other ^{3}H scales. Cells incubated in HEPES buffer (pH 8.5) containing 20% sucrose released large amounts of ^{3}H-labeled material. The extensive release of ^{3}H-lysine

masked any correlation with the peaks of ^{14}C activity representing the outer membrane proteins. The ^{3}H profile of material released during incubation of cells in the presence of 20 mM Mg^{2+} corresponded closely to the ^{14}C- outer membrane protein profile. When cells were incubated in the presence of Ca^{2+} many peaks of ^{3}H activity were present, of which one or two appeared to correspond to outer membrane proteins. Small quantities of heterogeneous material were released during incubation at pH 6.0. Two peaks of ^{3}H-activity appeared to comigrate with ^{14}C- outer membrane proteins.

DISCUSSION

N. gonorrhoeae strain JW-31 autolyzes readily when suspended in HEPES buffer (pH 8.5). Autolysis is reduced in the presence of divalent cations or at an acid pH (6.0) (4, 5, 19). Peptidoglycan hydrolysis alone cannot explain these different rates of autolysis (19). Although an acid pH or divalent cations protect against autolysis, viability is greatly reduced, as might be expected in view of the extensive hydrolysis of PG. Therefore, conditions which stabilize cells against autolysis still permit PG hydrolysis and cell death.

Divalent cations and an acid pH may prevent autolysis by stabilizing the gonococcal outer and/or inner membrane. Elmros et al. (4) observed that 4 mM divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}) inhibited lysis in Tris(hydroxymethyl)aminomethane (Tris) - hydrochloride buffer (pH 7.2) at 22° C. Partial protection was provided by 0.5 mM Mg^{2+}, while 4 to 16 mM Mg^{2+} showed increasing stabilization of the

cells. Also, cells were increasingly more stable in four different buffers as the pH was lowered. These data coupled with the lysis of gonococci in EDTA without the addition of lysozyme led these researchers to suggest that the outer membrane rather than peptidoglycan was largely responsible for mechanical stability.

Guymon and Sparling (8) found that strains of N. gonorrhoeae which possess the nonspecific resistance locus ery exhibit reduced rates of autolysis when suspended in phosphate buffer at pH 7.0, and reduced sensitivity to high concentrations of lysozyme when Mg^{2+} was depleted. Mutation at the nonspecific drug hypersensitivity locus, env, led to more rapid autolysis of cells at pH 7.0 and increased sensitivity to lysozyme in the absence of Mg^{2+}. The addition of 3 mM Mg^{2+} to phosphate buffer (pH 7.0) was sufficient to prevent autolysis of env and wild type cells. It was suggested that an alteration in the cell envelope of env mutants resulted in decreased stability and increased permeability under adverse conditions, while an ery mutation produced increased stability and decreased permeability. Guymon et al. (9) later showed that certain antibiotic-resistant and -sensitive gonococcal mutants contained variable amounts of a 52,000 molecular weight outer membrane protein and exhibited variable degrees of peptidoglycan cross-linking. Thus, divalent cations can have a positive effect on cell stability and permeability of the gonococcal cell wall.

The outer membrane of N. gonorrhoeae contains phospholipase A which is stimulated by divalent cations and which exhibits optimal activity at pH 8.0 to 9.0 (17). Under these conditions, divalent

cations would appear to have a negative effect on the gonococcal outer membrane. However, these same conditions, when compared to an alkaline buffer lacking divalent cations, reduce autolysis and prevent the leakage of nucleic acids.

To simulate the hypertonic conditions present during a gonococcal infection, 20% sucrose was added to HEPES buffer. The release of ^{3}H-adenine- and ^{3}H-arginine-labeled material was measured to investigate the extent of loss of membrane integrity. It was assumed that release of ^{3}H-arginine-labeled material without concomitant release of ^{3}H-adenine-labeled material indicated loss of outer and/or periplasmic proteins, with retention of cytoplasmic membrane integrity. Cells incubated in HEPES buffer (pH 8.5) containing 20% sucrose released a large quantity of both ^{3}H-adenine- and ^{3}H-arginine-labeled material, suggesting that the gonococci were undergoing extensive lysis with accompanying release of cytoplasmic and structural components. The addition of Mg^{2+} or Ca^{2+} apparently stabilized the cytoplasmic membrane (decrease in release of ^{3}H-adenine-labeled material) and reduced the amount of ^{3}H-arginine-labeled material released from cells. Approximately 20% of the ^{3}H-arginine-labeled material was released without a concomitant decrease in turbidity. However, this loss may contribute to the decrease in viability observed under these "stabilized" conditions. Similarly, an acid pH prevents cytoplasmic membrane damage and significantly reduces loss of ^{3}H-arginine-labeled material. This correlates with the data presented in Table 1 showing that gonococci incubated in buffer at pH 6.0 retained their viability 8 times better than cells incubated in buffer at pH 8.5 containing

Mg^{2+}. Although divalent cations and an acid pH both appear to stabilize the cytoplasmic membrane, the data on maintenance of viability under these conditions suggest that different mechanisms are involved.

The material released during incubation in HEPES buffer was further characterized. The increase in protein released from cells incubated in the presence of Ca^{2+}, as compared to that released in the presence of Mg^{2+} or at pH 6.0, is probably a result of phospholipase A activation. Senff et al. (17) have shown that gonococcal phospholipase requires Ca^{2+} for activity. Cacciapuoti et al. (3) further demonstrated that Ca^{2+} stimulated the hydrolysis of phosphatidylethanolamine and phosphatidylglycerol when strain JW-31 was suspended in HEPES buffer (pH 8.5), while Mg^{2+} stimulated hydrolysis of phosphatidylethanolamine alone. The phospholipase could be releasing outer membrane and/or periplasmic proteins into the supernatant, thus accounting for the 2.0 to 2.5-fold increase in protein. When incubated in HEPES buffer (pH 8.5), strain JW-31 released a considerable amount of material. It is not surprising, therefore, that more protein, KDO, and phosphate are found in the supernatant. There was no preferential release of any of these compounds under any of the conditions tested.

The ^{3}H-labeled material released during incubation under various conditions and a ^{14}C-labeled outer membrane preparation were electrophoresed simultaneously to determine if any of the released protein corresponded to outer membrane proteins. If our hypothesis that divalent cations and an acid pH are stabilizing the cytoplasmic membrane was correct, the ^{3}H and ^{14}C SDS-polyacrylamide gel profiles

would be similar. This situation was observed when cells were incubated at pH 8.5 in the presence of Mg^{2+}. In the presence of Ca^{2+} many proteins other than outer membrane proteins were released. This would agree with the data presented in Table 3 showing that cells released 2.0 to 2.5 times as much protein in the presence of Ca^{2+} as in the presence of Mg^{2+}. These non-outer membrane proteins may be periplasmic proteins or proteins loosely bound to the exterior of the cytoplasmic membrane. It was not surprising to see large amounts of ^{3}H-labeled protein released at pH 8.5, since the cells are lysing and releasing cytoplasmic components. The ^{3}H peaks produced at pH 6.0 are generally small, which is in agreement with the data presented in Tables 2 and 3. Some ^{3}H peaks may comigrate with ^{14}C-outer membrane proteins. Although Mg^{2+}, Ca^{2+}, and an acid pH have a similar stabilizing influence, it appears that at pH 6.0 less ^{3}H-labeled protein is released that comigrates with the major peak of ^{14}C-outer membrane protein (protein I).

LiCl extraction of gonococcal outer membrane may not produce a preparation representative of the total, intact outer membrane protein spectrum. Extrinsic and loosely bound proteins may be removed during the extraction procedure, leaving only the tightly bound intrinsic proteins associated with the membrane matrix. Also, some outer membrane proteins may contain little or no lysine, and thus would not be efficiently labeled with either ^{3}H- or ^{14}C-lysine. However, neither of these factors would significantly alter the interpretation of the data.

From the data presented we conclude that divalent cations stabilize the cytoplasmic membrane of N. gonorrhoeae. Divalent cations are also known to be responsible for outer membrane integrity (7, 15). Acid pH inhibits autolysis, but this stabilization may occur via a different mechanism than divalent cation stabilization.

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Table 1. Effect of pH and Mg^{2+} on autolysis, PG hydrolysis and viability

Conditions	Rate of autolysis[a] ($k \times 10^{-3}$)	PG hydrolysis[a]	Percent viability[b]
50 mM HEPES, pH 8.5	17.0	20	0.1
50 mM HEPES, pH 8.5 + 10 mM Mg^{2+}	<0.2	19	1.5
50 mM HEPES, pH 6.0	<0.2	17	12

[a]Data from Wegener et al. (19).

[b]Determined after 2 hr incubation.

Table 2. Release of ^{3}H-adenine- and ^{3}H-arginine-labeled material from N. gonorrhoeae strain JW-31 incubated in 50 mM HEPES buffer containing 20% sucrose

	Percent ^{3}H released after 60 min incubation			
Label	pH 8.5	pH 8.5+ 20 mM $MgCl_2$	pH 8.5+ 20 mM $CaCl_2$	pH 6.0
[8-^{3}H]-adenine	68.3	6.3	1.3	2.9
[2,3-^{3}H]-arginine	77.6	19.8	21.6	1.2

Table 3. Characterization of material released by N. gonorrhoeae strain JW-31 incubated in 50 mM HEPES buffer containing 20% sucrose[a]

Conditions	Total mg. material released	(% dry wt of cells)	Total mg protein released	(% dry wt of cells)	Total µg KDO released	(% dry wt of cells)	Total µg phosphate released	(% dry wt of cells)
pH 8.5	32.42	(90)	13.292±1.128	(37)	376.56±65.82	(1.0)	1263.20±2.90	(3.5)
pH 8.5 +20 mM Mg^{2+}	6.40	(18)	0.372±0.116	(1.0)	26.92±11.18	(0.1)	95.97±4.23	(0.3)
pH 8.5 +20 mM Ca^{2+}	6.17	(17)	0.863±0.019	(2.4)	17.98±1.48	(0.1)	84.17±6.33	(0.2)
pH 6.0	4.95	(14)	0.307±0.059	(0.9)	7.24±0.96	(<0.1)	107.53±3.35	(0.3)

[a]Gonococci (36 mg dry wt) were suspended in 50 mM HEPES buffer containing 20% sucrose under the conditions described above. After 60 min of incubation at 37° C, the cells were sedimented by centrifugation and the material released into the supernatant characterized as described in Materials and Methods.

Figure 1. Percent of initial turbidity and percent of initial cell-associated ^{3}H-activity released into the supernatant when N. gonorrhoeae strain JW-31 was incubated in 50 mM HEPES buffer.

A. pH 8.5. ○, turbidity; □, ^{3}H-activity in supernatant; △, TCA-insoluble ^{3}H-activity in supernatant.

B. pH 6.0. (○, □, △) and pH 8.5 + 20 mM Mg^{2+} (●, ■, ▲). ○, ● -turbidity; □, ■ - ^{3}H-activity in supernatant; △, ▲ -TCA-insoluble ^{3}H-activity in supernatant.

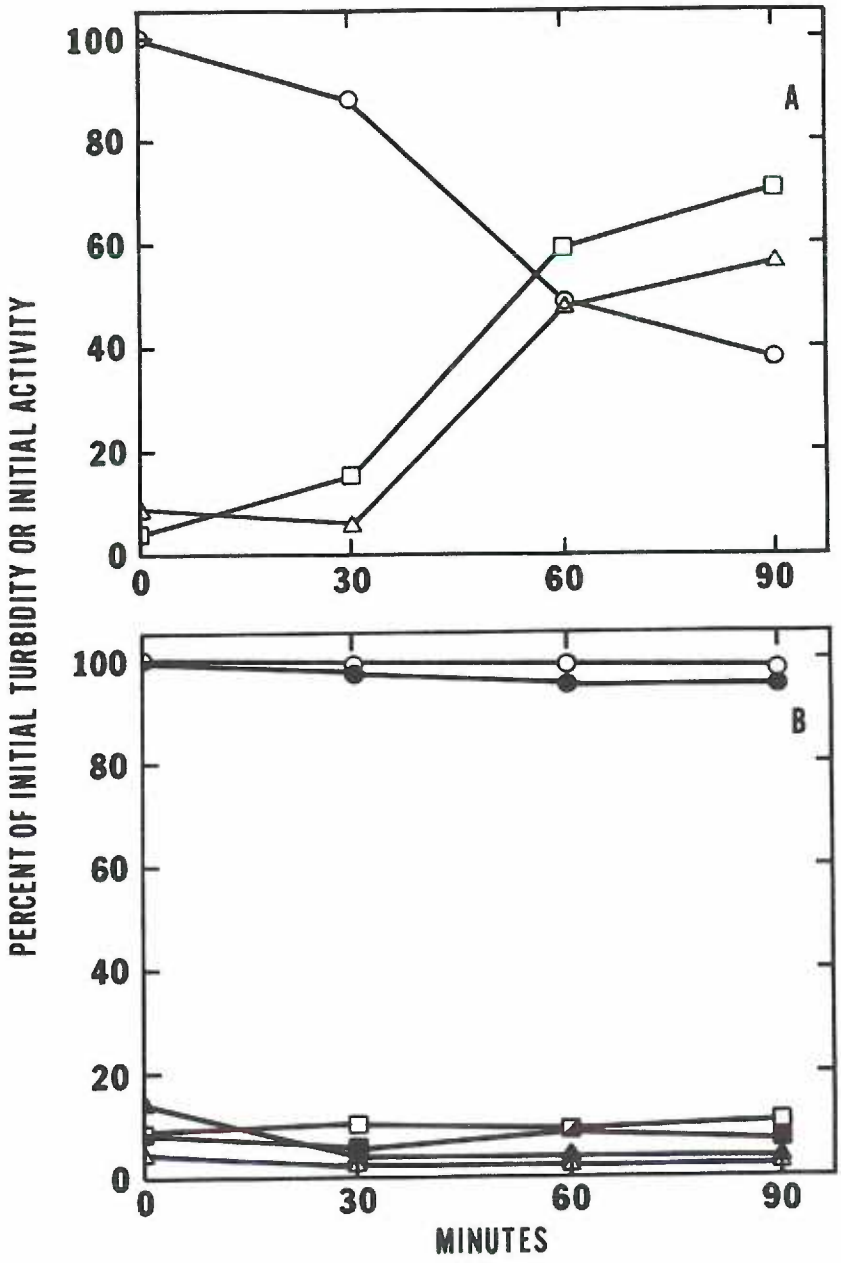
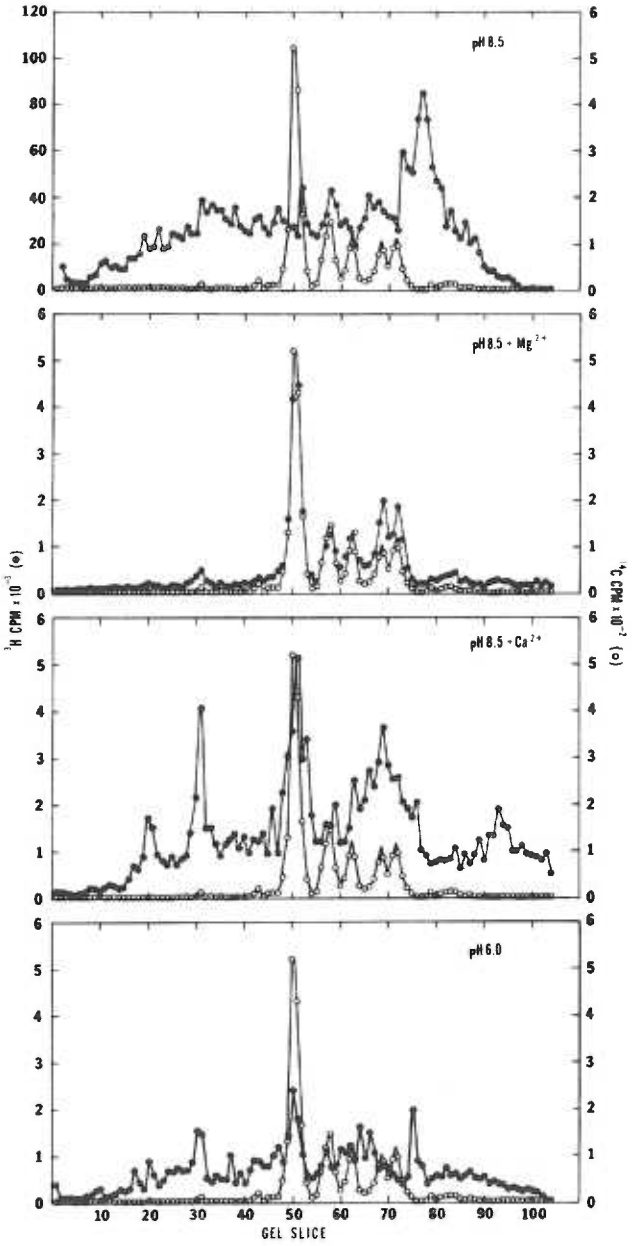

PERCENT OF INITIAL TURBIDITY OR INITIAL ACTIVITY
100
80
60
40
20
0
A
0
30
60
90
100
80
60
40
20
0
B
0
30
60
90
MINUTES

Figure 2. SDS-PAGE of a mixture of ^{14}C-lysine-labeled outer membrane proteins of N. gonorrhoeae strain JW-31 and ^{3}H-lysine-labeled material released from strain JW-31 suspended in 50 mM HEPES containing 20% sucrose and additions as indicated. Mg^{2+} and Ca^{2+} were added at a final concentration of 20 mM.


pH 8.5
pH 8.5 + Mg 2+
pH 8.5 + Ca 2+
pH 6.0
^{3}H CPM x 10^{-3} (●)
^{14}C CPM x 10^{-2} (○)
GEL SLICE

Manuscript 3

Effect of dissolved oxygen on outer membrane protein composition of *Neisseria gonorrhoeae* grown in continuous culture

INTRODUCTION

Environmental factors have been shown to alter the chemical composition, metabolism, cell envelope structure, and pathogenicity of Neisseria gonorrhoeae. Growth pH can affect the hexose content of a number of gonococcal strains (9), and alter the pathways by which glucose is dissimilated (9). Hebeler et al. (4) observed that a peptidoglycan-associated protein(s) was synthesized by gonococci grown at pH 6.0 but not at pH 7.2 or 8.0. The iron content of the growth medium also affects the gonococcal outer membrane protein composition (10). Gonococci grown in vitro and in vivo differ with respect to killing by normal human serum and complement (11). Also, organisms grown in vivo are more infective for guinea pig chambers than those cultivated in vitro (11).

Most studies on N. gonorrhoeae use organisms grown in batch culture. This method exposes the growing bacteria to an ever-changing environment. Investigations on environmental effects on gonococci require maintenance of a constant physiological state, which can be achieved by means of a chemostat. This study examines the effect of dissolved oxygen on the outer membrane protein profile of N. gonorrhoeae strain JW-31 grown in continous culture.

MATERIALS AND METHODS

Bacterial strains and growth conditions. N. gonorrhoeae strain JW-31 was used in this study. The properties and maintenance of this

strain have been previously described (8). Cultures were characterized as to colonial color and opacity according to the typing system of Swanson (13).

The basal medium used was that described previously (3), except 0.5% yeast extract (Difco) replaced the growth factor supplement. Glucose, the limiting substrate, was added at a final concentration of 0.051% (2.83 mM). Inocula were prepared by suspending the growth from a GC agar (Difco) plate in basal medium and inoculating 50 ml of fresh basal medium as previously described (3). Log phase cells were harvested and the cell pellet resuspended in the medium described above. The chemostat culture vessel was inoculated to an initial turbidity of 25 Klett units.

Gonococci were grown at 37° C at a dilution rate of 0.12 h^{-1} and a dissolved oxygen (DO) level of either 4% or 54% in a model C-30 chemostat (New Brunswick Scientific Co., New Brunswick, N.J.) equipped with a 405 ml volume culture vessel. Dissolved oxygen was monitored with a model M1016-0298 oxygen probe (New Brunswick Scientific Co.). During steady state growth the pH remained constant at both DO levels. Foaming was controlled by the periodic addition of Antifoam C (Sigma Chemical Co., St. Louis, Mo.).

Outer membrane extraction. Steady state cells were collected on ice over a 6-12 h period. The cells were centrifuged at 4° C (10,000 x g, 10 min) and the pellet was resuspended in 0.15 M NaCl and placed in tared centrifuge tubes. After centrifugation (12,000 x g, 10 min), the cell pellet was resuspended in 1 M LiCl (20 ml/g wet

weight of cells) and extracted by shaking with glass beads for 2 h at room temperature on a reciprocal shaker. The extracted cell suspension was centrifuged at 12,000 x g for 10 min at 4° C. The supernatant was centrifuged at 30,000 x g for 45 min at 4° C. The 30,000 x g supernatant was centrifuged at 85,000 x g for 2.5 h at 4° C in a Beckman L5-65 ultracentrifuge (type 30 rotor). The 85,000 x g pellet was washed twice with 0.15 M NaCl, then resuspended and centrifuged at low speed (12,000 x g, 5 min). The resulting supernatant was removed and stored at 4° C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done according to Laemmli (7). The gels contained 7.5% acrylamide. The protein content of gonococcal outer membrane preparations was determined by the method of Bradford (1), modified by using a commercial reagent (Bio-Rad Laboratories, Richmond, Calif.). Outer membranes were diluted in sample buffer to equal protein concentrations. Samples were heated at 100° C for 1, 3, 5, 10, or 30 min, or at 37° C for 2 h. Samples (50 µg protein) were placed on the gels and electrophoresed at 1 mA/gel through the stacking gel, then at 2 mA/gel through the separating gel. The gels were stained with Coomassie brilliant blue (16) and destained in acetic acid:methanol:H_2O (7.5:5.0:87.5) at 60° C in the presence of Bio-Rex RG501-X8 resin (Bio-Rad Laboratories). The apparent molecular weights of the outer membrane proteins were determined by comparing their relative mobilities with those of standard proteins of known molecular weights (MW) (Figure 1).

The standards included bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), and chymotrypsin (MW 25,700).

RESULTS

The outer membrane protein profiles of N. gonorrhoeae strain JW-31 grown at 54% and 4% DO were compared (Fig. 2, gels 1 and 2). Membrane preparations were solubilized at 100° C for 5 min, and identical amounts of protein were subjected to SDS-PAGE. Gel 2 (4% DO cells) has quantitatively less protein in bands B (MW 67,000), C_1 (MW 52,000), E_2 (MW 30,000), F_1 (MW 26,000), and F_2 (MW 25,000).

The effect of heating (100° C) time on the outer membrane protein profile of cells grown at 54° DO is shown in Figure 3 (gels 1-5). The principal outer membrane protein, located at band D, has an apparent molecular weight of 35,000. Three heat-modifiable proteins observed at bands A (MW 110,000), F_1 (MW 26,000), and F_2 (MW 25,000) decrease in intensity with increased time of heating. Band E_2 (MW 30,000) increases in intensity with increased heating time. Incubation of the membrane preparation at 37° C for 2 h (gel 6) prior to SDS-PAGE resulted in a gel profile similar to that seen with membrane preparations heated at 100° C for 1 min.

No differences in colony type or color/opacity characteristics were observed when JW-31 grown in the presence of different levels of DO was streaked onto GC agar (Difco) plates. Gonococcal colonies were P^-, light, and opaque/transparent.

DISCUSSION

Strain JW-31 grown in continuous culture at 54% DO contained greater amounts of certain outer membrane proteins than cells grown at 4% DO. These quantitatively different proteins were both heat-modifiable and -stable (MW range 25,000 to 67,000). Norqvist et al. (10) also observed that certain environmental factors influenced the outer membrane protein composition of N. gonorrhoeae. Three outer membrane proteins (MW 76,000, 86,000, 97,000) were apparently induced when gonococci were grown under conditions of iron-deficiency. Russell et al. (12) noted that a major outer membrane protein of N. sicca exhibited heat-modifiability depending on whether cells were grown in complex or defined medium. The data presented here indicate that the level of dissolved oxygen in the growth medium can affect the outer membrane protein composition of N. gonorrhoeae.

Heat-modifiable outer membrane proteins have been reported in N. gonorrhoeae by other investigators. Heckels (5) observed that one of the major outer membrane proteins had an apparent MW of 24,000 at 37° C, whereas at 100° C this protein had a MW of 29,500. Walstad et al. (15) reported that dark-colored gonococcal colonies contained an outer membrane protein of 28,000 MW when membranes were solubilized at 100° C for 5 min in the presence of 5 M urea. If both urea and boiling were omitted, this protein migrated with an apparent MW of 20,000. We found that strain JW-31 grown at 54% DO possessed 4 outer membrane proteins that were heat-modifiable. Three of these proteins

are in the MW range (25,000-30,000) of other gonococcal outer membrane proteins reported to exhibit heat-modifiable behavior. The high molecular weight heat-modifiable protein (MW 110,000) observed may have been an aggregate. A similar heat-modifiable high MW aggregate has been reported in N. meningitidis (2).

James and Swanson (6) found that urethral cultures from males tended to contain darker, more opaque colonies than did those from female cervices. They suggested that a host-factor component was selecting a certain gonococcal variant (genetic change). The finding that quantitative changes in gonococcal outer membrane proteins occurred without concomitant changes in colony characteristics suggests that such differences may also be due to phenotypic changes brought about by an environmental alteration.

Swanson (14) observed via ^{125}I-labeling that opaque colony types possessed increased amounts of outer membrane proteins in the 24,500 to 28,000 MW range as compared to transparent colony preparations. Swanson has not reported the heat-modifiability of these proteins. We observed that proteins in this MW range demonstrated heat-modifiable behavior. One of these proteins may be related to the heat-modifiable protein described by Walstad et al. (15).

Continuous culture provides a means to study the effects of environmental factors on gonococci grown in vitro. Since environmental parameters probably affect the ability of N. gonorrhoeae to cause disease, studies with continuous culture may provide information on in vivo phenomena.

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Figure 1. Relative molecular weights of the outer membrane proteins of N. gonorrhoeae strain JW-31. Symbols: ◯, relative mobility of known standards; □, relative mobility of outer membrane proteins. Letter designations refer to Figure 2.

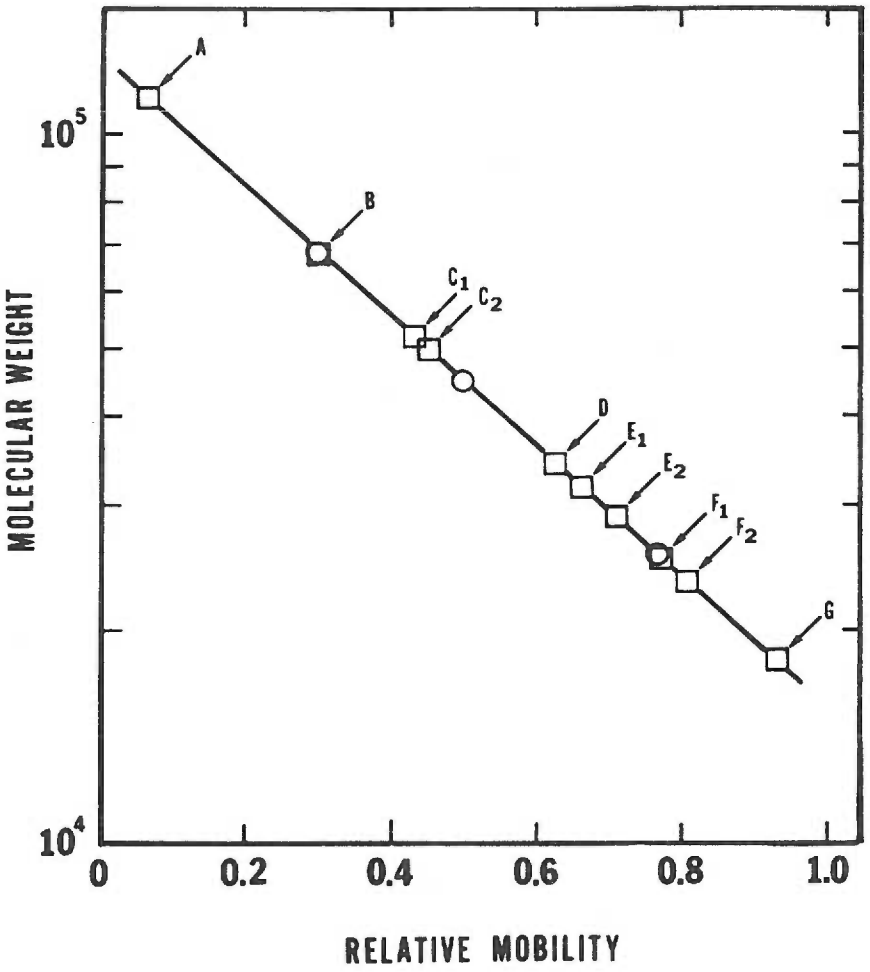

A
B
C1
C2
D
E1
E2
F1
F2
G
10^5
10^4
MOLECULAR WEIGHT
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 2. Comparison of outer membrane protein profiles of N. gonorrhoeae strain JW-31 grown in continuous culture at 54% (gel 1) and 4% (gels 2 and 3) DO. Samples were heated at 100° C for 5 min (gels 1 and 2) or at 37° C for 2 h (gel 3), as described in Materials and Methods. Protein-containing bands of interest are designated by letters A through G.

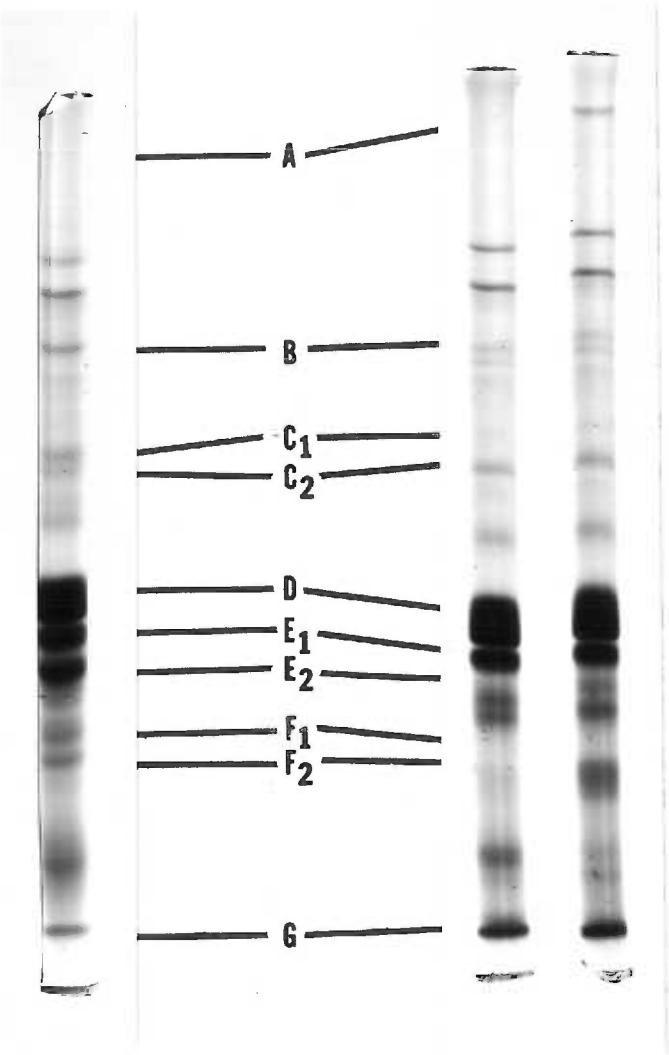
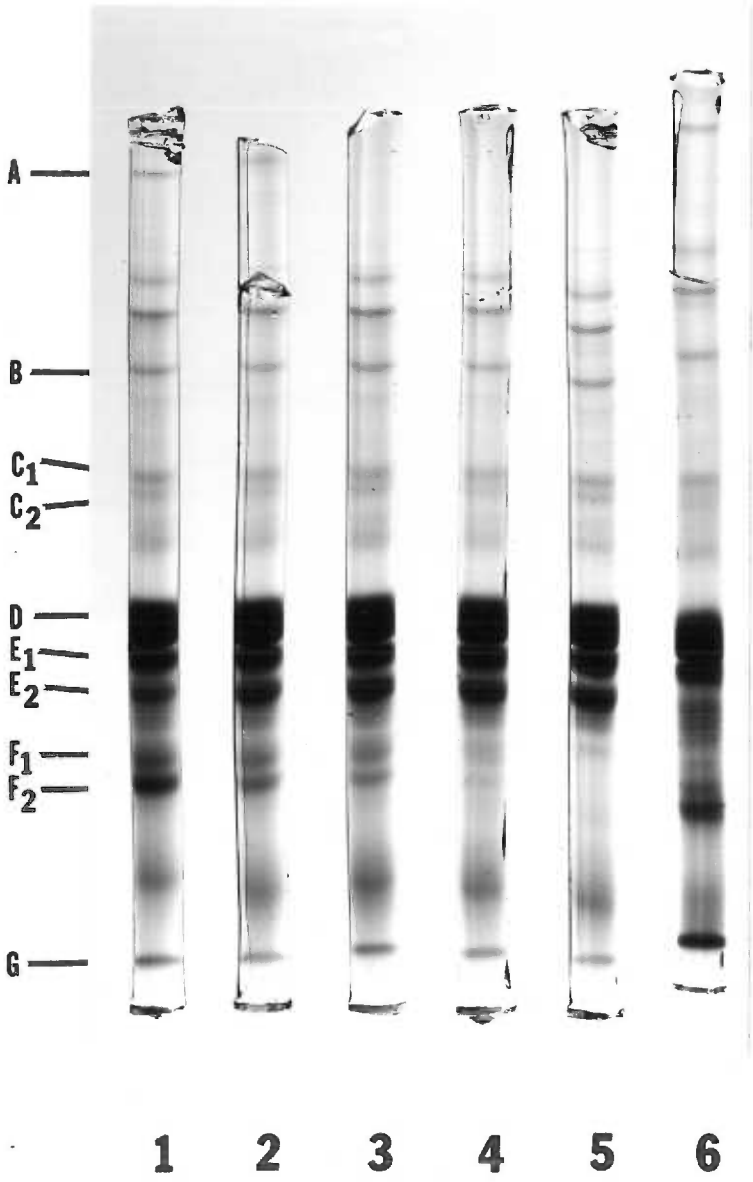

A
B
C1
C2
D
E1
E2
F1
F2
G
1
2
3

Figure 3. Effect of heating time on the outer membrane profile of N. gonorrhoeae strain JW-31 grown in continuous culture at 54% DO. Samples were heated at 100° C for 1 (gel 1), 3 (gel 2), 5 (gel 3), 10 (gel 4) or 30 min (gel 5) or at 37° C for 2 h (gel 6), as described in Materials and Methods. Protein-containing bands of interest are designated by letters A through G.


A
B
C1
C2
D
E1
E2
F1
F2
G
1
2
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5
6

DISCUSSION AND SUMMARY

Neisseria gonorrhoeae is but one of a number of microorganisms that cause sexually-transmitted diseases. Individuals previously infected with N. gonorrhoeae are susceptible to reinfection. The purported absence of protective immunity following a natural infection is one reason why gonorrhea has become the world's most prevalent bacterial disease. Thus, development of a gonococcal vaccine is receiving much attention at the present time. Current areas of study include gonococcal surface components (pili, LPS, outer membrane proteins) (5, 13, 14, 16, 17, 23, 25, 26, 33), attachment of organisms to eucaryotic cells (5, 15, 24, 25, 27, 28, 34), invasion of epithelial cells (34, 35, 36, 37), and interactions of gonococci with host defense systems (1, 4, 14, 18, 29-32, 36).

Several recent investigations have studied various aspects of outer membrane proteins of N. gonorrhoeae (13, 14, 16, 17, 25, 26, 33). To date, no one has reported a nearest-neighbor analysis of the major proteins of the gonococcal outer membrane. Such analyses have been performed with E. coli outer membrane proteins through the use of chemical cross-linking reagents and two-dimensaionl SDS-PAGE (19-22). In E. coli the predominant outer membrane protein (protein I) is cross-linked to itself, with resultant formation of dimers, trimers, and low yields of high multimers (9, 19-22).

Isolated outer membranes of N. gonorrhoeae strains F62 and 1362 were treated with a cleavable cross-linking reagent and subjected to

two-dimensional SDS-PAGE (MS 1). The resultant gel patterns resembled those seen with cross-linked E. coli outer membrane proteins in that dimers and trimers of the predominant outer membrane protein (protein I) were observed. However, in isogenic colony types from two different strains, evidence was obtained suggesting that protein I was also linked to the second-most prevalent outer membrane protein (protein II). Although both of these organisms (E. coli and N. gonorrhoeae) possess a "typical" gram-negative outer membrane, they are known to differ in at least one respect: E. coli contains a lipoprotein molecule which is thought to play a role in anchoring the outer membrane to the peptidoglycan (2, 3). This lipoprotein may form a complex with protein I that serves as a transmembrane pore (6). Lipoprotein reportedly stabilizes an inner core of three protein I molecules (6). In. N. gonorrhoeae, protein I may also form diffusion channels through the outer membrane. The gonococcus does not possess a lipoprotein-like molecule (12, 39), therefore protein II may stabilize an inner core of three protein I molecules. If so, protein II would likely be cross-linked to protein I. No evidence was obtained which indicated that protein II was cross-linked at the level of trimer. Perhaps the frequency of such an event was so low that the quantity of protein II at the level of trimer was undetectable. Alternatively, when protein II is cross-linked to protein I, the conformation of protein I may change so as to inhibit cross-linking to another molecule of protein I. In addition, cross-linking of protein I to protein I (dimer form) may restrict further linkage of the dimer to a molecule of protein II, but not to another molecule of protein I.

Further studies will have to be performed to determine the conformation of protein I and protein II complexes in the outer membrane matrix.

Cross-linking of low molecular weight outer membrane proteins was observed in N. gonorrhoeae strain 1362 (T1). Two proteins (14,000 and 19,000 MW) appeared to be cross-linked in a dimer form. This was the only difference observed in two-dimensional gel patterns when cross-linked outer membranes from isogenic colony types of two strains were compared. It must still be determined whether this phenomenon is strain-dependent.

The gonococcus is a very fragile microorganism which autolyzes rapidly when suspended in buffer at an alkaline pH (11). This characteristic has implications in the transport and isolation of gonococci in clinical specimens. In addition, such fragility may be important in the pathobiology and antigenic presentation of the organism in vivo. Divalent cations at an alkaline pH stabilize gonococci against autolysis (7, 8, 38). However, even though the cells are stabilized in the presence of divalent cations, cellular components are released into the environment (MS 2). Magnesium ions appeared to be most effective at stabilization, but even in the presence of this cation integral outer membrane proteins were released. An acid pH (pH 6.0) stabilizes the cells but also allows loss of cellular components. This suggests that gonococci in vivo may be shedding potentially antigenic and/or chemotactic molecules. Mucous surfaces and/or inflammatory exudate may provide an osmotically favorable environment for

gonococci that have become fragile following loss of structurally important surface components. High osmolality may permit these organisms to survive and multiply under conditions that would otherwise be inhibitory. Peptidoglycan fragments, membrane fragments, and IgA protease could be released from gonococci and contribute to the development of disease. The study on autolysis of N. gonorrhoeae presented herein demonstrates that gonccocci do release proteins into the environment, even when the cells are apparently stabilized. The results further suggest that the cytoplasmic membrane of the gonococcus is stabilized in the presence of divalent cations and at an acid pH. Divalent cations may be responsible for retention of certain substances or may be critical in maintaining membrane conformation. However, outer membrane proteins may be exposed or released during gonococcal multiplication in a changing in vivo environment, and may be involved in the initiation and/or maintenance of infection.

N. gonorrhoeae is a human pathogen which has become exquisitely adapted to its environment. In vivo, gonococci must react to changing environmental conditions (for example, pH, nutrient supply, competing microflora, inhibitory agents). In batch culture, the gonococcus also experiences variable conditions. Continuous culture, whereby bacteria are maintained in a constant environment at a given steady state, is more conducive to studies on effects of environmental parameters.

The effect of dissolved oxygen on outer membrane protein composition of N. gonorrhoeae strain JW-31 was examined (MS 3). The outer membrane protein profile, visualized by SDS-PAGE, showed that the

outer membrane pattern remained relatively constant when gonococci were grown at either 4% or 54% DO. However, significant quantitative differences were observed. Three of these proteins were in the molecular weight range of other reported heat-modifiable gonococcal outer membrane proteins (13, 33). The high molecular weight heat-modifiable outer membrane protein may be similar to one observed in N. meningitidis (10). Quantitative changes in protein bands were observed when outer membrane preparations from 4% and 54% DO cells were compared, yet nochanges were noted in colony type or color/opacity characteristics of JW-31 grown at different levels of DO. This suggests that outer membrane protein alterations can result from phenotypic changes occurring in response to environmental alterations.

Swanson (26) observed that opaque gonococci contain "colony opacity-associated proteins" in the 24,000 to 30,000 MW range. N. gonorrhoeae strain JW-31 (opaque/transparent) also exhibited proteins in this molecular weight range. Gonococcal heat-modifiable outer membrane proteins previously reported have molecular weights in the range observed for opacity-associated proteins (13, 26, 33). Although Swanson has not reported that his opacity-associated proteins are heat-modifiable, I suggest that the two categories of protein may overlap or be identical. The observation of Walstad et al. (33) of a heat-modifiable protein associated with dark gonococcal colonies supports this hypothesis.

This thesis examines several aspects of outer membrane proteins of N. gonorrhoeae. However, the role of these proteins in vivo has

yet to be determined. Do these proteins play a role in initial interaction with the host? Are they exposed on the surface during gonococcal infection? Are these proteins released *in vivo*, and if so, what effect do they have in the host? To what extent does the outer membrane protein composition vary (if at all) in response to *in vivo* environmental changes? Answers to such questions will aid in understanding the pathogenicity of *N. gonorrhoeae* and may assist in the development of gonococcal prophylaxis.

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Appendix A: Determination of optimal acrylamide concentration for resolution of control and cross-linked gonococcal outer membrane proteins.

Two-dimensional SDS-PAGE of outer membrane proteins treated with cross-linking reagents presents certain difficulties. Electrophoresis in the first dimension should produce good separation of non-cross-linked proteins without excluding from the separating gel the cross-linked high molecular weight (MW) complexes. These high MW complexes must enter the first dimensional gel in order to be analyzed by electrophoresis in the second dimension. Thus, it was important to determine the concentration(s) of acrylamide which would fulfill these requirements.

DTSP-cross-linked and control outer membrane preparations (60 µg protein) from N. gonorrhoeae strain F62 (T4) were applied to one-dimensional SDS-polyacrylamide gels (2) containing either 5%, 7.5%, or 10% acrylamide (MS 1). Figure 1 shows the results obtained following electrophoresis of outer membrane proteins on gels containing different acrylamide concentrations. When untreated outer membrane proteins were subjected to electrophoresis on 5% acrylamide gels, all proteins banded in the lower 40-50% of the gel; most of the major outer membrane proteins migrated together at the buffer front. With 7.5% acrylamide gels, proteins with a MW greater than 35,000 were separated; protein I, protein II, and outer membrane proteins with a MW less than 30,000 were not distinctly separated. An identical outer

membrane preparation subjected to electrophoresis on 10% acrylamide gels resulted in excellent distribution of protein bands throughout the gel. In addition, protein I and protein II were clearly separated and migrated approximately to the midpoint of the gel. Proteins with a MW less than 30,000 were also clearly resolved. From examination of untreated outer membranes, 10% acrylamide gels yielded optimal separation of proteins.

DTSP-treated outer membranes of strain F62 (T4) were subjected to SDS-PAGE on 10% acrylamide gels. Good separation of proteins was observed, but it appeared from the dark area at the top of the gel that some high MW material may have been excluded. The exclusion limit for these gels, calculated from the mobility-molecular weight curve, was approximately 150,000 MW. The protein bands seen in the top portion of the gel of cross-linked outer membrane had apparent MW's of 66,000, 93,000, and 120,000. F62 (T4) protein I has an apparent MW of 34,000 and protein II has an apparent MW of 32,000. The new band at 66,000 in the cross-linked preparation has an apparent MW that corresponds perfectly to that of a dimer of protein I-protein II. The new bands with MW's of 93,000 and 120,000 would correspond to trimers and tetramers. The calculated MW range for trimers and tetramers of protein I, protein II, or combinations thereof would be 96,000 to 102,000 MW and 128,000 to 136,000 MW, respectively. Chemical cross-linking may prevent these complexes from unfolding completely during sample preparation, and thus lead to anomalous MW determinations (1, 3, 4).

It appears that 10% polyacrylamide provides good separation of outer membrane proteins and allows cross-linked complexes of protein I, up to the level of tetramer, to enter the gel. The disadvantage in using this acrylamide concentration is that cross-linked complexes with a MW greater than 150,000 will not be resolved in the second dimensional gel. The products of greatest interest were complexes of protein I and protein II, and since the formation of tetramers and higher multimers is minor, the advantages of using 10% acrylamide gels outweighed the disadvantages. Further experiments utilizing cross-linked gonococcal outer membranes were performed with 8.75% or 10% polyacrylamide gels.

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Figure 1. One-dimensional SDS-PAGE analysis of control and cross-linked N. gonorrhoeae strain F62 (T4) outer membranes. A, control preparation, 5% acrylamide; B, control preparation, 7.5% acrylamide; C, control preparation, 10% acrylamide; D, DTSP-treated preparation, 10% acrylamide.

A B C D

Appendix B: Effect of heating time and reducing agent on the electrophoretic mobility of outer membrane proteins of Neisseria gonorrhoeae in SDS-PAGE.

The cleavable cross-linking reagent used for nearest-neighbor analysis of outer membrane proteins of Neisseria gonorrhoeae was dithiobis (succinimidyl propionimidate) (DTSP) (MS1). This bifunctional reagent contains a disulfide bond which can be cleaved by a reducing agent (β-mercaptoethanol). When chemical cross-linking is used in conjunction with two-dimensional SDS-PAGE, proteins are subjected to electrophoresis in the first dimension in the absence of reducing agent. The first dimensional gels are then exposed to reducing agent prior to electrophoresis in the second dimension. Several investigators (1, 4) have observed that the disulfide bonds introduced via chemical modification are heat-labile. Therefore, during preparation of cross-linked samples for SDS-PAGE in the first dimension, the outer membrane proteins must be solubilized in the absence of β-mercaptoethanol (β-ME) and must be heated at 100° C for no more than 45 sec (Dr. Steve Gutteridge, personal communication). However, before the first dimensional gels were electrophoresed in the second dimension they were exposed to 5% (v/v) β-ME in buffer at 100° C for 30 min. Exposure to a reducing agent and variable heating times could affect the electrophoretic mobility of outer membrane proteins, and thus lead to confusing or unexpected results on second dimensional

SDS-PAGE analysis. Therefore, the effects of reducing agent and heating time on the relative mobilities of gonococcal outer membrane proteins were examined.

N. gonorrhoeae strains F62 and 1362 were used in these studies. Outer membrane proteins from strain F62 did not exhibit heat-modifiable or β-ME-modifiable behavior when subjected to SDS-PAGE (data not shown). This correlates with previous results of two-dimensional analysis of cross-linked F62 outer membrane proteins, which show a diagonal line of non-cross-linked proteins and no off-diagonal spots other than those arising from cleaved cross-linked complexes.

Strain 1362 (T4) outer membrane proteins exhibited alterations with different heating times and in the presence or absence of β-ME (Fig. 1). No differences were observed with proteins having an apparent MW greater than 35,000. However, protein II had an apparent MW of 32,500 to 33,500 when heated in the presence of β-ME at 37° C for 2 h or at 100° C for 30 sec to 30 min (gels 1-7). When an identical preparation was heated at 100° C for 5 min in the absence of reducing agent (gel 8), protein II had an apparent MW of 30,000. Preparations heated at 37° C for 2 h or at 100° C for 30 sec and 1 min exhibited a diffuse staining band at 29,000 MW (gels 1-3) which decreased in intensity with increased time of heating (100° C for 3, 5, 10, 30 min) (gels 4-7). Differences in intensity of protein bands were also observed when gels 1-3 were compared to gels 4-7. The apparent molecular weights of these bands in gel 8 (no β-ME) were 21,000 and 19,500. The calculated difference may or may not be significant.

Second dimensional slab gels of 1362 (T4) further demonstrate the effects of heating time and exposure to reducing agent. On slab gels of both control and treated outer membranes, low molecular weight proteins migrate off-diagonal (skew to the left), suggesting that these proteins migrated faster in the second dimension than in the first. This observation is in agreement with the previous data showing that 21,000-22,500 MW proteins may migrate faster (19,500-21,000 apparent MW) if exposed to β-ME. Second dimensional slab gels of 1362 (T1) outer membranes (treated and untreated) do not show skewing of proteins in the low MW region as did gels of 1362 (T4). However, there is some horizontal smearing of protein spots in this area that may be the result of heat- or β-ME-modification.

Heat-modifiable outer membrane proteins have been demonstrated previously in N. gonorrhoeae (2, 3). Such heat-modifiable behavior becomes important when chemical cross-linking is combined with two-dimensional SDS-PAGE. In addition, membrane proteins are usually solubilized in buffer containing SDS and β-ME prior to SDS-PAGE. In this study, the presence or absence of β-ME was responsible for introducing variability in the electrophoretic mobility of certain outer membrane proteins of some strains of N. gonorrhoeae. Thus, the examination of the effects of reducing agent and heating time is an important prerequisite to two-dimensional SDS-PAGE analysis of chemically cross-linked outer membrane proteins.

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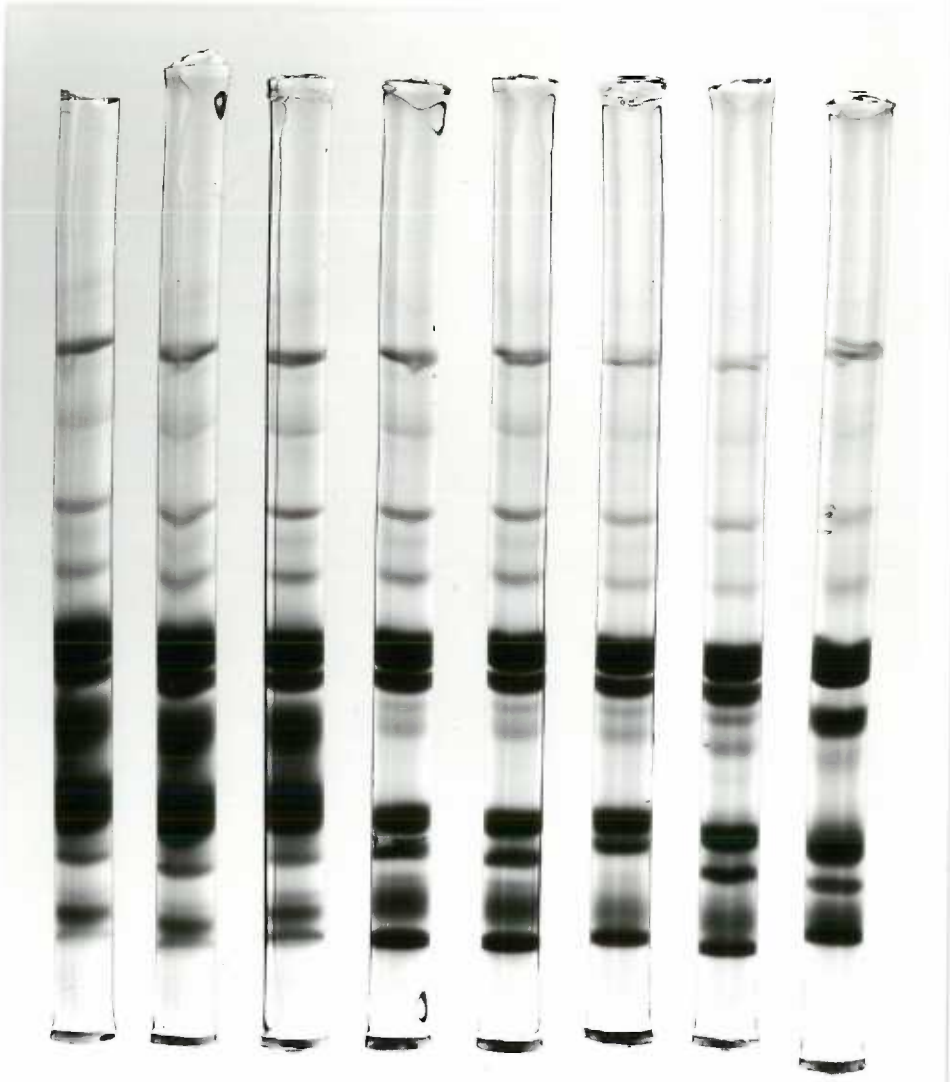
Figure 1. Effect of heating time and reducing agent on the SDS-PAGE profile of N. gonorrhoeae strain 1362 (T4) outer membrane proteins. Samples were heated at 37° C for 2 h (gel 1) or at 100° C for 30 sec (gel 2), 1 min (gel 3), 3 min (gel 4), 5 min (gels 5 and 8), 10 min (gel 6), or 30 min (gel 7). Samples were prepared in the presence (gels 1-7) or absence (gel 8) of β-mercaptoethanol.



1 2 3 4 5 6 7 8

Appendix C: Two-dimensional SDS-PAGE of molecular weight standards.

During the course of experiments performed on gonococcal outer membrane proteins, I have frequently observed smearing of protein bands upon SDS-PAGE. On several occasions, smearing of protein bands in the second dimensional slabs was quite extensive. Therefore, it was necessary to determine if this problem was a result of the two-dimensional SDS-PAGE system used, or if it was a characteristic of the outer membrane proteins.

Proteins of known molecular weight were solubilized at 100° C for 5 min in buffer containing β-ME and then subjected to the same two-dimensional SDS-PAGE procedure that was utilized in analysis of chemically cross-linked complexes (MS 1). Figure 1 shows that the protein standards migrated in the second dimensional gels as distinct spots with no apparent smearing. Thus, the two-dimensional SDS-PAGE procedure employed for analysis of chemically cross-linked complexes of outer membrane proteins of N. gonorrhoeae was not artifactually producing smearing of outer membrane proteins.

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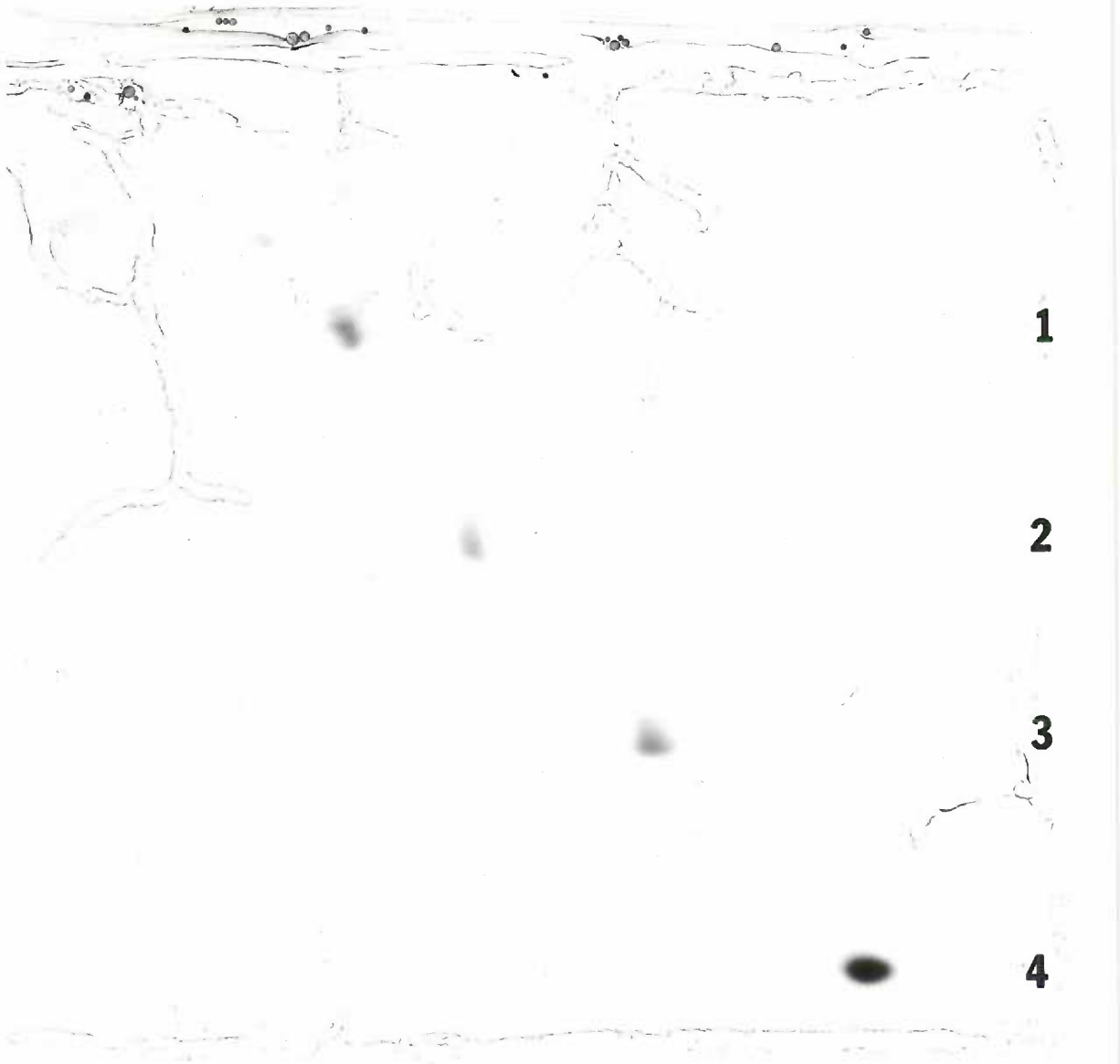
Figure 1. Two-dimensional SDS-PAGE analysis of molecular weight standards. Standards used: 1) bovine serum albumin (67,000 MW), 2) ovalbumin (43,000 MW), 3) carbonic anhydrase (30,000 MW) and 4) myoglobin (17,000 MW).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