MEMBRANE PROTEOLIPIDS AND IRON-REGULATED PROTEINS OF *NEISSERIA GONORRHOEAE*

by

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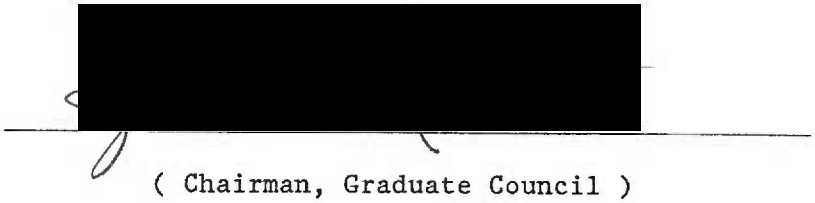

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PREFACE

This dissertation is comprised of manuscripts which have been prepared or accepted for publication. The manuscripts have been divided based on their emphasis on different membrane components of N. gonorrhoeae, i.e. membrane proteolipids, and iron-regulated proteins. A comprehensive review of the literature with relevant references precedes these manuscripts.

ABSTRACT

Isogenic strains of Neisseria gonorrhoeae were grown in medium supplemented with sub-inhibitory concentrations of [^{3}H]-palmitic or [^{3}H]-oleic acid (10 μCi/ml). Cells were harvested at the end of the exponential growth phase and the cell envelope isolated and analyzed by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fluorograms indicated that membranes from each of the strains contained many labeled components. Identical fluorograms were observed when cells were labeled with either [^{3}H]-palmitic or [^{3}H]-oleic acid. Exhaustive extraction of the membranes with chloroform-methanol (1:1) did not affect the banding pattern observed on the fluorograms. The bands labeled with [^{3}H]-palmitic or [^{3}H]-oleic acid consisted of proteins, since these bands disappeared when the delipidated envelope fraction was treated with proteinase K prior to SDS-PAGE. Proteinase K-treated delipidated membranes were extracted with chloroform-methanol-water, and the extract was analyzed by thin-layer chromatography. A radioactive ninhydrin-positive spot (ca. 29% of total radioactivity) which did not correspond to any of the known gonococcal phospholipids was observed. Extraction of this material and subsequent alkaline hydrolysis resulted in the liberation of labeled fatty acids. The modification of fatty acid to membrane proteins was not due to the contaminations of noncovalent interaction of lipopolysaccharide or phospholipid to cell envelopes. The results provide evidence for covalent modification of numerous membrane proteins by fatty acids. Among the gonococcal membrane proteolipids identified were the Mtr-associated protein

and protein III; protein I and protein II were not covalently modified by lipids.

Pulse-chase experiments showed that the radioactivity in the fatty acid moieties of these proteolipids increased rather than decreased during a 4 h chase period suggesting the presence of a large pool of acyl donors such as phospholipids. Fatty acid acylation of membrane proteins was decreased in cells treated with either cerulenin or chloramphenicol suggesting that de novo fatty acid synthesis was required and that there was no significant pool of proacyl proteins within the cell. In addition, the uptake and incorporation of exogenous fatty acids as well as the fatty acid acylation of proteins was affected by respiratory chain poisons and temperature indicating that the process is both enzymatic and energy-dependent. However, globomycin, a specific inhibitor of signal peptidase II, had no effect on the labeling profiles of membrane proteolipids. [^{14}C]-glycerol, [^{3}H]-palmitoyl-phosphatidylethanolamine, and [^{3}H]-palmitoyl-phosphatidylglycerol were not taken up nor incorporated into membrane proteolipids. The proteolipid profiles among strains of N. gonorrhoeae as well as from other Neisseria species exhibited a great degree of variability.

N. gonorrhoeae and N. meningitidis express a 37-Kdal iron-regulated protein (MIRP) that binds approximately 1 mole of Fe^{3+} per mole of protein. A similar-sized MIRP has been observed in N. lactamica and in some strains of N. cinerea; other strains of N. cinerea and N. subflava have a 36.5-Kdal MIRP. Polyclonal antiserum to the gonococcal MIRP reacted with MIRPs of the Neisseria species in Western blots and ELISAs. To examine the structural

heterogeneity of these proteins, purified MIRPs were radioiodinated and digested with trypsin, and the ^{125}I-peptides were resolved by high-voltage thin-layer electrophoresis and ascending thin-layer chromatography. Autoradiographs indicated that the MIRPs shared a common set of peptides. The MIRP of each species also possessed unique peptides. Surface-exposed peptides of the gonococcal MIRP were determined as above following radioiodination of whole cells. Eight peptides were labeled when intact cells were used, compared with more than 17 peptides when the purified protein was labeled. Thus, probably only a small portion of the molecule is surface exposed.

INTRODUCTION

During the last three decades, our knowledge of the biosynthetic process of bacterial lipoproteins and the nature of fatty acid-protein linkage has been generated mainly from the studies of Braun's lipoprotein in Escherichia coli. To date, with additional lipoproteins discovered in other Gram-negative and Gram-positive bacteria, the exact function and physiological significance of the lipoprotein still remains to be answered. There have been few, if any, studies on proteolipids in Neisseria gonorrhoeae, a Gram-negative diplococcus. Previous investigators considered the outer membrane to be less rigid than that of E. coli because it lacked lipoprotein(s). It was only recently that the discovery of peptidoglycan-associated protein(s) in N. gonorrhoeae provided indirect evidence for the presence of membrane proteolipids. Although this protein(s) was proposed to be analogous to Braun's lipoprotein, very limited data were available about its biochemical nature and structure. This finding prompted us to search for direct evidence to support the existence of membrane proteolipids in N. gonorrhoeae as well as in other Neisseria species.

The study was initiated by investigating the uptake and incorporation of exogenous long-chain fatty acids by N. gonorrhoeae. The first portion of this dissertation focused on: (1) identification of gonococcal membrane proteolipids; (2) establishing evidence for the covalent modification of membrane proteins by fatty acids; (3) determining the nature of the linkage between protein and fatty acid; (4) investigating the biosynthesis of

proteolipids; and (5) determining the presence of proteolipids in other Neisseria species.

A considerable effort has been made to identify several gonococcal surface components and to study their relevance in the host-parasite interaction. The expression of a 37,000 dalton major iron-regulated protein (MIRP) under iron-limiting conditions and by all strains of pathogenic Neisseria species has prompted the speculation that this protein may play a key role in iron acquisition. Structural studies to understand its function and potential role in virulence have been described in the second portion of the dissertation including: (1) purification of MIRP from different Neisseria species; (2) comparison of the structural heterogeneity of different MIRPs using two-dimensional peptide mapping; (3) determination of the surface-exposed regions of gonococcal MIRP; and (4) iron uptake from ^{55}Fe-transferrin by gonococci.

LITERATURE REVIEW:

I. Gonococcal infection

A. Historical aspects

Gonorrhea, an infection of humans caused by Neisseria gonorrhoeae, is almost always transmitted by sexual contact. The clinical manifestations of gonorrhea in men were recognized in accient Chinese, Japanese, Egyptian, Roman, and Greek writings, as well as in the Old Testament (201). The first scientific observations on gonorrhea are attributed to Hippocrates (460-355 BC), the "Father of Medicine". He dissected infected urethrae and observed their inflammation and discharge. He called it "strangury" and was in no doubt that it "resulted from indulgence in the pleasures of Venus". It was Galen (130-200AD), the "Prince of Physicians", who coined the word "gonorrhea" by which he meant a "flow of semen" under the mistaken impression that it was due to spermatorrhea. The term "clap" first appeared in a manuscript of 1378 written by John of Arderne, surgeon to Richard II and Henry IV. At that time, it was defined as "a certain inward heat and excoriation of the urethra". The origin of this term, by far the commonest used in the English speaking world, is obscure.

N. gonorrhoeae, the causative agent of gonorrhea, was first described in detail by Albert Neisser in 1879, for whom the genus is named. Leistikow and Loeffler, in 1882, were the first to report the cultivation of the gonococcus. Bumm showed that the gonococcus fulfilled Koch's postulates in 1885 (44). It was not until the 1930s that effective antimicrobial therapy in the form of sulfonamides was first used. However, gonococci rapidly became resistant to sulfonamides. The introduction of penicillin in 1943 restored control of the sulfonamide-resistant strains. Since its introduction,

penicillin has proved highly effective and has markedly decreased the serious consequences of the disease. In 1976, strains of N. gonorrhoeae were isolated which contained plasmids encoding for beta-lactamase. These strains have continued to increase in frequency, and in some areas have markedly reduced the effectiveness of penicillin. Thus, the increasing resistance to penicillin and other available antibiotics coupled with a large reservoir of asymptomatic females and males who unknowingly transmit the disease to their sexual partner(s) make gonorrhea one of the most prevalent communicable bacterial diseases today.

B. Epidemiology

Gonorrhea is a disease of worldwide importance. Humans are the only natural host for N. gonorrhoeae. The increase in cases during and shortly after World War II (1941-1946) was followed by a marked decline in incidence after the introduction of penicillin therapy. However, the annual incidence of reported gonorrhea in the United States tripled between 1963 and 1975 (6). This rising incidence coincided with the introduction of oral contraceptives and intrauterine devices (IUDs) that contributed to increased sexual freedom and to a decreased use of condoms and spermicidal preparations and facilitated the spread of gonorrhea. There were about one million cases of gonorrhea reported each year from 1975 to 1980 (50). In 1983, 900,435 cases of gonorrhea were reported to the Centers for Disease Control (CDC). In terms of incidence, this represents 357.6 cases per 100,000 population (51). However, after 10 years of decreasing incidence, there was a 4% increase in incidence observed during 1985. Since that time the incidence has decreased.

The incidence and prevalence of gonorrhea are highest in young adults (peak age incidence is 20-24 for men, 18-24 for women), the second highest attack rates occur in the 15-19 year old age group. The increases in the incidence of gonorrhea have resulted from: 1) an increased number of population at risk, i.e., sexually active young adults; 2) changing behavioral and cultural values that are reflected in more liberal attitudes; 3) the advent and popularity of oral contraceptives and IUDs; 4) more intensive contact tracing of patients; 5) development of an inexpensive transport medium for the use of clinicians without laboratory facilities; 6) the introduction of legal requirements for laboratories to report positive cultures; and 7) the evolving resistance to antimicrobial agents, e.g., the appearance of

penicillin-producing N. gonorrhoeae (PPNG) in 1976 as well as chromosomally mediated penicillin, tetracycline, and spectinomycin resistance during the past few years. However, the validity of gonorrhea reporting is somewhat compromised by the low reporting percentage from private physicians (it is estimated an additional 1 to 1.5 million cases of gonorrhea are diagnosed each year, but are not reported). When all of these factors are taken into consideration, the incidence of gonorrhea has actually leveled off in the early 1980's, which may reflect the success of current intense public health programs of surveillance and therapy. Also, it may reflect either a decreasing sexual adventurism by a public increasingly concious of "new" and "incurable" STDs (such as genital herpes and AIDS), or a decreased sexual promiscuity in the population known to be most at risk.

C. Clinical manifestations

The clinical spectrum of gonorrhea is broad. It includes asymptomatic, symptomatic, and complicated infections of several anatomic sites and predominantly involves mucosal surfaces lined by columnar epithelial cells, such as the urethra, endocervix, pharynx, conjunctiva, and rectum. These infections may sometimes progress to salpingitis (PID), bacteremia, perihepatitis, or septic arthritis, as well as rarer complications such as meningitis and endocarditis.

1. Urethral infection in men

Acute anterior urethritis is the most common manifestation of gonorrhea in men. A recent study (88) demonstrated that the usual incubation period of gonococcal urethritis was 2 to 5 days in ca. 80% of men with uncomplicated gonorrhea. Acute epididymitis occurred in 37 (20%) of 183 patients with untreated gonococcal urethitis (220). However, this condition is now uncommon in industrialized countries. Inflammatory urethral stricture is a potential complication of untreated gonococcal urethritis, but many strictures that developed in the pre-antibiotic era were probably related to repeated infections or to caustic "therapeutic" urethral irrigations (220). Strictures are rare today if effective therapy is instituted promptly. The ID_{50} for gonococci (number of colony formimg units necessary to produce infection in half the subjects) was about 10^3 organisms among a group of male volunteers undergoing experimental urethral inoculation (36). The likelihood that a man will become infected after a single exposure to an infected woman is estimated to be 20 to 35 percent (108,110,180). The males who are asymptomatic or ignore symptoms are of critical importance to the epidemiology of the disease

as they serve as reservoirs and may be at increased risk for disseminated disease (108).

2. Urogenital infection in women

The endocervical canal is the primary site of urogenital gonococcal infection in women, followed in descending order by the urethra, anal canal, and pharynx. The symptoms of primary gonococcal infection in women are less specific than those in men, and vary with both the clinical setting and site of infection (10). The most common symptoms are those of lower genital tract infection and include: abnormal vaginal discharge (cervicitis), dysuria and frequency (urethritis or skenitis), labial pain or swelling (acute bartholinitis), anorectal discomfort, intermenstrual uterine bleeding and/or lower abdominal pain (pelvic inflammatory disease) (55,175). The above symptoms may occur in any combination and range in intensity from barely noticeable to severe. The incubation period for urogenital gonorrhea in women is less certain and is more variable than in men, but most develop local symptoms within 10 days of exposure (225,305). The assessment of symptoms and signs in women with gonorrhea is confounded by the high prevalence of coinfection with Chlamydia trachomatis, Trichomonas vaginalis, Candida albicans, Gardnerella vaginalis, and other organisms (9).

3. Anorectal infection

The rectal mucosa is infected in 35 to 50 percent of women and homosexual men with gonorrhea, and is the only infected site in about 5% of women and 40% of homosexual men (87,143,144,159,299). In women, most anorectal infections are correlated with the duration of endocervical infection (144), suggesting that infection is usually due to perineal

contamination with cervicovaginal exudate. There is no question that rectal intercourse is of direct importance to the developement of rectal gonococcal infection among homosexual men. Gonococci isolated from homosexual men are more likely to carry the mtr locus than gonococci from heterosexuals (205). This locus confers resistance to several antibiotics as well as a selective advantage for survival in the hydrophobic rectal environment. The reported symptoms of anorectal gonococcal infection range from minimal anal pruritus, painless mucopurulent discharge, and scant rectal bleeding, to symptoms of overt acute proctitis, including severe rectal pain, tenesmus, and constipation (85,143,144).

4. Pharyngeal infection

Among patients with gonorrhea, the pharynx is infected in 3 to 7 percent of heterosexual men, 10 to 20 percent of hetersexual women, and 10 to 25 percent of homosexual men. The pharynx is the sole site of infection in less than 5 percent of patients, regardless of gender or sexual orientation (38,87,126,315,317). Oral-genital contact is clearly the major behavioral factor predisposing to gonococcal infection of the throat. Pharyngeal gonococcal infection is acquired more efficiently by fellatio than by cunnilingus (245,315). However, over 90% of these infections are asymptomatic (38,315). The clinical and epidemiologic significance of pharyngeal gonococcal infection is uncertain. However, patients with gonococcal pharyngeal infection may be at increased risk for development of disseminated infection (86,315).

5. Neonatal infection

Gonococcal infection of the newborn may involve the conjunctiviae,

pharynx, respiratory tract, or anal canal. Conjunctival infection is usually contracted during passage through an infected birth canal and is often bilateral; without treatment blindness may result. The risk of infection increases with prolonged rupture of the fetal membranes (108). With silver nitrate prophylaxis, the risk of transmission from an infected mother to her infant is probably less than 2% (7). Gonococcal conjunctivitis is rare in adults and usually occurs in patients with anogenital gonorrhea by autoinoculation (108).

6. Pelvic inflammatory diseases (PID)

Spread of N. gonorrhoeae upward from the cervix results in acute endometritis, salpingitis, and peritonitis, which are collectively termed acute PID. Approximately 10 to 15 % of infected women develop gonococcal PID (69,231,293). PID is a recurrent disease, with previous episodes being a significant predisposing factor (70). About 90% of the initial cases are associated with gonococcal infection of the endocervix (232). However, gonococci can be recovered from fewer women with recurrent PID. PID has been associated with the use of intrauterine contraceptive devices (IUDs). The risk of developing PID after contracting gonorrhea is estimated to be 3 to 4 times higher if an IUD is in place (70,268,296). Gonococcal PID is responsible for the majority of the economic cost of infections caused by N. gonorrhoeae. The infertility rate caused by PID has been calculated to be 13% after one attack, 36% after two, and 75% after three or more. Furthermore, the risk of ectopic pregnancy increases seven-fold after one attack of PID (314).

7. Disseminated gonococcal infection (DGI)

Hematogenous dissemination of gonococci from a primary site is a rare event. Prevalence studies indicate that about 1% (ranging from less than 0.5% to 3%) of patients with mucosal infection develop DGI. The usual clinical manifestation of DGI include: papulopustular or necrotic skin lesions on the extremities, asymmetrical arthralgias, tenosynovitis, or arthritis involving a small number of joints. Occasionally, endocarditis or meningitis may result (106). With the advent of antimicrobics and the emphasis on treating symptomatic infections in men, DGI became relatively more common in women and possibly in homosexual males (18,37,106,138). About half of the DGI cases in women occur either between 5 days before the onset of menses and the cessation of menses during pregnancy (37,106,138). Generally, the incubation period for DGI is between 7 and 30 days. Gonococcal strains producing disseminated infection are likely to express a Protein I belonging to a particular serogroup (WI or Protein IA) (41,46,250). Although Protein IA strains are usually serum-resistant (125), there is no evidence suggesting that the type of Protein I determines serum resistance. Nearly all DGI strains resist the complement-antibody-mediated bactericidal action of normal human serum (39,258). Also, most isolates from disseminated infections produce transparent colony phenotypes (300) which might be a selective advantage due to their decreased association with neutrophils (153). Many DGI strains have growth requirements for arginine, hypoxanthine, uracil, and are known as Arg^- Hyx^- Ura^- (AHU^-) auxotypes (145,146). This auxotype has also been associated with asymptomatic mucosal infections in men (54). In addition, AHU strains from DGI are almost always highly sensitive to penicillin (316). The host's environment is another important factor in the development of DGI. The most clearly identified host factor is a deficiency

or abnormality of one of the late acting complement components that include C_5, C_6, C_7, or C_8 (39,40,161,223) and which are essential for the bactericidal action of serum. Hormonal influences in women may also be important host factors since most cases of DGI occur close to the menstrual period or during pregnancy (18,37,106,138).

II. Biology of the gonococcus

A. General characterization

Lestikow and Leoffler, in 1882, were the first to report the cultivation of N. gonorrhoeae on artificial medium. In 1885, the etiological role of the gonococcus in gonorrhea was definitely established when Bumm was able to cultivate the organism and determine that genital inoculation of human subjects caused typical disease.

N. gonorrhoeae is an aerobic gram-negative diplococcus which usually occur in pairs with flattened adjacent sides, a characteristic that is responsible for their kidney or coffee bean appearance in microscopic preparations. The cells produce no endospores and are nonmotile. Cell division is in two planes at right angles, sometimes resulting in the formation of tetrads. Individual cell size may vary from 0.6 to 1.5 um, depending on the source of the isolate and age of the culture. Older cells tend to autolyze in culture. Although it is possible to grow the gonococcus on chemically defined media, it nevertheless is a fastidious organism with complex growth requirements. Free iron is an essential growth factor. Starch, cholesterol, or albumin should be added to media to neutralize the inhibitory effects of fatty acids. The optimal temperature for growth is 35°C to 37°C; growth is stimulated by 5% to 10% CO_2. The gonococcus produces acid from glucose but not maltose, a characteristic used to distinguish it from the meningococcus. Both organisms possess catalase and cytochrome oxidase activities. The gonococcus is quite fragile and can not survive adverse environmental conditions, such as drying, chilling,and exposure to unfavorable pH for extended periods outside the human host.

B. Colonial morphology

As early as 1904, differences were noted in the colonial appearance of N. gonorrhoeae (166). The modern era of gonococcal research can be traced backed to the pioneering work of Kellogg and associates in the 1960s (139,140), in which they related colonial morphology to virulence of the organisms. The morphology of colonies on clear agar were classified into four types (T1,T2,T3, and T4). In 1971, Jephcott and Reyn (128) reported the identification of a fifth colonial type, T5. T1 and T2 colony types predominated in isolates from the urethra of males (139,140), the endocervix of females (267) and from the rectum (148). The morphology of these colony types is small, dense , and virulent. Only by selective passage can T1 or T2 colonial forms be maintained indefinitely and still retain the ability to cause typical anterior urethritis when injected into male volunteers (140) or to cause death when injected intravenenously into chicken embryos (17). However, by non-selective in vitro passage the T3 and T4 colony variants which are larger, more granular, and avirulent will appear.

C. Pili variation

Pili are present on both pathogenic and non-pathogenic Neisseria spp. (128). While T1 and T2 colony variants have pili on their surfaces, they are absent on T3 and T4 colony variants (128,289). Recently, the piliated colony types T1, T2 and nonpiliated colony types T3, T4 have been designated as P^+ and P^-, respectively (290). Gonococcal pili are linear aggregates of a single kind of protein subunit (pilin), which has a molecular weight ranging between 17,000 to 21,000, depending on the strain from which the pili were derived (236). Nearly all freshly isolated gonococci have pili (140,267). However, with repeated subculture in vitro, they are rapidly lost (140,289). Nonpiliated cells may "switch back" to pilus expression (phase varation). The mechanism of pilus-phase variation frequently involves chromosomal rearrangements and deletions within pilus structural genes (189,262). Gonococcal pili from different strains are antigenically diverse, sharing no more than 25% of their antigenicity (36). In addition, a single gonococcal strain is capable of expressing multiple chemically and antigenically distinct types of pili (154,155,246), although a single organism apparently produces only a single type of pilus at one time (287). Piliation has also been reported to be associated with competence for genetic transformation (11,17).

D. Opacity variants

Dark and light colored variants of the colonial types of N. gonorrhoeae were first observed by Diena et al. (62). Swanson (279,280) also reported that gonococcal colonies differed in their opacity when observed with light directly reflected from the substage mirror of a dissecting microscope: some appeared transparent like water droplets, some were opaque like ground glass, and others colonies were intermediate in appearance. He later used the terms "transparent" and "opaque" for the light and dark colonial variants, respectively. The current nomenclature designates opaque colonies of intermediate darkness as O^{+}, those which are very dark as O^{++}, and the transparent colonies as O^{-} (290). These optical properties were independent of piliation, and were apparently related to the degree of cellular aggregation within a colony. Gonococci change from the transparent to the opaque phenotype (or vice versa) with a frequency estimated to be ca. 10^{-3} per cell division (177). Opaque colonies tend to clump in liquid medium, have abundant zones of intracellular adherence and are more sensitive to trypsin than transparent colonies. The change from the transparent to the opaque form is accompanied by the acquisition of one or more additional outer membrane proteins ranging in molecular weight between 24,000 to 30,000 and which also exhibit heat-modifiable behavior (282). The predominance of opacity variants in several forms of gonococcal infection have been determined. Gonococci recovered from blood and joint fluid, urethral mucosa of asymptomatic males, uterine or cervical specimens taken at menstruation, and fallopian tube or peritoneal specimens usually form transparent colonies (66,122,123). In contrast, urethral swab specimens from male patients with symptomatic

urethritis and cervical specimens obtained at midcycle usually form opaque colonies. Thus, the transparent-colony phenotype is associated with invasive disease even though the mechanisms behind this are unknown.

E. Cell envelope

The cell envelope of the gonococcus is similar in ultrastructure to those of other Gram-negative organisms and consists of a lipopolysaccharide (LPS)-containing outer membrane, an intermediate layer of peptidoglycan (or murein), and a cytoplasmic (or inner) membrane. Several soluble proteins are located in a compartment between the two membranes known as periplasmic space. Pili are also anchored in the cell envelope. Together they form the physical and functional barrier between the inside of the cell and its environment. The cell envelope of Gram-negative bacteria is more highly differentiated than that of Gram-positive bacteria suggesting that they might employ different strategies in coping with their environment (204).

1. Capsule

The presence of capsules on N. gonorrhoeae has been a controversial issue. Encapsulation is suggested by the finding that meningococci have a polysaccharide capsule that provides a basis for serogrouping. Recently, several reports indicated that freshly isolated strains of N. gonorrhoeae also possessed a capsule when examined by light microscopy using India ink or by electronmicroscopy of cells exposed to hyperimmune serum (99). Although the capsule was most evident on recent isolate or on organisms grown in vivo (121,235), it was also present on the surface of laboratory strains. Environmental and nutritional factors are important in capsule synthesis (99). Cells from all gonococcal colony types produced capsules (121,235). The capsule was loosely associated with the cell surface and was easily removed by mild shearing (121). Polysaccharide capsules are often polyanionic and are believed to have an antiphagocytic function by complexing divalent cations in the immediate environment (328). Thus, the observation of

extracellular polyphosphate (a polyanion) in gonococci (212) has led to speculation that it functions as a capsule. Evidence against the presence of a capsule was reported by Melley et al. (187) who employed an electron microscopic India ink technique, which required neither specific anticapsular antibody nor staining of specific capsular carbohydrates. No true capsules were definitively identified by this method. However, two types of false capsules were observed on N. gonorrhoeae either grown in vitro or obtained from human urethral exudates. These artifacts were found to be caused either by separation of the outer membrane or by a surrounding membrane of unknown origin (possibly derived from a phagocytic cell), and might easily be misinterpreted as a capsule.

Wheat germ agglutinin (WGA) agglutinates non-capsulate strains of N. meningitidis by binding to a N-acetylglucosamine moiety on the LPS ; WGA failed to agglutinate capsulate strains of all serogroups tested (251). In contrast, all gonococcal strains were strongly agglutinated by this lectin. This observation was also taken as evidence against a gonococcal capsule. It has not been possible to isolate the capsular material of N. gonorrhoeae. Thus, the chemical nature of the capsule and homo- or heterogeneity of capsular types are still unknown. The issue of gonococcal encapsulation will to be settled when more compelling evidence is available.

2. Cytoplasmic membrane

The cytoplasmic membranes (CM) of Gram-negative bacteria are characteristic of biological membranes in that they are composed of a phospholipid bilayer throughout which intrinsic and extrinsic proteins are distributed. In general, the CM is considered to play a role in the transport of nutrients, in oxidative phosphorylation, and in the synthesis of

phospholipids, peptidoglycan, LPS, periplasmic and membrane proteins. Moreover, it is probably involved in cell division and serves as an anchor for DNA during replication. Johnston and Gotschlich (132) separated the cytoplasmic membrane from the outer membrane of N. gonorrhoeae by isopycnic sucrose density centrifugation of osmotically ruptured spheroplasts. The chemical and enzymatic composition of the isolated CM were reported to be similar to E. coli or Salmonella typhimurium in terms of their buoyant density (1.141 g/cm^3) and the specific enzyme activities present, e.g., succinic dehydrogenase, D-lacate dehydrogenase, and NADH oxidase. Relatively little is known about the gonococcal CM. Rodriguez and Saz (237) investigated the uptake of [^{14}C]-penicillin by N. gonorrhoeae and found that membranes derived from penicillin- susceptible cells bound 140 to 200 nmol of [^{14}C]-penicillin per gram of membrane protein; relatively nonsusceptible strains bound only 18 to 59 nmol. The very low level of [^{14}C]-penicillin bound by the cell wall indicated that binding occurred in the CM. Polyacrylamide gel electrophoresis of the gonococcal CM revealed 13 protein bands, 8 of which could be labeled with radioactive penicillin. Miller et al. (195) analyzed the isolated CM from two spectinomycin-susceptible and three spectinomycin-resistant clinical isolates of N. gonorrhoeae. Their CM preparations revealed a multibanded electrophoretic pattern of 21 to 25 proteins with some strain-specific variation. A major protein with an apparent molecular weight of 24,000 was present in the spectinomycin-sensitive isolates but was absent in the spectinomycin-resistant clinical isolates. The 24k protein comprised ca. 7% of the total CM proteins in spectinomycin-sensitive strains. The highly autolytic nature of the gonococcus, exposure to antibiotics during therapy, and exposure to lysozyme

found in leukocytes all increase in vivo exposure of CM constituents. CM proteins may also be important in antibiotic resistance and as possible antigen sources for vaccine production (202,247).

3. Peptidoglycan

Peptidoglycan (PG) is responsible for the shape of the cell and for maintenance of bacterial integrity. PG represents 1 to 2% of the dry weight of the gonococcus. The chemical composition of gonococcal PG is similar between different cell types with a molar ratio of 1:1:2:1:1 for muramic acid, glucosamine, alanine, glutamic acid, and diaminopimelic acid (DAP), respectively (95). The PG of N. gonorrhoeae RD5 has a high turnover rate of approximately 50% per generation and follows first-order kinetics. Rosenthal et al. (239) demonstrated that PG turnover in exponentially growning N. gonorrhoeae was accompanied by the release of soluble PG fragments into the medium. Four major types of soluble PG have been tentatively identified: (1) cross-linked, bisdisaccharide peptide dimer; (2) uncross-linked disaccharide peptide monomer; (3) free peptide; and (4) free disaccharide. Further characterization of the predominant monomer fraction revealed that it was composed of two distinct disaccharide peptides (265). The major (80%) component of the monomer appeared to be N-acetyl-glucosaminyl-beta-1-4, -1,6-anhydro-N-acetylmuramyl-L-alanyl-D-glutamyl-Meso-diaminopimelic acid (anhydro-monomer), the remainder was the corresponding disaccharide tetrapeptide containing a C-terminal D-alanine (Chalaropsis monomer). The presence of these different soluble PG fragments indicated that turnover and release of PG by growing gonococci was mediated by both hexosaminidase and N-acetylmuramyl-L-alanine amidase activities (239).

Guymon et al. (81) determined the relative degree of PG cross-linking

among isogenic strains of N. gonorrhoeae. They observed that the presence of genetic loci that governed low-level non-specific resistance to several antibiotics was correlated with an increase in the extent of PG cross-linking. However, this alteration in cross-linking may be a secondary effect of other as yet undetected changes in cell envelope structure. Rothenthal et al. (243) found that the percent of PG cross-linking reflected the percentage of DAP residues that were involved in peptide cross-linking bonds. Although there were subtle strain- and medium-dependent differences in percent of cross-linking, the extent of cross-linking among gonoccal strains (36-44%) was slightly higher than that of most other Gram-negative bacteria. The presence of O-acetyl groups on some of the amino sugar residues (O-acetylation) has been shown to confer resistance of PG to the degradative action of hen egg white (HEW) lysozyme (27,240). The relation between lysozyme sensitivity of gonococcal PG and the extent of O-acetylation was investigated by Rosenthal et al. (240). They observed that PG from strain RD5 (a highly autolytic strain) was completely degraded by HEW lysozyme and was not O-acetylated. In contrast, extensively O-acetylated PG was isolated from strains that were markedly resistant to digestion by HEW lysozyme. Subsequent studies showed that extensively O-acetylated gonococcal PG was resistant to the PG-degrading enzymes present in human serum or derived from human polymorphonuclear leucocytes (241). Swim et al. (294) confirmed the correlation between the lysozyme-resistance and extent of O-acetylation. Lear and Perkins (158) examined the maturation of PG with regard to cross-linking and to O-acetylation. They found that cross-linking occurred very rapidly (84% within 3 minutes) during the synthesis of PG in growing cultures of N. gonorrhoeae. However, rapid O-acetylation occurred during a 10 minute period,

at which time only about 60% of the maximum value had been reached. Thus, the O-acetylation of PG in N. gonorrhoeae was a slower process than cross-linking, indicating that subunits already incorporated into pre-existing PG must undergo acetylation. The mechanisms by which gonococci regulate the extent of PG O-acetylation have been recently investigated (242). O-Acetylation is markedly increased by bacteriostasis associated either with the stationary phase of growth or induced by inhibitors of protein synthesis, e.g., chloramphenicol, tetracycline, and streptomycin. Bacteriostatic conditions may be encountered in vivo by gonococci. The resulting increase in O-acetylation not only promotes the persistence of gonococcal PG in vivo but may also potentiate the pathobiological consequences of PG-host interactions.

Dougherty (64) used reverse-phase HPLC to examine the structure of muramidase-digested gonococcal PG. He observed a substantial number of new PG subunits; substantial differences in these newly resolved PG subunits were observed between penicillin-susceptible and highly resistant gonococcal strains. Whether these newly described subunits participate in PG-host interactions remains to be elucidated.

4. Outer membrane

The outer membrane (OM) of gram-negative bacteria has been studied for many years. A great deal is known, especially about the OM of E. coli and S. typhimurium. Thus, most studies dealing with pathogenic Gram-negative organisms use these organisms for comparison. The major constituents of the OM are proteins, lipopolysaccharide (LPS) and phospholipids. LPS is associated with the outer surface of the OM, while phospholipids are asymmetrically distributed between the inner and the outerleaf of OM and also between the OM and CM. The weight ratios between these components, however,

mean very little, as they are strongly affected by the lengths of the carbohydrate chains of LPS. Johnston and Gotschlich (132) osmotically ruptured gonococcal spheroplasts and used isopycnic sucrose centrifugation to separate the OM from the CM. The purified gonococcal OM (buoyant density=1.219 g/cm^3) consisted of 48.6-58.2% protein, 0.29-0.31 mg phospholipids/mg protein, and 1.19-1.21 mg LPS/mg protein. This composition is also similar to those reported for other Gram-negative bacteria. Because of its direct contact with the host's environment, the OM may act as the first line of defense against the immune mechanisms of the host. Therefore, the biochemical composition of the gonococcal OM has been the subject of much investigation.

a. Phospholipids

Phospholipids are important in the structure and function of biological membranes. In bacteria, phosphatidylethanolamine and phosphatidylglycerol are the most common phospholipids, whereas phosphatidylcholine is only rarely found. This pattern distinguishes prokaryotic membranes from eukaryotic membranes. Sud and Feingold (273) determined that the phospholipid composition of N. gonorrhoeae consisted of 70% phosphatidylethanolamine and 20% phosphatidylglycerol; surprisingly, small amounts of phosphatidylcholine and traces of cardiolipin were also present. Between 8.2 and 10.2% of the dry weight of gonococci are lipids (15), and more than 80% of the lipids are phospholipids. Growing and stationary phase cells were similar in both content and composition of phospholipids except for phosphatidylcholine, which increased two- to five-fold in stationary-phase cells. The fatty acids of the phospholipids were characterized by two major acids, palmitic (C16:0) and a C16:1, with myristic (C14:0) and a C18:1 fatty

acid present in smaller amounts. Cyclopropane fatty acids and fatty acids with more than 18 carbons, generally found in Gram-negative bacteria (8,137), were not detected in N. gonorrhoeae. Other investigators (264,273,320) have also quantitatively analyzed the phospholipid composition of N. gonorrhoeae and confirmed that its phospholipid composition was similar to that of other Gram-negative bacteria. However, they were unable to detect phosphatidylcholine in either exponential of stationary-phase cells of recent isolates or laboratory strains of N. gonorrhoeae (15,264), even when the procedure described by Sud and Feingold (273) was used. Phosphatidylcholine was only found in cells of Branhamella catarrhalis.

Osborn et al. (217) reported that although the two membranes of S. typhimurium contained equal amounts of phospholipids, the OM was enriched in phosphatidylethanolamine and the CM was enriched in phosphatidylglycerol and diphosphatidylglycerol (or cardiolipin). A rapid exchange between the phospholipids of the two membranes was subsequently demonstrated by Jones and Osborn (133) who fused lipid vesicles with intact cells of S. typhimurium. Lugtenberg and Peters (169) studied the lipid composition of the membranes of E. coli and observed that the OM was also enriched in phosphatidylethanolamine. However, in contrast to S. typhimurium, phosphatidylethanolamine often represented over 90% of the OM phospholipids of E. coli. The reason for the enrichment of the OM with phosphatidylethanolamine might be that it forms stable bilayers with LPS (74). Detailed studies have shown that the phosphatidylethanolamine of the OM contained more saturated fatty acids (169) and less of the molecular species with two unsaturated fatty acids (118) than the phosphatidylethanolamine of the CM. Phospholipids were also found to be asymmetrically distributed between the CM and OM of N. gonorrhoeae.

Wolf-Watz et al. (320) reported that cardiolipin was located primarily in the OM and that the CM had a slightly higher proportion of phosphatidylglycerol relative to other phospholipids. The phospholipid composition of N. gonorrhoeae changed during growth in liquid medium. As cells entered the stationary phase, the concentration of phosphatidylethanolamine decreased slightly and was accompanied by a concommitant increase in lysophosphatidylethanolamine (15). The proportion of phosphatidylglycerol declined about 40% throughout growth while that of cardiolipin increased three-fold. The growth inhibition resulting from the production of lysophosphatidylethanolamine may partially explain the short survival of most gonococcal cultures (306). With phospholipids from all sources, saturated fatty acids are esterified at the C-1 and unsaturated fatty acids at the C-2 of the glycerol moiety. Although phospholipid biosynthesis in E. coli has been extensively studied, there is relatively little known concerning the regulation and function of prokaryotic phospholipids.

b. Lipopolysaccharide (LPS)

LPS are characteristic components of the cell envelope of Gram-negative bacteria. They are located in the outer leaflet of the OM and are heat stable amphiphathic molecules, with a hydrophilic, polysaccharide portion and a hydrophobic portion, lipid A. LPS is anchored in the bacterial cell through its lipid A component. The lipid A is covalently linked through 2-keto-3-deoxyoctulosonic acid (KDO) to a core oligosaccharide (R-core). The polysaccharide portion is usually divided into two subregions, i.e., the R-core and a polymer consisting of repeating carbohydrate units (O-specific chains). The structure of lipid A is relatively invariable and the R-core sugars to which it is attached are similar in different species. In contrast,

the O-specific chains have a variable composition and structure, which confer O antigenic specificity and are referred to as O-antigens. The O-antigen and most of the R-core sugars can be lost through mutations leaving lipid A linked to an incomplete R-core. These LPS molecules are referred to as R (rough)-type since on solid medium they give rise to rough colonies with serrated edges. Organisms possessing LPS molecules with complete structures form smooth colonies on solid medium and are called S (smooth)-type. Lipid A and KDO components appear to be essential for cell viability. LPS are also known as endotoxins because of their importance as mediators of toxicity in the host (68). They are thought to be responsible for the resistance of a wide range of Gram-negative bacteria to various antibiotics because they form a highly effective permeability barrier.

LPS is easily released upon mild chemical treatment of whole organisms (222). Spontaneous release of LPS from gram-negative bacteria during growth has also been documented (222), and seems to be a general phenomenon. Mild acid hydrolysis of purified LPS was shown by Westphal and Luderitz (312) to generate a toxic lipid A fraction and a nontoxic polysaccharide fraction. The demonstration that mutant Salmonella strains that lacked the capacity to synthesize polysaccharide-containing LPS (141,167) were equally as toxic and pyrogenic as their parent strains established the dominant role of lipid A in the endotoxicity of bacterial endotoxins (313). Free LPS has been found in culture supernatant of N. meningitidis (60), and a number of other Neisseria spp. (129,130). The LPS was released by a process described by DeVoe and Gilchrist as "blebbing" (60); free LPS isolated from culture medium is identical with cell-bound LPS.

The standard procedure for the isolation of S-LPS from S. typhimurium

is the phenol-water method. This method is also applicable for Ra and Rb LPS; however, Rc, Rd, and Re LPS are isolated in low yields by this method. Therefore, a more hydrophobic solvent mixture, phenol-chloroform-petrolium ether (PCP) was developed for the extration of R-LPS (75). Early studies on gonococcal LPS used a number of extration methods (171,173,298). In 1957, Tauber and Garson (297) extracted endotoxin from gonococci by treating dried bacterial cells with 0.05M sodium hydroxide. The preparation had a low toxicity for mice. However, extraction with phenol-water yielded a highly toxic preparation for mice (298). On the basis of its chemical composition, which consisted of 43% saccharide, 13.8% hexosamine, and 28.4% lipid, they called the endotoxin a lipopolysaccharide phosphoric acid ester. Maeland (172) found that the crude LPS preparations obtained by extraction of gonococci with either aqueous ether, alkali, trichloroacetic acid,or by heating contained an antigenic polysaccharide determinant called a, and a protein determinant called b. Purified phenol-water extracted LPS possessed the activity of a but not that of b, whereas LPS obtained by aqueous-ether extraction consisted of 90% protein and about 3% of carbohydrate and lipids. The sugars found were glucose, galactose, glucosamine, and heptose. Among the 16 amino acids detected, lysine, arginine, aspartic acid, glutamic acid, alanine, valine, and leucine were present in the largest amounts. In addition, KDO was detected in the phenol-water extracted endotoxin but not in the aqueous-ether extracted endotoxin. These data indicated that LPS was mainly carbohydrate and lipid in nature. Perry et al. (221) isolated and compared the composition of LPS from gonococcal type T1 and T4 cells. On mild hydrolysis, LPS from T4 colony type cells gave a lipid A and a core oligosaccharide composed of 2-amino-2-deoxy-D-glucose, D-glucose, D-galactose,

L-glycero-D-manno-heptose and 3-deoxy-D-manno-octulosonic acid. These sugars appeared to be common to all of the strains examined. LPS from T1 colony type cells consisted of lipid A and a high-molecular-weight O polysaccharide that exhibited considerable differences in glycose composition for each strain examined. These investigators proposed that T4 organisms produced a common R-type LPS, whereas T1 cells produced a common S-type LPS with structurally heterologous O-antigen side chains. In another study, Stead et al. (269) extracted the LPS from 5 strains of N. gonorrhoeae by the phenol-water method and showed that all preparations contained glucose, galactose, glucosamine, heptose, 2-keto-3-deoxyoctulosonic acid and phosphate. The fatty acids consisted primarily of beta-OH-10:0, beta-OH-12:0, beta-OH-14:0, 12:0, 14:0, 16:0. 16:1, 18:0 and 18:1. In contrast to the previous study, they were unable to detect significant differences between the LPS of virulent and avirulent gonococci, or between penicillin-sensitive and -resistant strains. These investigators concluded that gonococcal LPS appeared to lack O-antigen side chains. Wiseman and Caird (318) examined the LPS composition of isogenic colonial variants from 38 strains. Colonial types 1 to 5 contained glucose, mannose, galactose, N-acetylneuraminic acid, KDO, glucosamine, and galactosamine. In contrast to avirulent colonial type 3 to 5, virulent types 1 and 2 contained no rhamnose. Most notably, the concentration of glucose, galactose, and mannose varied from strain to strain, but virulent types 1 and 2 generally contained a greater total glycose concentration and higher mannose/KDO or galactose/KDO ratios than avirulent types 3 to 5. No qualitative difference between T1 and T4 LPS, apart from the presence of rhamnose in the latter, was observed. In addition, the composition of glycoses in T1 colony phenotypes were found to be identical except for some

variation in the amount present. These results were also inconsistent with the results of Perry et al. (221). Fatty acid composition of lipid A was similar to that found by Stead et al. (269), except that OH-10:0, OH-12:0, 16:1 and 18:1 were not detected. The basis of these difference is unclear, differences in strains, culture medium, growth conditins, and analytical techniques are likely responsible for the variation. As pointed out by Morse (202), these data must be accepted with caution since isogenic colonial variants were not used. Also, it is not certain whether the colonial stability of the organisms was assessed at the end of the growth cycle prior to extraction of the LPS.

Leghorn hens were immunized with R-type gonococcal LPS and the antiserum used for rapid identification of gonococci from primary isolates and secondary cultures (304). This antiserum recognized 249 out of 251 primary gonococcal isolates in a slide agglutination test and was specific for N. gonorrhoeae. It did not recognize N. meningitidis, other Neisseria spp., and organisms of different genera with the exception of certain streptococci. When immunized with the same R-type LPS isolated from a T4 colonial variant, protection against infection with both homologous and heterlogous gonococcal T1 isolates was observed in both the mouse intracerebral challenge model and the chicken embryo neutralization test (61).

The importance of the delineation of the antigenic structure of gonococcal LPS was emphasized after the demonstration that antibody to gonococcal LPS is bactericidal (233,302). Maeland (172) first demonstrated the multispecificity of antigenic determinants of the polysaccharide component of gonococcal LPS. Six different determinants were identified in the LPS of three different gonococcal strains. Apicella (1-4) confirmed this

multispecificity, and further, showed that these determinants were an integral portion of the serogroup and non-serogroup regions of the polysaccharide component of gonococcal LPS. Each gonococcal LPS-derived polysaccharide contained a serogroup-specific determinant that was analogous to the O-specific antigen. Six different serogroups were recognized. In addition, these polysaccharides contained at least 2 sets of heterogeneous non-serogroup determinants. One set was common to all six gonococcal serogroup polysaccharides (common region), whereas the second set was shared by three of the six serogroups (variable determinants).

Application of monoclonal antibodies (McAb) has helped to further elucidate the antigenic and chemical structure of gonococcal LPS. McAb 3F11 recognizes a specific site common to gonococcal LPS which is partially shared by meningococcal LPS but not by E. coli, S. typhimurium or gonococcal strains that lack the common determinant. Using an ELISA inhibition assay, Apicella et al. (4) determined that the chemical structure of the common determinant contained a D-galactosamine-O-D-galactopyranosyl-(1-4)-D-glucopyranose moiety.

Four immunologically distinct acidic polysaccharides have been identified (2) and designated Gc1 through Gc4. Using these purified serogroup antigens and appropriately adsorbed antisera in a hemagglutination inhibition assay, Apicella (2) developed a serotyping system for gonococci. Of the 163 strains studied, 71.2% possessed one of the 4 Gc antigens, 12.3% possessed more than one polysaccharide antigen, and 16.5% could not be classified with these reagents. These studies indicated that purified Gc antigens contained common as well as serogroup-specific determinants and were variable between different gonococcal strains. Morse et al. (203) isolated a LPS mutant of N. gonorrhoeae using an R-type pyocin from P. aeruginosa in order to study the

relationship among phenotypic variation, biologic function, and the antigenicity of gonococcal LPS. The change in the chemical nature of the LPS was accompanied by the loss of pyocin sensitivity, an increase in serum sensitivity, and an alteration in lectin affinity. Guymon et al. (79) observed that spontaneous pyocin-resistance occurred at a frequency of ca. 10^{-6}. This observation indicated that the gonococcal LPS chemotype could mutate at a high frequency and suggested that strains of gonococci could apparently produce more than one type of LPS.

Evidence for environmental factors that affect the composition of LPS have been obtained by several groups (182,206,213). McDonald and Adams (182) reported that LPS isolated from N. sicca grown at a high aeration rate (high growth rate) had a greater content of hexosamine and KDO, and a higher galactosamine to glucosamine ratio than LPS isolated from the same organisms grown at a low aeration rate (low growth rate). Morse et al. (206) showed that environmental conditions could affect not only the chemical composition, but also serum resistance of N. gonorrhoeae. Growth of gonococci in continuous culture (chemostat) under glucose-limiting conditions resulted in growth rate-dependent changes in the LPS. The LPS from cells grown at a low dilution rate contained about eight-fold less serotype antigen than the LPS from cells grown at a high dilution rate. An increase in the amount of LPS serotype antigen was associated with a reduction in the accessibility of a McAb to a core LPS determinant, an increase in resistance to normal serum, and a decrease in cell surface hydrophobicity. It is likely that gonococci are growing under nutrient-limited conditions in vivo. The change in LPS altered the serum resistance and might ultimately play a role in affecting the in vivo survival and virulence of the gonococcus. Norrod et al. (213) demonstrated

that changes in gonococcal LPS could be induced by adding sodium sulfite to the growth medium and suggested that these changes were responsible for variation in colonial morphology. Studies using SDS-PAGE and silver staining indicated that the electrophoretic banding pattern of gonococcal LPS was similar to that of LPS from R-type Salmonella mutants (52,80,196,256). Structural heterogeneity, indicated by differences in electrophoretic mobilities of LPS preparations was observed in N. gonorrhoeae (196,256), and also in various non-pathogenic Neisseria spp. Yamasaki et al. (325) further showed that gonococcal LPSs were composed of multiple oligosaccharides that contained 14 to 27 glycose units and that were antigenically discrete. Heterogeneity of apparent electrophoretic mobilities among intact LPS reflects differences in the apparent electrophoretic mobilities of their constituent oligosaccharides. Mintz et al. (196) used an enzyme-linked immunosorbent inhibition assay to compare the antigenic properties of phenol-water extracted- and PCP-extracted-LPS. They found that PCP-extracted LPS contained substantially less serotype-specific antigen than did phenol-water extracted LPS. These results suggested that the PCP and phenol-water methods extracted different molecular species of LPS from N. gonorrhoeae and that it was important to standardize growth conditions and to select the proper extraction procedure when the chemical and antigenic properties of gonococcal LPS were under study. The terms "rough" and "smooth" have been applied to the LPS of Neisseria spp. by several investigators (3,221,230). Such terminology probably has little meaning when considered in the same context as enterobacterial LPS. Schneider et al. (256) introduced the term "lipooligosaccharides" (LOS) and suggested that it more accurately reflected the structure of gonococcal and meningococcal LPS since they lacked the

repeating O-side chains that were characteristic of members of the Enterobacteriaceae. These investigators also observed that LOS of individual strains of gonococci and meningococci were heterogeneous. A number of studies concluded that the antigenic expression of gonococcal LOS was complex and was regulated by a number of factors, including pyocin susceptibility, growth conditions, divalent cation interaction, and association with lipid A (174,257). Recently, Hitchcock et al. (105) criticized the accuracy of using the term oligosaccharide for the LPS from non-enteric mucosal pathogens because the biochemical structure of these molecules has not yet been fully elucidated. Introducing new terms will only cause confusion and misunderstanding at present time.

C. Outer membrane proteins

Gonococcal OM proteins are classified by their migration during SDS-PAGE. They have been divided into three distinct groups (181,228) that have been termed Protein(s) I, II, III, which is based on their relative abundance, molecular weight, and modified migration by either heat or reducing agent. Specific labeling of whole organism with ^{125}I by the Iodo-Gen procedure indicated that these proteins were all exposed on the external surface of the cell membrane (181,275,285).

1. Protein I (P.I)

Protein(s) I is present in all gonococcal lysates or OM preparations regardless of the colony phenotype. The molecular weight of P.I varies between 32,000 and 38,000 daltons in SDS-PAGE and is strain dependent. The pI of P.I varies between 5.2 to 8 (22). The migration of protein I is not altered by heat or reducing agents. P.I accounts for more than 60% of the total protein present in the OM and was named the major gonococcal OM protein

(MOMP) (132). Recent studies of the serological heterogeneity of P.I indicated that there were 9 major serotypes (41). Earlier studies suggested the presence of 16 serotypes (131). Such variation could be accomplished by changes in relatively small, surface-exposed regions of the molecule leaving the majority of the molecule unchanged. The ability to become cross-linked to peptidoglycan demonstrates that P.I is a transmembrane protein (97). P.I forms anion-specific channels in artificial planar lipid bilayers as well as liposome vesicles (326). Thus, P.I(s) function as porins (65).

Sandstrom et al. (248) performed peptide mapping studies of tryptic digests of P.I molecules and classified them into two serogroups that were designated P.IA and P.IB. Swanson (13,283) examined the two dimensional-tryptic peptide maps of iodinated P.Is from 10 gonococcal strains and segregated them into two pattern groups. One group consisted of P.Is with molecular weights of 33,000 or 34,000 and included P.Is that were susceptible in situ to cleavage by trypsin or chymotrypsin or both. P.Is belonging to this group were all P.IBs (248), and were typical of organism isolated from local genital infection (48). Protein I with molecular weights of 32,000 constituted the other group. P.Is belonging to this group were all P.IAs. They were not cleaved by exogenous trypsin or chymotrypsin, and shared little homology in ^{125}I-peptide maps with P.IB (248,283). P.IAs were typical of organisms isolated from disseminated gonococcal infections (48,100). Each particular gonococcal strain expressed only one P.I.

Cleavage of P.IB subunits in situ by trypsin or chymotrypsin yielded two membrane-bound fragments whose ^{125}I-peptides seemed to account for the entire P.IB subunit (25). This pattern suggested that the subunit was oriented with its terminal portions buried in the matrix of the OM, and its

central region exposed to the exterior of the organism as a loop-like structure, similar to a hairpin. P.IA subunits were cleaved by proteinase K to yield a single membrane-associated fragment which was slightly smaller than the intact P.IA subunit (13). This finding suggested that P.IA subunits had one of their termini exposed on the surface of the organism. P.IA and P.IB subunits are quite different in their surface exposures and orientations in the gonococcal OM. Blake and Gotschlich (22) purified these two species of P.I (34,000 and 32,000 daltons) using hexadecyltrimethylammonium bromide (CTB) together with N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent-3,14). The purified P.I reacted with antiserum prepared against the homologous organism. Upon treatment of the purified 34,000 dalton P.I with trypsin and chymotrypsin, proteolytic fragments were generated that were similar to those generated when intact gonococci were treated with these proteases. The amino acid composition and N-terminal amino acid sequences of these purified P.Is were similar to those of other major OM proteins from S. typhimurium and E. coli except they had a decreased content of hydroxyl-containing residues and completely lacked tryptophan.

Newhall et al. (209) investigated the organization of the OM proteins of N. gonorrhoeae using two dimensional SDS-PAGE together with cross-linking agents. They reported that P.I (MW:34,000) could be cross-linked: (i) to itself to form a trimeric complex; (ii) to a 28,000-molecular weight OM protein to form a bimolecular complex; and (iii) to the 28,000-molecular weight OM protein in a 3:1 ratio. The formation of these complexes was independent of colony type, pH during growth, and presence of genetic loci for drug resistance or hypersensitivity. A similar observation was obtained by Leith and Morse (163) who observed that, at least in certain

regions of the OM, P.I and P.III were found in close association, which may be important in antigenic presentation and interaction with the host. McDade et al. (181) examined the cross-linking results from serotype-specific OM vesicles (SSV) and suggested that PI and PIII interacted hydrophobically to form a trimeric unit in situ and that these two proteins seemed to be responsible for serotype specificity.

Wong et al. (321) analyzed one-dimensional peptide maps of P.I following digestion with either staphylococcal V8 protease or alpha-chymotrypsin. Six of eight P.I digests exhibited distinct peptide maps. These investigators suggested that the gene pool coding for the P.I is diverse and reflected the complexity of the evolution of N. gonorrhoeae. Furthermore, strains of different serotypes possessed identical P.I peptide patterns suggesting that P.I alone may not be capable of imparting serotypic specificity.

Judd (135) used surface peptide mapping to compare the surface exposure of OM proteins. His results demonstrated that the P.I molecule of each gonococcal strain studied had unique iodinatable peptides exposed on the surface of whole cells and OMs. Many more radiolabeled peptides were seen in surface peptide maps of P.Is from radioiodinated OMs than in those from radioiodinated whole cells. The author suggested that P.I may possess strain-specific antigenic determinants and that the quantity and quality of these determinants may be different in OM vesicles than in whole cells.

Serological classification of N. gonorrhoeae based on P.I has been described by several groups (248,249,307). Wang et al. (307) divided gonococci into three groups, termed A, B, and C. Sandstrom and Danielsson (249) also serologically classified gonococci into three groups which they

designated WI, WII, and WIII. Serogroup WI corresponded to serogroup A, WII corresponded to serogroup B, and WIII to serogroup C. Sandstrom et al. (248) further analyzed the tryptic peptide map of P.I molecules and found that strains belonging to serogroup WI possessed P.IA, whereas strains belonging to serogroup WII and WIII possessed P.IB. Development of McAb directed against epitopes on gonococcal P.I molecules (295) facilitated further serological resolution among strains of N. gonorrhoeae. Gonococcal isolates have been assigned to serovars by their patterns of reactivity with either six P.IA-specific or six P.IB-specific McAb reagents (147). These studies have greatly enhanced the analysis of patterns of strain transmission and the evaluation of the effectiveness of gonorrhea control measures. Recently, with the use of oligonucleotide probes derived from the known amino-terminal sequence of the P.I (Carbonetti and Sparling, 1987) as well as P.IB-specific McAbs to screen a lambda gt11 bank of gonococcal DNA (Gotschlich et al. 1987), both P.IA and P.IB have been cloned and characterized. The gene sequence predicts P.I with characteristics typical of the porins of other Gram-negative bacteria. Hybridization analysis suggested that there is a single structure gene for P.I and that it is homologous between P.IA and P.IB.

2. Protein II (P.II)

A second class of OM proteins initially described by Swanson (280) as "leukocyte-association" factor have been designated Protein(s) II (P.II). Heckels (96) isolated the OMs from several strains of N. gonorrhoeae by extraction with aqueous lithium acetate and observed that P.IIs exhibited heat modifiability with subunit molecular weights being apparently larger when samples were solubilized by boiling as compared to solubilization at 37˚C to 60˚C. P.II was not present in every gonococcal strain. Swanson (282)

observed an association between these heat modifiable proteins with molecular weights varying from 24,000 to 30,000 by SDS-PAGE, colony opacity, and the property of intra-gonococcal aggregation. Thus, P.II(s) were also designated as "colony opacity-associated proteins", and were found to be more subceptible to hydrolysis by trypsin than P.I. The presence of these proteins was independent of the state of piliation of the organism. Studies using I^{125}-tryptic peptide mapping indicated a relatively high degree of structural homology among the P.II(s) from opaque colony forms of different gonococcal strains (284). Results of cleavage with proteolytic enzymes indicated that only one end of the protein was membrane-associated; the other end was exposed on the surface (25).

Lambden (153) characterized five different P.II molecules from opacity variants derived from a single gonococcal strain P9. The apparent molecular weight of these P.IIs ranged from 27,500 to 29,000. Each variant contained either none, one, or two P.IIs. All variants showed increased adhesion to buccal epithelial cells and were less sensitive to the bactericidal action of serum than the prototype transparent strain. Swanson (286) also identified and compared five different P.IIs from strain JS3. Four of these five were consistently associated with colony opacity. The electrophoretic migration for each of the five P.IIs from strain JS3 differed with regard to apparent molecular weight and the effects of temperature as well as 2-mercaptoethanol.

The high prevalence of colony opacity variants suggests that gonococcal populations were capable of presenting a variety of surface components to their external environment. Swanson and Barrera (287) used immunoprecipitation or coagglutination with whole gonococci and immunoblotting

to assess the comparative antigenicities of 11 different P.II moieties from four different strains. They determined that immunization with gonococci possessing a single P.II species elicited the formation of antibodies directed mainly at the homologous P.II when assessed by either immunoprecipitation or immunoblotting; however, immunization with isolated P.II elicited formation of cross-reactive antibodies with all (nearly all) P.II species as assessed on immunoblots. These antibodies mainly recognized the homologous P.II by immunoprecipitation. The authors suggested that all P.II moieties shared antigenic determinants, but these common antigens were not generally accessible on the organisms surfaces for interaction with antibody molecules; and, in general, the surface-exposed antigens of different P.II differed from one another.

Judd (136) examined the primary structure relationships and the surface exposure of five P.IIs of N. gonorrhoeae strain JS3 by surface peptide mapping. The results demonstrated that P.IIs were unrelated to either P.I or P.III in structure but were closely related to one another, sharing about two-thirds of the peptides generated by alpha-chymotrypsin. However, the variable peptides were not always among the exposed peptides, suggesting that the structural differences in the P.IIs occurred at a discrete site (or sites) of the P.II molecules and not randomly throughout the protein. By altering various regions of the P.II, the gonococcus can present a changing and confusing array of immunogenic sites which may be a mechanism by which gonococci evade the immune response of the host.

Blake and Gotschlich (24) purified and partially characterized a P.II. The amino acid composition of P.II(s) indicated a larger amount of arginine than P.I, which may account for its high susceptibility to enzymatic

cleavage by trypsin, and to a pI ranging from pH 9.0 to 9.5. P.II appeared to migrate as monomers during molecular sieve chromatography. This is in contrast to P.I, which exist as trimers in solution. Draper et al. (67) also purified and compared several different P.IIs (MW from 30,000 to 32,000) by their apparent molecular weight, amino acid content, isoelectric point, and by peptide and epitope mapping. Amino acid analysis showed that the P.IIs had a similar basic and hydrophobic amino acid composition. The isoelectric points were very basic (pI $\geq$ 8.5) and were similar for all variants. Peptide mapping indicated that some portions of the molecule were structurally conserved and others were variable. Epitope mapping with P.II-specific immune serum showed that the P.IIs within a single strain, or from different strains, contained common antigenic determinants. Each P.II also contained unique antigenic regions. Schwalbe (260) compared the sequence of the first 10 N-terminal amino acids of 3 P.IIs from one strain. Considerable conservation was observed, but the differences in primary sequence indicated that these proteins were probably the products of different structural genes. Results from genetic transformation experiments suggested that there were multiple P.II genes in the gonococcal genome. Salit et al. (246) reported that expression of Op protein (P.II) may be genetically linked to pilus production in some gonococcal strains. Stern et al. (270) subsequently cloned an Op gene to examine the possible linkage between Op gene(s) and pilus gene(s). They found that the Op genes mapped near the pilus expression site. Genomic blotting experiment with an Op gene probe revealed complex hybridization patterns but little heterogeneity among the genes of Op variants. These investigators speculated that colonial variation involving the Op protein is based upon recombination and/or gene conversion at the site(s) of Op

expression, in contrast to the pilus variation system, in which changes in expression were resulted from substantial genomic rearrangements. Zak et al. (327) observed that the antigenic variation of P.II commonly occurred during natural infection. Complete sets of isolates and sera from eight groups of sexual partners were analyzed. No particular correlation was noted between the expression of a different molecular weight P.II and the site of isolation. Antibodies to P.II were highly specific, reacting with only one of the P.II types produced by a single strain. These results suggested that the host immune response and tissue tropisms may be the important factor in antigenic variation. Black et al. (21) identified six electrophoretically distinct forms of P.IIs (designated P.IIa through P.IIf) within a single strain and isolated colonial variants that expressed only one of the six different P.II forms. Two McAb that bound specifically and differentially to P.IIb-expressing variants and P.IIa-expressing variants respectively were bactericidal. Analysis of the P.II profiles of survivors of antibody killing indicated that multiple P.II species may be expressed on a single bacterium and that P.II switching in the gonococcus was not random. Recently, Schwable et al. (261) studied P.II variation among isolates from an outbreak caused by a single gonococcal strain. At least seven distinct P.IIs were observed; no single protein or combination of proteins predominated in the different isolates, and there was no association of P.II profile with site of isolation. Gonococci recovered from the same patient at different times had different P.II profiles. Similarily, Lammel et al. (156) studied five male-female consort pairs infected with the same strain of N. gonorrhoeae and found that they often had different antibody reponse to p.IIs and other gonococcal antigens. Thus, it is evident that P.II variation occurred in vivo.

3. Protein III (P.III)

The reduction-modifiable migration of one of the gonococcal OM proteins (P.III) was first described by McDade et al. (181). P.III has a molecular weight of 30,000 in the absence of mercaptoethanol and 31,000 in the presence of this reducing agent. P.III can be cross-linked to P.I within membranes using bifunctional reagents (163,181). From cross-linking experiments, it appeared that P.I exists as a trimer with one P.III molecule closely associated with this trimer. Exactly how this structure is arranged in the OM is not known. Wong et al. (321) used one-dimensional peptide analysis to show that the staphylococcal V8 protease- and chymotrypsin-generated peptides of P.III from several different serological strains were identical. Antiserum raised against P.III of one strain cross-reacted with P.III of all strains regardless of serotype. They concluded that P.III was a species-specific protein and was identical in all strains of gonococci. Judd (134) used ^{125}I-peptide mapping to study the comparative structures of the 30,000- and 31,000-dalton subunit forms of P.III from four different strains. He found that both the total and surface-labeled I^{125}-peptides of P.III were vitually identical. Swanson et al. (291) studied the antigenic properties of P.III by using a McAb in Western blot transfer and immunoprecipitation reactions. All three gonococcal strains examined exhibited identical reactivities of their P.III with this McAb. Both P.III and P.I were present in the immunoprecipitates regardless of the gonococcal strain used. This study confirmed previous cross-linking experiments suggesting that P.III and P.I were closely associated in the membrane of the gonococcus and that P.III was a surface-exposed antigenic moiety common to diverse strains of gonococci. The surface radioiodinated

peptide maps of P.III from whole gonococcal cells and from isolated OMs have also been examined and compared (135). P.IIIs appeared to have the same portion of the molecule exposed on the surface of different gonococci or OMs. However, different peptide fragments were seen in the surface peptide maps of P.IIIs from radiolabeled OMs than were seen in those from radiolabeled whole cells. The immunological significance of this observation remains obscure.

4. Protein IV

Swanson (285) observed a 44,000 dalton protein present on the surface of gonococci following radioiodination and immunoprecipitaion with rabbit sera against whole gonococci. Zak et al. (327) also found several patient sera contained antibodies to a common surface protein with a molecular weight of 43,000 that was present in all strains tested and suggested that this protein be designated as Protein IV. The terminology has not been well accepted and the characterization and significance of this protein remains to be elucidated.

5. Iron-regulated proteins

Norqvist et al. (215) observed the presence of several high molecular weight proteins (76,000, 86,000 and 97,000 daltons) in OM protein profiles when gonococci were grown under iron starvation. These iron-regulated proteins were heterogeneic with respect to both relative migration and the number produced between different strains. Only one protein with an apparent molecular weight of 97,000 appeared to be conserved among all gonococcal strains analyzed. The observation regarding the iron-regulated proteins of N. gonorrhoeae was confirmed by Mietzner et al. (193). In addition, these investigators identified a new iron-regulated protein with an apparent molecular weight of ca. 37,000 which was common to all gonococcal

strains examined. This protein comigrated with P.I under normal Laemmli gel conditions. By increasing the ionic strength of the separating gel buffer, they were able to separate P.I and the 37,000 dalton protein. The 37K protein stained poorly with Coomassie blue. However, when a silver stain was used, this protein appeared as a major component of the gonococcal OM. Most of the other iron-regulated proteins varied in their expression between strains which indicated that they were not coordinately regulated. Peptide maps showed that the 37,000 protein was distinct from P.I but was identical between strains of the WI and WII serogroups. Similar observations were reported by West and Sparling (311) who examined the expression of these iron-regulated proteins during growth in the presence of several different iron sources. Mietzner et al. (194) have subsequently purified the 37,000 dalton protein and showed that it had a pI > 9.35. Analysis of peptide map of the 37,000 dalton protein from meningcocci and gonococci suggested that these proteins shared several common peptides. Western blotting and an enzyme-linked immunosorbent assay were used to examine the heterogeneity of the antigenically-related 37,000 dalton proteins from 57 strains representing species of nine Neisseria and Branhamella catarrhalis (192). The data indicated that the antigenically-related 37,000 dalton proteins were conserved among the strains of N.gonorrhoeae, N. meningitidis, N. lactamica, and N. cinerea tested. Preliminary studies on patients suffering from gonococcal infection indicated that there was an antibody response to the 37,000 dalton protein and supports the contention that gonococci may produce this protein in vivo. The conservation of these antigenically related 37,000 dalton proteins, particulary among the pathogenic members of the genus Neisseria, implies that they may play a key role in iron acquisition and serve a common function in

pathogenesis.

6. H.8 antigen

Cannon et al. (47) reported the isolation of a McAb that recognized a determinant common to all gonococci and meningococci, but only rarely to other nonpathogenic Neisseria species. This determinant designated H.8 antigen, was found to be a surface-exposed, protease-sensitive, and heat modifiable OM component distinct from previously described gonococcal OM proteins. The evidence that H.8 antigen was distinct from P.II or pilin was primarily indirect, in that the presence and electrophoretic mobility of the antigen was unaffected by demonstrated differences in possession of P.II or pili. Also, there was no binding of the H.8 specific McAb to purified pilin. The H.8 antigen failed to stain with Coomassie blue and only faintly stained with a silver stain. Black (20) subsequently cloned the H.8 antigen gene into the lambda phage Sep6 which should facilitate an investigation into the clinical utility of H.8 antigen and a determination of its possible function in gonococcal pathogenesis. Recently, Strittmatter et al. (271) analyzed the methyl-ester derivatives of purified, hydrolyzed H.8 by gas liquid chromatography. Two lipoidal components were found suggesting that the H.8 antigen may be a proteolipid.

7. Outer membrane protein-macromolecular complex (OMP-MC)

Newhall et al. (209) described an OM protein-macromolecular complex (OMP-MC) which when purified by gel filtration and analyzed by SDS-PAGE exhibited an apparent molecular weight of 800,000. This complex comprised 10 to 13% of the total protein mass of isolated OMs. However, after reduction and alkylation of this complex, subunits with an apparent molecular weight of 76,000 were recovered. OMP-MC was detected in all gonococcal

strains tested but not in several other Gram-negative species. Hanson et al. (89) further characterized the complex and reported that the OMP-MC had a pI of 7.6 and was a homopolymer of 10 to 12 apparently identical 76,000-dalton subunits. They also confirmed that OMP-MCs isolated from various strains of N. gonorrhoeae shared structural homology.

III. Components of gonococcal cell envelope and their relationship to pathogenicity

The bacterial cell surface is a mosaic of macromolecules that constitutes the interface between the organism and its external environment. As such, these surface molecules collectively exert an important influence on the interaction of the bacterium with host tissues, cells, and molecules. In order to cause disease, a pathogen usually (i) enters the host, (ii) multiplies in host tissues, (iii) resists or fails to stimulate host defenses, and (iv) damages the host. The microbial products responsible for these processes are the determinants of pathogenicity (or virulence), and many of them are surface components. The mechanisms by which these essential steps in disease production are accomplished are very complex, several determinants are usually involved in the overall effect. Similar to other mucosal pathogens, the gonococcus also has various strategies that allow it to live on different mucosal surfaces, invade tissue, and evade multiple host defenses.

A. Components that contribute to entry to the host

It is generally accepted that bacteria must attach to host cells before they can cause infection. Attachment is mediated by cell-surface structures termed "adhesins". The distribution of specific receptors for adhesins determines many of the tissue and species tropisms observed for many bacteria (274). Gonococcal surface structures that mediate attachment have received considerable attention because interrupting attachment to the mucosal epithelium may be critical for prevention of infection. Gonococcal pili are the primary mediators of adherence to epithelial cells. Piliated gonococci attach more successfully than nonpiliated variants to human amniotic cells (278), sperm (120), erythrocytes (42), buccal epithelial cells (229), neutrophils (229), vaginal epithelial cells (175), and nonciliated cells in fallopian-tube organ cultures (223). Cells from tissue sites of natural gonococcal infection bound more pili per square micron than cells from sites that were not usually infected (219). This suggested a greater concentration of pilus receptors or differences in the chemistry of receptors on cells from common sites of infection. The pilus receptor(s) has yet to be fully characterized. However, the attachment of pili to Chinese hamster ovary cells was reduced by pretreating the cells with exoglycosidases or trypsin indicating that the receptor is a glycolipid or glycoprotein or both (43).

Gonococcal pili generally contain two methionine residues, one at position 7 and the second at about position 92. CNBr treatment yields three fragments, CNBr-1, which is the N-terminal heptapeptide; CNBr-2, a peptide of about 90 amino acids, and CNBr-3, the carboxy-terminal peptide of about 80 amino acids. CNBr-2 was found to be immunorecessive and to encompass a highly conserved region that mediates receptor binding. CNBr-3 was immunodominant

and comprised a variable region that confered type-specific antigenicity (244,259).

Gonococcal opacity-associated proteins (p.IIs) are alsoe involved in mediating attachment as suggested by the observation that selected opacity variants adhered better to some tissue culture cells than transparent colony forms even when the former were nonpiliated and the latter were piliated (124). Swanson and colleagues (142,292) demonstrated that attachment via P.II(s) was independant of pili. Lambden and co-workers confirmed the previous observation by showing that different P.II variants of strain P9 behaved differently with regard to their adherence to buccal cells, erythrocytes, and polymorphonuclear leukocytes (153). Moreover, variations in P.II are sometimes accompanied by changes in the structure, antigenicity, and function of pili (246). The presence of P.II is generally accompanied by intergonococcal aggregation (280), which is an additional kind of interaction, that may be important in promoting continuing gonococcal infections by enhancing retention of progency gonococci at a mucosal site to which their parent or sister organisms are attached (124). Thus, gonococci possess the ability to modulate their adhesion properties to cope with different anatomic/environmental niches that are encountered in the human host. This modulation might derive from either a gain or loss of particular P.IIs as well as from structural changes in pili with resultant changes in their biological behaviors.

B. Components that contribute to multiplication *in vivo*

An essential attribute for pathogenicity is the ability to multiply in the environment of the host. The requirements for multiplication usually are nutrients, minerals, the appropriate Eh and pH. Iron is essential for bacterial growth and to ensure the supply of iron, many microbial species synthesize iron chelators called siderophores, especially under conditions of iron deficiency (208,276). Recent data does not support the existence of a siderophore-mediated iron-uptake system among the pathogenic *Neisseria* spp. (214,311). Gonococci possess the ability to utilize the iron bound to transferrin as well as in heated human serum for growth, whereas the growth of most commensal *Neisseria* spp. is inhibited by transferrin (191). With respect to lactoferrin, approximately 53% of selected gonococcal isolates and 24% of commensal *Neisseria* were able to use it as a iron source (190). If lactoferrin and transferrin are "nonspecific" defense mechanisms, one may conclude that gonococci are often able to overcome the effects of such nonspecific defenses. It has been suggested that the ability to utilize mucosal lactoferrin as a source of essential iron may contribute to the ability of gonococci to cause symptomatic urethritis or salpingitis (190). Also, the inability of AHU^- strains of *N. gonorrhoeae* to utilize lactoferrin-bound iron has been linked to the high association of these strains with asymtomatic infections (35). Although the exact mechanisms by which gonococci obtains iron from the host have not been characterized, the induction of iron-regulated proteins under iron-stressed conditions are thought to play an important role.

C. Components that interfere with host defenses

Many pathogens are apparently well equipped to compete with local antibodies. Local immunoglobulins that develop during gonococcal infections are mainly secretory IgA as well as specific IgG antibodies (35). Virtually all pathogenic Neisseria spp., but none of the nonpathogenic Neisseria spp. produce one of two IgA_1 proteases that cleaves IgA_1 at the hinge region, releasing intact Fc and Fab fragments (226). The clinical importance of the gonococcal IgA_1 proteases is of some uncertainty. Koomey et al. (150) constructed IgA_1-protease deficient gonococcal strains by in vitro mutagenesis. Cooper et al.(53) subsequently examined the virulence of these IgA_1-protease deficient strains in a human fallopian tube organ culture. No differences were detected in the ability of the mutants to attach, damage, and invade human fallopian tube mucosa when compared to the parental strains, suggesting that IgA_1 protease might not be an critical determinant in the initial encounter with previously uninfected human mucosa.

The functional characteristics of P.I have been studied by Blake et al. (23,170). They reported that the asymmetric insertion of purified P.I into lipid bilayers resulted in the formation of a pore. Similar results were observed when whole gonococci were added to the artificial lipid planar bilayer system. Furthermore, using human red blood cells as a model membrane to assess the orientation of the inserted P.I, they determined that the transfer of P.I into lipid bilayers was not the result of P.I releasing from the gonococcal OM, becoming soluble in the aqueous phase, and then inserting into lipid bilayer. Nor did it appear to result from a fusion between OM vesicles containing P.I and the lipid bilayer. Rather, a vectorial transfer of P.I from the OM directly into the lipid bilayer occurred as the OM of the

gonococcus approached the lipid bilayer. It was speculated that the transfer of P.I from the gonococcal OM to the mucosal epithelial cells is the process that alters the membrane potential and triggers the internalization of gonococci by these epithelial cells thus further facilitating the invasive capabilities of these organisms.

Epidemiologic evidence linking the ability of gonococci to cause disseminated infection with the presence of a particular antigenic type of P.I has also been obtained (48). Two pieces of preliminary data further implicate P.I as an active anticipant in the infectious process. One is that P.I interacts with the intracelluar regulator, calmodulin (252). The other is that P.I-treated PMNs released 70% less beta-glucuronidase and approximately 45% less lysozyme than untreated PMNs (23).

Another striking feature of N. gonorrhoeae is its ability to undergo substantial alteration in cell-surface characteristics during both natural infections and during growth in vitro. One of these changes is the expression of pilus protein. The gonococcus apparently possess both an on-off switch for pilus production and a whole repertoire of genes to express many serologically distinguishable pilin types through substanial genomic rearrangements. Protein II is another gonococcal surface antigen that can undergo both phase and antigenic variation. Stern et al. (270) showed that the gonococcal chromosome contains multiple copies of DNA sequences that hybridize to a cloned P.II gene. They detected no major rearrangements in the chromosome of colony variants differing in P.II expression, but found minor differences in sequence between the expressed and unexpressed forms of the P.II gene. The mechanism of regulating P.II gene expression has yet to be determined. The proximity of genes for P.II and pilus protein raised the possibility that

there may be common elements in the regulation of the two proteins. The ability of N. gonorrhoeae to present a variety of new antigenic sites might play a crucial role in helping the organism to adapt to different environmental conditions as well as to evade the immune surveillance of the host.

A high proportion (ca. 49%) of gonococci in urethral exduate from male patients with acute gonorrhea were found to be associated with polymorphonuclear phagocytes (49). Electron microscopic observations indicated that the majority of these organisms were intracellular and while many were in the process of disintegration, others appeared morphologically intact (216,308). Gonococci could also be detected in the buffy coat cells of patients with disseminated gonorrhea by a fluorescent antibody staining technique (319). The intracellular survival and multiplication of gonococci in human PMNs from peripheral blood and from urethral exudate have been demonstrated using penicillin (303) or spectinomycin (49) plus fresh human serum to kill extracellular organisms. The cytotoxicity of gonococci for human peripheral blood PMNs tested in vitro was shown to be caused by a factor or factors produced by viable gonococci and not by LPS per se. However, these factors and the mechanism by which gonococci interfere with intracellular digestion by phagocytes is still not clear. Several different strategies have been suggested by which gonococci resist phagocytosis and/or digestion: (i) to reduce or inhibit chemotaxis. For instance, gonococci isolated from patients with disseminated infection activate complement slowly and are poor stimulators of chemotaxis as compared to gonococci isolated from uncomplicated infection (59); (ii) to decrease phagocyte membrane fluidity. Senff et al. (263) determined that P^+ but not P^- gonococci decrease PMN membrane

fluidity following incubation with these cells. Since membrane fluidity is essential for ingestion, they suggested this might contribute to the ability of piliated gonococci to avoid phagocytosis; and (iii) to reduce the release of primary granules from phagocytes. The oxidative metabolism of neutrophils was stimulated far less by virulent gonococci than by ingested avirulent organisms. It was suggested that survival of attached gonococci on the surface of oxidatively active neutrophils might be due to the failure of the neutrophil to release myeloperoxidase from the primary granule. However, there were also data which suggested that the problem resulted from the inability of the neutrophil to incorporate attached organisms into the confines of a phagosome where oxidative matabolites could exert their toxic effects in the proper milieu (63).

Environmental factors have been shown to alter the chemical composition, metabolism, and cell envelope structure of batch-grown N. gonorrhoeae (93,215). Thus, they might also be important factors which contribute to pathogenicity. Using continuous culture, Leith et al. (164) observed that the concentration of dissolved oxygen affected gonococcal OM protein composition. In addition, Morse et al. (206) demonstrated that resistance of gonococci to killing by normal human serum could be modulated by the nature of the growth condition in a chemostat. These reports indicated that gonococci were able to adapt to different environmental conditions in vitro, even though the environmental conditions were set to resemble those encountered in vivo.

Evidence that gonococci interact with the human host and to turn the host's response to it's advantage have also been obtained. McCutchen et al. (180) as well as others (234) have reported the presence of an IgG antibody in

normal human serum that is able to block serum-mediated killing of gonococci. This blocking antibody apparently bound to gonococcal OM proteins and may be important in helping gonococcal disseminate (234). Morse et al. (205) observed that a large proportion of gonococcal strains isolated from the rectum and urethra of homosexual males possessed the Mtr-phenotype when compared to matched isolates from the rectum and urethra of heterosexual females or the urethra of heterosexual males. Strains expressing the Mtr-phenotype were more resistant to growth inhibition by fecal lipids than were non-Mtr strains. These data suggested that the mtr locus conferred a selective advantage in host environments that were rich in hydrophobic compounds.

D. Components that contribute to host damage

Elucidation of the mechanisms by which gonococci produce mucosal disease has been hampered by the lack of a suitable animal model. However, the development of techniques for maintaining human fallopian tubes in organ culture (183) has provided a useful *in vitro* model for studying the interaction of gonococci with human genital mucosa. Gonococcal damage to human fallopian tube mucosa was associated with attachment of gonococci to nonciliated mucosal cells, loss of ciliated cells from the mucosa, and subsequent invasion of nonciliated cells by gonococci (309). Using fallopian organ culture, McGee *et al*. (184) observed that P^- gonococci damaged tissues, although at a slower rate than P^+ gonococci, a result which indicated that factors in addition to pili were involved in virulence. Filter-sterilized supernatant from organ cultures infected with *N. gonorrhoeae* rapidly damaged fresh organ cultures in a similar fashion (185). Thus, some of the damage to the mucosa seemed to be mediated by one or more subcellular toxic factors. Melly *et al*. (186) examined the characteristics of this toxic activity and suggested that gonococcal LPS might be the principle mediator of the damage to the fallopian tube mucosa. Gregg *et al*.(78) confirmed that damage to the organ culture occurred with purified LPS and showed that the lipid A component was essential.

Evidence that peptidoglycan (PG) is important in pathogenesis is accumulating. Petersen *et al*. (224) first reported the capacity of gonococcal PG to consume complement in human sera. Melly *et al*. (188) showed that two PG fragments (anhydro-monomer and Chalaropsis-monomer) caused sloughing of ciliated cells from the mucosa of human fallopian tubes in organ culture that resembled the damage observed during active gonococcal infection and that

produced by fiter-sterilized toxic supernatant fluids from gonococcal-infected organ cultures.

Fleming et al. (71) studied the arthropathic activity of purified O-acetyled PG (O-PG) and non-O-acetylated PG (non-O-PG) in male lewis (LEW/N) rats. They observed that macromolecular O-PGs caused more extensive paw swelling than did their non-O-PG counterparts, suggesting that persistence of hydrolase-resistant and extensively O-acetylated PG may be important for the optimal expression of arthropathic activity. The ability of purified gonococcal PG to induce arthritis in this animal model supports the hypothesis that PG plays a role in the pathogenesis of gonococcal arthritis.

In summary, gonococcal virulence is the result of many different attributes that contribute to different steps in the complicated series of events we recognize as infection. Expression of virulence also depends on a large number of host variables, including nonspecific and specific immune defenses. Interruption of the process of infection is undoubtedly possible at many different steps. Unfortunately, relatively little is known about the precise roles played by gonococcal surface antigens or components in pathogenesis due due to the complicated structure of gonococcal cell surface.

VI. Membrane proteolipids --- an overview

A. Introduction/Definition

The primary structure of many proteins in their "mature" functional state includes various types of chemical substituents that have been added onto the polypeptide backbone. Among these substituents are oligosaccharides, nucleosides, phosphate, acetyl, formyl, and methyl groups. Their addition to the polypeptide occurs both during nascent chain biosynthesis and later as the protein reaches its final destination. In addition, membrane proteins from a number of enveloped viruses and animal cells contain small amounts of tightly-bound lipid. The lipid consists predominantly of long-chain fatty acids and appears to be linked covalently through an ester bond to hydroxy-amino acids in the polypeptide chain. This modification appears to influence the interaction of some proteins with membranes. The covalent attachment of lipid to proteins in higher organisms was first reported over 30 years ago. Our knowledge of similar proteins in bacteria was mostly based on the discovery by Braun in 1969 of a "lipoprotein" in the E. coli cell wall (32). Folch-Pi and Lees (72) were the first to describe the presence of a protein material in brain myelin that was insoluble in water and aqueous solvents, but soluble in chloroform:methanol mixtures. It was assumed that the proteins involved were combined with a lipid moiety that gave the complex its particular lipid-like solubility. To designate this new type of substances, the term "proteolipid" was coined to indicate that they were lipoproteins with some of the physical properties of lipids. Subsequently, various non-myelin proteins extractable from membranes by organic solvents were also classified as proteolipids (73). The terminology, therefore, was

based on an experimental procedure, applicable to proteins on the basis of their solubility in lipophilic solvents. Lees et al. (162) pointed out the difficulty in using an experimental procedure to classify proteins. Nevertheless, in the absence of functional criteria, the term proteolipid is useful for describing certain hydrophobic membrane proteins. In a more recent review, Schlesinger (253) proposed an alternative definition for proteolipid, namely a protein that contains a lipid moiety as part of its primary structure. Proteolipids defined in this way would be analogous to glycoproteins, phosphoproteins, and nucleoproteins which are terms based on structural features of their respective entities. Unfortunately, it is not feasible to use the term lipoprotein because of the human plasma lipoproteins. The term "lipoprotein" is well-entrenched in the literature to identify a water-soluble complex composed of specific proteins and lipids. As to the bacterial lipoproteins and most of the viral glycoproteins, the term is appropriate, but these modified proteins are not considered to be the same as classical proteolipids, since they contained bound lipids and were not extractable by organic solvents.

Schmidt (254) introduced the term "acylprotein" for all proteins whose primary structure is modified through covalently bound fatty acids whether they were soluble in organic solvent or not, and retained the definition of proteolipid originally introduced by Folch-Pi and Lees (72) for those proteins that were soluble in organic solvents. Since several different terms have been used to describe a protein with covalently attached fatty acids, we decided to adopt the definition of Schlesinger (253) for gonococcal membrane proteins that are modified with fatty acids. This was done in order to avoid the confusion with Braun's lipoprotein that is covalently bound to the peptidoglycan of E. coli (32).

B. Lipoprotein from the outer membrane of E. coli.

1. Characterization of peptidoglycan-bound lipoprotein

a. Isolation

Schnaitman (255) used cell disruption in a French pressure cell followed by membrane separation on a sucrose density gradient to isolate OM complexed to the peptidoglycan layer. Burnell et al. (45) modified this method to avoid the use of EDTA and lysozyme and obtained good separation of the OM and CM. The advantage of this modification was to separate the CM and OMs of heptose-less mutants, which could not otherwise be separated using the EDTA-lysozyme method (217). The method by which the cell envelope is isolated is of great importance in obtaining a pure peptidoglycan-lipoprotein complex. Treatment of cells or cell envelopes with NaOH, various detergents, or phenol before dissolving them in boiling 4% SDS greatly reduces the amount and the quality of the peptidoglycan-lipoprotein complex. Braun et al. (32) were the first to investigate the murein-lipoprotein of E. coli. Most of the components of the cell envelope (e.g., proteins, phospholipids, and LPS), are dissolved in boiling 4% SDS (30). The peptidoglycan-lipoprotein complex can be sedimented from the SDS-solubilized cell envelopes due to its high molecular weight (ca. 1-4 x 10^9). Thus, the isolation of peptidoglycan-lipoprotein complex greatly facilitated the studies on lipoproteins.

The peptidoglycan of E. coli consists of a polysaccharide backbone comprised of repeating units (N-acetylglucosamine-N-acetylmuramic acid) to which the peptide side chain (L-alaine-D-glutamic acid-meso-diaminopimelic acid-D-alanine) are linked through the lactyl group of the muramic acid. The peptide side chains form crosslinks between the polysaccharide chains (32).

Most of the structural studies on lipoprotein have used lipoprotein obtained by treating peptidoglycan with hen egg white lysozyme which cleaves only the glycosidic bonds between the C-1 carbon of N-acetylmuramic acid and the C-4 carbon of N-acetylglucosamine (19). The resulting products consist primarily of GlcNAc-MurNAc-L-Ala-D-Glu-meso-DAP-D-Ala, a dimer due to the cross linkage between the peptide side chain, and the lipoprotein containing 2-3 subunits of peptidoglycan. Lysozyme is apparently sterically hindered close to the lipoprotein attachment sites, so that more than one peptidoglycan subunit remains covalently bound to the lipoprotein.

b. Chemical characterization and spatial distribution

Braun et al. (34) digested the peptidoglycan-lipoprotein complex with trypsin and found that lysine was the only additional amino acid remaining with the peptidoglycan. The fact that lysine, which is not a constituent of the peptidoglycan, was found to be the terminal amino acid of the lipoprotein indicates that it represents the linkage between the peptidoglycan and the lipoprotein. The ratio of the amount of lysine to the known constituents of the peptidoglycan indicated that on the average, one lipoprotein molecule was covalently bound to every tenth repeating unit of the peptidoglycan. The main part of the lipid associated with the murein-lipoprotein complex was not removable by treatments supposed to disrupt non-covalent bonds, including strong detergent solutions such as hot 4% SDS and various organic solvents. The lipid component of lipoprotein does not contain beta-hydroxymyristic acid, the main constituent of the lipid A of the LPS, indicating that it was not due to the contamination by other cell wall components. The lipoprotein has an unusual amino acid composition consisting of about 63% polar amino acids;

neither glycine, cysteine, proline, phenyalanine, tryptophan, or histidine were present. On a weight basis, the lipoprotein accounted for more than 40% of the rigid layer (lipoprotein-peptidoglycan complex). The high content of lipoprotein made it conceivable that it was evenly distributed over the whole surface of the rigid layer. This was also suggested by electron micrographs of the rigid layer, in which a rough surface could be observed with a characteristic pattern, and also by finding that the 10^5 lipoprotein molecules per cell were spaced on average 103 Å apart along the polysaccharide chains of the peptidoglycan (310). From partial acid hydrolysates of pronase-digested peptidoglycan, the following peptides have been isolated: (1) diaminopimelyl-lysyl-arginine; (2) alanyl-glutamyl-diaminopimelyl-lysyl-arginine; and (3) glucosaminyl-muramyl-alanyl-glutamyl-diaminopimelyl-lysyl-arginine. Peptide 1 indicated that the lipoprotein was bound through the alpha-amino group of the presumably N-terminal lysine to the carboxyl group of diaminopimelic acid. Peptide 2 consisted of a peptide side chain of the murein to which the two amino acids of the N-terminal end of the lipoprotein, lysine and arginine were attached. Peptide 3 constituted a repeating unit of the peptidoglycan to which the peptide lysyl-arginine of the lipoprotein was bound.

The amino acid sequence of lipoprotein consists of 58 residues with an apparent molecular weight of 7,500. The repetitive nature of the sequence suggested a very conservative evolution in which a structural gene coding for 15 amino acids was duplicated once and then only half of this gene, coding for 7 amino acids was fused four times with the first 29 amino acids. Some deletions, but only a few exchanges of amino acids, have apparently occurred.

The lipid attachment site of the protein has also been analyzed by

combined partial alkali- and acid- hydrolysis of lipopeptides obtained by pronase digestion of the lipoprotein. Ser-Asx-Ala-Lys was found to be the sequence at the site of lipid attachment. When lipoprotein was extensively cleaved with subtilisin, only serine remained bound to the lipid. In addition, lipoprotein contained six serine residues whereas only four were found in the soluble peptides. Thus, the N-terminal sequence can be formulated as (Ser)-Ser-Asn-Ala-Lys (31). Hantke et al. (90) determined that glycerylcysteine at the N-terminal of the polypeptide chain was the attachment site of two ester-linked fatty acids. An additional fatty acid was amide-linked to the N-terminal group. The identification of this connecting link between the fatty acids and the polypeptide chain confirmed the covalent attachment of the lipid to the protein as proposed earlier. The amide-linked fatty acids were mainly palmitic acid (65%), palmitoleic acid (11%), and cis-vaccenic acid (11%). The ester-linked fatty acids were mainly palmitic acid (45%), palmitoleic acid (11%), cis-vaccenic acid (24%), cyclopropylene hexdadecanoic acid (12%), and cyclopropylene-octadecanoic acid (8%).

c. Conformation, location and possible function

Heat and detergent-treatment denatured lipoprotein. This denatured lipoprotein was usually resistant to trypsin and reacted with antiserum prepared against whole cells (29). The resistance towards trypsin and the unchanged antigenic specificity upon heating and treatment with SDS pointed to a stable secondary structure. In fact, measurements of the circular dichroism of purified lipoprotein suspensions revealed the spectrum of the molar ellipticity in the ultraviolet range at pH 7.6 was typical of a protein with about 80% alpha-helical content (29). Lipoprotein denatured by the isolation

procedure, resumed a highly ordered structure in aqueous solution after the SDS was removed. About 50% of the ellipticity of the lipoprotein was irreversibly lost under conditions (0.1M NaOH, 56°C, 1h) which cleaved the ester-linked fatty acids. Thus, the hydrophobic fatty acid residues may contribute to the stabilization of the highly ordered conformation. The pH stability of the lipoprotein is also remarkable. A short exposure to pH 12 was without effect, and between pH 4 and pH 10 lipoprotein could be stored for hours without a perceptible decrease in ellipticity. The distribution pattern of the hydrophobic amino acids in the amino acid sequence is very peculiar in that starting at the 4th amino acid (Asn), it can be seen that nonpolar residues occur constantly along the whole sequence at intervals of 4 and 3 residues, respectively, in a consistently alternating set. Since 3.6 residues make up one helical turn, all the hydrophobic amino acid residues would be aligned on one face of the helical rod. This proposed structural conformation is consistent with the previous circular dichroism findings (29). The ease with which the CM detached from the OM when cells are plasmolized suggested that the fixation of the CM to OM by the lipoprotein is unlikely. This kind of reasoning led to the hypothesis that the lipoprotein extended into the OM and firmly affixed the peptidoglycan layer to the OM. The problem was experimentally approached by solubilizing peptidoglycan labeled with radioactive diaminopimelate with lysozyme. The solubilized lipoprotein was also labeled, since two peptidoglycan subunits remained bound to lipoprotein. The CM and OMs were then separated by isopycnic sucrose density gradient centrifugation (217). More than 90% of the label was found in the OM (28). These results were also confirmed by using antibody prepared against lipoprotein both in passive hemagglutination and immune hemolysis inhibition

tests. Thus, it appeared that lipoprotein was affixed to the peptidoglycan layer and extended into the OM.

In aqueous solution, intermolecular interaction of lipoprotein along the hydrophobic face could lead to aggregation of molecules. Within the hydrophobic membrane environment, several alpha-helical rods could be grouped to form an ionic channel by intermolecular ionic interaction, between positively charged lysine and arginine residus and negatively charged aspartic and glutamic acid residues. The hydrophobic surface could also interact with other hydrophobic components of the membrane (fatty acids, hydrophobic regions of proteins). A possible structural role of the lipoprotein was to serve as an anchor for other membrane proteins and thus contribute to the stability of the cells (32). Although the distribution of amino acids in the lipoprotein is stongly repetitive, the attachment sites of the lipid and the peptidoglycan are not helical and do not fit into the repetitive design. These areas stick out of the helical portion of the molecule and probably serve as recognition sites for the enzyme system that transfers the lipid to the polypeptide chain and attaches the lipoprotein to the peptidoglycan. Based on the complete amino acid sequence, Inouye (113) proposed a three-dimensional molecular assembly model of the lipoprotein and suggested that the function of lipoprotein was to provide a tubular hydrophilic channel through the OM which served as a passive diffusion pore. So far, no experimental evidence favoring this hypothesis has been obtained.

Hirota *et al.* (104) isolated a mutant of *E. coli* which lacked both free and bound forms of lipoprotein due to a defect in producing mRNA active for their synthesis. The mutation leading to the loss of lipoprotein synthesis, referred to as *lpo*, seems to have arisen during production of an F'. This

mutant grew and divided normally and remained susceptible to bacteriophage lambda, ∅80, p1, p2, the T series, and f1,f2, and MS2 in its male derivatives. However, this mutant also exhibited some severe defects like increased production of OM vesicles, increased sensitivity to EDTA, cationic dyes, and sensitivity to detergents. In addition, there was considerable leakage of periplasmic enzymes, although the passive transport of a beta-galactoside was unchanged. Based on these physiological characteristics, it was suggested that lipoprotein was indeed involved in maintaining the integrity of the cell envelope structure by anchoring the OM to the peptidoglycan, but not in the vital processes of growth and division. Yamada et al. (323) reconstituted an ordered membraneous structure with purified major OM components and the lipoprotein-bearing peptidoglycan. The molecular organization of the reassembled membrane was the same as that of the intact cell envelope. These results suggested that another possible role of lipoprotein was in the assembly of the OM.

2. Murein-bound and -free forms of lipoprotein

Inouye (112) prepared double labeled E. coli cells by growth in medium containing [^{14}C]-arginine and [^{3}H]-histidine, and observed a major envelope protein with an apparent molecular weight of 7,500 on SDS-PAGE. This protein had a very high arginine to histidine ratio that was similar to what was previous found in the peptidoglycan-bound lipoprotein. Because trypsin or lysozyme had not been used in isolating the cell envelope, Inouye suggested that lipoprotein occurred also in a free form, not bound to peptidoglycan. The occurrence of a free form of lipoprotein was subsequently confirmed by additional labeling experiments coupled with amino acid analysis of the

extracted protein band from SDS polyacrylamide gels (114). In contrast to the free form of lipoprotein, the bound form could only be solubilized by 1% SDS after lysozyme-treatment of the cell envelope. The bound form exhibited a slightly higher molecular weight than the free form on SDS-PAGE due to the additional peptidoglycan fragments attached to it. Pulse-chase experiments with [^{3}H]- or [^{14}C]-arginine showed that the free form was not a degradative product of the bound form, but rather was a precursor of the bound form. The reaction between the two forms was reversible and there was a dynamic equilibrium between the two forms of proteins in the cell envelope. The reaction appeared to be energy independent. Inouye et al. (310) purified the free form of lipoprotein on a large scale. Hirashima et al. (103) further investigated the chemical properties of the free form and found that it contained the same amount of palmitic acid as the bound form but had neither diaminopimelic acid nor glucosamine. The ratio of the free form to the bound form of the lipoprotein in the cell envelope was estimated to be 2:1. According to Braun and Rehn (32), there were ca. 2.5 x 10^{5} molecules of peptidoglycan-bound lipoprotein per cell. Thus, the total number of lipoprotein molecules per cell would be 7.5x10^{5} making it the most abundant membrane protein in E. coli.

3. Biosynthesis of lipoprotein

Hirashima et al. (102) studied the biosynthesis of lipoprotein in a histidine auxotroph of E. coli under conditions of histidine starvation. These conditions led to the synthesis of lipoprotein as the only protein of the cell envelope synthesized de novo since the polypeptide chain of the lipoprotein lacked histidine. After 1 h of histidine starvation, synthesis of lipoprotein was almost unaffected; after 4 h the rate of biosynthesis decreased to 30%. Lipoprotein synthesis was inhibited by tetracycline and chloramphenicol indicating that the lipoprotein was synthesized on ribosomes de novo. In contrast to tetracycline and chloramphenicol, rifampcin (200 ug/ml) had little effect on the biosynthesis of the lipoprotein, suggesting that lipoprotein mRNA was extraordinarily stable with a half life of about 11.5 min (160). The observation that lipoprotein synthesis was more resistant to puromycin than that of other cytoplasmic and envelope proteins strongly indicated that it had a unique biosynthetic system. About 750,000 lipoprotein molecules are synthesized per cell in one generation. The lipoprotein mRNA may be the most abundant mRNA in E. coli. Thus, the isolation of this mRNA appeared to be feasible, and indeed it was purified as 7S RNA and shown to direct the synthesis of lipoprotein in a cell-free system; the lipoprotein was identified by immunoprecipitation and peptide mapping. Pirtle (225) sequenced the first 89 nucleotides at the 5' terminus of the lipoprotein mRNA and found that there were 38 nucleotides preceding the initiation codon (AUG) in the untranslated region at the 5' end of the mRNA. Halegoua et al. (83) characterized a new form of the lipoprotein of the E. coli OM that is produced when cells are treated with toluene. This new protein had the same carboxyl-terminal structure as the lipoprotein, but also contained an extra

sequence at the amino-terminal end. The amino-terminal residue of the new protein was found to be methionine in contrast to the glycerylcysteine observed in the lipoprotein. There were ca. 18 to 19 amino acid residues in the extra region that was enriched in hydrophobic amino acids (61-63%). The function of this extra region is unknown. However, since the region was enriched in hydrophobic amino acids, it most likely plays a role in the assembly mechanism of the lipoprotein in the OM. These hydrophobic sequences may also function as a signal peptide in directing the transfer of the lipoprotein across the membrane. These investigators proposed that the new protein be called "prolipoprotein". It was interesting that among the 5 amino acids which are missing in the lipoprotein (i.e., glycine, phenylalanine, tryptophan, proline, and histidine), glycine was the only amino acid found in the extra region. These glycine residues may have a significant role in the assembly mechanism of the lipoprotein in the OM.

Inouye et al. (116) analyzed the product synthesized in a cell-free system directed by the purified mRNA and found that the cell-free product had an extension of 20 additional amino acid residues at the amino terminus of the lipoprotein. The complete amino acid sequence of this extra region of the putative precursor, prolipoprotein, was determined. This sequence had several unusual features: (i) the extended region was basic and positively charged at neutral pH because it contained two lysine but no acidic amino acid residues; (ii) the region contained 3 glycine residues which were not present in the lipoprotein; it is particularly interesting that the last amino acid residue of the extended region is glycine, where a specific enzyme must process the prolipoprotein to produce the lipoprotein; (iii) sixty percent of the amino acid residues in the extended region were hydrophobic, in contrast to 38% in

the lipoprotein; and (iv) the distribution of these hydrophobic amino acids along the peptide chain was completely different from their periodic distribution in the lipoprotein. The extended region can be divided into four separate sections on the basis of the amino acid arrangement: 2 hydrophobic and 2 hydrophilic segments. Although a model was proposed for prolipoprotein attachment to the CM, it is still difficult to formulate the mechanism by which the peptide extension promotes the attachment of the prolipoprotein to the surface of the CM and the translocation across the CM into the OM. It should be noted that some secretory proteins in eukaryotic systems are also formed from precursors which have 16 to 20 additional amino acid residues at their amino termini (26). The accumulation of prolipoprotein with toluene treated cells indicated that the processing enzyme(s) for the prolipoprotein were inhibited or inactivated by toluene.

Hussain et al. (110) observed that prolipoprotein was found almost exclusively in the CM following treatment with the cyclic peptide antibiotic, globomycin. Globomycin was thought to be a substrate analogue of the signal sequence in prolipoprotein (117), or alternatively, it may bind to the diglyceride moiety in prolipoprotein, thereby interfering with its processing and translocation. In vivo labeled prolipoprotein could be immunoprecipitated with anti-lipoprotein immunoglobulin and the label could be chased to the lipoprotein both in vitro and in vivo. Globomycin inhibited the chase. The prolipoprotein contained both glycerol and fatty acid residues; no free sulfhydryl group was detected suggesting that the prolipoprotein possessed a glyceride which was covalently bound to the cysteine residue in the peptide, and that the removal of signal peptide occurred after the modification. In addition, the data also suggested that the processing enzyme (signal

peptidase) could cleave the glycyl-glycerylcysteine peptide linkage and the modification of the cysteine residue with glyceride was essential for the processing. Ito et al. (119) reported that prolipoprotein accumulated in E. coli strain MM18 cells containing malE-lacZ hybrid protein after induction with maltose. The prolipoprotein was not modified and lacked covalently linked glyceride (301). When the cell envelope from strain MM18 containing unmodified prolipoprotein was incubated in the presence of detergent with [2-^{3}H]glycerol-labeled cell envelope from E. coli strain JE5505 lacking murein-lipoprotein, incorporation of radioactivity into both prolipoprotein and processed mature lipoprotein was observed. Likewise, when [^{3}H]-palmitate-labeled JE5505 cell envelope was incubated in the presence of detergent with the MM18 cell envelope containing unmodified prolipoprotein, radioactivity was incorporated through an ester linkage into prolipoprotein and into mature lipoprotein through both ester and amide linkages. These results indicated that glyceryltransferase, O-acyltransferase, signal peptidase, and N-acyltransferase were involved in prolipoprotein modification and processing in vitro. Glyceryltransferase was inactive at pH 5.0; signal peptidase was active at this pH provided that the prolipoprotein had been modified by glyceryltransferase and O-acyltransferase. These results strongly suggested that the modification of prolipoprotein by these two enzymes precedes, and may in fact be a prerequisite for, the processing of prolipoprotein by signal peptidase. However, whether the insertion of nascent prolipoprotein into the CM occurs cotranslationally or post-translationally in vivo remains unknown. Yamada et al. (324) partially purified the signal peptidase from E. coli cells harboring a plasmid that carried the gene for this enzyme (lspA). The enzyme was localized to the CM and was active against

glyceride-containing precursors of the peptidoglycan-associated lipoproteins and many additional membrane proteins, but was not active against the unmodified precursor of the major lipoprotein. Lin et al. (164) characterized a novel mutant with an altered murein-lipoprotein. All of the post-translational modifications of prolipoprotein were aborted in this mutant due to the single amino acid substitution of aspartic acid for glycine at the 14th position of the prolipoprotein. The amount of murein-bound lipoprotein found in this mutant was greatly reduced when compared to the wild-type strain. This interesting finding verified the importance of glycine in the assembly of lipoprotein as previously mentioned, and also suggested that modification of the lipoprotein may be required for the assembly of lipoprotein into the cell envelope. Wu et al. (322) isolated globomycin-resistant mutants of E. coli. Approximately 2 to 5% of these mutants synthesized structurally altered lipoprotein. The majority of these mutants contained unprocessed and unmodified prolipoprotein. One mutant contained modified, processed, but structurally altered lipoprotein. Mutants containing lipid-deficient prolipoprotein or lipoprotein also showed increased resistance to globomycin. These results suggested that the inhibition of processing of modified prolipoprotein by globomycin may require fully modified prolipoprotein as the biochemical target of this novel antibiotic. Failure to isolate mutants containing cleaved but unmodified lipoprotein among globomycin-resistant mutants was consistent with the possibility that modification of pro-lipoprotein preceded the removal of the signal sequence by a unique signal peptidase.

Challopadhyay et al. (51) studied the biosynthesis of the diglyceride moiety of murein-lipoprotein by pulse labeling with [2-^{3}H]glycerol for 5 min

followed by a chase period. After a 1 h chase, there was a 10-to 15-fold linear increase in the incorporation of [2-^{3}H]glycerol into both the free and bound forms of murein lipoprotein indicating that the lipid moiety in lipoprotein was derived biosynthetically from one of the major phospholipid species in E. coli. Mutants defective in phosphatidylserine synthase and cardiolipin synthase were used to identify the putative precursor for the diglyceride moiety in lipoprotein. It appeared that neither phosphatidyl-ethanolamine nor cardiolipin was the precursor, because a moderate to severe decrease in the synthesis of either of these two lipids did not produce a decrease in the incorporation of pulse-labeled [2-^{3}H]glycerol into lipoprotein. Thus, phosphatidylglycerol was probably the donor of the diglyceride moiety in lipoprotein. However, there is no mutant that is defective in phosphatidylglycerol synthesis with which to directly verify this assumption. Biosynthetic studies of lipoprotein in cerulenin-treated cells showed that the incorporation of [2-^{3}H]glycerol into phospholipid proceeded at a rate equal to about 29% of that in the untreated cells, with more than 96% of the radioactivity recovered as phosphatidylglycerol. Under these condition, the nonacylated glycerol moiety of phosphatidylglycerol was exclusively labeled. These observation and the fact that phosphatidyl-ethanolamine was not labeled in cerulenin-treated cells suggested that the synthesis of phosphatidylglycerol from labeled sn-glycerol-3-phosphate and unlabeled cytidine diphosphodiglyceride is due to turnover of preexisting phosphlipid. In addition, the observation that cerulenin treatment enhanced the pulse-labeling and subsequent chase of [2-^{3}H]glycerol into the free form of murein lipoprotein suggests that the nonacylated glycerol moiety of phosphatidylglycerol was the immediate precusor of the glycerylcysteine

in lipoprotein. This is further supported by the observation that carbon 1 rather than carbon 3 of sn-glycerol was involved in the thioether linkage.

Biosynthesis of the acyl moieties in the murein-lipoprotein of E. coli was studied by pulse-chase experiments with [9,10-^{3}H]palmitic acid (150). A linear increase in [^{3}H]palmitate in both the ester- and amide-linked fatty acids of free form of lipoprotein suggested that the fatty acids in lipoprotein were derived from precursors present in a large pool, such as the acyl moieties of phospholipids. Double labeling of lipoprotein in medium containing [9,10-^{3}H]palmitic acid and [^{14}C]-arginine during growth of glycerol requiring mutants of E. coli revealed a severe inhibition of [9,10-^{3}H]-palmitic acid incorporation into the newly synthesized lipoprotein of glycerol-starved cells. In addition, periodate oxidation and dansylation of the free form lipoprotein synthesized by cerulenin-treated E. coli indicated that it still contained the amide-linked and at least one ester-linked fatty acids. These results indicated that the fatty acids in lipoprotein were not derived directly from acyl-CoA or acyl-ACP, but from the preexisting fatty acids of the phospholipids. Lai et al. (150) provided more direct evidence supporting the hypothesis that the acyl moieties in phospholipids were the precursors of the fatty acids in murein-lipoprotein by fusion of [^{3}H]-palmitate-labeled phospholipid vesicles with intact cells of an fadD mutant of E. coli. This mutant was used to prevent the reutilization of free fatty acids released during fusion and the subsequent chase. A linear increase was observed in the incorporation of [^{3}H]-palmitate into both the ester- and amide-linked fatty acids in lipoprotein during a 3 h chase after the fusion. Furthermore, there was no apparent difference among the three

major phospholipid species (phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin) in their ability to serve as the donor of acyl moieties for lipoprotein.

Recently, Hayashi et al. (91) found that the export of lipoprotein was affected in both SecA and SecY mutants of E. coli that were defective in the secretion of a number of outer membrane and periplasmic proteins. In both mutants, the accumulated prolipoprotein was unmodified with glycerol and was localized in the CM. These investigators concluded that the early steps in protein export were common to prolipoprotein and non-lipoprotein precursors. The pathways for the export of these two groups of precursor proteins diverge with regard to the modification and processing reactions which are late events in the export process.

Based upon previous studies, the post-translational modifications of prolipoprotein and biosynthesis of lipoprotein can be summarized in 5 steps: (i) the transfer of glycerol from the nonacylated glycerol moiety of phosphatidylglycerol to the sulfhydryl group of cysteine in prolipoprotein; (ii) transacylation of both saturated and unsaturated fatty acids from membrane phospholipids to the glycerolcysteine in prolipoprotein to give rise to the ester-linked fatty acids in lipoprotein; (iii) the proteolytic cleavage of the signal peptide at the amino-terminus of prolipoprotein; (iv) transacylation of a fatty acid from a phospholipid to the alpha-amino group of cysteine to acylate the amino-terminus of lipoprotein (free form of lipoprotein); and (v) covalent attachment of part of the mature and modified lipoprotein to peptidoglycan, presumably by transpeptidation (bound form of lipoprotein).

4. Peptidoglycan-associated lipoprotein

After incubation of whole cells or cell envelopes of E. coli strain B at 60˚C in 2% SDS and subsequent centrifugation, the pellet was found to consist of Braun's lipoprotein which was covalently attached to the peptidoglycan and another protein with an apparent molecular weight of 36,500 (OmpF protein). The latter protein could be removed from the peptidoglycan-lipoprotein complex by incubation in 2% SDS at temperature of 70˚C (238), or by incubation at 37˚C in 2% SDS containing 0.5M NaCl (207). Several porins such as the OmpF and OmpC protein of E.coli K-12 and S. typhimurium were also discovered under similar conditions. Since these proteins were not covalently bound to peptidoglycan and did not cross-react immunologically with Braun's lipoprotein, they were designated as peptidoglycan-associated proteins. Mizuno (197) observed a protein with an apparent molecular weight of 21,000 in the peptidoglycan-containing fraction of E. coli K-12 when the peptidoglycan was prepared by differential extraction of the cell envelope with 2% SDS at two different temperatures (30˚C for 1h followed by 50˚C for 1h). This 21K protein and the free form of Braun's lipoprotein were recovered in the soluble fraction after the second extraction, whereas other porins were retained in the insoluble fraction. The 21K protein was subsequently purified and analyzed for its amino acid and fatty acid content. Among the fatty acids found was hexadecanoic acid (C16:0), which accounted for 80 to 90% of the total fatty acids. The molar ratio of fatty acid:protein was 3:1. However, the amino acid composition was very different from that of Braun's lipoprotein in that Pro, Cys, Phe, His and Trp are present in the 21K peptidoglycan-associated protein. The incorporation of [^{14}C]-palmitic acid and [2-^{3}H]glycerol confirmed that this peptidoglycan-associated protein was actually a lipoprotein. It has been suggested that this protein is an

essential component of the cell envelope of Gram-negative rod-shaped bacteria, since all nine Gram-negative bacteria examined ,including both enteric and non-enteric bacteria, contained a protein with an apparent molecular weight of 21,000.

5. Additional membrane lipoproteins

Ichihara et al. (111) showed that E. coli contained several new species of cell envelope lipoproteins, in addition to the above mentioned Braun's lipoprotein and peptidoglycan-associated lipoprotein, when grown in medium containing [^{3}H]-glycerol or [^{3}H]-palmitic acid. Four of these new lipoproteins were localized to the OM, while other two species were found in the CM. The location of one species is unknown. Since the amounts of these new lipoproteins were small, especially when compared to Braun's lipoprotein which is the smallest lipoprotein on the basis of molecular weight, the possibility that they were artifical oligomeric derivatives of Braun's lipoprotein (BLP) or peptidoglycan-associated lipoprotein (PAL) was examined. This possibility was ruled out by the following evidence: (i) the new lipoproteins did not react with either the anti-BLP or the anti-PAL sera; and (ii) the proteins were present in a lpp mutant that lacks the structural gene for BLP. When cells were treated with globomycin, all of the newly found lipoproteins, as well as BLP and PAL, accumulated as precursor forms. All of the precursors contained glycerol and fatty acid(s). These results suggested that the structures of "signal" regions of all lipoprotein precursors and mechanisms of their processing might be similar to each other. On the other hand, E. coli might possess one species of signal peptidase that was specific for all the lipoproteins. To date, there are at least nine different lipoprotein species in E. coli. However, it is unclear whether the new

lipoprotein(s) interact with the peptidoglycan layer, or whether they also exist covalently bound to the peptidoglycan layer. The small quantities of these lipoproteins in a cell suggests that they are important funtionally rather than structurally.

C. Occurrence of lipoprotein in Gram-negative bacteria other than E. coli

The occurrence of lipoprotein in bacteria other than E. coli was first investigated for the murein-bound form (33). Lipoprotein in an amount equal to that in E. coli was found in three strains of Salmonella spp. The only differences in the amino acid composition observed was in the methionine content; E. coli lipoprotein contained two residues whereas Salmonella lipoprotein contained only one. In Serratia marcescens, there was as much lipoprotein bound to murein as in E. coli. However, the amino acid composition differed from that of E. coli in that only one methionine and only one threonine residue was present. In addition, histidine was present in somewhat variable amounts (up to one residue). In other instances, lipoprotein from Shigella and Citrobacter were found to react with the E. coli anti-lipoprotein serum (30). Interestingly, no murein-lipoprotein was found in Proteus mirabilis, P. vulgaris and Pseudomonas fluorescens. Hallegoua et al. (82) identified the free form of lipoprotein by SDS-PAGE in E. coli, S. typhimurium and S. marcescens. The identification of the protein band as lipoprotein (MW 7,200) was achieved by immunoprecipitation of the lipoprotein from the envelope suspensions of Salmonella and Serratia with antisera prepared against the free form of the E. coli lipoprotein. P. aeruginosa also contains a protein of the same size as the lipoprotein, but it is not certain whether the protein is the same structural protein as the lipoprotein from E. coli since it failed to react with the anti-lipoprotein serum (82).

The existence of a free form of the lipoprotein (MW 7,200) in P. mirabilis remains uncertain. This finding is consistent with previous studies by Braun (33) who fail to detect covalently-bound lipoprotein in this species. In contrast, Gmeiner et al. (77) used a similar methodology and were able to

isolate the covalently-linked lipoprotein from P.mirabilis and to compare it with the Braun's lipoprotein of E. coli. Amino acid analysis revealed: (i) the same set of amino acids was present except for methionine which was absent in the lipoprotein of P. mirabilis; (ii) the ratio of lipophilic and hydrophilic amino acid was essentially the same, although the P. mirabilis lipoprotein contained more acidic and fewer basic residues; (iii) P. mirabilis lipoprotein was composed of 60 amino acid residues as compared with 58 residues in the Braun's lipoprotein; and (iv) 55% and 59% of the lipoprotein molecules from E. coli and P. mirabilis, respectively, were linked to peptide cross-linked peptidoglycan dimers. Fatty acid analysis of the P. mirabilis lipoprotein gave 1.71 mol ester-linked and 1.14 mol amide-linked (mainly palmitic acid) fatty acids per mol lipoprotein. Although the lipoprotein from E. coli and P. mirabilis had a similar composition and molecular weight, they behaved differently in SDS-PAGE under various conditions. Also, there was a striking difference in the quantity of covalent lipoprotein in early stationary phase E. coli (1 lipoprotein per 10-12 subunits of peptidoglycan) and P. mirabilis (1 lipoprotein per 80 subunits of peptidoglycan). Gmeiner (76) has also reported and characterized a new murein-associated lipoprotein in the OM of P. mirabilis with an apparent molecular weight of 15,000. Because of its prominent appearence in the OM, it was regarded as a major OM protein in this organism. The amino acid composition of this new lipoprotein suggested that it was not closely related to the 7,300 dalton lipoprotein since it lacked histidine, proline, glycine, methionine and phenylalanine. Also, this low molecular weight lipoprotein was completely absent in exponential-phase wild type cells. However, the mode of fatty acid linkage and the fatty acid composition indicated that both lipoproteins might carry

the same type of lipid. Since a lipoprotein-free mutant of E. coli is able to assemble the OM in vivo (112), Hazumi et al. (92) suggested that the murein-associated lipoprotein might play an important role in this assembly process. The suggestion might be even more pertinent for P. mirabilis, where the covalently linked lipoprotein is completely absent in exponential-phase cells, whereas the new murein-associated lipoprotein occurs in much larger quantities in the OM of this organism than in E. coli.

Heilmann(98) reported that a (lipo)-protein covalently linked to the peptidoglycan was found in the murein of P. aeruginosa. Furthermore, Martin and his coworkers reported that particles, which were protein in nature, were observed on negatively stained murein of P. aeruginosa (176), and also suggested the presence of an analogous (lipo)-protein in this organism in a smaller amount. These findings are contradictary to the previous report by Braun (33).

In the course of characterizing the OM of P. aeruginosa, six major OM proteins, designated as proteins D,E,F,G,H and I were identified (198). Mizuno (199) has further purified and analyzed protein I. This protein had an apparent molecular weight of 8,000 and accounted for about 20% of total OM proteins. The protein lacked proline, valine, isoleucine, phenylalanine, tryptophan, and half-cystine and contained 0.89 mol of fatty acids (predominately C16:0) per mol of protein. In an in vivo labeling experiment, [2-^{3}H]glycerol was also incorporated into protein I. These results suggested that this protein was analogous to the free form of the Braun's lipoprotein. However, considerable differences were observed in the amino acid composition and the fatty acid content. Although the bound form of protein I exists in a smaller amount, it is not yet clear whether the glycerol

residue of protein I is present as glycerylcysteine at the amino terminus or what linkages are involved in the attachment of fatty acid to this protein.

Lugtenberg et al. (168) reported that all the Enterobacteriaceae strains tested contained one or more peptidoglycan-associated proteins, while P. aeruginosa, which does not belong to the family Enterobacteriaceae, contained no peptidoglycan-associated protein, i.e., no proteins remained associated with the peptidoglycan after extraction of the cell envelope at 60°C with 2% SDS. Hancock and Naikaido(84) showed that the solubilization of the OM proteins of P. aeruginosa in SDS solution at either 37°C or 100°C had no significant effect on the protein profile. In contrast, Mizuno et al. (200) found that two of the major OM proteins (proteins F and H) of P. aeruginosa remained with the peptidoglycan after cell envelopes were extracted with 2% SDS solution at 35°C. In addition, protein F showed anomalous migration on an electrophorogram that depended on the solubilizing conditions or pretreatment with trichloroacetic acid. However, no proteins remained with the peptidoglycan at temperature greater than 55°C. Protein H (MW 21,000) has subsequently been purified and found to be a novel peptidoglycan-associated lipoprotein. The E. coli 21K lipoprotein and protein H had similar amino acid compositions, but there was no apparent correlation between them and that of Braun's lipoprotein. It was speculated that the 21K protein of E. coli and protein H of P. aeruginosa were structurally and functionally equivalent in the cell envelope of these different species, since these proteins had essentially the same properties with the only difference being their quantity present in the cell envelopes; i.e., protein H was one of the major constituents of the OM of P. aeruginosa, while the 21K protein of E. coli seemed to be a rather minor component. The exact role of these

peptidoglycan-associated lipoproteins is still not clear. The conserved repetitive amino acid sequence of the Braun's lipoprotein suggests that there is a high evolutionary selection pressure preventing amino acid replacement or, since the lipoprotein probably plays a structural role, to the lack of neeed for considerable environmental adaptation in order to fulfill its function. Whatever the reason may be, it is feasible to expect the presence of lipoprotein-related structures in other species that are unrelated to E. coli. Recently, membrane proteolipids have been described in the members of the Mycoplasmataceae (Mycoplasma capricolum) (56) and Acholeplasmataceae (Acholeplasma laidlawii) (57), which are characterized by their lack of a peptidoglycan layer. Thus, the membrane proteolipids found in these cell wall-less bacteria strongly suggest that they play a major role in maintaining the structural integrity of the membrane.

D. Proteolipids found in Gram-positive bacteria

Beta-lactamases fall into three classes (127) that are distinguishable on the basis of size, substrate specificity, and sequence homology. Class A enzymes have a molecular weight of about 30,000, are preferentially active on penicillins, and share considerable homology with one another. Within this class are secreted penicillinases of the Gram-positive organisms Bacillus licheniformis, B. cereus, S. auerus and the periplasmic R6K beta-lactamase (formally called TEM) carried on plasmid pBR322 in E. coli (277). Beta-lactamases of this class, in contrast to the other two classes, exist both as a membrane-bound hydrophobic form as well as a soluble exoenzyme. Though the membrane-bound penicilinase can be converted to the exoenzyme, kinetic studies suggest that it is not an obligatory intermediate (157). Nielsen (210) has shown that the amino-terminal extension responsible for membrane anchorage in B. licheniformis carries a lipophilic modification which is analogous to the amino-terminus of the Braun's lipoprotein in E. coli. Smith et al. (266) identified the precursor of this penicillinase which has an apparent molecular weight of 36,000, but most interestingly the attachment of lipid moiety and the cleavage of the precursor can occur while the peptide chain is growing and being secreted. Globomycin prevents the net incorporation of both glycerol and palmitate into membrane-associated penicillinase without affecting total penicillinase production. Under the same conditions, globomycin does not prevent the attachment of palmitate or glycerol to the E. coli prolipoprotein but inhibits processing of the modified precursor to the mature lipoprotein. The difference can be explained that in B. licheniformis, globomycin blocks the derivatization of prepenicillinase and renders the unmodified precursor more available to the peptidase that normally

produces exopenicillinase. Lai et al. (151) also observed that B. licheniformis membrane penicillinase produced in E. coli (λpen) is a lipoprotein that contained a cysteinyl glyceride thioether linkage. However, the incorporation of [2-^{3}H]glycerol into the membrane penicillinase was low. DNA sequence analysis of the structural gene for this penicillinase revealed a tetrapeptide sequence of Leu-Ala-Gly-Cys within the amino-terminal part of the prepenicillinase. The same tetrapeptide also occurred in the signal sequence of the prolipoprotein of E. coli, and the cysteine residue in the tetrapeptide of prolipoprotein was modified to form glyceride-cysteine which became the amino-terminus of Braun's lipoprotein. The same modification was also found in the membrane penicillinase of S. aureus and B. cereus (211). These results suggested that the presence of the proper signal sequence was essential for the modification reaction to take place in both Gram-negative and Gram-positive bacteria.

E. Peptidoglycan-associated proteins of N. gonorrhoeae

Using electron microscopy Wolf-Watz et al. (320) observed that the OM of N. gonorrhoeae appeared more loosely associated with the peptidoglycan layer than was the case for E. coli. Amino acid analysis of the peptidoglycan indicated the presence of minor amounts of aspartic acid, glycine, ane threonine. The first evidence for peptidoglycan-associated protein(s) in N. gonorrhoeae was obtained by Hebeler et al. (93,94) who observed that growth pH markedly influenced the composition of the cell envelope. No accessory polymers were identified in association with the peptidoglycan component when cells were grown at pH 7.2 and pH 8.0. However, when cells were grown at pH 6.0, the amount of peptidoglycan increased 2 to 8 fold and contained an accessory protein(s) which accounted for 42% of the weight of the isolated peptidoglycan-protein complex. N. gonorrhoeae strain CS-7 produced large amount of peptidoglycan-associated protein when grown at both pH 6.0 and pH 7.2; all amino acids were apparently increased by an average factor of 1.5 at low pH. This increase in the amount of total proteins could be due to either an increased rate of synthesis or to a slower rate of degradation at pH 6.0. After digestion of the CS-7 peptidoglycan-protein complex with lysozyme, a major band with apparent molecular weight of 11-13,000 was observed with Coomassie blue on SDS-PAGE. It was proposed that this protein(s) was covalently attached to the peptidoglycan in a fashion analogous to the Braun's lipoprotein of E. coli. However, it has not been established whether there is a lipid associated with this protein or what residue in the peptidoglycan serves as the binding site for the protein. In addition, the exact physiological significance of this protein(s) remains to be answered. Presumably it plays a role in anchoring the peptidoglycan to the envelope.

Recently, Hill et al. (101) reported the presence of a 14K peptidoglycan-associated protein in N. gonorrhoeae strains JS1, JS3 and 638. ^{125}I-surface labeled gonococcal strains were subjected to differential SDS detergent extraction and a possible close association of several radiolabeled proteins was found; primarily P.I, P.III and a low molecular weight protein of approximately 14 kilodaltons. Trypsin treatment of purified murein sacculi prepared from radiolabeled cells with lysozyme, suggested that P.I and possibly the 14K protein were covalently associated with the cell wall. The 14K protein has been partially purified. Although the chemical nature of this protein has yet to be determined, the data suggest it might well be a peptidoglycan-associated lipoprotein. Recently, two additional gonococcal membrane proteins have been found to contain fatty acids. One is the H.8 antigen which has an apparent molecular weight ranging from 22,000 to 27,000 (272). The other gonococcal surface proteolipid was reported to have an apparent molecular weight of ca. 20,000 and a high content of glutamic acid. This proteolipid contains three unidentified lipoid components (ca. 5.7% of the total protein content), and was considered to be the determinant responsible for gonococcal resistance to killing by human phagocytes (218).

H.8 antigen forms a cone-shape band during SDS-PAGE that does not stain with Coomassie blue. Purified H.8 is free of C16:1, and C:16:0, as well as 3-OH-C14:0, 3-OH-C12:0, and C12:0 fatty acids, indicating that there was no contamination with gonococcal phospholipids or LPS. However, two lipid components not previously identified or described in the gonococcus were detected. Analysis by gas/liquid chromatography indicates that these components have an Rf value comparable to a carbon chain of C10 or C11 and

2-OH-C14:0 fatty acid.

H.8 and the enterobacterial lipoprotein lack phenylalanine and tryptophan as well as have similar Coomassie staining properties (272). However, there was no incorporation of [^{14}C]-glycerol into the H.8 antigen, suggesting that the substituted glycerol anchoris not present or that gonococci do not transport glycerol. Although cysteine was not detected by amino acid analysis, incorporation of [^{35}S]-cysteine has been demonstrated. There is no DNA homology between the lipoprotein gene and the gonococcal genome. Based on DNA sequence of the cloned H.8 gene, a lipoprotein leader consensus sequence and an amino-terminal cysteine were found. It is possible that the lipid constituent may be amide-linked to the amino-terminal cysteine. However, the information concerning the role of the H.8 protein in neisserial pathogenesis as well as whether it might be functionally analogous to Braun's lipoprotein is still unclear. Recently, H.8 antigen has also been purified from N. meningitidis group B (16). A preliminary analysis indicates the presence of a C9 and C11 straight-chain fatty acids lipid component. In addition, an epitope on the H.8 antigen that binds to a monoclonal antibody is labile to treatment with either 1 M HCl or 0.1 M NaOH at room temperature. The N-terminal 39-amino acid proline- and alanine-rich peptides were identical in both gonococcal and meningococcal H.8 antigen suggests that they might undergo the same mechanism for post-translational cleavage and coupling of lipids.

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Manuscript 1.

Membrane Proteolipids of *Neisseria gonorrhoeae*.

I. Covalent Modification of Membrane Proteins with Lipids

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ABSTRACT

Neisseria gonorrhoeae strains FA19 (wild-type envelope phenotype), FA171 (Mtr phenotype), and BR87 (Env phenotype) were grown in liquid medium supplemented with sub-inhibitory concentrations of either [^{3}H]-palmitic acid (10 μCi/ml), [^{3}H]-oleic aicd (10 μCi/ml), [^{3}H]-acetate (5 μCi/ml), or a [^{3}H]-amino acid mixture (7 μCi/ml). Cells were harvested at the end of the exponential growth phase, and the cell envelopes were isolated and analyzed by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fluorograms indicated that the membranes from each of the strains contained many labeled components. Identical fluorograms were observed when cells were labeled with either [^{3}H]-palmitic or [^{3}H]-oleic acid. Exhaustive extraction of the membranes with chloroform-methanol (1:1) removed phospholipids but did not affect the banding pattern observed on the fluorograms. The bands that were labeled during growth with [^{3}H]-palmitic or [^{3}H]-oleic acid consisted of proteins, since these bands disappeared when the delipidated envelope fraction was treated with proteinase K prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteinase K-treated delipidated membranes were extracted with chloroform-methanol-water, and the extract was analyzed by thin-layer chromatography. A radioactive ninhydrin-positive spot (ca. 29% of total radioactivity) which did not correspond to any of the known gonococcal phospholipids was observed. Extraction of this material and subsequent alkaline hydrolysis resulted in the liberation of labeled fatty acids. The possibility of a noncovalent interaction between lipopolysaccharide and membrane proteins was eliminated

by: (i) prolonged boiling of the membrane preparations as well as hot phenol extraction prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, (ii) urea-EDTA gel electrophoresis, and (iii) by probing Western blots with a monoclonal antibody reactive with gonococcal lipopolysaccharide. The possibility of anchoring membrane proteins through phospholipids instead of fatty acids was also ruled out by treatment with phospholipase prior to SDS-PAGE. The results provide evidence for covalent modification of numerous membrane proteins by fatty acids. Among the gonococcal membrane proteolipids identified were the Mtr-associated protein and protein III; protein I and protein II were not covalently modified by lipids.

INTRODUCTION

Covalent binding of fatty acids to membrane proteins has been documented in both procaryotic and eucaryotic cells (25). Among procaryotes, the only protein for which the precise structure of the lipid-protein linkage has been characterized is Braun's lipoprotein in Escherichia coli (4,5). Similar findings have been reported with regard to the association of lipid with membrane-bound beta-lactamases in gram-positive organisms, such as Bacillus licheniformis, B. cereus, and Staphylococcus aureus (22,23,33). To avoid confusion with Braun's lipoprotein, the term "proteolipid" has now been adopted for these acylated proteins since they contain a lipid moiety as part of their primary structure (25). To date, a lipoprotein covalently attached to peptidoglycan as described in E. coli has not been demonstrated in Neisseria gonorrhoeae. This report describes the uptake and incorporation of labeled fatty acids into gonococcal membrane proteins. We have examined gonococcal membrane proteins labeled during growth in medium containing [^{3}H]-palmitic or [^{3}H]-oleic acid. To our knowledge, the results reported here are the first to provide evidence for covalent modification of membrane proteins by long-chain fatty acids in gonococci. Our data support the existence of membrane proteolipids in N. gonorrhoeae.

MATERIALS AND METHODS

Organisms. Isogenic strains of N. gonorrhoeae with known genotypes were kindly provided by P. F. Sparling (University of North Carolina, Chapel Hill). These strains were FA19 (wild type), FA171 (mtr-2), and BR87 (env-2 str-7 penA2 mtr-2 penB2). Nonpiliated (P-) transparent (Op-) or opaque (Op+) colonies were used in the experiments as indicated.

Media and radioactive labeling. Cells grown overnight on GC agar (Difco laboratories, Detroit, MI) were used to inoculate a liquid medium (20) which was supplemented with $NaHCO_3$ (0.42 g/liter), glucose (5 g/liter), and a growth factor supplement similar to IsoVitaleX (BBL Microbiology System, Cockeysville, MD) (1%, vol/vol). The cultures were incubated at 37`C in a gyratory shaker, and growth was monitored with a Klett-Summerson colorimeter (Klett Manufacturing Co., NY) with a no. 54 filter. At the begining of exponential growth, 10 μCi of [9,10-^{3}H]-palmitic acid (23.5 Ci/mmol, New England Nuclear Corp., Boston, Mass.) or [9,10-^{3}H]oleic acid (2.9 Ci/mmol, New England Nuclear Corp.) or 5 μCi of [^{3}H]sodium acetate (6 Ci/mmol, New England Nuclear Corp.), or 7 μCi of [^{3}H]-amino acid mixtures (glycine, proline, arginine, lysine, and tyrosine) (1 mCi/ml, New England Nuclear Corp.) was added per ml of culture. Cultures were harvested at early stationary phase of growth by centrifugation at 10,000 x g for 15 min.

Uptake and incorporation of [^{3}H]-palmitic acid. [^{3}H]-Palmitic acid (10 μCi/ml) was added to the growth medium at the beginning of the exponential growth phase. Samples (200 μl) were removed and transferred to micro-

centrifuge tubes at 20-min intervals. After centrifugation for 2 min, the cell pellets were washed, resuspended in 100 µl of 10 mM N-2-hydroxyethyl-piperazine- N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 20 mM $MgCl_2$, and transferred to a scintillation vial containing 2 ml of absolute ethanol. Radioactivity was determined after the addition of 10 ml of scintillation fluid (Beckman Ready-Solv MP). The supernatant fluid from each wash was pooled and counted as described above. To determine the distribution of [^{3}H]-palmitic acid or [^{3}H]-acetate, the labeled cells were chemically fractionated by the procedure described by Morse et al. (21).

Crude membrane preparations. Labeled gonococci were washed and resuspended in 10 mM HEPES buffer (pH 7.4) containing 0.1% (vol/vol) protease inhibitor (10 mM phenylmethylsulfonyl fluoride in isopropanol). Cell suspensions were disrupted by sonication for 30 s (five times). This was followed by incubation for 15 min at 37°C in the presence of 40 µg of Deoxyribonuclease per ml, 40 µg of Ribonuclease per ml, and 1 mM $MgCl_2$. Membranes were collected by centrifugation (40,000 x g) for 40 min at 4°C. The membrane pellet was washed, suspended in a small volume of the same buffer, and stored at -20°C.

Binding of [^{3}H]-palmitic acid to cell membranes. A crude membrane preparation (2.5 or 5.0 µg protein/ml) from N. gonorrhoeae strain FA171 was incubated at 37°C for 1 h with [^{3}H]-palmitic acid (10 µCi/ml) in a final volume of 150 µl. At 15-min intervals, samples (30 µl) were removed and centrifuged (40,000 X g) for 30 min at room temperature. Membrane pellets were washed once with 10 mM tris-hydroxymethylaminomethane (Tris)-

hydrochloride buffer (pH 7.0). Radioactivity was determined by liquid scintillation spectrometry. Duplicate samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorographic analysis. Phosphlipids were extracted by suspending the membrane pellets in 2 ml of chloroform-methanol (1:1) and stirring vigorously for 30 min at room temperature. Supernatants from the extracts were concentrated under nitrogen and quantified by thin-layer chromatography (7).

Delipidation. Crude membranes (approximately 500 μg of protein) from cells labeled during growth in medium containing either [^{3}H]-fatty acid or [^{3}H]-acetate were extracted by a modification of the method of Hirashima et al. (11), by stirring in 4 ml of chloroform-methanol (1:1) and incubating for 20 min at room temperature. The delipidated membranes were collected by centrifugation (40,000 x g) for 60 min at 4°C. After repeating the extraction, the membrane pellets were dried under nitrogen and suspended in sample buffer (18). Supernatant from each extract was also dried under nitrogen and the radioactivity determined as described previously.

LPS extraction of delipidated membranes. LPS was extracted by a modification of the method described by Inzana (13). [^{3}H]-Palmitic acid-labeled gonococcal membrane proteins (500 μg) were delipidated as described above, dried under nitrogen, resuspended in 200 μl of water, and transferred to a 1-dram vial containing a stir bar. An equal volume of hot (65-70°C) 90% phenol was added, and the mixture was stirred vigorously at 65-70°C for 15 min. The suspension was chilled on ice until the temperature dropped to about 10°C, and then was centrifuged at 12,000 x g for 15 min. The upper aqueous

phase was transferrin to a 10 ml conical centrifuge tube. The phenol phase was reextracted with 200 ul of water and the aqueous phases were combined. The radioactivity in both phases was determined after the addition of 10 ml of scintillation fluid.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Crude membranes were solubilized by boiling for 5 min in final sample buffer. Samples were electrophoresed on a 10% polyacrylamide slab gel by the method of Laemmli (15), modified by the addition of 70 mM NaCl to the separating gel (18). After staining with Coomassie blue G250 (Bio-Rad Laboratories, Richmond, CA) in distilled water-methanol-acetic acid (5:5:2) and destaining in 10% acetic acid, the gels were treated with En^{3}Hance (New England Nuclear Corp.) for 30 min, washed, and dried. Fluorograms were obtained after exposing the gels to preflashed Kodak X-Omat AR films with Cronex intensifying screens (Dupont, Wilmington, Del.) at -70˚C.

The effect of temperature and the addition of 2-mercaptoethanol (2-ME) on proteolipids. Nonpiliated-opaque colonies of N. gonorrhoeae strain FA171 were grown in liquid medium containing [^{3}H]-palmitic acid (10 μCi/ml) for ca. 2 generations. Cell envelopes were prepared and solubilized in final sample buffer (either with or without 2-ME) at 100˚C for 5 min, or at 37˚C for 3 h prior to SDS-PAGE and fluorography.

Phospholipase digestion. Exponential phase cells labeled with [^{3}H]-palmitic acid (approx. 200 μg of protein in 40 μl of 10 mM HEPES buffer, pH 7.2) were incubated at 100˚C for 10 min. After cooling to room temperature, 40 μl of

phospholipase A_2 (378 U/ml, Sigma Chemical Co. St. Louis, MO) or phospholipase C (13,913 U/ml, Sigma Chemical Co.) were added and the suspensions incubated at 35˚C for 6h. At the end of the incubation period, final sample buffer was added and the mixture was incubated at 100˚C prior to SDS-PAGE. Labeled bands were visualized by fluorography. A control containing labeled cells and buffer, but not phospholipase, was also included.

Proteinase K digestion, alkaline methanolysis, and thin-layer chromatography. Delipidated crude membranes (ca. 500 µg of protein) were solubilized by incubation at 100˚C for 5 min in 1 ml of a solution containing 10 mM Tris-hydrochloride (pH 7.4), 5 mM EDTA, 1% SDS (wt/vol), and 10 mM $CaCl_2$. The proteins were digested by treatment with 200 µg of proteinase K per ml (Bethesda Research Laboratories, Inc., Gaithersburg, MD) for 72h at 37˚C. Lipids were extracted from the digest by the method of Bligh and Dyer (2). The extracts were dried under nitrogen and suspended in chloroform-methanol (2:1). One half of the sample was analyzed immediately by thin-layer chromatography on silica gel plates (Whatman LK6, Whatman Chemical Separation Inc., Clifton, NJ) using a solvent system of chloroform-methanol-water (65:25:4). Lipid standards were used as controls. Lipids were visualized by exposure of the plate to iodine vapor. Ninhydrin (0.3%, wt/vol) in butanol was used to identify amino-containing compounds. The remainder of the sample was mixed with 1 ml of 5 N NaOH, containing 90% methanol, and was incubated at 70˚C for 60 min. It was then acidified by the addition of 0.2 ml of 6 N HCl and extracted twice with ether. The ether extract was dried under nitrogen and analyzed by thin-layer chromatography as described above.

To determine how much [^{3}H]-palmitic or [^{3}H]-oleic acid was extracted

from the delipidated membranes by alkaline hydrolysis, 0.5 ml of 0.1 M KOH in 90% methanol was added to the solubilized proteins (ca. 200 µg) and the mixture incubated at room temperature for 0 to 3 h. Samples of the reaction mixture were removed at various time intervals, neutralized with 6N HCl, and extracted 3 times with petroleum ether. The release of alkaline-labile radioactivity was determined as described above. A portion of the extracts were also applied to silica gel thin layer plates and developed in hexane-diethyl ether-acetic acid (80:20:1) to separate fatty acids and their methyl esters.

Peptide mapping. The method of Border and Jarvinen (3) was used for peptide mapping of labeled crude membrane proteins. Proteins were first separated by electrophoresis on a 10% polyacrylamide gel. After completion of the electrophoresis, a gel lane was excised, equilibrated in stacking gel buffer and then transferred at right angles onto a second slab gel (15% polyacrylamide). S. aureus V8 protease (1-2 µg) (Sigma Chemical Co.) in sample buffer was overlayed on the gel, and electrophoresis in the second dimension was carried out. This was followed by the preparation of fluorograms as described above.

Western blot with LPS-specific monoclonal antibody. SDS-PAGE gels of membrane preparations from N. gonorrhoeae strains FA171 and FA19 were electroblotted onto nitrocellulose according to the method of Burnette (6). Bands corresponding to LPS were identified after incubation with a LPS-specific monoclonal antibody designated 06B4 (obtained from M.S. Apicella, SUNY-Buffalo) that recognizes a common determinant on the oligosaccharide moiety of

the LPS. Bands corresponding to LPS were visualized after incubation with protein A-Horseradish peroxidase (HRP) (Polysciences, Inc., Warrington, PA) and staining with the HRP color developing reagent (Bio-Rad laboratories, Richmond, CA). Protein bands were visualized after staining with amido black (0.1% in 45% methanol and 10% acetic acid) for 3 minutes and destaining with deionized water.

Glassware. All glassware was rinsed with chloroform-methanol (2:1) before use.

RESULTS

Uptake and incorporation of [9,10-^{3}H]-palmitic acid. The concentration of [^{3}H]-palmitic acid added to the growth medium in these experiments did not affect the growth rate of any of the strains examined (data not shown). The kinetics of palmitic acid uptake by N. gonorrhoeae strains FA171, FA19, and BR87 are shown in Fig. 1. The uptake was linear during the first 25 min of incubation, with maximum uptake occurring by 50 min. All three strains examined show similar rates of uptake. Cells of N. gonorrhoeae FA171 were chemically fractionated after 2 h of growth in medium containing either [9,10-^{3}H]-palmitic acid or [^{3}H]-acetate. The results (Table 1) showed that 93.4% of the labeled palmitic acid was associated with the lipid-containing cell fractions (ethanol-soluble and ethanol-ether-soluble fractions). Negligible amounts of radioactivity were found in the other cell fractions with the exception of 5% of total counts that were associated with the protein- and cell wall-containing cell fractions (papain soluble and residue fractions). In contrast, 81% of the [^{3}H]-acetate was associated with the lipid-containing fractions, and 14.5% with the protein- and cell wall-containing fractions.

Binding of [^{3}H]-palmitic acid to cell membranes. Direct binding of labeled palmitic acid to crude membranes of N. gonorrhoeae strain FA171 was examined. The kinetics of binding at 37°C is shown in Fig. 2. Binding of labeled palmitic acid to cell envelopes was rapid, with maximum amounts of fatty acid bound after 15 min of incubation, and was proportional to the amount of membrane present. Excess [^{3}H]-palmitic acid was added to insure saturation

of binding sites. Incubation temperatures of 25`C or 4`C did not have a significant effect on the binding of [^{3}H]-palmitic acid to cell envelopes (data not shown). Lipid analysis indicated that after 60 min of incubation, greater than 93% of the radioactivity added remained as free fatty acids, and less than 1.5% was associated with phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). These results were also consistent with fluorograms of the membranes which did not reveal any labeling of membrane proteins (data not shown).

Delipidation. Exhaustive delipidation of membranes with chloroform:methanol (1:1) removed 98.8% of the [^{3}H]-palmitic acid and 98.4% of the [^{3}H]-oleic acid (Table 2). The majority of the extracted lipids were phospholipids, since membrane proteolipid profiles on fluorograms were not affected (data not shown). Greater than 99% of the [^{3}H]-acetate-labeled components were removed after 3 extractions (Table 3). Lipopolysaccharide was not effectively removed by this procedure as it could still be visualized on the fluorogram of delipidated [^{3}H]-acetate-labeled membranes (Fig. 6).

LPS extraction of delipidated membranes. Delipidated membranes of N. gonorrhoeae strain FA171 labeled during growth in medium containing either [^{3}H]-palmitic or [^{3}H]-acetate were extracted with hot phenol and the radioactivity in both the phenol and aqueous phases was determined. The results (Table 4) indicated that the amount of radioactivity extracted into the LPS-containing aqueous phase was very low. [^{3}H]-Acetate appeared to label LPS better than [^{3}H]-palmitic acid.

Membrane profiles of [^{3}H]-amino acid-labeled gonococci. N. gonorrhoeae strain FA19 was grown in liquid medium containing [^{3}H]-amino acids (7 μCi/ml) for ca. 2 generations. The cell envelopes were examined by SDS-PAGE and fluorography as described above. The membrane protein profiles of cells labeled with [^{3}H]-amino acids were compared to those labeled with [^{3}H]-palmitic acid (Fig. 3). Substantially more peptides were labeled during growth with [^{3}H]-amino acids than during growth with [^{3}H]-palmitic acid. Protein I was heavily labeled with [^{3}H]-amino acids but not with [^{3}H]-palmitic acid. In contrast, phospholipids were preferentially labeled by [^{3}H]-palmitic acid.

Membrane profiles of [^{3}H]-palmitic acid-, [^{3}H]-oleic acid-, or [^{3}H]-acetate-labeled gonococci. N. gonorrhoeae strains FA171, FA19, and BR87 were grown for ca. 2 generations in medium containing either [^{3}H]-palmitic acid, [^{3}H]-oleic acid, or [^{3}H]-acetate. The cell envelope fractions were delipidated, and analyzed by SDS-PAGE as described above. The membrane protein profiles visualized by Coomassie blue staining (Fig. 4A) were compared with those in the fluorogram (Fig. 4B) of the same gel. Identical fluorograms were observed between whole cells and membrane preparations as well as between fluorograms of Coomassie blue-stained and non-stained gels that were prepared from cells labeled with either [^{3}H]-palmitic or [^{3}H]-oleic acid (data not shown). The apparent molecular weights of the major proteolipids estimated by comparison with known protein standards were 47,000, 44,000, 39,500, 32,000, 31,000, 30,500, 30,000, and 26,000. Qualitative and quantitaive differences in fatty acid-labeling were also observed between the membrane profiles of strains FA171, FA19, and BR87. The

major proteolipid of strain FA19 had an apparent molecular weight of 30,500 and was unique to this strain. The predominant proteolipid common to strains BR87 and FA171 had an apparent molecular weight of 31,000 (Fig. 4B). These proteolipids were not apparent in Coomassie blue-stained gels. Strain FA171 exhibited an increased amount of a protein with an apparent molecular weight of 47,000 (Fig. 4A). A similar increase in the 47,000-dalton protein was observed in the fluorogram. Protein III from the three strains examined had an apparent molecular weight of 32,000 and was visualized by both Coomassie blue staining and fluorography. Protein I, on the other hand, could be visualized only by Coomassie blue staining; it had an apparent molecular weight of 35,500 in strain BR87 and 34,000 in strains FA171 and FA19. To determine the relative amounts of the different proteolipids present in the gonococcal membrane, the Coomassie blue-stained gel and the fluorogram of strain FA171 were scanned with a laser scanning densitometer (model SL-2DUV, Zeineh, Fullerton, CA). Figure 5 indicates that the 31,000 and 47,000 dalton proteins are the two major proteolipids in the membrane of strain FA171.

The fluorogram from delipidated [^{3}H]-acetate-labeled cell membranes also revealed several protein-containing bands (Fig. 6). The Mtr-associated protein, protein I, protein III, and a protein with a molecular weight of 39,500 daltons were predominantly labeled. In addition, LPS could also be visualized as an arc-shaped band toward the bottom of the gel. Phospholipids labeled with [^{3}H]-acetate were effectively removed during the delipidation process.

Effect of temperature and reducing agents on the migration of membrane proteolipids. The effect of heating and reducing agents on the migration of

the membrane proteolipids was examined using cell envelopes obtained from P-Op+ colonies of N. gonorrhoeae strain FA171. Protein II is a heat modifiable protein with a molecular weight (24,000-30,000) that appears to increase when samples are solubilized at 100°C instead of at 37°C (30) (Fig. 7). Similarly, two proteolipids were affected by temperature. A proteolipid with an apparent molecular weight of ca. 180,000 daltons was observed in fluorograms when membranes were solubilized at 37°C but disappeared when membranes were solubilized at 100°C. Another proteolipid, with an apparent molecular weight of 33,000 daltons, was not observed when membranes were solubilized at 37°C but was observed when membranes were solubilized at 100°C. After heating at 100°C for 5 min, protein II migrated on Coommassie blue-stained gels with an apparent molecular weight of 30,000 daltons. Furthermore, the fluorogram indicated that heating at 100°C had no effect on the intensity of the 30,000 dalton proteolipid, suggesting that it was a proteolipid which comigrated with Protein II.

Protein III is a reduction-modifiable protein (17). It has a molecular weight of ca. 31,000 daltons in the absence of beta-mercaptoethanol (2-ME) and 32,000 in the presence of this reducing agent (Fig. 7A). Fig. 7B indicates that in addition to Protein III, the migration of a 39,500 dalton proteolipid was also affected by 2-ME. The change in apparent molecular weight due to 2-ME was subtle, but could still be visualized on both the Coomassie blue-stained gel and the fluorogram. The Mtr-associated protein appeared to be unaffected by either temperature or reducing conditions.

Proteinase K treatment and alkaline methanolysis of delipidated membranes. A membrane preparation of N. gonorrhoeae FA19 was delipidated and solubilized

prior to digestion with proteinase K at 37°C for 3 days. The digests were extracted with chloroform-methanol-water, and a portion was subjected to alkaline hydrolysis before analysis by TLC. TLC analysis of the lipid extract of the proteinase K digest yielded a radioactive ninhydrin-positive spot (ca. 29% of total radioactivity) that did not correspond to any of the known gonococcal phospholipids (Table 5). Subsequent treatment of the lipid extract with alkali resulted in the liberation of labeled fatty acids. The proteinase K-treated delipidated envelope fraction was also analyzed by SDS-PAGE. The bands which labeled with either [^{3}H]-palmitic acid or [^{3}H]-oleic acid (Fig. 4B) disappeared after proteinase K treatment (data not shown). In addition, several bands which were labeled with [^{3}H]-palmitic acid were found to correspond to those which were labeled with [^{3}H]-amino acids (Fig. 3). These data suggest the presence of a protein component in the labeled bands.

Approximately 88% of the [^{3}H]-palmitic acid and 83% of the [^{3}H]-oleic acid was released after treatment of delipidated membranes of N. gonorrhoeae strain FA171 for 2 h with KOH/methanol (Table 6). In addition, 79% of the palmitic acid released during alkaline hydrolysis was in the form of its methyl ester; the balance of radioactivity comigrated with the free fatty acid standard (Table 7). These results suggest that most of the fatty acids are covalently linked to membrane polypeptides through an ester linkage.

Phospholipase digestion. [^{3}H]-Palmitic acid-labeled exponential phase cells of strain FA171 were treated with either phospholipase A_2 or phospholipase C for 6h at 35°C prior to SDS-PAGE and fluorography. The fluorogram (Fig. 8) indicated that phospholipase treatment had no affect on the SDS-PAGE profiles

of membrane proteolipids. Both phospholipases were enzymatically active under these conditions since the intensity of the bands corresponding to labeled phospholipid was greatly reduced after treatment.

Peptide mapping. Limited proteolysis was used to obtain peptide maps of the [^{3}H]-palmitic acid-labeled proteolipids from N. gonorrhoeae strain FA171. The results (Fig. 9) indicate that many of the proteolipids are susceptible to cleavage with V8 protease. However, many of the cleavage fragments observed in the Coomassie blue-stained gel have no corresponding spot on the fluorogram. This suggests that fatty acid acylation is not homogeneous throughout the protein, but occurs at discrete sites.

Western blot with LPS-specific monoclonal antibody. Membrane preparations from N. gonorrhoeae strains FA19 and FA171 were subjected to SDS-PAGE, transferred to nitrocellulose and probed with LPS-specific monoclonal antibody 06B4, followed by histochemical staining with horseradish peroxidase. The bands corresponding to LPS (purple color) were located toward the bottom of the gel (Fig. 10). Bands corresponding to protein that did not react with the monoclonal antibody retained their blue color due to staining with amido black.

DISCUSSION

The absence of a lipoprotein analogous to that of E. coli, which links the peptidoglycan with the outer membrane, is thought to be responsible for the release of outer membrane blebs by N. gonorrhoeae (9,23). Nevertheless, evidence to date cannot rule out the presence of unbound proteolipids in gonococci. Hebeler et al. (8) demonstrated the existence of a protein-peptidoglycan complex in gonococci and suggested that a protein(s) was covalently attached to the peptidoglycan in a fashion analogous to that of the lipoprotein of E. coli. These reports prompted us to investigate the presence of gonococcal membrane proteolipids by following the uptake and distribution of exogenous labeled fatty acids. The uptake and incorporation of exogenous fatty acids by N. gonorrhoeae has been generally overlooked because of their concentration-dependent inhibitory action on the growth of this organism (14). Our results indicated that the gonococcal outer membrane was very permeable to fatty acids and that, at sub-inhibitory concentrations, gonococci could efficiently assimilate exogenous fatty acids.

Chemical fractionation of [^{3}H]-palmitic acid-labeled cells or [^{3}H]-acetate-labeled cells (Table 1) revealed that most of the radioactivity was associated with the lipid-containing cell fractions. Therefore, it was assumed that the labeled fatty acids were primarily incorporated into cell membranes that comprise most of the cellular lipid. A small portion of the incorporated palmitic acid or acetate was associated with the protein- and cell wall-containing fractions. Fluorograms of these membranes after SDS-PAGE revealed that the acetate was primarily incorporated into phospholipids and

lipopolysaccharide, while the fatty acids were primarily incorporated into phospholipids and proteolipids. Further evidence for the incorporation of fatty acids into proteolipids was obtained by exhaustive delipidation of the [^{3}H]-palmitic acid-labeled membrane preparations with chloroform-methanol (1:1), which removed the phospholipids, but did not alter the banding patterns observed by either Coomassie blue staining or fluorography. Furthermore, digestion of the delipidated membranes with proteinase K liberated acylated amino acids, which could be extracted into chloroform-methanol and visualized by their ninhydrin reaction after thin-layer chromatography. Owing to the difficulties of purifying these membrane proteolipids, it was not possible to carry out a quantitative fatty acid analysis by gas-liquid chromatography; the identity and stoichiometry of the acyl chains could not be established.

The exact nature of this protein-lipid association is unclear; although it was not affected by exhaustive delipidation with chloroform:methanol, it was susceptible to alkaline methanolysis which resulted in the conversion of 79 percent of the [^{3}H]-palmitic acid to its methyl ester. This supports the conclusion that most of the palmitic and oleic acid was covalently attached to protein through ester linkages. Whether the remaining fatty acids, that were resistant to alkaline methanolysis, were present in more stable ester or some other linkage remains to be established. It was not possible to distinguish between direct acylation of the protein by the fatty acid, or an indirect acylation through a glycerol moiety.

The results of these experiments have provided four lines of evidence that support the presence of membrane proteolipids in N. gonorrhoeae: (i) fatty

acids remained bound to protein through a linkage that was resistant to extraction with chloroform-methanol; this association was also stable after prolonged boiling and during electrophoresis on urea-EDTA gels (data not shown); (ii) protease treatment of the membranes did not liberate free fatty acids, but instead yielded an acylated amino-containing compound(s); (iii) free fatty acids (and fatty acid methyl esters) were released by alkaline methanolysis; and (iv) the proteins were localized in the cell membrane fraction.

It is unlikely that the radioactivity incorporated into membrane proteins was derived from catabolic products of palmitic acid or oleic acid, since little if any radioactivity was associated with protein I, which is the most abundant envelope protein (11). Likewise, this radioactivity did not result from contamination with labeled Lipopolysaccharide which has been reported to be tightly associated with several gonococcal outer membrane proteins (12). The finding that Protein I was not labeled by tritiated fatty acids is consistent with the hypothesis that the labeling of proteins was not due to contamination with lipopolysaccharide. Also, phenol extraction removed lipopolysaccharide without affecting the protein labeling patterns observed on fluorograms. Contamination with labeled lipopolysaccharide was further ruled out by prolonged boiling of the membrane preparations (up to 30 min) and by electrophoresis on urea-EDTA gels. Both procedures are known to dissociate lipopolysaccharide from proteins and did not alter the banding patterns seen in fluorograms. In addition, Western blots probed with a gonococcal lipopolysaccharide-specific monoclonal antibody showed a positive reaction with lipopolysaccharide only.

To determine whether these fatty acids were bound to proteins as phospholipids, [^{3}H]-palmitic acid-labeled cells were treated with either phospholipase A_2 or phospholipase C prior to SDS-PAGE and fluorography. The results indicated that treatment with phospholipases did not affect the labeling pattern of the membrane proteolipids, and thus ruled out the possibility of covalent binding of phospholipids to membrane proteins.

It was possible that the binding of fatty acids to proteins was an artifact that occurred during extraction and analysis by SDS-PAGE. To rule out this possibility, cell membranes were incubated at 37°C for 1 h with [^{3}H]-palmitic acid and analyzed by SDS-PAGE and fluorography. Proteins could not be visualized on fluorograms, thus eliminating the possibility of nonspecific binding of fatty acids to proteins.

To date, protein acyltransferases have not been purified from gonococci. In our experiments, the same labeling pattern observed, with [^{3}H]-saturated and unsaturated long chain-fatty acids, suggested that these fatty acids might use the same acyltransferase or that the acylating reaction is non-specific. However, different protein acyltransferases with similar specificities cannot be ruled out at the present time.

The results of the peptide mapping experiments indicated that acylation occurred at discrete sites of only certain membrane proteins. It is therefore likely that the acylation of these proteins is important in their function. A number of possibilities have been proposed for the role of protein acylation

among different organisms: (i) anchoring the protein in the lipid bilayer (27); (ii) facilitating membrane fusion (26); (iii) modifying the local conformation of proteins in membranes (16); and (iv) translocating a protein to the lipid bilayer (32).

In gonococci, the physiological significance of proteolipids is not clear. Presumably the bound fatty acids may anchor proteins in the lipid bilayer, thus performing a structural role. Alternatively, some of the proteolipids may play a role in envelope permeability by affecting the protein-protein interaction within the membranes. The latter may hold true for the Mtr-associated protein which is a proteolipid. The acquisition of mtr-2 by gonococci has been shown to confer nonspecific resistance to multiple antibiotics, dyes, and detergents (8,28), and also to be accompanied by a sevenfold increase in the amount of a 52,000 dalton outer membrane protein, as well as an increase in the extent of peptidoglycan cross-linking (8). When we compared N. gonorrhoeae strain FA171 with its isogenic strains FA19 and BR87, We observed a similar increase in a 47,000 dalton membrane protein, analogous to the previously reported 52,000 dalton Mtr-associated protein; the difference between the molecular weights reported is likely due to the different SDS-PAGE systems used by individual laboratories.

Recently, two gonococcal membrane proteins have been found to contain fatty acids. One of them is the H.8 antigen which has a signal peptide characteristic of Braun's lipoprotein and contains C:10 or C:11 as well as 2-OH-14:0 fatty acids (29). The other gonococcal proteolipid has an apparent molecular weight of 20,000 daltons and contains three unidentified lipid

components (24) that are estimated to account for ca. 5.7% of this proteolipid. The 20,000 dalton proteolipid has a high content of glutamic acid and is postulated to be responsible for the resistance of gonococci to killing by human phagocytes. It is yet to be established whether these fatty acid components are connected with the ability of the 20,000 dalton protein to inhibit the bactericidal activity of the phagocytes. The lipid components found in the purified H.8 antigen have not been described previously in the gonococcus, so artifacts or degraded products arising from purification and derivatization procedures have to be taken into consideration. We have observed a 19,500 dalton proteolipid on the fluorograms of [^{3}H]-palmitic acid-labeled cells. It is not known whether this minor proteolipid corresponds to either one of the proteins described above.

Two gonococcal proteins (MAb2, MAb3) that are similar in apparent molecular weight to protein III have recently been reported (1). We were unable to determine whether the proteolipid which we suspected to correspond to protein III was actually MAb2 or MAb3.

The results of this study provide evidence for the covalent modification of certain gonococcal membrane proteins by long-chain fatty acids. Although the significance of protein acylation is not understood, the proposed roles mentioned above would make it crucial to the survival of gonococci in vivo.

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TABLE 1. Incorporation of [^{3}H]-palmitic acid and [^{3}H]-acetate by *N. gonorrhoeae* strain FA171.

Cell fraction	Incorporation (%)	
	[^{3}H]-Palmitic acid	[^{3}H]-acetate
Whole cells	100	100
Cold tricholroacetic acid soluble	1.4	1.4
Ethanol soluble	89.6	75.9
Ethanol-ether soluble	3.8	5.1
Hot trichloroacetic acid soluble	0.2	3.1
Papain soluble	0.1	4.5
Residue	4.9	10.0
Recovery (%)	100	100

TABLE 2. Delipidation of membranes of N. gonorrhoeae strain FA19 grown in medium containing either [^{3}H]-palmitic or [H]-oleic acid.[a]

Fraction	Distribution of radioactivity (%)	
	[^{3}H]-Palmitic acid	[^{3}H]-Oleic acid
Extract I	94.4	92.1
Extract II	3.7	5.8
Extract III	0.5	0.4
Extract IV	0.2	0.1
Delipidated membranes	1.2	1.6
Recovery (%)	100	100

a. Equal amounts of membrane proteins (500 µg) were extracted with chloroform:methanol (1:1); 100% corresponds to 1.7 x 10^5 CPM for [^{3}H]-oleic acid and 3.4 x 10^6 CPM for [^{3}H]-palmitic acid-labeled membranes.

TABLE 3. Delipidation of membranes of N. gonorrhoeae strains FA171, FA19, and BR87 grown in medium containing [^{3}H]-acetate.[a]

Fraction	Distribution of radioactivity (%)		
	BR87	FA19	FA171
Extract I	90.7	95.9	92.6
Extract II	5.5	2.4	4.5
Extract III	3.4	1.1	2.3
Pellets	0.4	0.6	0.6
Recovery (%)	100	100	100

a. Equal amounts of membrane proteins (500 µg) were extracted with chloroform:methanol (1:1); 100% corresponds to 2.2 x 10^6 CPM for strain BR87, 1.9 x 10^6 CPM for strain FA19, and 2.0 x 10^6 CPM for strain FA171.

TABLE 4. Phenol extraction of delipidated membranes of N. gonorrhoeae strain FA171.[a]

Label	Distribution of radioactivity (%)	
	Phenol phase	Aqueous phase
[^{3}H]-acetate	98.2	1.8
[^{3}H]-palmitic acid	99.5	0.5

a. Delipidated membranes of N. gonorrhoeae strain FA171 grown in medium containing either [^{3}H]-palmitic acid or [^{3}H]-acetate were extracted with hot phenol. The radioactivity from both the phenol and aqueous phases was determined by liquid scintillation spectrometry.

TABLE 5. Effect of proteinase K treatment and alkaline methanolysis on delipidated membranes of [^{3}H]-palmitic acid-labeled *N. gonorrhoeae* strain FA19.

Component[a]	Alkaline methanolysis (%)	
	Before[b]	After[c]
FFA + FA methyl ester	0.7	93.3
PE + PG	30.9	0.6
Ninhydrin-positive spot (not PE or PG)	28.6	0.4
Others	39.8	5.7

a. Components were identified by comparison with known lipid standards chromatographed on the same thin-layer chromatography plate. Abbreviations: FFA, free fatty acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; LPE, lysophosphatidylethanolamine.

b. Refers to the relative proportion of the component prior to alkaline methanolysis.

c. Refers to the relative proportion of the component after alkaline methanolysis.

TABLE 6. Release of [^{3}H]-palmitic and [^{3}H]-oleic acid from delipidated membranes by alkaline hydrolysis.[a]

Time of incubation (min)	Radioactivity in organic phase (%)	
	[^{3}H]-Palmitic acid	[^{3}H]-Oleic acid
30	48.6	37.9
60	72.5	55.8
120	88.0	82.6
180	85.7	79.7

a. Membranes from N. gonorrhoeae FA171 were delipidated with chloroform:methanol (1:1), solubilized, and treated with 0.1 M KOH in 90% methanol. At various time intervals, samples were removed and extracted with petroleum ether as described in Materials and Methods.

TABLE 7. Distribution of palmitic acid and palmityl methyl ester after treatment of labeled membranes with 0.1 M KOH in 90% methanol.[a]

Time of incubation (min)	Distribution of radioactivity (%)	
	Palmitic acid	Palmityl methyl ester
0	94.6	5.4
60	24.7	75.3
120	20.9	79.1

a. [^{3}H]-Palmitic acid-labeled membranes (200 µg) from N. gonorrhoeae strain FA171 were delipidated and subjected to alkaline hydrolysis. Samples were removed at various time intervals, extracted, and applied to silica gel thin layer plates. Chromatograms were developed in hexane-diethyl ether-acetic acid (80:20:1) to separate the fatty acids from their methyl esters.

FIG. 1. Uptake of [^{3}H]-palmitic acid by N. gonorrhoeae strains FA171 (△--△), FA19 (○—○), and BR87 (□···□). [^{3}H]-Palmitic acid (10 μCi/ml final concentration) was added at the beginning of the exponential growth phase. Samples (200 μl) were removed at 20-min intervals, and the amount of radioactivity associated with the cell pellets was determined. Results are expressed as a percentage of the total radioactivity added.

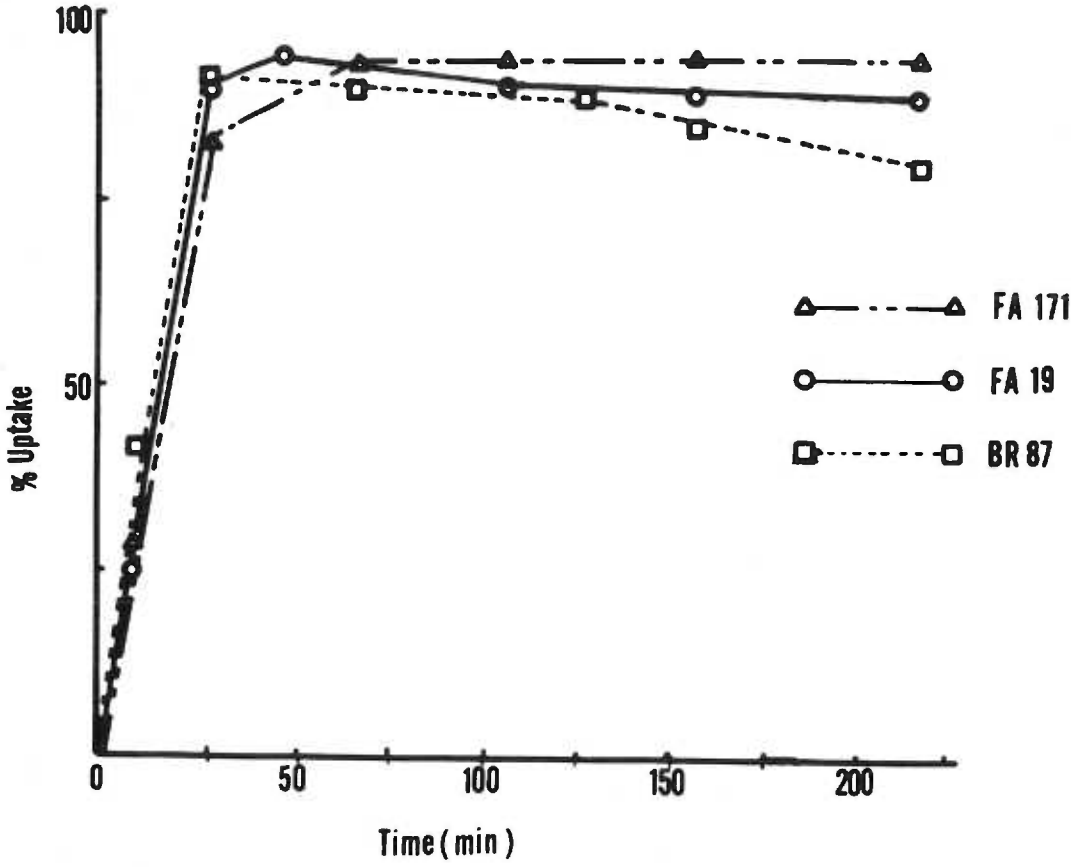

100
50
% Uptake
0
50
100
150
200
Time (min)
FA 171
FA 19
BR 87

FIG. 2. Binding of [^{3}H]-palmitic acid by cell membranes of N. gonorrhoeae strain FA171. Cell membrane preparations containing 2.5 (■—■) or 5.0 μg protein/ml (○—○) were incubated with [^{3}H]-palmitic acid (10 μCi/ml final concentration) in a final volume of 150 μl at 37°C for 1h. Samples (30 μl) were removed at 15-min intervals, and the amount of radioactivity associated with the membranes was determined. Results are expressed as a percentage of the total radioactivity added.

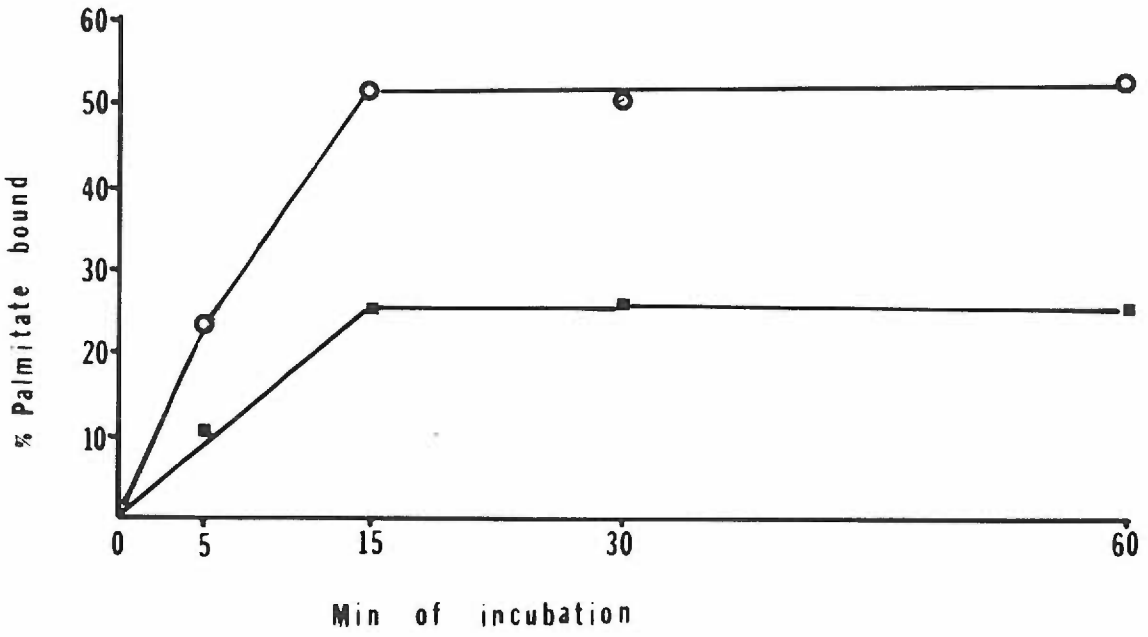

60
50
40
30
20
10
% Palmitate bound
0
5
15
30
60
Min of incubation

FIG. 3. Membrane protein profiles of N. gonorrhoeae strain FA19 labeled with [^{3}H]-palmitic acid (lane A) or [^{3}H]-amino acids (lane B). The proteins were separated by SDS-PAGE (10% acrylamide gel) and visualized by fluorography. Approximately 10^6 CPM were added to each lane. PI, protein I; PIII, protein III; MTR, Mtr-associated protein; PL, phospholipids.

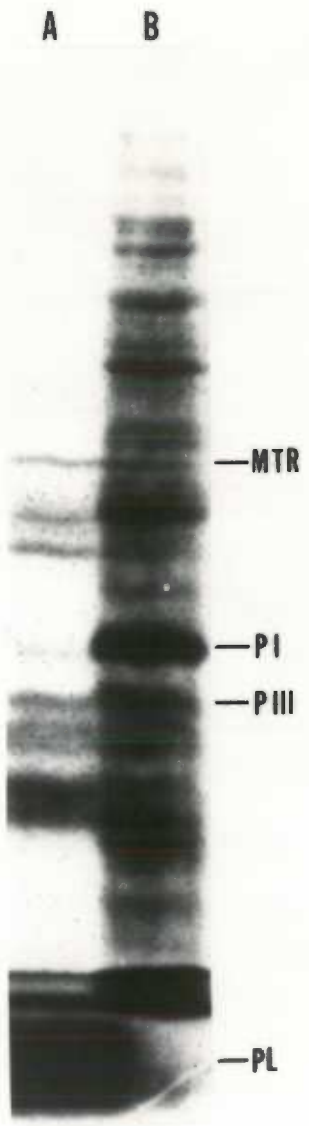

A
B
—MTR
—PI
—PIII
—PL

FIG. 4. Membrane protein profiles of N. gonorrhoeae strains FA171, FA19, and BR87 labled with [^{3}H]-palmitic acid. The proteins were separated by SDS-PAGE (10% acrylamide gel) and visualized by (A) Coomassie blue staining or (B) fluorography. Equal amounts of radioactivity (1.3×10^{5} cpm) were added to each lane. PI, protein I; PIII, protein III; MTR, Mtr-associated protein.

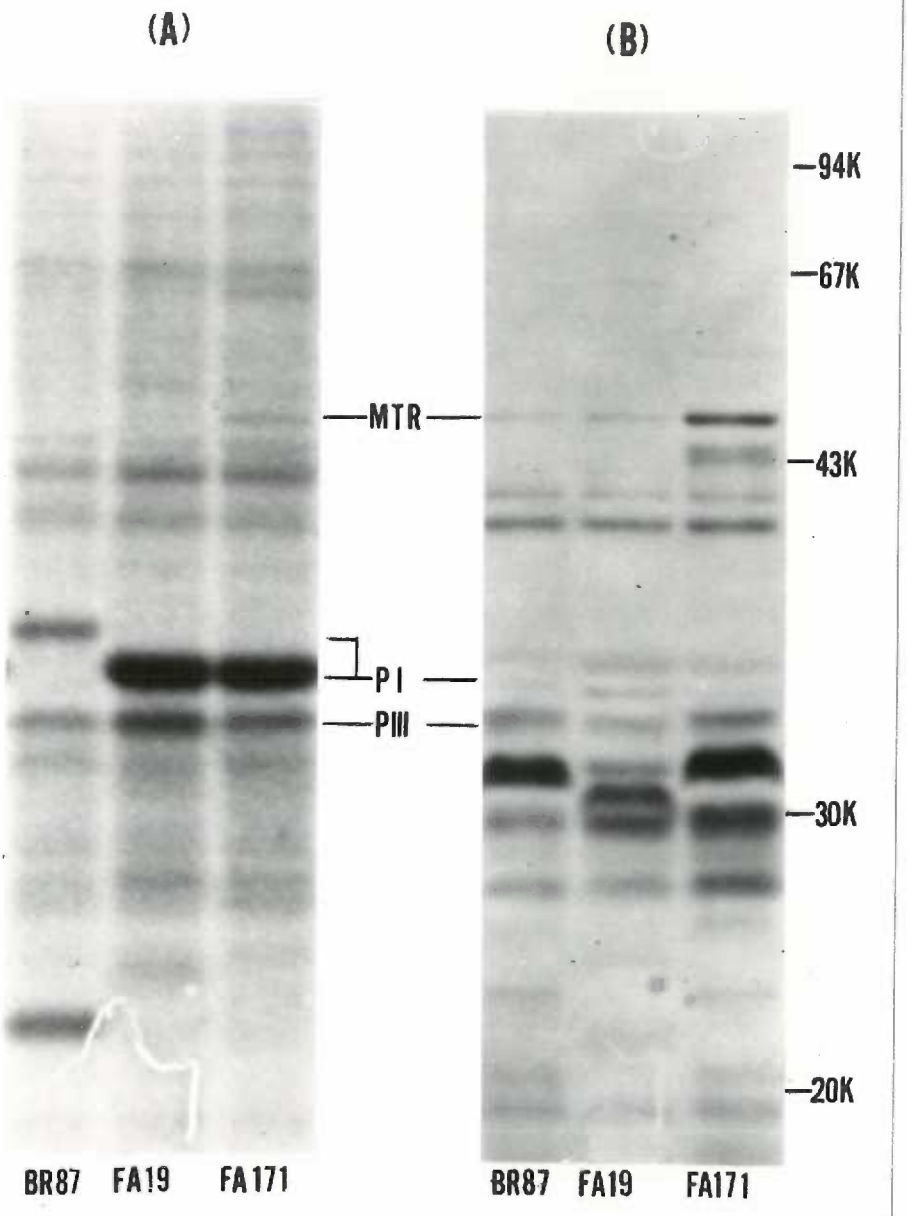

(A)
(B)
MTR
PI
PIII
94K
67K
43K
30K
20K
BR87
FA19
FA171
BR87
FA19
FA171

FIG. 5. Membrane protein profiles of N. gonorrhoeae strain FA171 labeled during growth in medium containing [^{3}H]-palmitic acid. The proteins were separated by SDS-PAGE (10% acrylamide gel) and visualized by Coomassie blue staining and fluorography. The Coomassie blue-stained gel (solid line) and the fluorogram (dashed line) were analyzed by scanning densitometry. Arrows marked A, B, and C correspond to the locations of protein III, protein I, and the Mtr-associated protein, respectively.

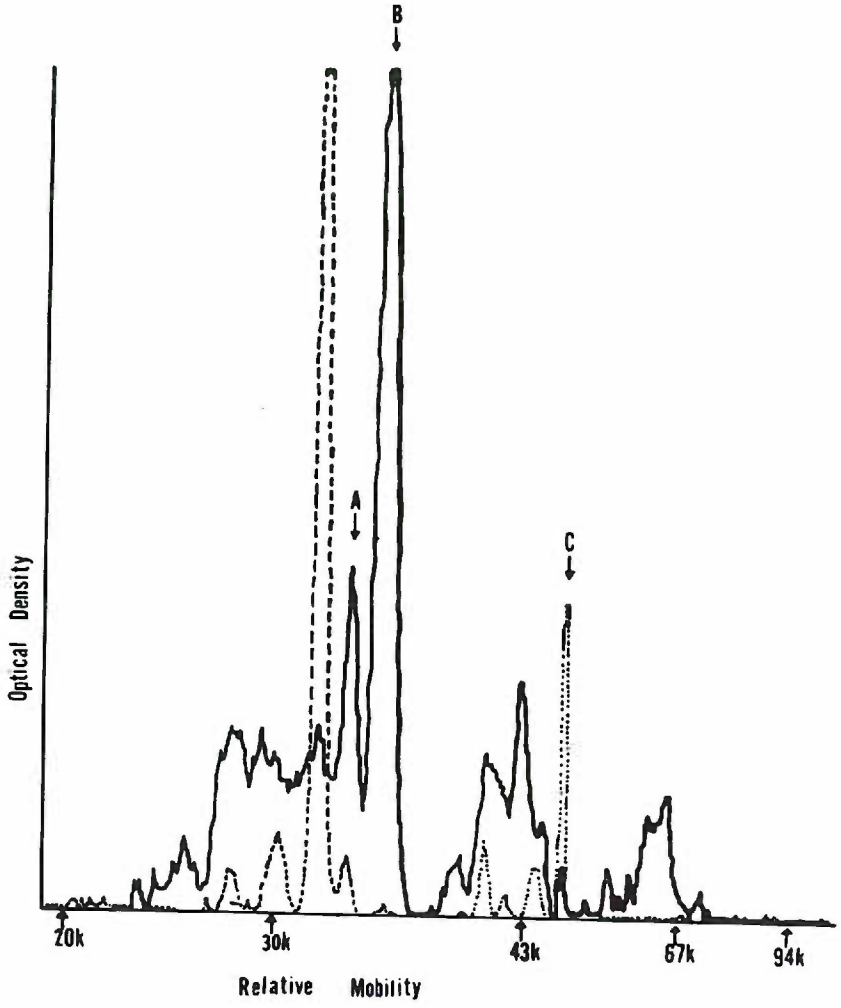

B
A
C
Optical Density
20k
30k
43k
67k
94k
Relative Mobility

FIG. 6. Fluorogram of SDS-PAGE profile of delipidated-membrane from N. gonorrhoeae strains FA171, FA19, and BR87 labeled during growth in medium containing [^{3}H]-acetate. The proteins were separated by SDS-PAGE (12% acrylamide gel) and visualized by fluorography. Equal amounts of radioactivity (ca. 10^{5} CPM) were loaded in each lane. LPS, lipopolysaccharides.

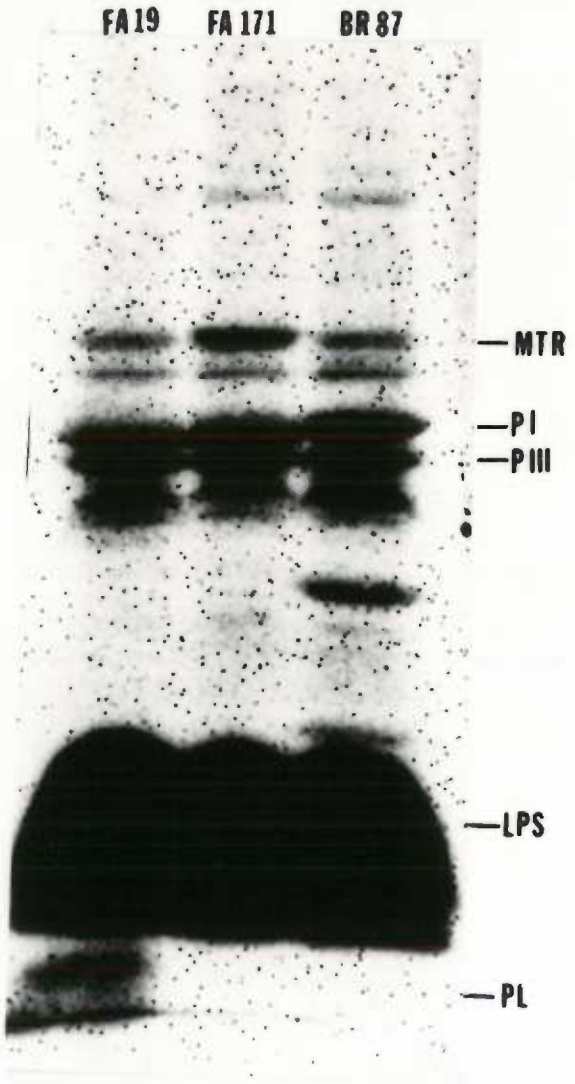

FA 19
FA 171
BR 87
MTR
PI
PIII
LPS
PL

FIG. 7. Effect of temperature and reducing agents on the migration of proteolipids in SDS polyacrylamide gels. N. gonorrhoeae strain FA171 (P- Op+) was grown in liquid medium containing [^{3}H]-palmitic acid (10 μCi/ml) for ca. 2 generations. Cell envelopes were solubilized in final sample buffer (+ 2-ME or - 2-ME) at 100`C for 5 min or at 37`C for 3h prior to SDS-PAGE (10% acrylamide gel). Bands were visualized by (A) staining with Coomassie blue or by (B) fluorography.
*, indicates observable difference.

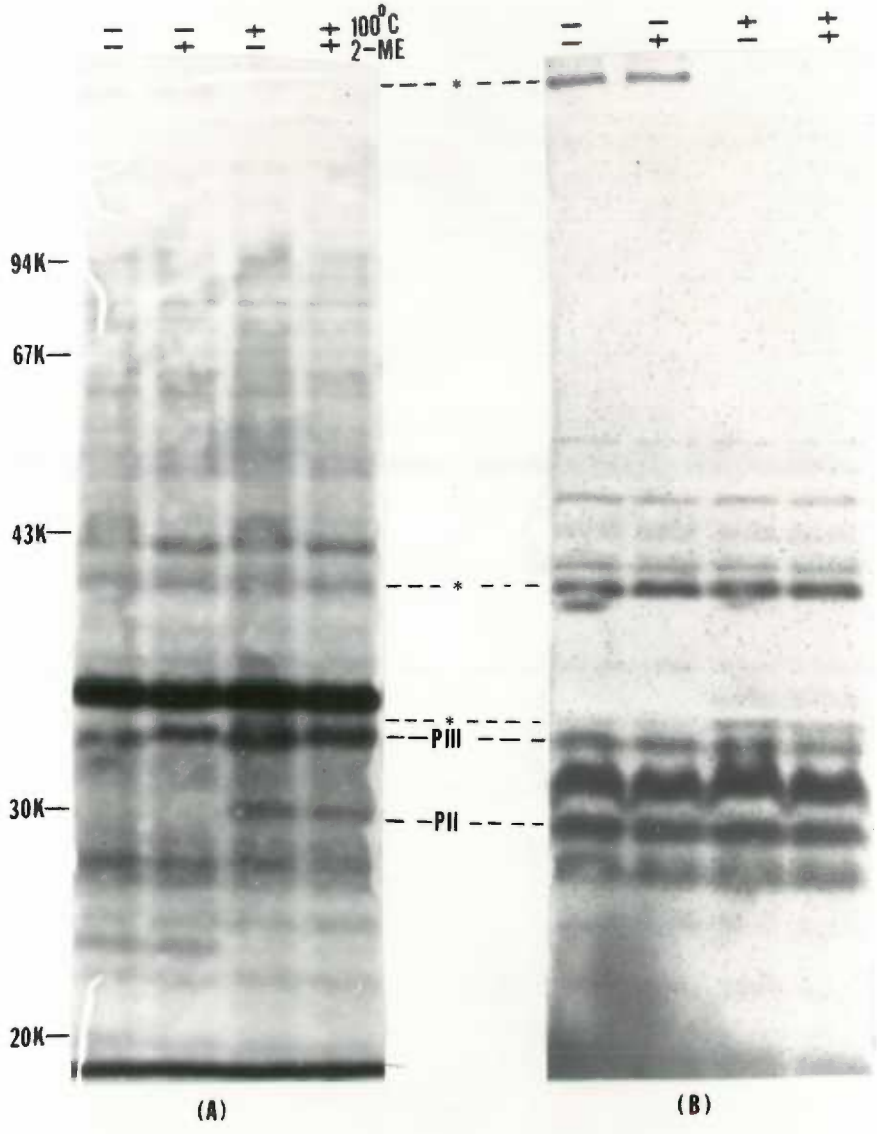

100°C
2-ME
94K
67K
43K
30K
20K
PIII
PII
(A)
(B)

FIG. 8. Effect of phospholipase treatment on membrane proteolipids. Cells of N. gonorrhoeae strain FA171 labeled with [^{3}H]-palmitic acid (ca. 100 µg of total protein) were incubated with either phospholipase A_2 or phospholipase C for 6h at 35°C prior to SDS-PAGE (10% acrylamide gel) and fluorography. Lane A: control without phospholipase; Lane B: whole cells incubated with phospholipase A_2 (690 U/mg); Lane C: whole cells incubated with phospholipase C (3200 U/mg). An equal amount of protein (50 µg) was used in each lane.

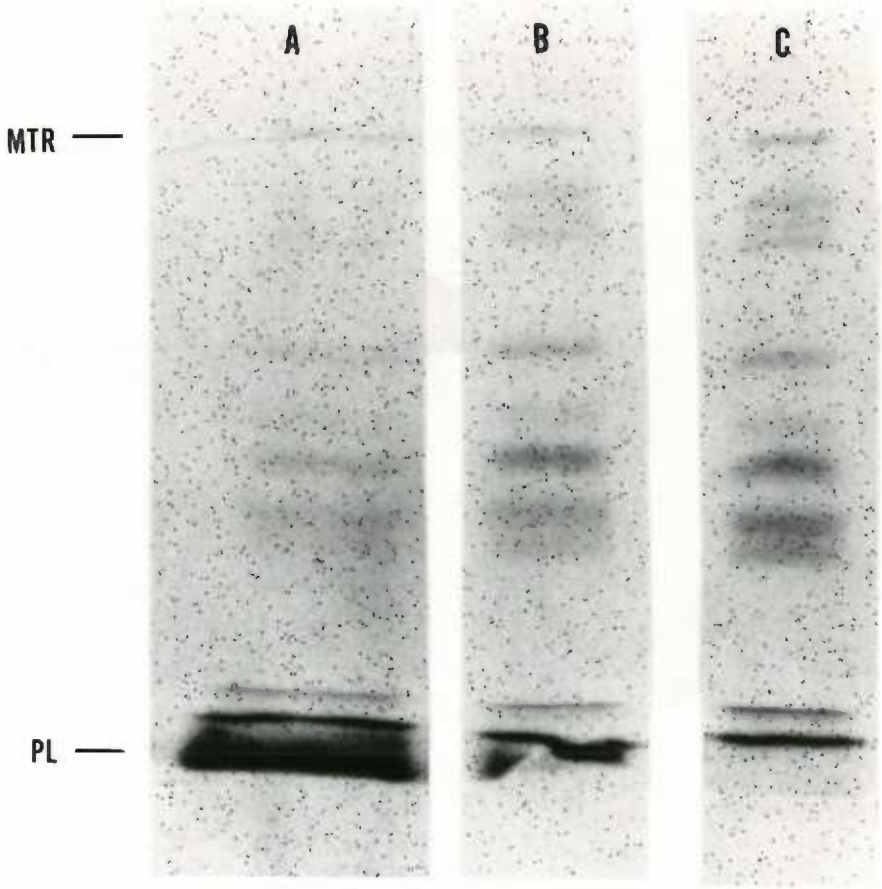

A
B
C
MTR
PL

FIG. 9. Limited proteolysis of membrane proteins from N. gonorrhoeae strain FA171. The electrophoretic separation of the [^{3}H]-palmitic acid-labeled membranes from N. gonorrhoeae FA171 was performed by SDS-PAGE (10% acrylamide gel) in the first dimension from left to right (A), the separation of the V8 protease digestion products was performed in the second dimension from top to bottom, and visualized by staining with Coomassie blue (B) or by fluorography (C).

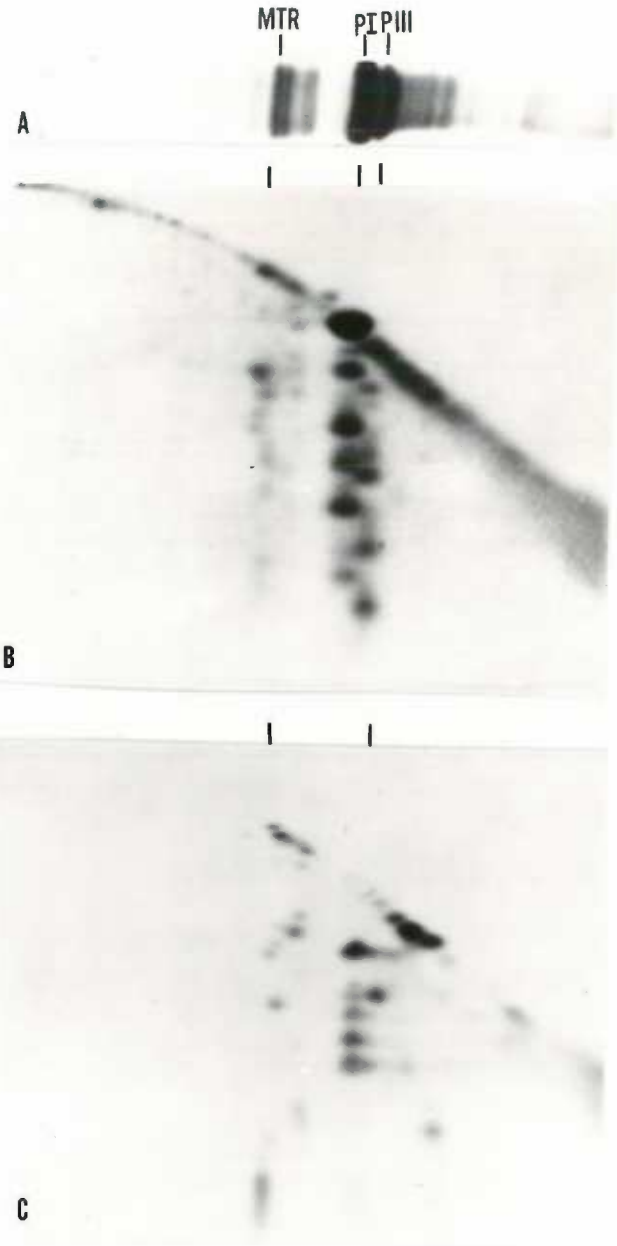
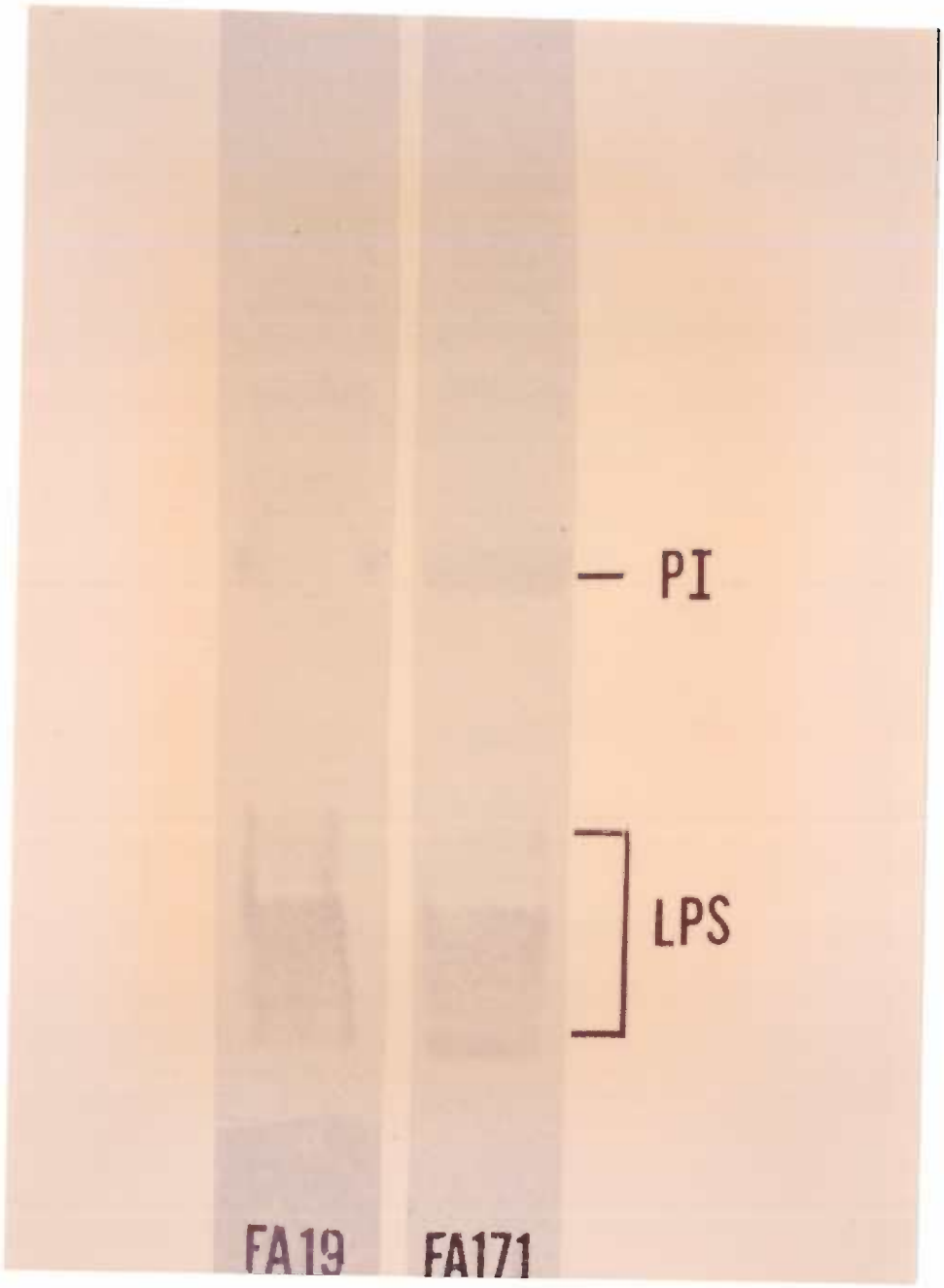

MTR
PI
PIII
A
B
C

FIG.10. Western blot of membrane preparations from N. gonorrhoeae strains FA19 and FA171. Membrane preparations from gonococcal strains FA19 and FA171 were transferred to nitrocellulose and probed with LPS-specific monoclonal antibody 06B4. Bands reacting with the LPS-specific antibody were located toward the bottom of the gel and were not associated with any membrane proteins, including protein I.


PI
LPS
FA19
FA171

Manuscript 2.

Membrane Proteolipids of *Neisseria gonorrhoeae*. II. Studies on the Biosynthesis of Proteolipids

Cheng-Yen Chen and Stephen A. Morse

ABSTRACT

Neisseria gonorrhoeae is able to incorporate sub-inhibitory concentrations of palmitic, oleic, and arachidonic acids directly into membrane phospholipids and proteolipids. Palmitic or oleic acid was primarily incorporated into phosphatidylethanolamine (PE) and phosphatidylglycerol (PG); while arachidonic acid was preferentially incorporated into PE. The incorporation of fatty acids was directly proportional to growth and appeared to be non-specific. Similar rates of uptake of exogenous free fatty acid uptake were observed regardless of the envelope phenotype. In contrast, when tritiated fatty acids bound to human serum albumin or to bovine serum albumin were used, isogenic strains with different envelope phenotypes exhibited different rates of uptake. Mtr strains exhibited a slower rate of fatty acid uptake in comparison with Env or wild type strains. The differences in the rate of uptake among isogenic strains of N. gonorrhoeae was related to their sensitivity to growth inhibition by free fatty acids.

Fatty acids were also incorporated into proteolipids. Pulse-chase experiments showed that the radioactivity in the fatty acid moieties of these proteolipids increased during a 4h chase period, suggesting the presence of a large pool of acyl donors such as phospholipids. Fatty acid acylation of membrane proteins was decreased in cells treated with either cerulenin or chloramphenicol suggesting that de novo fatty acid synthesis was required and that there was no significant pool of proacyl proteins within the cell. In addition, the uptake and incorporation of exogenous fatty acids, as well as the fatty acid acylation of proteins, was affected by respiratory chain

inhibitors and temperature indicating that the process is both enzymatic and energy-dependent. No accumulation of proteolipid precursors could be detected since globomycin, a specific inhibitor of signal peptidase, had no effect on the labeling profiles of gonococcal membrane proteolipids. The proteolipid profiles among strains of N. gonorrhoeae as well as from other Neisseria spp. exhibited a great degree of variability.

INTRODUCTION

Proteins covalently modified by fatty acids have been observed in a wide variety of procaryotic and eucaryotic cells. One such protein is the murein lipoprotein of Escherichia coli, discovered and extensively examined by Braun and Rehn (1). The amino terminus of this protein is an N-acylated cysteine residue with a diglyceride moiety covalently attached to the sulfhydryl group through a thioester linkage (5). The glycerol moiety of the lipoprotein is derived from the nonacylated glycerol moiety of phosphatidylglycerol (2). Acyl moieties of phospholipids appear to be the precursors of the diglyceride-linked fatty acid in this protein (10). It has been proposed that glyceride-cysteine at the amino-termini may be a ubiquitous feature of acyl proteins (26), and that the modification of the protein by glyceride may be a prerequisite for the processing of the protein by signal peptidase (24). Precursors of lipoproteins have been shown to accumulate within cells when the signal peptidase is blocked with the cyclic peptide antibiotic globomycin (17).

In the previous manuscript, we showed that several membrane proteins of Neisseria gonorrhoeae were covalently modified by saturated and unsaturated fatty acids through ester linkages. Gonococci were able to efficiently incorporate sub-inhibitory concentrations of exogenous fatty acids into their phospholipids and proteolipids. In this manuscript, we have investigated the factors that affect the uptake and incorporation of free and albumin-bound fatty acids, as well as their effect on the formation of gonococcal proteolipids. In addition, we have examined the relationship between the

degree of uptake and cell envelope phenotype, the presence of a proteolipid precursor(s), and the presence of membrane proteolipids among other Neisseria species. The results indicate that the phenomenon of fatty acid acylation is widely distributed among Neisseria species and that the mechanism of processing proteolipids in N. gonorrhoeae might be different from that of lipoprotein in E. coli.

MATERIALS AND METHODS

Organisms. The strains used in this study are listed in Table 1. These strains were kindly provided from the following collections: J. S. Knapp and R. J. Arko (Sexually Transmitted Diseases Laboratory Program, Centers for Disease Control, Atlanta, GA); D. Hollis (Division of Bacterial Diseases, CDC); H. Schneider (Walter Reed Army Institute of Research, Washington, D.C.) and P. F. Sparling (University of North Carolina, Chapel Hill). Nonpiliated (P-) transparent (Op-) colonies were used in all experiments.

Chemicals and fatty acids. Palmitic, oleic, and arachidonic acid were obtained from Sigma Chemical Co., St. Louis, MO., and were the highest grade available. All were in the free acid form and were dissolved in ethanol and sterilized by filtration. Fatty acid-free human serum albumin and bovine serum albumin (containing less than 0.2 mg fatty acid/g protein) were purchased from Miles Laboratories Inc., Naperville, IL. Cerulenin (Sigma Chemical Co.) was dissolved in ethanol immediately prior to use. Globomycin was a gift from Dr. M. Arai of Sankyo Co. Ltd. Japan.

Media and radioactive labeling. Cells grown overnight on GC agar (Difco Laboratories, Detroit, MI) were used to inoculate a liquid medium (17) that was supplemented with $NaHCO_3$ (0.42 g/liter), glucose (5 g/ml), and a growth factor supplement similar to IsoVitaleX (BBL Microbiology System, Cockeysville, MD) (1%, vol/vol). The cultures were incubated at 37°C in a gyratory shaker, and growth was monitored with a Klett-Summerson colorimeter (Klett Manufacturing Co., NY) with a No. 54 filter. At the beginning of

exponential growth, 10 μCi of [9,10-^{3}H]-palmitic acid (23.5 Ci/mmol, New England Nuclear Corp., Boston, MA), or [9,10-^{3}H]-oleic acid (2.9 Ci/mmol, New England Nuclear Corp.), or 0.6 μCi of [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (87.2 Ci/mmol, New England Nuclear Corp.), or 4 μCi of [1,3-^{14}C]-glycerol (48.6 mCi/mmol, New England Nuclear Corp.) was added per ml of culture. Samples of the cultures were removed at various time intervals and centrifuged (12,000 rpm, 10 min). The cell pellet was washed and the percentage of uptake and distribution of radioactivity in the various cellular lipids determined as described below.

Inocula and cultural conditions. A 2% (vol/vol) inoculum of a cell suspension from an overnight culture was used to inoculate a chemically defined medium (19) containing various concentrations of free fatty acids. The initial turbidity was adjusted to 25 to 35 Klett units (ca. 7 x 10^{7} CFU/ml). Control flasks containing ethanol but no fatty acids were included for each fatty acid tested. The concentration of ethanol used (4 μl/ml) did not inhibit the growth in control flasks. Cultures were incubated at 37°C in a New Brunswick gyratory shaker.

Lipid extraction. Fatty acid-labeled cell pellets were suspended in 10 ml of a 1:1 (vol/vol) mixture of chloroform-methanol and shaken for 60 min at 25°C to extract cellular lipids. The insoluble material was removed by centrifugation, and the supernatant dried under nitrogen at 25°C with nitrogen. The resulting residue was dissolved in a minimal amount of chloroform-methanol-water (2:1:0.1) and quantitatively applied to thin layer silica gel plates (Whatman LK6, Whatman Chemical Separation Inc., Clifton,

NJ). Lipid standards were used as controls. Chromatograms were developed in a mixture of chloroform-methanol-water (65:25:4). Lipids were visualized by exposure of the plate to iodine vapor. Phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) were confirmed by staining with ninhydrin in butanol (0.3%, wt/vol). Lipid-containing spots were scraped from the plates and placed into scintillation vials. One ml of methanol-water (2:1) and 10 ml of scintillation fluid (Beckman Ready-Solv MP) were added to the vials, and the radioactivity determined by liquid scintillation spectrometry. No significant quenching was observed in the presence of unlabeled phospholipids or fatty acids.

Pulse-chase experiments. [^{3}H]-Palmitic acid (5 μCi/ml) was added to an early log phase culture of N. gonorrhoeae strain FA171. After 5 min of incubation at 37°C, the culture was harvested, washed twice with growth medium, and resuspended in 60 ml of fresh medium containing unlabeled palmitic acid (0.43 μM). The cultures was incubated at 37°C and samples (6 ml) were removed at 0, 20, 40, 60, 90, 120, 150, 180, and 240 min. Cells were harvested by centrifugation for 4.5 min in an Eppendorf centrifuge. Washed cell pellets were extracted for lipid analysis, and were analyzed by SDS-PAGE and fluorography. Radioactivity associated with the cell pellets from each time interval was determined as described above.

Uptake of [^{3}H]-palmitoyl phosphatidylethanolamine or [^{3}H]-palmitoyl phosphatidylglycerol. [^{3}H]-Palmitoyl PE and PG were prepared in the following manner: A 50 ml culture of N. gonorrhoeae strain FA171 was grown for ca. 2 generations in medium containing [^{3}H]-palmitic acid (10 μCi/ml).

Cells were harvested by centrifugation and the phospholipids extracted and separated by TLC as described above. Spots corresponding to [^{3}H]-palmitoyl-PE or -PG were scraped from a TLC plate and placed in a scintillation vial with 2 ml of chloroform:methanol (2:1). The vials were flushed with nitrogen and incubated at room temperature for 2 h before centrifugation. The solvent layers were removed and concentrated under nitrogen.

Binding of [^{3}H]-palmitic acid to human or bovine serum albumin. Complexes of free fatty acid with human (HSA) or bovine serum albumin (BSA) were prepared in the following manner: 100 µl of [^{3}H]-palmitic acid (0.27 mCi/mmol) were added to 10 ml of chemically defined medium (pH 7.4) containing 0.5% fatty acid-free serum albumin (wt/vol). The reaction mixture was at 37°C in a gyratory shaking water bath operated at 150 rpm. At 10-min intervals, samples (0.5 ml) were removed and transferred to Eppendorf centrifuge tubes, containing 0.5 ml of cold 10% trichloroacetic acid (TCA), and incubated on ice for 30 min. Precipitates were collected by centrifugation and washed twice with 0.5 ml of cold 5% (wt/vol) TCA. The precipitates were resuspended in 2 ml ethanol, transferred to a scintillation vial, and 10 ml scintillation fluid was added. Radioactivity was determined by scintillation spectrometry as described above.

Uptake of HSA bound-[^{3}H]-palmitic acid. Tne HSA-[^{3}H]-palmitic acid complex was added to exponential phase cells of N. gonorrhoeae strains FA19, FA171, BR87, or AHU14 as described above. Time 0 refers to the time that the labeled complex was added. The final concentration of albumin was 1.25 mg/ml

of chemically defined medium. Cultures were incubated at 37°C in a gyratory water bath and samples (5 ml) were taken at 0, 30, 60, 120, 180, 240, and 360 min. Turbidity was measured as described above. A portion of each sample was used for lipid analysis and determination of viable counts. Cells were removed by centrifugation, the pellets washed once with the same medium, and radioactivity determined as described above.

Inhibition of fatty acid uptake by unlabeled BSA. N. gonorrhoeae strain AHU14 was grown in chemically defined medium for ca. 1 generation. Aliquots (3 ml) of the cell suspension were added to 2 ml of prewarmed medium containing unlabeled-BSA (0.025, 0.1, 0.5, 1.25, 2.5, 3.75, or 5 mg/ml) and incubation at 37°C continued for 30 min in a gyratory water bath, before adding 1 ml of the albumin-[^{3}H]-palmitic acid complex (5 mg BSA/ml of medium). After 30 min incubation at 37°C, cell pellets were collected, washed, and radioactivity determined. The different molar ratios of BSA : BSA-[^{3}H]-palmitic acid used were 0:1, 0.01:1, 0.04:1, 0.2:1, 0.5:1, 1:1, 1.5:1 and 2:1.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Whole cells were solubilized by boiling for 5 min in final sample buffer. Samples were electrophoresed on a 10% polyacrylamide slab gel by the method of Laemmli (9), modified by the addition of 70 mM NaCl to the separating gel (15). After staining with Commassie blue G250 (Bio-Rad Laboratories, Richmond, CA) in distilled water-methanol-acetic acid (5:5:2) and destaining in 10% acetic acid, the gels were treated with En^{3}Hance (New England Nuclear Corp.) for 30 min, washed, and dried. Fluorograms were obtained by exposing the gels to preflashed sheets of Kodak X-Omat AR film

with Cronex intensifying screens (Dupont Co., Wilmington, DL) at -70˚C.

Effect of temperature on the uptake of [^{3}H]-palmitic acid. Cultures of N. gonorrhoeae strain FA171 were grown in chemically defined medium at 37˚C for ca. 1 generation. Cells were harvested by centrifugation, washed once with medium, resuspended, and dispensed into 3 flasks. Each of the flasks contained fresh medium supplemented with [^{3}H]-palmitic acid (5 μCi/ml), which had been preincubated at 4˚C, 25˚C, and 37˚C, respectively. After the addition of cells, incubation was continued at these same temperatures. Samples were removed at 0, 5, 15, 30, and 45 min. The time 0 sample refers to the sample taken immediately after resuspension. Growth was monitored by Klett-Summerson colorimetry; radioactivity associated with the cell pellet was determined as described above.

Effect of cerulenin on the growth of N. gonorrhoeae. Cerulenin (2.5, 10, 20, or 40 μg/ml final concentration) was added to early log phase cultures of N. gonorrhoeae strain FA171 growing in chemically defined medium. After 30 min of incubation at 37˚C, [^{3}H]-palmitic acid or [^{3}H]-oleic acid (5 μCi/ml) was added and incubation continued for an additional 2.5 h. Control experiments were carried out in parallel in the absence of cerulenin. Aliquots of cells were removed at 30 min-intervals. Cell membranes were prepared and examined by SDS-PAGE and fluorography. Phospholipids were also extracted and analyzed by TLC as described above.

Effect of respiratory-chain inhibitors and chloramphenicol on the uptake of [^{3}H]-fatty acids. Sodium azide and potassium cyanide were dissolved in

water and Antimycin A and chloroamphenicol were dissolved in ethanol prior to use; their final concentraions in growth media were of 5 mM, 0.5 mM, 8 μM, and 10 μg/ml, respectively. After the addition of these inhibitors to gonococcal cultures in the exponential phase of growth, incubation at 37°C was continued for 5 min before the addition of either [^{3}H]-palmitic acid, [^{3}H]-oleic acid, or HSA-bound [^{3}H]-palmitic acid. Samples were removed at different time intervals and radioactivity associated with the cell pellets was determined as described above. Cell pellets were also analyzed by SDS-PAGE and fluorography.

Effect of globomycin on the growth of N. gonorrhoeae. Globomycin was dissolved in methanol and added to log-phase cultures of N. gonorrhoeae strain FA171 to a final concentration of 5, 10, 20, or 40 μg/ml of chemically defined medium. The carbon source added to the medium was either glucose (0.2% or 0.5%, wt/vol) or pyruvate (1%, wt/vol). When glucose was used, cells were incubated with globomycin for 5 min before the addition of [^{3}H]-palmitic acid; in medium containing pyruvate, the incubation with globomycin was increased to 30 or 60 min. Following the addition of [^{3}H]-palmitic acid, samples wered removed hourly up to 4 h. A control consisting of cells growing in medium without globomycin was included. At the end of the incubation period, cells were collected, washed, and analyzed by SDS-PAGE and fluorography.

RESULTS

Effect of fatty acids on the growth of N. gonorrhoeae. Addition of either palmitic acid, oleic acid, or arachidonic acid to the chemically defined medium at the time of inoculation markedly inhibited the growth of N. gonorrhoeae strains FA19, FA171, and BR87. The relationship between the concentration of fatty acid needed to inhibit growth by 50% (IC_{50}) and the chain length of the fatty acid is shown in Table 2. The inhibition of growth was dependent upon the concentration (data not shown), the chain length, and the degree of saturation of the fatty acid. Inhibitory activity decreased as the fatty acid chain length decreased. The degree of inhibition was also depedent upon the cell envelope phenotype. An Mtr strain (FA171) was more resistant to the three fatty acids than the isogenic wild type (FA19) and Env (BR87) strains. The Env strain was the most sensitive to growth inhibition by fatty acids.

Lipid analysis of N. gonorrhoeae strain FA171 labeled with [^{3}H]-fatty acids. The uptake of exogenous fatty acids has been shown to be very rapid (previous manuscript). Isogenic gonococcal strains with different cell envelope phenotypes exhibited similar rates of uptake. The uptake of [^{3}H]-palmitic acid was dependent upon the temperature of incubation. At 4˚C, there was little uptake of [^{3}H]-palmitic acid. Uptake increased at 25˚C and 37˚C by 10% and 25%, respectively (data not shown). In order to determine whether the exogenous fatty acids were further metabolized, cellular lipids of N. gonorrhoeae strain FA171, labeled during growth with either [^{3}H]-palmitic acid, [^{3}H]-oleic acid, or [^{3}H]-arachidonic acid, were

extracted and analyzed by thin layer chromatography. The results (Table 3-5) indicated that gonococci efficiently incorporated exogenous fatty acids into cellular phospholipids even at the very early stage of uptake; an equilibrium in the distribution of the fatty acid was reached by ca. 15 min. The cellular phospholipids were composed primarily of ca. 70% PE and 20% PG. The uptake and incorporation of [^{3}H]-palmitic or [^{3}H]-oleic acid was similar, with ca. 70% of the radioactivity associated with PE and ca. 20% with PG. [^{3}H]-Arachidonic acid was incorporated more slowly, as indicated by the substantial activity associated with the free fatty acid fraction, and preferentially into PE with < 10% of the radioactivity associated with PG. Fluorographic analysis indicated that [^{3}H]-palmitic acid or [^{3}H]-oleic acid labeled membrane proteolipids (previous manuscript); however, proteolipids were not labeled during growth in medium containing [^{3}H]-arachidonic acid (data not shown).

Labeling of proteolipids by [^{3}H]-palmitic acid. Pulse-chase experiments with [^{3}H]-palmitic acid were performed to follow the turnover of acyl moieties. N. gonorrhoeae FA171 was incubated for 5 min in medium containing [^{3}H]-palmitic acid. Cells were harvested, resuspended in fresh medium containing unlabeled palmitic acid and chased for 4 h. The total radioactivity associated with cell pellets did not increase during the 4 h chase period (data not shown). Radioactivity associated with the non-lipid fraction increased over five-fold during the chase period (Fig. 1). Furthermore, radioactivity associated with proteolipids as observed on fluorograms also increased with time (Fig. 2a). Interestingly, the predominant proteolipid appeared to increase in molecular weight over the 4 h

chase period from 31,000 daltons to ca. 35,000 daltons. The increase in molecular weight was proportional to the number of generations (Fig. 2b). The phospholipid pool decreased gradually to 50% in ca. 3.5 generations, whereas the fatty acid pool decreased rapidly to below 50% within 0.2 generations. Due to very rapid turnover rate of fatty acids, it is not known whether the free fatty acid pool was directly used to acylate cellular proteins or whether the fatty acids were first incorporated into phospholipids, which then served as the donors of acyl groups.

Incorporation and metabolism of [^{3}H]-palmitoyl-PE, or [^{3}H]-palmitoyl-PG by N. gonorrhoeae strain FA171. To test whether phospholipids functioned as acyl donors for proteolipids, cells from N. gonorrhoeae strain FA171 were grown for 6 h in a complex medium supplemented with either [^{3}H]-palmitoyl-PE or [^{3}H]-palmitoyl-PG. The labeled phospholipids were at least 95% pure as determined by TLC (data not shown). Neither [^{3}H]-palmitoyl-PE nor -PG was incorporated efficiently by gonococci; however, [^{3}H]-palmitoyl-PG was incorporated ca. 3 times more efficiently than [^{3}H]-palmitoyl-PE (20% versus 7% over 3 h) (data not shown). The results (Fig. 3a and 3b.) indicated that the [^{3}H]-palmitic acid label was exchanged between PE and PG as well as between the phospholipid and free fatty acids. The radioactivity in PE increased Less than 2-fold during 3 h incubation with [^{3}H]-palmitoyl-PG (Fig. 3b), whereas the radioactivity in PG increased more than 10-fold during the same incubation time with [^{3}H]-palmitoyl-PE (Fig. 3a). This suggested that [^{3}H]-palmitoyl-PG was not as metabolically active as [^{3}H]-palmitoyl-PE in serving as a donor of palmitic acid. Fluorograms prepared from these tritiated phospholipid-labeled cells revealed no labeled protein bands. This

was likely due to either the poor uptake or to the low specific radioactivity of the labeled phospholipid. The uptake of [^{14}C]-glycerol by N. gonorrhoeae strain FA171 also occurred at an even slower rate (data not shown).

Uptake of [^{3}H]-palmitic acid bound to HSA. The ratio of HSA to [^{3}H]-palmitic acid was adjusted so that all of the [^{3}H]-palmitic acid was bound and that the concentration of HSA used would not affect the growth of gonococci (data not shown). Isogenic strains of N. gonorrhoeae with different cell envelope phenotypes differed in their ability to take up [^{3}H]-palmitic acid when it was bound to HSA. Fig. 4a indicates that the amount of [^{3}H]-palmitic acid taken up by gonococci continued to increase during the 5 h incubation period. The Mtr phenotype (strain FA171) exhibited the slowest rate of [^{3}H]-palmitic acid uptake, and the DGI (AHU14) strain exhibited the fastest rate, while both the wild type (strain FA19) and Env (strain BR87) phenotypes had similar rates of uptake. This is consistent with the percentage uptake of [^{3}H]-palmitic acid plotted versus colony forming units (CFU) (Fig. 4b).

Samples were also removed at various time intervals during the experiment and analyzed for the distribution of the [^{3}H]-palmitic acid (Fig. 5). Strain AHU14 incorporated [^{3}H]-palmitic acid into cellular lipids more rapidly than the other strains, conversely, FA171 exhibited the slowest incorporation.

The uptake studies were repeated using dual labeled I^{125}-HSA-[^{3}H]-palmitic acid. The results indicated that HSA was not internalized by gonococci (data not shown). Similar uptake and incorporation was also observed when BSA-[^{3}H]-palmitic acid was used (data not shown). Since no

differences were observed, the BSA complex was used in subsequent experiments.

Inhibition of uptake by unlabeled BSA. Exponential phase cells of N. gonorrhoeae AHU14 were incubated at 37°C for 30 min with various concentration of BSA prior to the addition of the BSA-[^{3}H]palmitic acid complex. The results (Fig. 6) indicated that free BSA inhibited the uptake of [^{3}H]-palmitic acid from the BSA-[^{3}H]-palmitic acid complex. The degree of inhibition was directly proportional to the unlabeled BSA added. The percentage of inhibition observed was close to the theoretical value suggesting that gonococci interact with BSA prior to the removal and incorporation of the bound fatty acid.

Effect of inhibitors on the uptake of free or BSA-bound [^{3}H]-palmitic acid. The results in Table 6 demonstrate the effect of 5 mM sodium azide on the uptake of free or BSA-bound [^{3}H]-palmitic acid. Uptake of free fatty acid appeared to be an energy-independent process. However, uptake of the BSA-bound [^{3}H]-palmitic acid was energy-dependent. After 15 min of incubation in the presence of sodium azide, the uptake and incorporation of BSA-bound [^{3}H]-palmitic acid was reduced to ca. 10% of that observed in control cells. A similar inhibition of uptake was also observed when either 8 uM antimycin A or 0.5 mM KCN were used (data not shown). In addition, the intensity of proteolipid bands on fluorograms was also reduced (data not shown).

Addition of chloramphenicol (10 µg/ml) reduced growth ca. 40% after 2 h of incubation. Under these conditions, phospholipid synthesis was not inhibited and the [^{3}H]-palmitic acid associated with chloramphenicol-treated cells was

slightly decreased when compared with a control culture. However, the proteolipid profiles on fluorograms of chloroamphenicol-treated cells exhibited a weaker band intensity than proteolipid profiles of cells grown in absence of chloramphenicol (Fig. 7).

Effect of cerulenin and globomycin on proteolipid biosynthesis. To determine the effect of de novo fatty acid synthesis on the incorporation of exogenous fatty acids into proteolipids, cultures of N. gonorrhoeae strain FA171 growing in chemically defined medium were treated with various concentrations of cerulenin prior to the addition of [^{3}H]-palmitic acid. The results in Fig. 8 indicate that cerulenin markedly inhibited the growth of N. gonorrhoeae and that the degree of inhibition was dependent on the concentration of cerulenin. At a concentration of 20 μg cerulenin/ml, the growth rate was initially decreased by about 25%; complete inhibition of growth occurred by 90 min after the addition of cerulenin. Proteolipid synthesis was also affected by cerulenin. Fluorographic analysis of SDS-polyacrylamide gels of cell membrane preparations indicated that the labeling of proteolipids by [^{3}H]-palmitic acid in cerulenin-treated cells was reduced when compared to that of the control cells (Fig. 9) even though the total radioactivity associated with both were similar (data not shown). The inhibition of fatty acid incorporation into proteolipids was proportional to the amount of cerulenin added. The incorporation of [^{3}H]-palmitic acid into phospholipids was also markedly inhibited by the addition of cerulenin (Fig. 10). The amount of [^{3}H]-palmitic acid incorporation into PE and PG was reduced to about 30% of the level observed in control cells after 2 h incubation; the ratio of PE to PG was similar in the presence and absence of cerulenin.

The proteolipid profile was not affected by the carbon source (glucose or pyruvate), globomycin concentration (8-fold high than that used with E. coli), and preincubation time with globomycin (12 times longer than that used with E. coli). A concentration of 10 μg globomycin/ml had no effect on the growth of N. gonorrhoeae. However, when the concentration of globomycin was increased to 20 μg/ml or to 40 μg/ml, growth after 4 h was inhibited ca. 30% and 60%, respectively (data not shown).

Proteolipid profiles of other Neisseria spp. Strains representing various Neisseria spp. (Table 1) were examined for the presence of proteolipids following growth in medium containing [^{3}H]-palmitic acid. The results (Fig. 11) indicate that fatty acid acylation of proteins occurred widely among other Neisseria species. In addition, the labeling pattern appeared to be very heterogeneous with each species possessing either one or more major proteolipids. No one proteolipid with the exact same molecular weight was observed in all Neisseria spp. examined.

Discussion

The sensitivity of N. gonorrhoeae to free fatty acids in agar has been known since the work of Ley and Mueller in 1946 (12). Walstad et al. (25) confirmed that the substances produced by N. gonorrhoeae, that inhibited the growth of gonococcal or meningococcal strains, were long-chain fatty acids and phospholipids. Subsequently, the detection of phospholipase A activity in the outer membrane of N. gonorrhoeae (22) supported the hypothesis that free fatty acids were generated by a stepwise degradation of phopholipids during growth. The inhibitory effect of fatty acids is due to their ability to interfere with the membrane-associated functions of oxidative phosphorylation and electron transport (16). Ironically, the outer membrane of N. gonorrhoeae is more permeable than that of other Gram-negative bacteria to hydrophobic dyes and fatty acids, owing to the presence of phospholipid bilayer regions capable of interacting with these molecules (13). Miller et al. (16) showed that the inhibition of gonococcal growth by fatty acids increased with chain length. Fatty acids are present in human vaginal secretions (14), animal tissue, and plasma (6,11); their effect on N. gonorrhoeae in vivo is not clear.

Gonococci can be divided into one of three cell envelope phenotypes (Mtr, wild type, Env) based on their sensitivities to various hydrophobic molecules (13,20). The Mtr phenotype exhibits resistance to hydrophobic dyes, detergents, and antibiotics, whereas the Env phenotype exhibits hypersensitivity to these compounds. Strains with the wild-type envelope phenotype exhibit intermediate levels of resistance. However, auxotrophic strains requiring arginine, hypoxanthine, and uracil (AHU^-) for growth possess the

wild-type envelope phenotype but are as sensitive to fatty acids as are strains with the Env phenotype.

Different degrees of sensitivity to fatty acids could be mediated either by the degree of phospholipid exposure on the surface, thus affecting membrane permeability, by a decreased level of the susceptible target site(s), or by an increased affinity for the inhibitory fatty acids. In this study, we have shown that N. gonorrhoeae can incorporate both saturated and unsaturated fatty acids into membrane phospholipids as well as into some of its membrane proteins. The uptake of exogenous free fatty acids is very rapid indicating that the gonococcal outer membrane is quite permeable to these hydrophobic compounds. Strains with different cell envelope phenotypes exhibited similar rates of [^{3}H]-palmitic acid or [^{3}H]-oleic acid uptake as well as a similar distribution of the labeled fatty acids into their lipids. The uptake of exogenous free fatty acids appears to be non-specific and energy-independent. The effect of temperature on the uptake of palmitic acid indicated that membrane fluidity was also important.

Less than 0.01% of the fatty acids in plasma are present in a free form, under normal physiological conditions (4); the rest are tightly bound to albumin in a noncovalent complex. Therefore, we examined the uptake of albumin-bound fatty acids by N. gonorrhoeae. In contrast to the uptake of free fatty acids, isogenic gonococcal strains with different envelope phenotypes exhibited different rates of uptake of fatty acids bound to albumin. Mtr strains exhibited a slower rate of uptake as compared with Env or wild type strains. The differences in the ability of gonococci to acquire

fatty acids is correlated with differences in their sensitivity to the inhibitory effect of free fatty acids. The uptake of albumin-bound fatty acids was an energy-dependent process. The uptake was due primarily to a transfer of palmitic acid from albumin to the cell, not to incorporation of the intact palmitate-albumin complex. Inhibition of uptake by unlabeled serum albumin suggested that contact between the cell and the albumin was necessary. Thus, the differences observed in the uptake and incorporation of fatty acids bound to serum albumin may be due to differences in the ability of the various envelope phenotypes to bind serum albumin per se.

Gonococci were able to rapidly incorporate [^{3}H]-arachidonic acid. Most of the incorporated arachidonic acid was found in PE. The metabolism of arachidonic acid has been well documented (3). Through the action of 5-lipoxgenase, physiological or chemical stimuli such as leukotriene B4 and 5-monohydroxyeicosatetraenoic acid are formed. Both are chemotatic factors for neutrophils. Their availability in vivo may be reduced since gonococci are able to incorporate arachidonic acid. This may be another possible strategy that allows gonococci to modify the inflammatory process. The significance of arachidonic acid incorporation into PE is presently unknown. The observation that proteolipids were not labeled during growth in medium containing [^{3}H]-arachidonic acid suggested that this fatty acid, or phospholipids containing it, are not involved in protein acylation in N. gonorrhoeae.

There are five intermediates in the maturation of the E. coli lipoprotein (26). The primary gene product is a prolipoprotein that contains a 20-residue

amino-terminal signal peptide. The glycerol moiety is then transferred from PG to the cysteine sulfhydryl group followed by the esterification of two fatty acids to the glycerylcysteine. The signal peptide is then removed by signal peptidase II to form an apolipoprotein. The last step in lipoprotein maturation is the transfer of an additional fatty acid to the new amino terminus. The fatty acid composition of the protein-bound diglyceride is similar to that found in membrane phospholipids and palmitic acid is the major amide-linked fatty acid. In addition, fatty acid turn over at the 1-position in PE is initiated by the transfer of acyl moieties to the lipoprotein (7). However, the biochemical events responsible for the post-translational acylation of the lipoprotein are less clear.

Cerulenin, an antibiotic isolated from culture filtrates of Cephalosporium caerulens, inhibits de novo fatty acid synthesis in a variety of organisms, including bacteria, fungi, and yeasts (21) by acting as a non-competitive inhibitor of the enzyme of fatty acid chain elongation. Gonococcal proteolipids were underacylated when cells were treated with cerulenin suggesting that de novo fatty acid synthesis was required for one or more of the steps in their synthesis.

Pretreatment of cells with choloramphenicol resulted in a decrease in palmitate incorporation suggesting that fatty acid acylation occurred during protein synthesis, and ruled out the presence of a pool of acylated pro-lipoproteins. Furthermore, the continued palmitate incorporation in the absence of protein synthesis suggested that the acylation of proteins by fatty acids is probably a late post-translational modification.

Globomycin has been shown to inhibit the cleavage of the signal peptide of prolipoprotein in E. coli, resulting in its accumulation (10,26); this inhibitory effect was specific in that no other precursors of the major membrane proteins accumulated. In E. coli this suggested that all lipoproteins have a similar chemical structure in the region that is to be cleaved by signal peptidase. Alternatively, E. coli might possess one species of signal peptidase that is specific for all the lipoproteins. The addition of globomycin had no effect on the profiles of gonococcal proteolipids, suggesting that gonococci may be different from E. coli in their recognition peptide sequences for the signal peptidase or in the specificity of their signal peptidase(s).

Biosynthesis of the acyl moieties in the proteolipids of N. gonorrhoeae was studied by pulse-labeling with [^{3}H]-palmitic acid and subsequent chase. A linear increase in the radioactivity associated with proteolipids during a 4 h chase, suggested that the fatty acids in proteolipids were derived from a precursor(s) present in a large pool, such as the acyl moieties in phospholipids. However, the uptake of [^{3}H]-palmitoyl-PE or -PG was inefficient. Thus, we were unable to determine whether gonococci prefer a particular phospholipid as the acyl donor. The results of pulse-chase experiments did not exclude the possibility that either free fatty acids, acyl-CoA, or acyl-ACP (acyl carrier protein) was the immediate acyl donor for the proteolipids. However, the results of subsequent experiments with cerulenin suggested that the free fatty acid pool was not the direct precursor of the acyl moiety of proteolipids. Furthermore, it has been shown that in E. coli,

the concentration of free fatty acids is very low, even in a mutant deficient in fatty acid degradation (8,18).

The reason for the increase in the molecular weight of the predominant proteolipid over the 4 h chase period, as well as during different growth phases, is not clear at the present time. Presumably, a continuous acylation of the preexisting proteolipid with fatty acids affected its mobility during SDS-PAGE. The presence of fatty acids may also be responsible for the observation that most of the proteolipids were poorly stained with Coomassie blue (23).

The inability of gonococci to take up and incorporate [^{14}C]-glycerol into proteolipids, as well as the resistance of proteolipids to treatment with phospholipases (previous manuscript), suggested that a glycerol moiety was not present. These results are consistent with the recent finding that gonococcal H.8 antigen is a lipoprotein which may not contain glycerol (23). Our results have led to the hypothesis that the fatty acids are covalently linked to the side-chains of the hydroxyl-containing amino acids (serine, threonine, tyrosine) of the protein, mostly by simple ester bonds.

Our knowledge of the biosynthetic process of gonococcal proteolipids and the nature of the fatty acid-protein linkage is still limited. The function(s) of fatty acid acylation are only beginning to be understood. These fatty acid moieties may be involved in protein-protein and/or protein-lipid interactions within membranes, possibly by influencing protein folding or protein orientation. A more specific or metabolic role for these

proteins remains to be determined. The intracellular pathways that proteolipids follow during their maturatuion, as well as the characteristics of a protein that enable it to serve as a substrate for fatty acid acylation remain unclear. Our results indicate that gonococcal proteolipids share some of the characteristics of the E. coli lipoprotein, and that fatty acid acylation is common to a variety of proteins among Neisseria species. Whether each of these proteins contains structural similarities, or shares amino acid recognition sequences required for acylation, remains to be determined.

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TABLE 1. Designation and source of strains used in this study

Species	Strain designation	Source
N. gonorrhoeae	FA171, FA19, BR87	P. F. Sparling
	AHU14	S. A. Morse
N. meningitidis (serogroup B)	E6942	R. J. Arko
N. lactamica	D7256	D. Hollis
N. cinerea	30016	J. S. Knapp
N. subflava	B886	D. Hollis
N. perflava	4926	H. Schneider
N. mucosa	1500	D. Hollis

TABLE 2. Inhibition of growth of N. gonorrhoeae strains FA19, FA171, and BR87 by free fatty acids.

Fatty acid	Chain length	IC_{50} (μM)		
		BR87	FA19	FA171
Palmitic acid	C16:0	16	26	57
Oleic acid	C18:1	2.6	3.6	13
Arachidonic acid	C20:4	2.1	4.5	21

a. IC_{50} refers to the concentration of fatty acids that produced a 50% decrease in cell yield.

TABLE 3. Distribution of [^{3}H]-oleic acid in the lipids of N. gonorrhoeae strain FA171.[a]

	Distribution of radioactivity (%)					
	0 min	15 min	30 min	60 min	120 min	180 min
Lipid[b]	(0)[c]	(0.2)	(0.4)	(0.8)	(1.7)	(2.5)
FFA	8.3	4.7	4.1	3.1	2.5	3.4
PE	70.3	72.6	76.5	73.2	74.8	66.2
PG	17.1	18.4	16.4	21.0	20.1	27.2
LPE	0.5	0.4	0.9	0.2	0.8	0.3
Uptake (%)	27.3	83.7	94.6	100.0	100.0	100.0

a. Gonococci were grown in chemically defined medium with a final concentration of 10 μCi [^{3}H]-oleic acid/ml. Time 0 sample refers to the sample taken immediately after adding the labeled fatty acid.

b. FFA, free fatty acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; LPE, lysophosphatidylethanolamine.

c. Generations.

TABLE 4. Distribution of [^{3}H]-palmitic acid in the lipids of N. gonorrhoeae strain FA171.[a]

	Distribution of radioactivity (%)					
	0 min	15 min	30 min	60 min	120 min	180 min
Lipid	(0)[b]	(0.2)	(0.4)	(0.8)	(1.7)	(2.5)
FFA	17.3	4.1	3.1	3.2	2.6	1.8
PE	60.7	72.1	77.9	72.4	70.3	66.3
PG	18.1	18.9	13.8	20.2	21.2	26.4
LPE	0.3	0.2	3.5	0.2	4.2	0.2
% Uptake	21.2	82.3	93.8	100.0	100.0	100.0

a. Gonococci were grown in chemically defined medium with a final concentration of 10 μCi [^{3}H]-palmitic acid/ml.

b. Generations.

TABLE 5. Distribution of [^{3}H]-arachidonic acid in the lipids of *N. gonorrhoeae* strain FA171.[a]

	Distribution of radioactivity (%)					
	0 min	15 min	30 min	60 min	120 min	180 min
Lipid	(0)[b]	(0.2)	(0.3)	(0.7)	(1.3)	(2.0)
FFA	23.8	19.1	18.6	11.9	14.0	12.4
PE	69.0	77.7	68.9	68.3	78.3	77.4
PG	5.9	3.9	9.0	12.8	5.8	8.6
LPE	1.0	1.1	1.9	3.1	1.1	0.9
% Uptake	58.4	94.0	100.0	100.0	100.0	100.0

a. Gonococci were grown in chemically defined medium with a final concentration of 0.6 μCi [^{3}H]-arachidonic acid/ml.

b. Generations.

TABLE 6. Effect of NaN_3 on the uptake of [^{3}H]-palmitic acid or BSA bound-[^{3}H]-palmitic acid by N. gonorrhoeae strain AHU14.[a]

	Radioactivity of uptake (CPM/CFU)			
	[^{3}H]-Palmitic acid		BSA-[^{3}H]-palmitic acid	
	NaN_3		NaN_3	
Time (min)	-	+	-	+
0	2.2×10^{-4}	2.2×10^{-4}	0.7×10^{-7}	0.7×10^{-7}
10	2.5×10^{-4}	2.1×10^{-4}	25.2×10^{-7}	2.4×10^{-7}
20	2.6×10^{-4}	2.2×10^{-4}	43.4×10^{-7}	4.4×10^{-7}

a. NaN_3 (final concentration of 5 mM) was added to a log phase culture of N. gonorrhoeae. Cells were incubated for 5 min before adding [^{3}H]-palmitic acid or BSA bound-[^{3}H]-palmitic acid (0.5 μCi/ml). Incubation was continued in the presence of NaN_3. CPM: counts per min; CFU: colony forming units; - : without, and + : with NaN_3.

FIG. 1. Distribution of radioactivity in N. gonorrhoeae strain FA171 pulse-chase labeled with [^{3}H]-palmitic acid. Five μCi/ml of [^{3}H]-palmitic acid was added to an early log phase culture. After 5 min of incubation at 37°C, the culture was harvested by centrifugation (10,000 rpm for 5 min at room temperature), washed twice with growth medium, and then resuspended in fresh medium containing unlabeled palmitic acid (0.43 μM). Cultures were incubated at 37°C and samples were removed at 0, 20, 40, 60, 90, 120, 150, 180, and 240 min for lipid analysis. Radioactivity incorporated into fatty acid (FFA), phospholipids (PE +PG), and non-lipid fraction was determined as a percentage relativeto time zero.

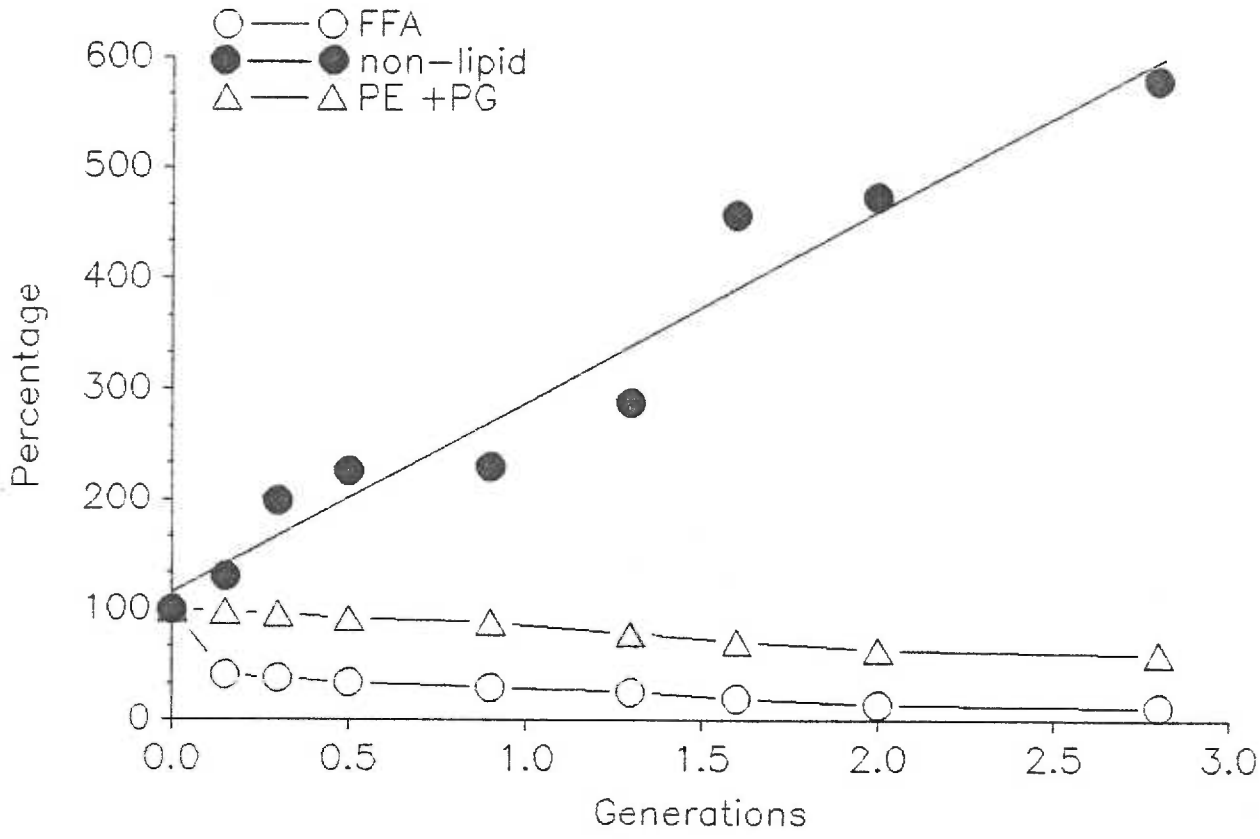
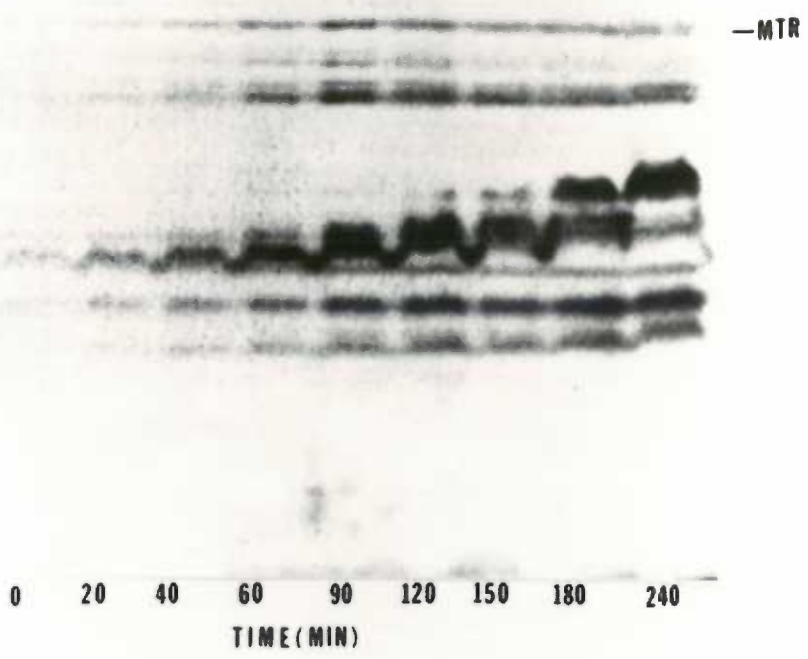

FFA
non-lipid
PE +PG
600
500
400
300
200
100
0
Percentage
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Generations

FIG. 2. Labeling of proteolipids by [^{3}H]-palmitic acid during chase period. Early exponential phase culture of N. gonorrhoeae strain FA171 was pulse labeled with [^{3}H]-palmitic acid for 5 min at 37°C and chased with unlabeled palmitic acid for 4 h. Cell pellets were prepared from samples taken at 0, 20, 40, 60, 90, 120, 150, 180, and 240 min and solubilized in final sample buffer at 100°C for 5 min prior to SDS-PAGE (10% acrylamide gel). (a). Fluorogram. (b). The increase in molecular weight of the major proteolipid during the chase period vs. generation time.

(a)



(b)

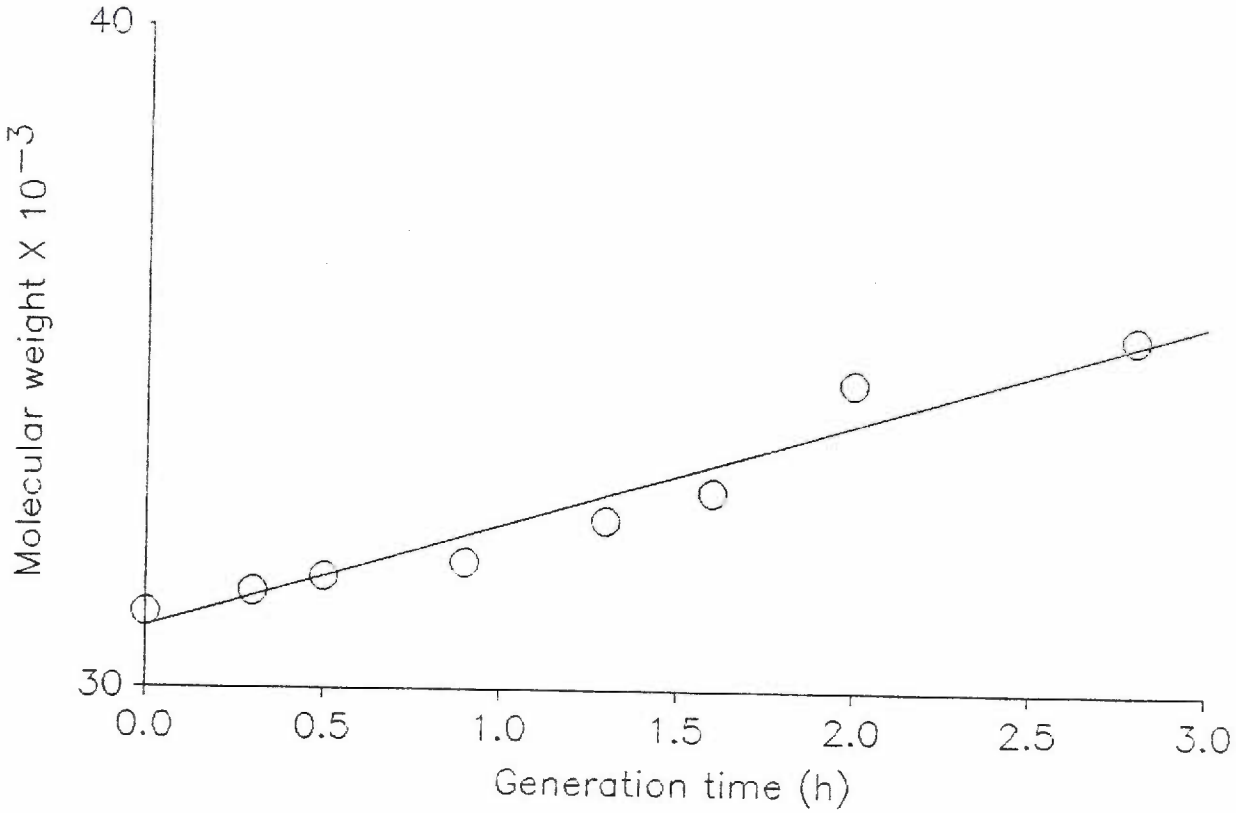
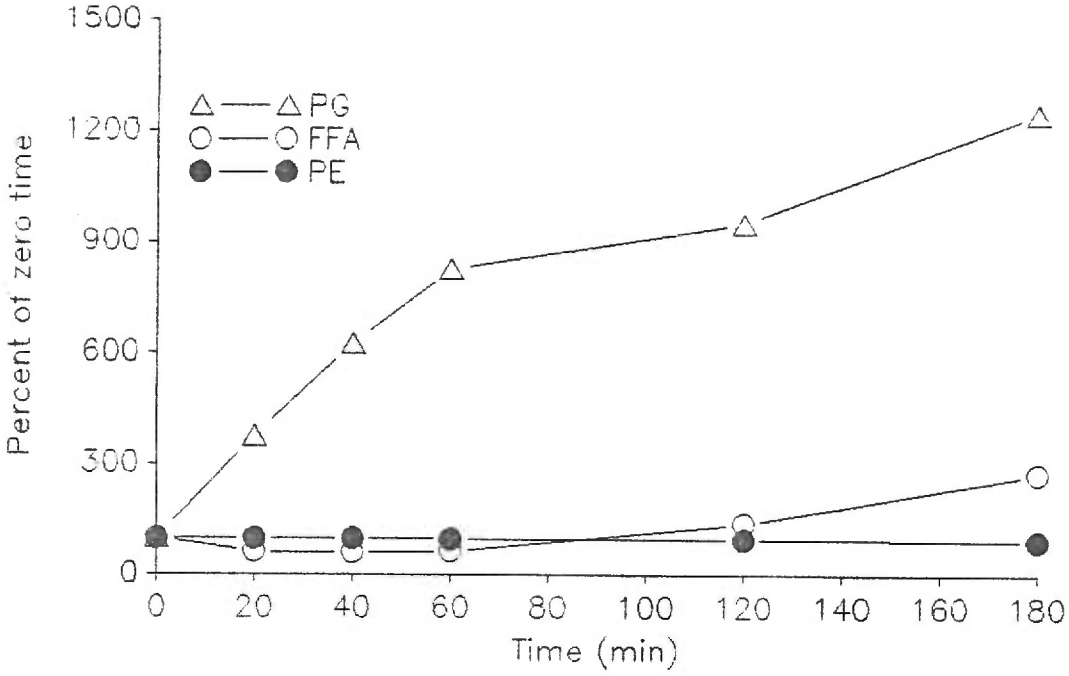

40
30
Molecular weight X 10^{-3}
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Generation time (h)

FIG. 3. Distribution of radioactivity in the lipids of N. gonorrhoeae strain FA171 labeled with (a) [^{3}H]-palmitoyl-PE or (b) [^{3}H]-palmitoyl-PG. Labeled phospholipid (ca. 6 to 7 X 10^{5} CPM) was added to the medium at the beginning of the exponential growth phase. Samples were removed and transferred to microfuge tubes at 0, 20, 40, 60, 120, and 180 min. After centrifugation, the cell pellets were washed, and the lipid extracted. The distribution of radioactivity into each lipid species was expressed as a percentage relative to time zero.

(a)


1500
1200
900
600
300
0
Percent of zero time
PG
FFA
PE
0
20
40
60
80
100
120
140
160
180
Time (min)

(b)

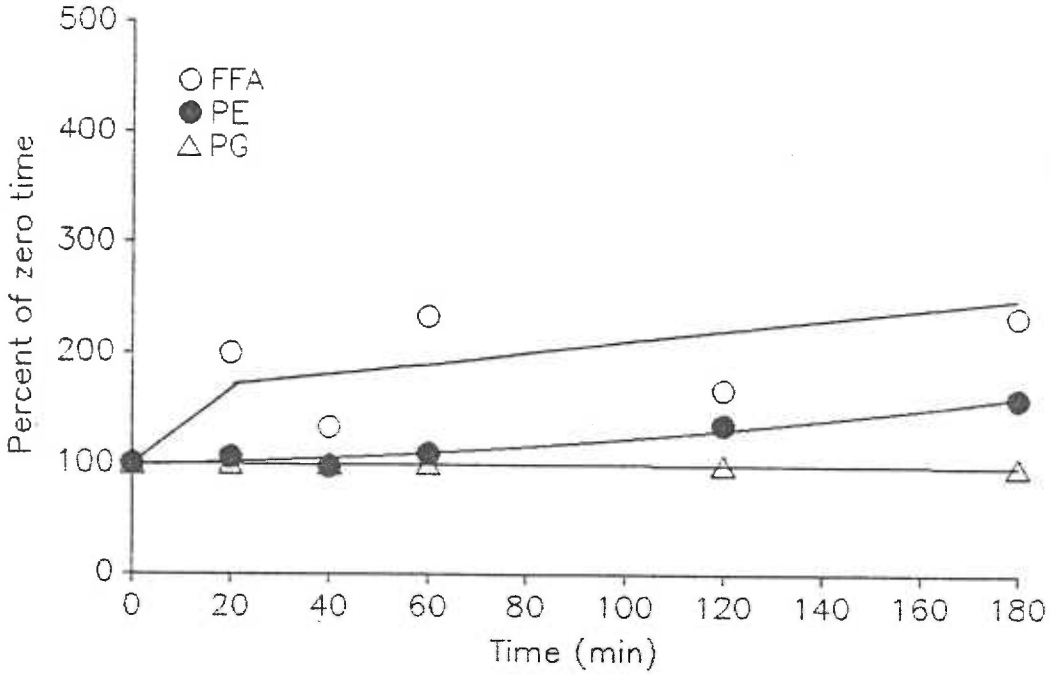
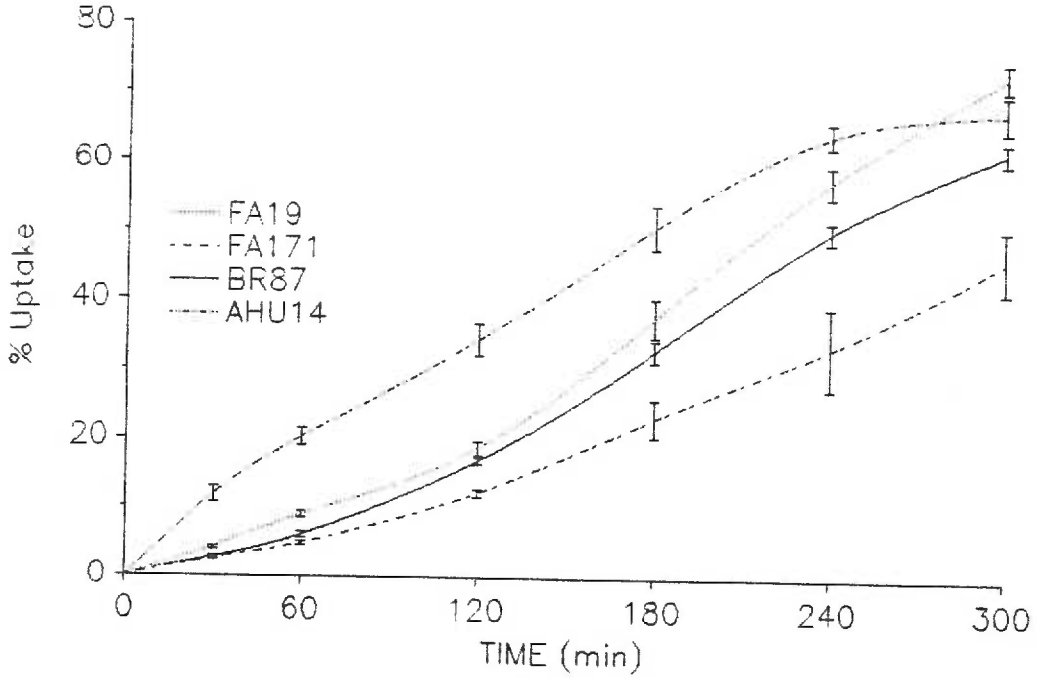

500
400
300
200
100
0
Percent of zero time
FFA
PE
PG
0
20
40
60
80
100
120
140
160
180
Time (min)

FIG. 4. Uptake of HSA bound-[^{3}H]-palmitic acid by N. gonorrhoeae strains FA171, FA19, BR87, and AHU14. HSA-[^{3}H]-palmitic acid complex was added to log phase cells. The cell suspensions were incubated at 37`C and samples collected at 0, 30, 60, 120, 180, 240, and 360 min. Time 0 refers to the time that the labeled complex was added. Percent uptake is expressed as a percentage of the total radioactivity added. Growth was monitored with a Klett-Summerson colorimeter with a No. 54 filter. (a). Uptake of [^{3}H]-palmitic acid over time. (b). Relationship of viable counts to uptake of [^{3}H]-palmitic acid.

(a)


80
60
40
20
0
% Uptake
FA19
FA171
BR87
AHU14
0
60
120
180
240
300
TIME (min)

(b)

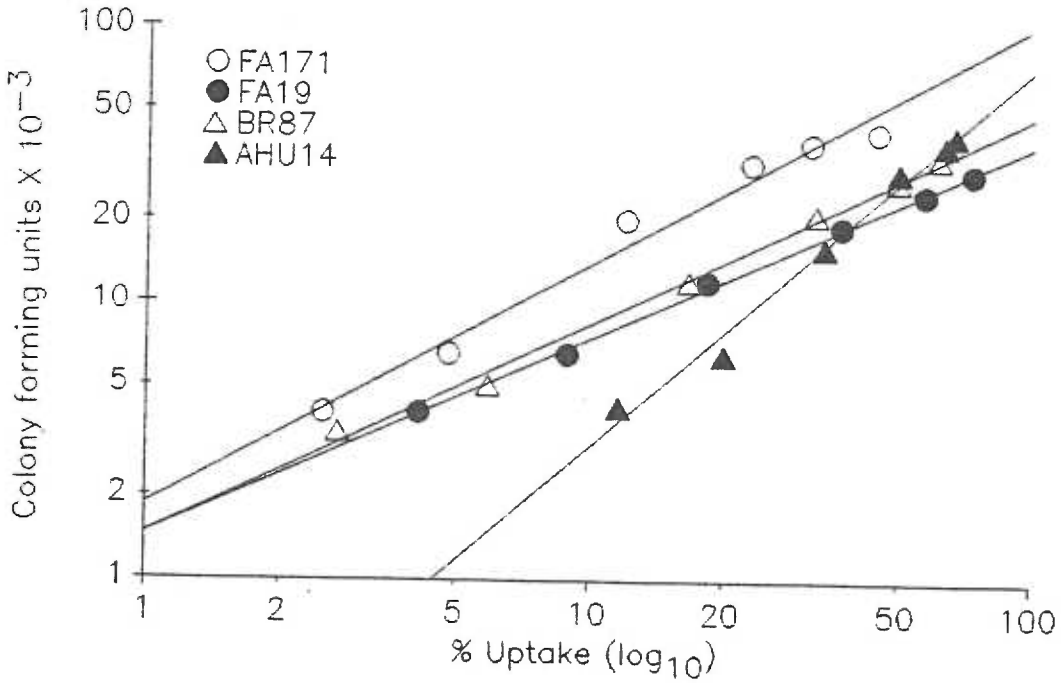

100
50
20
10
5
2
1
Colony forming units X 10^{-3}
FA171
FA19
BR87
AHU14
1
2
5
10
20
50
100
% Uptake (log_{10})

FIG. 5. Distribution of radioactivity in the lipids of N. gonorrhoeae strains FA19 (△—△), FA171 (●—●), BR87 (▲—▲), and AHU14 (○—○) during growth with HSA bound-[^{3}H]-palmitic acid. HSA-[^{3}H]-palmitic acid complex was added to exponential phase cells. Cultures were incubated at 37°C in a gyratory water bath, and samples were collected at various time intervals for lipid analysis. Distribution of [^{3}H]-palmitic acid in: (a). FFA; (b). PE; (c). PG.

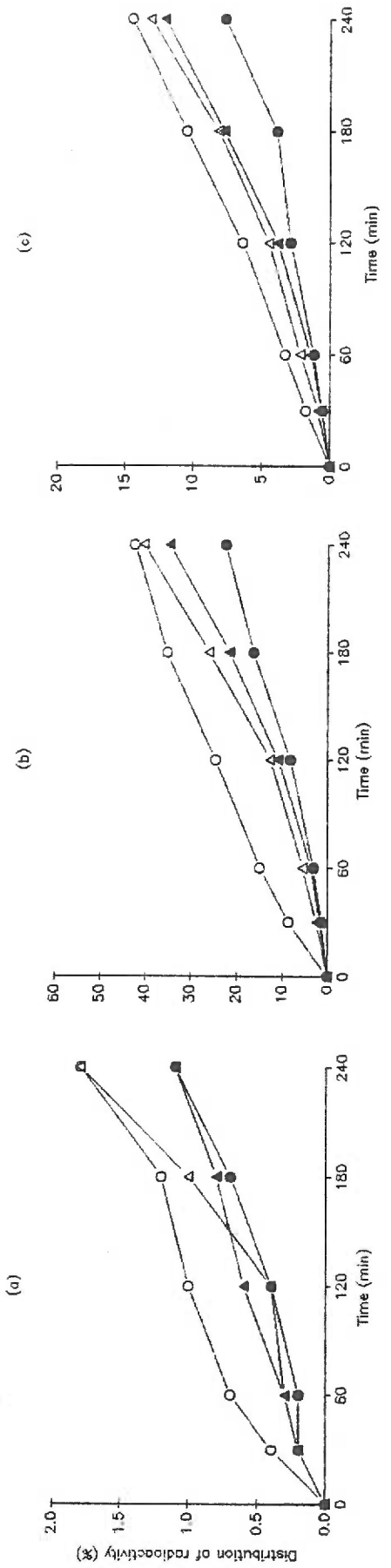

(a)
(b)
(c)
Distribution of radioactivity (%)
Time (min)
Time (min)
Time (min)
0.0
0.5
1.0
1.5
2.0
0
10
20
30
40
50
60
0
5
10
15
20
0
60
120
180
240

FIG. 6. Inhibition of BSA-[^{3}H]-palmitic acid uptake by unlabeled BSA. *N. gonorrhoeae* strain AHU14 was grown in chemically defined medium for *ca.* 1 generation. Aliquots of the cell suspensions were incubated in the presence of increasing amounts of unlabeled BSA. The reaction mixtures were incubated at 37°C for 30 min prior to the addition of BSA-[^{3}H]-palmitic acid complex.

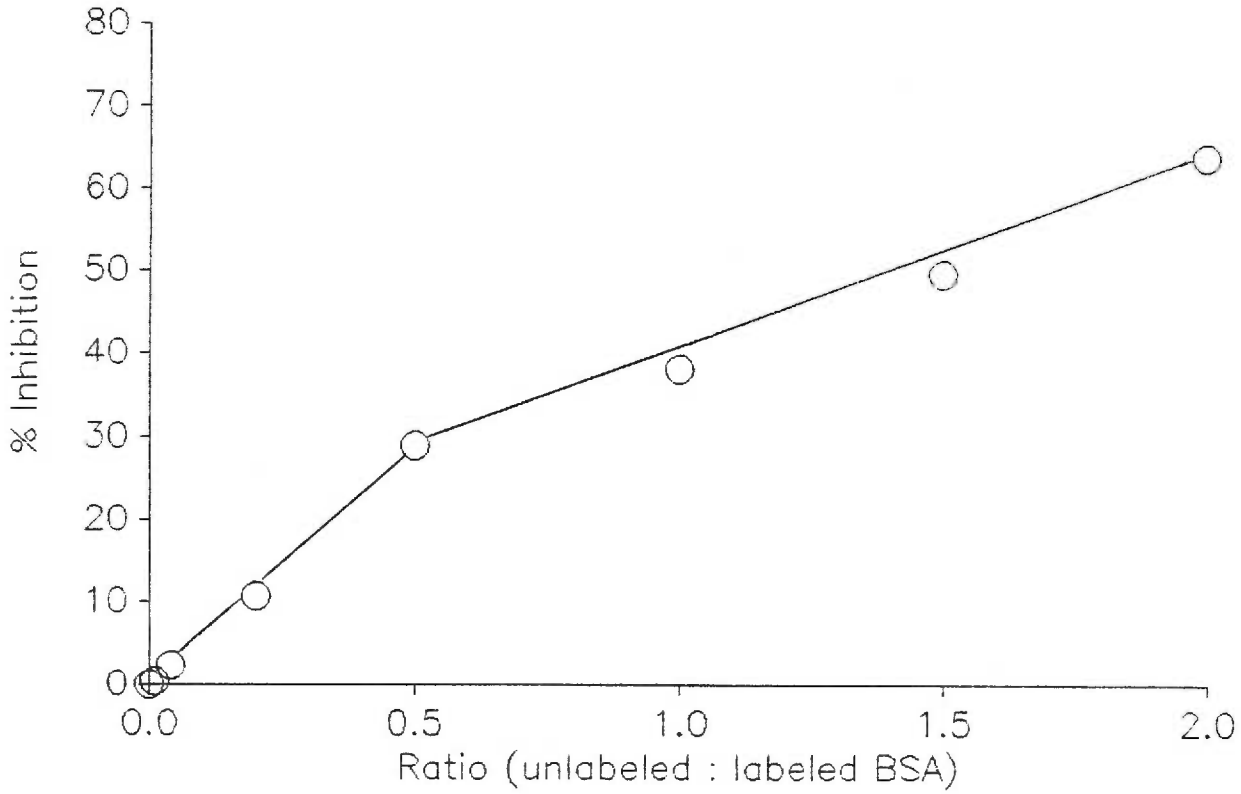

80
70
60
50
40
30
20
10
0
% Inhibition
0.0
0.5
1.0
1.5
2.0
Ratio (unlabeled : labeled BSA)

FIG. 7. Effect of chloramphenicol on the uptake of [^{3}H]-palmitic acid. Chloramphenicol (10 μg/ml) was added to a log phase culture of N. gonorrhoeae strain FA171 and incubation was continued for 5 min at 37°C before the addition of [^{3}H]-palmitic acid (5 μCi/ml). Samples were removed at 0, 120, and 240 min (lanes D, E, and F). Cell pellets were solubilized in final sample buffer prior to SDS-PAGE (10% acrylamide gel) and fluorography. The same experiment was carried out in the absence of chloramphenicol (lanes A, B, and C); equivalent amounts of protein (40 μg/ml) were loaded in each lane.

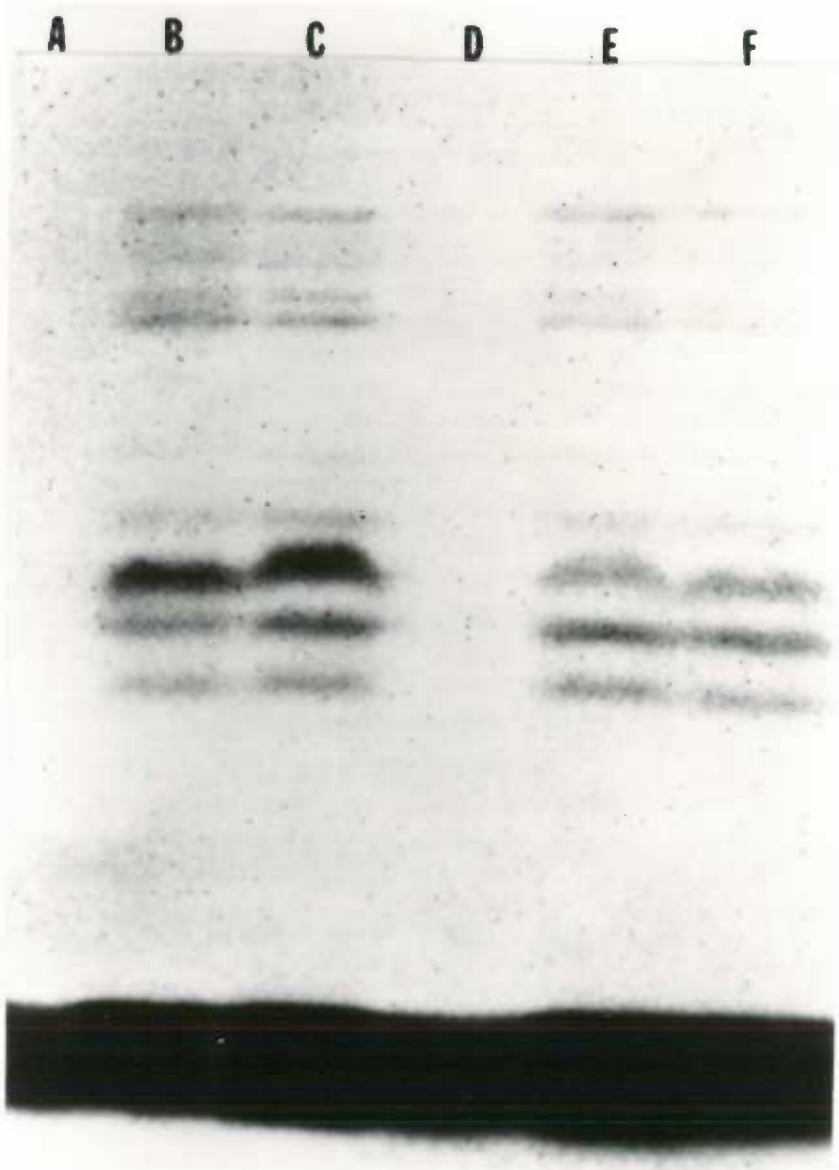

A
B
C
D
E
F

FIG. 8. Effect of cerulenin on the growth of N. gonorrhoeae strain FA171. Exponential phase cells were suspended in chemically defined medium containing different concentrations of cerulenin (numbers in parenthesis indicate μg/ml) to an initial turbidity of 50 klett units. After 30 min of incubation at 37°C, [^{3}H]-palmitic acid was added (final concentration: 5 μCi/ml). Growth was followed turbidometrically. Percent inhibition of growth was determined as a percentage relative to growth in the absence of cerulenin.

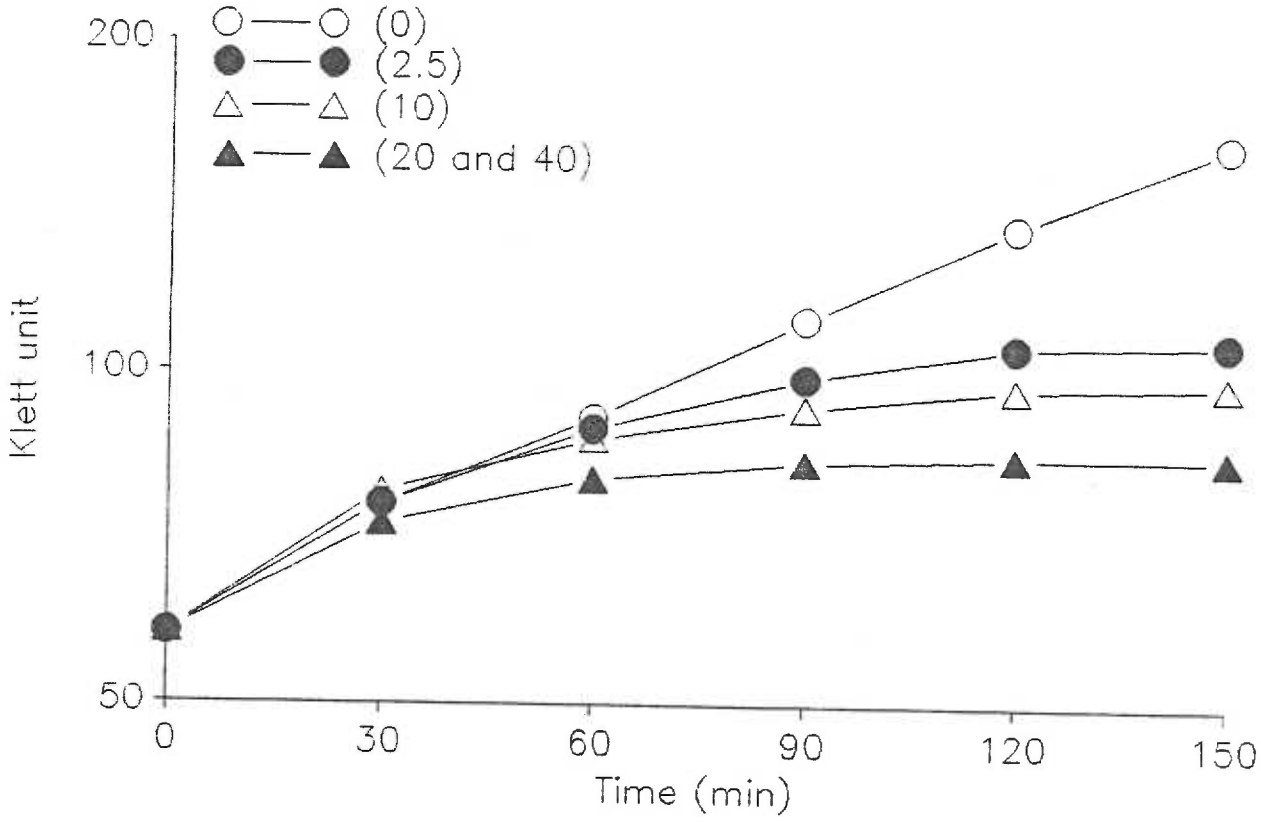

200
100
50
Klett unit
(0)
(2.5)
(10)
(20 and 40)
0
30
60
90
120
150
Time (min)

FIG. 9. Fluorograms of cerulenin-treated N. gonorrhoeae strain FA171 labeled with [^{3}H]-palmitic acid. Cerulenin was added to early log phase of N. gonorrhoeae strain FA171 growing in chemically defined medium. After 30 min of incubation at 37°C, [^{3}H]-palmitic acid (5 μCi/ml) was added and incubation continued for 2.5 h. Cells were harvested by centrifugation and cell membranes prepared. Equivalent amounts of protein were analyzed by SDS-PAGE (10% acrylamide gel) and fluorography. Lane A to F corresponds to 0, 2.5, 5, 10, 20, 40 μg/ml of cerulenin, respectively.

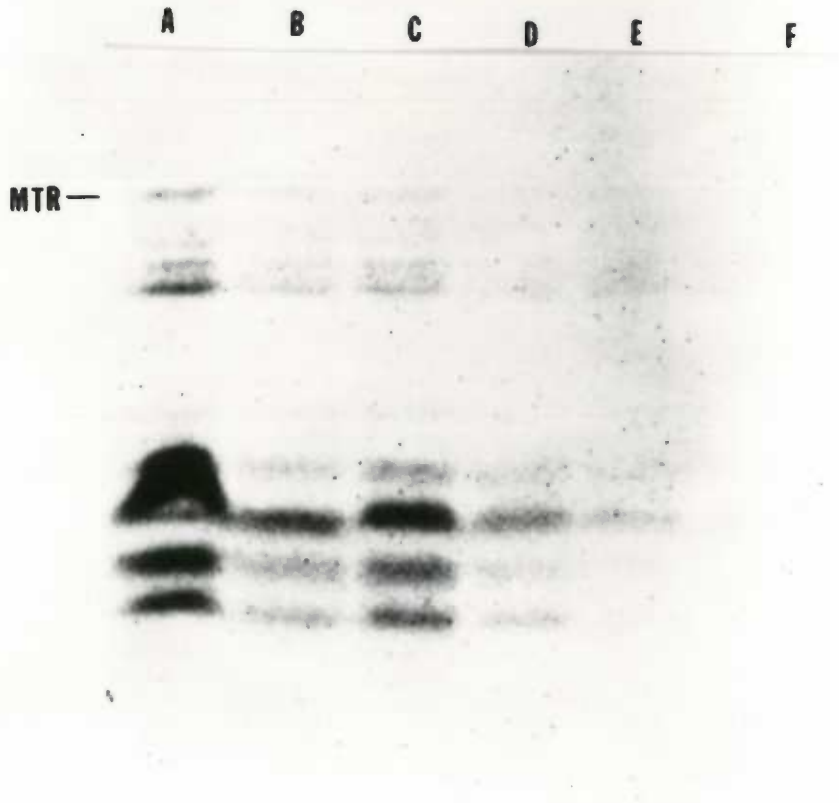

A
B
C
D
E
F
MTR—

FIG. 10. Distribution of [^{3}H]-palmitic acid in the lipids of N. gonorrhoeae strain FA171 treated with cerulenin. Cerulenin (10 µg/ml) was added to early log phase cultures growing in chemically defined medium for 30 min before the addition of [^{3}H]-palmitic acid (5 µCi/ml). Samples were collected at various time intervals for lipid analysis. (a). with cerulenin. (b). without cerulenin.

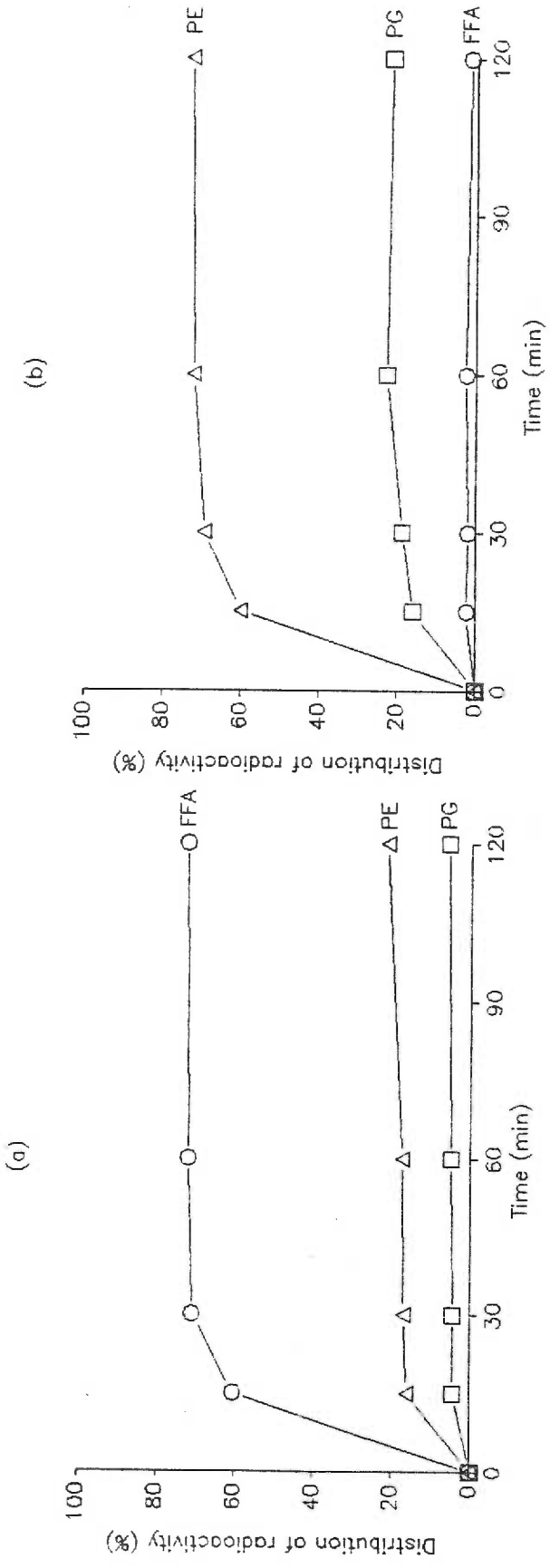
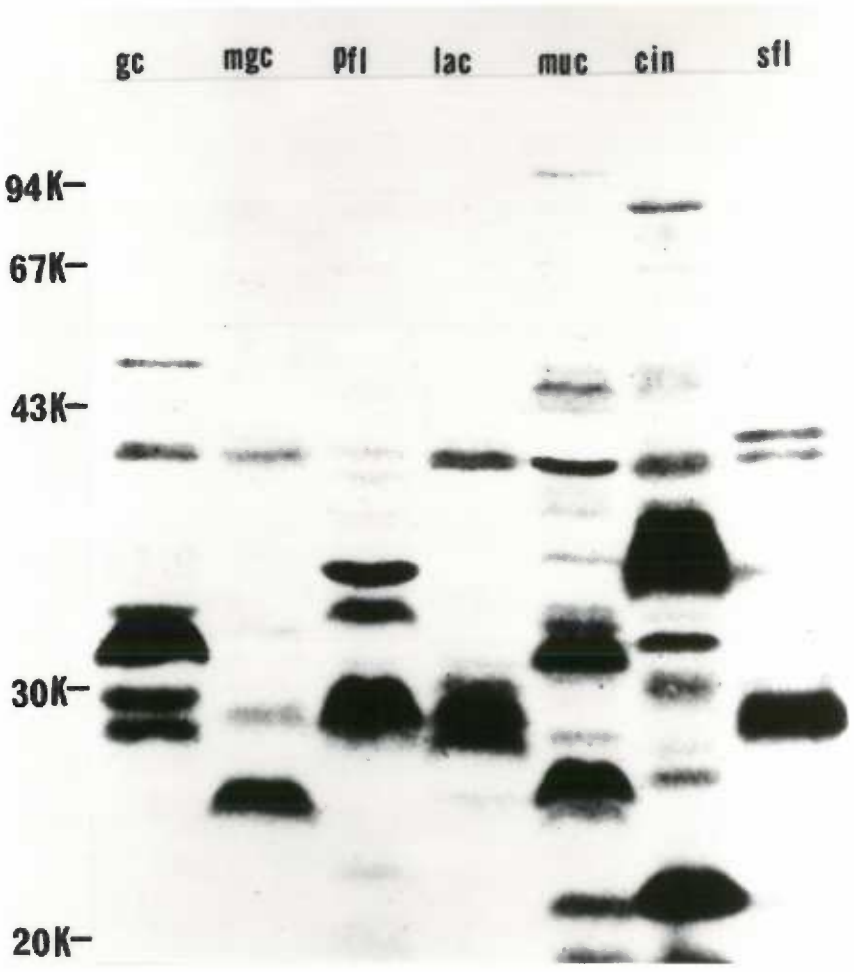

(a)
Distribution of radioactivity (%)
100
80
60
40
20
0
0
30
60
90
120
Time (min)
FFA
PE
PG
(b)
Distribution of radioactivity (%)
100
80
60
40
20
0
0
30
60
90
120
Time (min)
PE
PG
FFA

FIG. 11. Proteolipid profiles of other Neisseria spp. Strains representing various Neisseria spp. (Table 1) were grown in medium containing [^{3}H]-palmitic acid (10 μCi/ml) for ca. 2 generations. Equivalent amounts of cell pellets (40 μg/ml) were solubilized in final sample buffer prior to SDS-PAGE (10% acrylamide gel) and fluorography. gc: N. gonorrhoeae; mgc: N. meningitidis; pfl: N. perflava; lac: N. lactamica; muc: N. mucosa; cin: N. cinerea; sfl: N. subflava.


gc
mgc
pfl
lac
muc
cin
sfl
94K–
67K–
43K–
30K–
20K–

CONCLUSIONS AND FUTURE PERSPECTIVES

Many membrane proteins are modified either during or post translation. One type of modification is the covalent addition of fatty acids to the polypeptide chain. Fatty acid acylation seems to occur more frequently with membrane proteins that are in close association with lipid bilayers than with cytoplasmic or nuclear proteins. The discovery of a "lipoprotein" in the cell wall of E. coli by Braun has led to detailed structural studies showing that fatty acids were bound to the protein by both ester and amide linkages. The lipid consisted predominantly of long-chain fatty acids. A major advance in our knowledge of the biosynthesis of membrane lipoproteins was provided by the identification of the precursor form of murein lipoprotein, the prolipoprotein. The structure of prolipoprotein differs from that of mature lipoprotein in a number of ways. A series of post-translational modifications and processing reactions occurred before the assembly of the lipoprotein into the outer membrane. These modifications included the proteolytic removal of the signal peptide, the transfer of a glycerol moiety with or without fatty acids to the sulfhydryl group of cysteine, and the attachment of a fatty acid to the alpha-NH_2 group of cysteine to generate a fully modified and processed lipoprotein.

Presently, there are a substantial number of membrane proteins from enveloped viruses, eucaryotic cells, and Gram-negative bacteria that have been reported to contain covalently bound fatty acids. We have demonstrated that membrane proteolipids are also present in N. gonorrhoeae by labeling cells during growth in medium containing [^{3}H]-palmitic or -oleic acid, and

analysis by SDS-PAGE and fluorography. These results offer additional evidence that fatty acid acylation is a general phenomenon that occurs among widely diverse organisms. The fatty acids present on the gonococcal proteolipids were not removed by exhaustive extraction with organic solvents, phospholipase treatment, prolonged boiling, treatment with urea and EDTA, hot phenol extraction, or by SDS. They appeared to be attached by an ester linkage directly to the polypeptide chain. The fatty acid acylation occurred during the post-translational maturation of the protein. This post-translational attachment of fatty acids at specific sites on the protein may help to direct and anchor these proteins in the cell membrane. Nevertheless, the role that fatty acids play in the structure of proteolipids is not clear. How membrane proteins are directed and inserted into specific membrane locations is still a unresolved problem in membrane biology.

Our knowledge of the intracellular pathways that proteolipids follow during their maturation, as well as the characteristics of a protein that enable it to serve as a substrate for fatty acid acylation remains to be determined. The lack of nucleotide sequence homology between the gene encoding Braun's lipoprotein and gonococcal genomic DNA, the absence of a glycerol moiety, and the insensitivity to globomycin treatment suggest that there are unique features of proteolipid biosynthesis in N. gonorrhoeae. On the other hand, pulse-chase experiments indicated that Braun's lipoprotein and the proteolipids of N. gonorrhoeae shared a common pathway by utilizing phospholipids as the acyl donor. Consequently, it will be of interest to further examine and compare the differences as well as the similarities between the biosynthsis of Braun's lipoprotein and gonococcal proteolipids.

Elucidation of the precise chemical structure of the acylation site will depend upon the purification of gonococcal membrane proteolipids, which remains a difficult task. Determination of the intracellular location of proteolipids and identification of the specific polypeptide acyltransferases will also help to delineate the biosynthetic pathway of proteolipids and their role(s) in pathogenesis and membrane biology.

The function(s) of fatty acid acylation are only beginning to be understood. Palmitylation of integral membrane proteins is thought to be involved in protein-protein and/or protein-lipid interactions within membranes, possibly by influencing protein folding or protein orientation. The diversity of proteolipids demonstrated in our study suggest that covalent modification by fatty acids may serve a variety of functions depending on the type of protein to which they are attached. For example, the fatty acids on the Mtr-associated protein may contribute to a decrease in membrane permeability, while the fatty acids on protein III may provide an anchor for protein I.

The recent discovery that the H.8 antigen of pathogenic Neisseria species was a proteolipid and the subsequent cloning of the gene have provided an excellent opportunity to broaden our knowledge of the biochemical and biophysical characteristics of proteolipids. Furthermore, genetic analysis of the H.8 antigen could provide an insight into its function, its biosynthetic pathway, and possibly its association with virulence.

Manuscript 3.

Use of Two-Dimensional Peptide Mapping to Elucidate the Primary Structure and Surface Exposed Regions of the Major Iron-Regulated Protein of *Neisseria gonorrhoeae* and other *Neisseria* species.

Cheng-Yen Chen and Stephen A. Morse

ABSTRACT

Neisseria gonorrhoeae and N. meningitidis express a major iron-regulated protein (MIRP) with an apparent molecular weight (MW) of 37,000 daltons. This protein binds approximately 1 mole of Fe^{3+} per mole of protein and probably functions in iron acquisition. A MIRP with a similar size has been observed in N. lactamica and in some strains of N. cinerea; other strains of N. cinerea were found to have a MIRP with a M.W. of 36,500 daltons. A 36,500 dalton MIRP was also expressed in one of five strains of N. subflava examined; the other four strains did not possess a MIRP. In spite of minor molecular weight differences, a polyclonal antiserum to gonococcal MIRP reacted with the MIRPs of N. gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea, and N. subflava in both Western blot and ELISA suggesting that these proteins shared conserved regions. In order to examine the structural heterogeneity of these proteins, MIRP from each species was purified by detergent extraction and ion-exchange chromatography. The purified MIRPs were radioiodinated and digested with trypsin. The resultant ^{125}I-peptides were then resolved by high-voltage thin-layer electrophoresis (TLE), followed by ascending thin-layer chromatography (TLC), and visualized by autoradiography. The proteins from all of the species examined shared a common set of peptides. However, the MIRP from each species also had unique peptides. The N-terminal amino acid sequences of these proteins was compared. The N-terminal 30 amino acids were identical for the proteins from N. gonorrhoeae, N. meningitidis, and N. lactamica; the sequence of the proteins from N. subflava and N. cinerea were also identical, but differed from those of the first group by 4 conservative amino acid substitutions. Surface-exposed peptides of the gonococcal MIRP

were determined by radioiodinating whole cells, extraction and purification of the MIRP, followed by trypsin digestion and analysis of the peptides by TLE and TLC. The results indicated that only 8 peptides were labeled compared with more than 17 peptides when the purified protein was labeled. Thus, it would appear that only a small portion of the molecule is surface-exposed.

INTRODUCTION

The membrane proteins of N. gonorrhoeae have been classified into several groups on the basis of their relative abundance, molecular weight (MW), and the effect of heat or reducing agents on their migration during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein I (P.I), the major outer membrane protein, is present in all gonococci regardless of colony phenotype (2). The apparent MW of P.I varies between 32,000 and 38,000 daltons in SDS-PAGE and is strain dependent. The migration of P.I is not altered by heat or in the presence of reducing agents. Two-dimensional tryptic peptide maps of iodinated P.Is from 10 gonococcal strains showed a significant degree of structural homology (26). Based on the presence or absence of additional ^{125}I-peptides, P.Is could be segregated into two pattern groups. One group consisted of P.Is with apparent MWs of 33,000 or 34,000; P.Is with MWs of 32,000 constituted the other group. The former group has been termed P.IA and the latter group P.IB. P.I isolated from different colony phenotypes (piliation, colony opacity) of a single strain showed no structural variations.

A second class of gonococcal outer membrane proteins, Protein II(s) (P.II), have been referred to as opacity-associated proteins (25). P.II is not expressed by every gonococcal strain. P.II(s) are heat-modifiable proteins with MWs varying from 24,000 to 30,000 by SDS-PAGE. The primary structure and the surface exposure of five P.IIs of N. gonorrhoeae strain JS3 by surface peptide mapping indicated that P.IIs were unrelated to either P.I or protein III (P.III) in structure, but were closely related to one another,

sharing about two-thirds of the peptides generated by alpha-chymotrypsin cleavage. The remaining third of the peptides varied with each P.II, resulting in unique portions of the molecule being exposed on the bacterial surface. The variable peptides were not always among the exposed peptides, suggesting that the structural differences occurred at a discrete site(s) of the P.II molecules and randomly throughout the protein, such alterations could probably present a variety of new immunodeterminants to the host during the course of infection (11).

Protein III (P.III) is another class of gonococcal outer membrane protein. This protein has an apparent MW of 30,000 in the absence of 2-mercaptoethanol and 31,000 in the presence of this reducing agent (15). ^{125}I-Peptide mapping indicated that all P.IIIs had very similar primary structures, regardless of the strain from which they were isolated, the source (i.e., whole cells or outer membranes), the reduction state, or the different P.I groups (9).

Mietzner et al. (19) described a common antigenic component that is present in all gonococci when grown under iron-limiting conditions. This major iron-regulated protein (MIRP) had an apparent MW of 37,000 (37K) and was not affected by either heat or reducing agents on SDS-PAGE. The presence of proteins antigenically-related to the gonococcal 37K protein were also found among the 40 strains of N. gonorrhoeae, N. meningitidis, N. lactamica, and N. cinerea examined by Western blots or ELISAs using rabbit monospecific antiserum and murine monoclonal antibodies against the gonococcal 37K MIRP (17). Seventeen strains representing other species of Neisseria and

Branhamella catarrhalis did not express this protein with the exception of one strain of N. subflava. While most of the Neisseria spp. expressed the 37K antigencially-related MIRP under iron-limiting conditions, some of the strains of N. cinerea as well as the strain of N. subflava expressed a MIRP with a slightly lower MW (36.5K).

In this study, two-dimensional peptide mapping and protein sequencing was used to examine the primary structures of the two different sizes of MIRP from several Neisseria species using either MIRP from whole cells or that purified by hexadecyltrimethylammonium bromide (CTB) extraction and cation-exchange chromatography. In addition, the results of surface labeling of gonococcal MIRP as well as peptide maps of MIRPs from gonococcal and meningococcal mutants that were unable to utilize iron from either transferrin or/and lactoferrin was investigated.

MATERIALS AND METHODS

Bacteria and growth condition. The strains used in this study are listed in Table 1. These strains were kindly provided from the following collections: P. F. Sparling and D. W. Dyer (University of North Carolina, Chapel Hill, NC), R. P. Williams (Baylor College of Medicine, Houston, TX), D. Hollis (Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA), and J. S. Knapp (Sexually Transmitted Diseases Laboratory Program, Center for Infectious Diseases, CDC). All strains were grown on GC medium base agar (Difco Laboratories, Detroit, MI) that contained a supplement (1%, vol/vol) similar to IsoVitaleX (BBL Microbiology Systems, Cockeysville, MD) except that the glucose and ferric nitrate were omitted. This medium was further supplemented with 0.5% (wt/vol) glucose and 25 μM Desferal Mesylate (Ciba-Geigy Corp., Summit, NJ). This medium is referred to as low-iron agar medium or liquid low-iron medium (excluding the agar). N. gonorrhoeae and N. meningitidis are unable to remove iron bound to Desferal (16). Inocula were prepared after growth for 16 to 20 h on GC agar medium supplemented with ferric nitrate. Cells were removed with a dacron swab and streaked heavily onto large petri dish plates (150 mm x 15 mm) containing 25 ml of low-iron medium. The inoculated plates were incubated at 37°C for 16 to 20 h in a humidified atmosphere containing 5% CO_2. Alternatively, the inoculated flasks were supplemented with 0.04% (wt/vol) $NaHCO_3$ and incubated at 37°C in a gyratory water bath.

Purification of MIRP. The procedure used for the purification of MIRP was that described by Mietzner et al. (18). Briefly, bacteria harvested from 1

liter (40 plates) of low-iron agar medium were resuspended in 40 ml of Davis A minimal medium (Difco Laboratories). After centrifugation (10,000 rpm, 15 min), the cell pellets were resuspended in 30 ml of 10mM N-2-hydroxyethyl-pipierazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 0.1% (vol/vol) protease inhibitor (10 mM phenylmethylsulfonyl fluoride in isopropanol) and were disrupted by two passages through a French pressure cell apparatus (1000 psi, room temperature) (SLM Instruments, Inc., Urbana, IL). The total protein from disrupted cells was determined by a modification of the Lowry method as described by Markwell et al. (14); the disrupted cell suspension was diluted to a final protein concentration of 1 mg/ml. Hexadecyltrimethylammonium bromide (CTB, Sigma Chemical Co., St. Louis, MO) was added at a detergent:protein ratio of 0.5 to 1. Final concentration of CTB was 0.05% (wt/vol). After incubation for 30 min at room temperature, the insoluble material was removed by centrifugation (48,000 x g) for 30 min at room temperature. CTB soluble proteins were then separated by cation-exchange chromatography (CM-Sepharose 6B-CL, Pharmacia, Piscataway, NJ). The MIRP-containing fractions were pooled and stored at -70°C. Fresh column packing was used for each purification.

Iodination of bacteria. Whole cells of N. gonorrhoeae strain FA19 were surface labeled with ^{125}I using Iodo-beads (Pierce Chemical Co., Rockford, IL) to facilitate the process. Gonococci were grown in low-iron liquid medium for ca. 2 generations. Cells were then harvested and washed once with 100 mM Tris-HCl buffer, pH 7.0. Cell pellets were resuspended in the same buffer and adjusted to an absorbance (A_{450}) of 2.0 (ca. 10^9 cells/ml). To 1 ml of the cell suspension, 200 μCi of $Na^{125}I$ (104 mCi/ml, New England Nuclear

Corp., Boston, MA) and 5 Iodobeads were added. The reaction was allowed to proceed for 15 min at room temperature. The reaction was stopped by removing the cell suspension from contact with the Iodobeads. After centrifugation, cell pellets were washed once with Tris-HCl buffer and prepared for SDS-PAGE. Alternatively, iodinated cells were mixed with unlabeled cells and extracted with CTB prior to gel electrophoresis and peptide mapping.

SDS-PAGE. SDS-PAGE was performed using the method described by Laemmli (13). The resolving gel consisted of 10% or 12% acrylamide (wt/vol) and contained 70 mM NaCl as described previously (19). Samples were solubilized at 100°C for 5 min in a final sample buffer consisting of 6.25 mM Tris buffer, pH 6.8, 2% SDS (wt/vol), 10% glycerol (vol/vol), 0.001% bromphenol blue (wt/vol), and 5% 2-mercaptoethanol (vol/vol). The gels were stained with a solution of 0.1% Coomassie blue (wt/vol) in water-methanol-acetic acid (5:5:2). A 10% acetic acid solution (vol/vol) was used to destain the gels. Alternatively, gels were stained with silver nitrate by the method of Tsai and Frasch (27) modified by the omission of the periodate oxidation step. The low molecular weight markers phosphorylase B (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (21K), and lysozyme (14.3K) (Bio-Rad Laboratories, Richmond, CA) were included with each gel.

Iodination and trypsin treatment of protein bands. The protein bands were excised from acrylamide gels and placed in siliconized tubes. After washing the gel slice with 10% methanol, the tubes were covered with parafilm and the film punctured to permit the moisture to evaporate. The dried gel slices were then transferred to disposable plastic tubes. To each tube, 20 µl of 0.5 M

phosphate buffer, pH 7.5, 5 ul of chloramine T (1 mg/ml, Sigma Chemical Co.), and 30 µl of ^{125}I (150 µCi) were added. The gels were allowed to absorb the liquid for ca. 1 h. One ml of sodium bisulfite (1 mg/ml) was added and the tube incubated at room temperature for 15 min to stop the reaction. The gel slice was removed, placed in dialysis tubing, and dialyzed against 10% methanol in distilled water (400 ml per slice) overnight at 4°C on a magnetic stirrer. Anionic exchange resin (Dowex 1X8-50, Sigma Chemical Co.) was added to the methanol solution to adsorb free radiolabeled iodine. The gel slices were transferred from the dialysis sac to a siliconized tube and dried under a heat lamp. To each tube, 400 µl of 0.2M $(NH_4)_2CO_3$ and 20 µl of TPCK-treated trypsin (8 mg/ml, Sigma Chemical Co.) were added. After incubation for 3 h at 37°C on a rotary shaker, an additional 20 µl of trypsin solution were added and incubation was continued overnight at 37°C. The solution containing the tryptic peptides was removed, lyophilized, and resuspended in 100 µl of distilled water. The peptide solutions were then transferred to siliconized screw-capped vials, lyophilized, and stored at -20°C.

^{125}I-Peptide mapping. The lyophilized tryptic ^{125}I-peptides were resuspended in distilled water to yield approximately 70,000 CPM/µl. A 1 µl-amount of this mixture was spotted onto a 10 x 10 cm cellulose-coated TLC plastic sheet without fluorescent indicator (EM Laboratories Inc., Elmsford, NY). High voltage thin-layer electrophoresis (TLE) was carried out at a constant 1000 V for ca. 10 min on a flatbed electrophoresis apparatus (LKB 2117 Multiphor, Bromma, Sweden) precooled to 4°C by a circulating cooling bath. Electrophoresis buffer (pH 2.1) was a solution of formic acid-acetic

acid-water (1:4:45). Progress of electrophoresis was monitored by using a mixture containing Rhodamine 6G, Orange II, and Fast green FCF (Analtech Inc., Newark, DL). The dye was spotted at the opposite end from the sample, and each TLC plate was removed when the dye front reached a preset mark. After electrophoresis, the sheet was air dried, turned 90°, and subjected to TLC using a solvent system of n-butanol-pyridine-water-acetic acid (13:10:8:2). Chromatography was allowed to proceed until the solvent front was within 3 to 5 mm from the top of the sheet. The sheets were dried and applied to X-ray film (XAR-5; Eastman Kodak Co., Rochester, NY). The autoradiograph exposure was enhanced by using a Cronex intensifying screen (Du Pont Co., Wilmington, DL) at -70°C.

One-dimensional peptide mapping and Western blotting. The MIRPs of several strains of N. gonorrhoeae isolated from urogenital infections, pelvic inflammatory disease, or disseminated infections were compared by the method described by Border and Jarvinen (3). Whole cell lysates were first separated by electrophoresis on a 10% polyacrylamide gel. After completion of the electrophoresis, the band corresponding to MIRP was cut out, equilibrated in stacking gel buffer and then transferred horizontally onto a second slab gel (15% polyacrylamide). S. aureus V8 protease (1-2 μg) (Sigma chemical Co.) in sample buffer was overlayed on the gel, and electrophoresis in the second dimension was carried out. The gel was electroblotted onto a nitrocellulose sheet according to the method of Burnette (4) and probed with polyclonal antiserum to gonococcal MIRP.

Transferrin preparation. Human serum transferrin (TF) (>90% purity,

substantially iron-free, Sigma Chemical Co.) was dissolved in 20 mM sodium bicarbonate and 40 mM Tris buffer (Trizma base; Sigma), pH 7.4, and dialyzed against the same buffer for 6 to 8 h. $^{55}FeCl_3$ (New England Nuclear Corp., Boston, MA) was complexed with a 10-fold molar excess of sodium citrate and incubated with apotransferrin in the Tris-bicarbonate buffer described above at room temperature for 30 min, followed by dialysis against the same buffer overnight. To achieve 30% saturation of the TF binding sites, 0.42 μg of iron was added per mg of apotransferrin (24). All glassware was washed with 6N HCl and thoroughly rinsed in deionized water before use.

Iron uptake from ^{55}Fe-TF by N. gonorrhoeae. N. gonorrhoeae strains FA19 (TF^+, LF^+) or FA6303 (TF^-, LF^-) was grown overnight on a chemically defined medium (CDM) (20) and suspended in 100 ml of fresh liquid CDM without the addition of ferric nitrate. This medium was further supplemented with $CaCl_2$ (0.37 g/ml), $NaHCO_3$ (0.42 g/ml), and 7.5 μM Desferal. The cultures were incubated at 37°C in a gyratory shaker, and growth was monitored with a Klett-Summerson spectrophotometer (Klett Manufacturing Co., NY) using a no. 54 filter. After reaching the mid-exponential phase of growth, the culture was used to inoculate 500 ml of CDM containing 25 μM Desferal and incubation was continued. The culture was harvested when it began to show signs of growth limitation and the cells resuspended in 10 ml of liquid CDM with ^{55}Fe-TF (30% Fe saturation, and 30 μM TF) as the sole iron source. After 30 min of incubation at 37°C in a gyratory shaker, cells were collected (12,000 rpm, 15 min at room temperature), washed, and the MIRP extracted with CTB and purified as described previously. Radioactivity associated with each fraction from the CM-sepharose chromatography was determined by liquid scintillation

spectrophotometry and protein profiles in the peak fractions were analyzed by SDS-PAGE.

N-terminal sequence analysis. Purified MIRPs were dialyzed for 48 h at 4°C against five changes of distilled water and lyophilized prior to this analysis. Automated Edman degradation of protein samples was performed on an Applied Biosystems (ABI, Foster City, CA) 470A gas phase protein sequencer, using the original manufacturer's program and a load of approximately 100-500 pmol of protein sample. All sample filters were pre-treated with biobrene-plus (ABI). The phenylthiohydantoins (PTH-amino acids) were identified by HPLC on a Brownlee PTH-C18 MPLC cartridge (2.1 x 220 mm) in an ABI model 120A liquid chromatograph system. The eluents were monitored at 269 nm and quantitative analysis was performed on an ABI model 900A data system using the manufacturer's standard software. All reagents for the gas-phase sequencer were purchased from ABI.

RESULTS

SDS-PAGE of whole bacteria and purified MIRP. The SDS-PAGE profiles of purified MIRP and whole-cell lysates from N. gonorrhoeae strains FA19 (lanes C, H, and I), FA6303 (lanes B, F, and G), and FA6342 (lanes A, D, and E) are shown in Fig. 1. MIRP was expressed only under iron-limiting conditions (D, F, and G) and not during growth under iron-replete conditions (E, G, and I). All three gonococcal strains had a MIRP with a similar apparent molecular weight (i.e., 37,000 daltons) whether observed in whole-cell lysates or in its purified form. The SDS-PAGE profiles of whole-cell lysates and purified MIRP from N. cinerea strains 33817 and 33837 grown on solid low-iron medium are shown in Fig. 2. The MIRP of N. cinerea strain 33817 had the same apparent molecular weight as the gonococcal 37,000-dalton MIRP (lane E), while the MIRP of strain 33837 had a lower apparent molecular weight (36,500 daltons). The SDS-PAGE profiles of whole-cell lysates and purified MIRP from N. meningitidis, N. lactamica, and N. subflava are shown in Fig. 3. Except for N. subflava, the MIRP from these Neisseria species appeared to be similar in apparent molecular weight to the gonococcal 37,000-dalton MIRP. The MIRP of N. subflava strain B886 had an apparent molecular weight similar to that of N. cinerea strain 33837. Regardless of apparent molecular weight, the MIRP from these Neisseria species reacted with a polyclonal antiserum to gonococcal MIRP on Western blots and in ELISA (8, unpublished observation).

Comparison of two-dimensional peptide maps of MIRP. After SDS-PAGE of purified MIRP from N. gonorrhoeae, N. meningitidis, N. cinerea, N. lactamica, and N. subflava, the bands corresponding to MIRP were excised from the

SDS-polyacrylamide gels and subjected to two-dimensional peptide mapping as previously described. Autoradiographs of these peptide maps are shown in Fig. 4. The ^{125}I-peptide maps of MIRP from these Neisseria species were very similar indicating that the primary structure of MIRP is highly conserved. Notwithstaning the shape of the spot and the relative radioemitting intensity, there were approxmately 15 common peptides (Fig. 5A) that were shared among the various MIRPs. Unique peptides were also associated with each species (Fig. 5B). However, most of them appeared as weaker intensity spots on autoradiographs. The peptide maps of MIRP from pathogenic Neisseria species (N. gonorrhoeae and N. meningitidis) were virtually identical. When the shape of the spot and the relative radioemitting intensity were taken into consideration, the peptide maps of the MIRP from the pathogenic species were slightly different from those of the commensal species. However, the peptide maps of the MIRP from the commensal species appeared similar to one another.

N-terminal amino acid sequence. The N-terminal amino acid sequence of the MIRP from five Neisseria species is shown in Fig. 6. The amino acid sequences through residue 30 were identical among N. gonorrhoeae strain FA19, N. meningitidis strain FAM2, and N. lactamica strain 1519. N. subflava strain B886 and N. cinerea strain 33837 also shared the same sequence with substituted amino acids at residues 13 (Ala→Thr), 16 (Val→Leu), 22 (Arg→Gln), and 30 (Leu→Ile). These substitutions resulted from a single base replacement and were the same in both N. subflava strain B886 and N. cinerea strain 33837.

One-dimensional peptide mapping and Western blotting. The MIRP from several

clinical isolates of N. gonorrhoeae obtained from either urogenital infections (UGI), pelvic inflammatory disease (PID), or disseminated infections (DGI) were compared following cleavage at the COOH-terminal side of aspartic and glutamic acid with S. aureus V8 protease (8), electroblotting, and probing with polyclonal antiserum to gonococcal MIRP (Fig. 7). The patterns exhibited by V8 protease-digested MIRP on Western blots were essentially identical between the gonococcal laboratory strain F62 (Lane B) and the clinical isolates (Lane D to G). N. subflava strain B886 and N. cinerea strain 33837, both of which had 36,500 dalton MIRPs, exhibited identical blot patterns (Lane A and C) that were different than that observed with the gonococcal strains.

Comparison of 36,500 dalton and 37,000 dalton MIRPs produced by N. cinerea. SDS-PAGE indicated that some strains of N. cinerea possessed a 36,500 dalton MIRP instead of a 37,000 dalton MIRP (Fig. 2). Tryptic peptide maps representing the two size classes of MIRP are shown in Fig. 8. These maps were almost identical except for a few peptides. This suggests that the 500 dalton difference in molecular weight might be due to a simple deletion. However, there were significant differences between the peptide map of the 36,500 dalton MIRP from N. subflava strain B886 and the 36,500 dalton MIRP from N. cinerea (Fig. 4) suggesting that a more complicated process might be involved.

Comparison of MIRPs from iron mutants of N. gonorrhoeae. The peptide maps of MIRP from ethylmethane sulfonate (EMS) mutagenized, streptonigrin-resistant mutants of N. gonorrhoeae that were unable to utilize either transferrin (TF^-) and/or lactoferrin (LF^-) were compared to that of the parental TF^+

LF^+ strain (Fig. 9). Unique peptides as indicated by arrows were observed with strain FA19 (TF^+, LF^+), FA6342 (TF^-, LF^+), and FA6303 (TF^-, LF^-) suggesting that there were minor structural variations.

Iron uptake from ^{55}Fe-TF by N. gonorrhoeae. In order to examine whether the minor structural variations of MIRP observed in gonococcal TF^- strains could affect its ability to obtain iron from TF, N. gonorrhoeae strains FA19 (TF^+, LF^+) and FA6303 (TF^-, LF^-) were grown in liquid low-iron CDM, followed by incubation with ^{55}Fe-TF as described above. The MIRP was subsequently purified and the amount of ^{55}Fe associated with the protein determined as described previously. Both strains FA19 (Fig. 10A) and FA6303 (Fig. 10B) showed similar elution profiles from the CM-sepharose chromatography with three major protein fractions (a, b, and c). ^{55}Fe co-eluted with protein in fraction a that was shown by SDS-PAGE to only contain MIRP (Fig. 10C). The amount of ^{55}Fe associated with the MIRP from each strain was similar. Fraction c also contained a minute amount of MIRP, presumably in a denatured form since no labeled iron was detected.

Surface-exposed peptides of MIRP from N. gonorrhoeae. After SDS-PAGE of cell lysates of surfaced-labeled whole cells of N. gonorrhoeae strain FA19, the band corresponding to MIRP was excised and subjected to peptide mapping as described previously. The results (Fig. 11) indicated that there were about 8 surface-exposed peptides; two of which were strongly radioemitting peptides. Extended exposure periods of up to 4 weeks revealed the weaker radioemitting peptides. Fig. 12. shows a composite of the MIRP peptide maps. Of the 15 peptides that were common to all MIRPs, five appeared in the surface peptide

maps as well (designated by solid black circles). There was also a spot (arrow) on the map of the surface-exposed peptides that did not correspond to a spot on the chloramine T-labeled maps.

DISCUSSION

The ability of pathogenic but not commensal Neisseria species to obtain iron from the transferrin and lactoferrin of the host has attracted a great deal of interest and may be an important factor that influences the virulence of these organisms (5,6,28). The absence of soluble siderphores as well as the observation that several proteins are regulated by the availability of iron have stimulated studies that have attempted to define the mechanism(s) by which pathogenic species of Neisseria acquire iron (1,19,22,29). Although the role of iron-regulated proteins in pathogenesis remains to be established, their expression appears to be one mechanism involved in iron-acquisition.

The presence of a 37,000 dalton MIRP in pathogenic Neisseria species grown under iron-limited conditions prompted the speculation that this protein plays a role in iron acquisition. Its subsequent purification from different Neisseria species provided an opportunity to analyze it further in an attempt to define its function(s) in vivo. Previous studies indicated that purified MIRP contains approximately 1 mole of Fe^{3+} per mole of protein and binds ^{55}Fe when whole cells of gonococci are incubated with ^{55}Fe-labeled transferrin as the only exogenous iron source (21). Purified MIRP from each of the Neisseria species examined exhibited isoelectric points of > 9.35 suggesting that these proteins may be very similar (data not shown). In the present study, we examined the structural heterogeneity of MIRP among different Neisseria species with respect to size, and to surface-exposure. To this end, we used the same technique of ^{125}I-peptide mapping that was employed to determine the primary structural relationships and the surface

exposure of gonococcal outer membrane proteins P.I, P.II, and P.III (9-11,25).

A polyclonal antiserum to the gonococcal MIRP reacted with the MIRPs of N. gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea, and N. subflava in both Western blots and ELISAs suggesting that these proteins shared conserved regions (17). Similar results were obtained when clinical isolates from either urogenital infections, pelvic inflammatory disease, or disseminated infections were examined. In addition, a polyclonal antiserum to the first ten N-terminal residues of the gonococcal MIRP (Asp-Ile-Thr-Val-Tyr-Asn-Gly-Gln-His-Lys) also reacted with the MIRPs from the above Neisseria species in Western blots indicating the similarity of the N-terminal sequences (data not shown). This similarity was subsequently confirmed by the N-terminal amino acid sequence analysis. The highly conserved N-terminal sequences of the MIRP from these Neisseria species suggested that this portion of the protein may have an essential function.

The peptide maps of MIRP from the various Neisseria species examined confirmed the existence of a high degree of structural homology, especially within the pathogenic Neisseria species. The N-terminal amino acid sequences of the gonococcal and meningococcal MIRP were previously found to be identical through the first 48 residues (18). Slight differences in amino acid composition previously reported (18) suggest that any differences between these MIRPs are minor. The peptide maps of the MIRPs from the commensal species exhibited more variation. The unique peptides were usually among the weaker radioemitting peptides suggesting that the primary structure was highly

conserved. A previous study showed that five monoclonal antibodies to gonococcal MIRP reacted uniformly on Western blots with MIRPs from pathogenic Neisseria species but not with those from commensal species (17) further indicating that there were common epitopes among pathogenic Neisseria species that were not found in commensal species. These minor differences among the MIRPs, including the variation in size, presumably reflect structural and not functional differences since all of these species were able to survive under iron-limiting conditions.

A few variable tryptic ^{125}I-peptides were observed among gonococcal TF^+ and TF^- strains. It is not clear whether these structural differences, presumably a result of EMS mutagenesis, contributed to the inability of these mutants to use TF as an exogenous source of iron. Since the MIRP isolated from gonococcal strain FA6303 (TF^-) acquired ^{55}Fe from ^{55}Fe-TF to about the same extent as its parental TF^+ strain (FA19), we conclude that the minor structural variation of MIRP in these mutants did not affect its ability to acquire iron from TF. However, it is not known whether the structural alterations might affect a subsequent step in iron acquisition.

The susceptibility of the MIRP to proteolytic cleavage following treatment of intact gonococci with the serine protease, lysosomal cathepsin G, had suggested that certain portions of the MIRP might be surface exposed (23). Our results confirmed that only a small portion of the gonococcal MIRP was surface exposed. Fewer peptides were observed in the surface peptide maps of gonococcal MIRP than in the chloramine T-^{125}I-peptide maps prepared from purified MIRP. Virtually all of the surface-labeled peptides could be found

in the chloramine T-^{125}I-peptide maps prepared from purified MIRP; however, there were differences in the extent to which certain peptides were labeled. Minor discrepencies were due to the use of Iodo-beads versus the chloramine T labeling procedure (data not shown). The inherent limitations of these methods may provide a somewhat incomplete impression about the surface-exposed areas of these proteins. Both chloramine T and Iodo-beads catalyze the iodination of tyrosyl residues under optimal conditions (3). Tryptophane, phenylalanine, and histidine are labeled to a lesser degree (12). Previous analysis has shown that there are 9 tyrosine, 10 phenylalanine, and 6 histidine residues in gonococcal MIRP (18). Peptides that do not contain these amino acids would not be labeled. In addition, some variation from run to run could be accounted for by temperature fluctuations during TLE and by the evenness with which TLC plates were saturated with running buffer. To further substantiate our findings, we are currently investigating the use of peptide mapping combined with high-performance liquid chromatography to isolate surface-exposed peptides for sequence analysis.

The highly conserved structure of MIRP together with its surface exposure and ability to bind iron could be useful properties for elucidating the unique and complex mechanism of iron acquisition utilized by N. gonorrhoeae.

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TABLE 1. Designations and sources of strains used in this study

Species	Strain designation	Source
N. gonorrhoeae	FA19 (TF^+, LF^+)[a]	P. F. Sparling
	FA6303 (TF^-, LF^-)	" "
	FA6342 (TF^-, LF^+)	" "
	F62	R. P. Williams
	12, 21 (DGI)	J. S. Knapp
	36-431, 36-433 (AHU^-)	" "
	82-045993, 83-041661 (PID)	" "
	83-033846, 83-015255 (UGI)	" "
N. meningitidis	FAM2, FAM11, FAM53, FAM58	D. W. Dyer
	serogroup A 1491	S. A. Morse
	serogroup B 1494	" "
	serogroup C 1495	" "
N. cinerea	33817, 33837	J. S. Knapp
N. lactamica	1519	D. Hollis
N. subflava	B886	D. Hollis

a. TF, transferrin; LF, lactoferrin. The + or - refers to the ability or inability to use TF or LF as source of iron.

FIG. 1. SDS-PAGE profiles of whole-cell lysates and purified MIRP of N. gonorrhoeae strains FA19, FA6303, and FA6342. Gonococci were grown on medium supplemented with ferric nitrate (+Fe) or on low-iron medium (-Fe) for 16 to 20 h. Whole cells (40 μg) or purified MIRP (5 μg) were solubilized in sample buffer at 100°C for 10 min and separated on 12% acrylamide gel. The gel was stained with Coomassie blue and destained with 10% acetic acid. Lane A to C: purified MIRPs of FA6342, FA6303, and FA19, respectively. Lane D, FA6342 (-Fe); Lane E, FA6342 (+Fe); Lane F, FA6303 (-Fe); Lane G, FA6303 (+Fe); Lane H, FA19 (-Fe); Lane I, FA19 (+Fe).

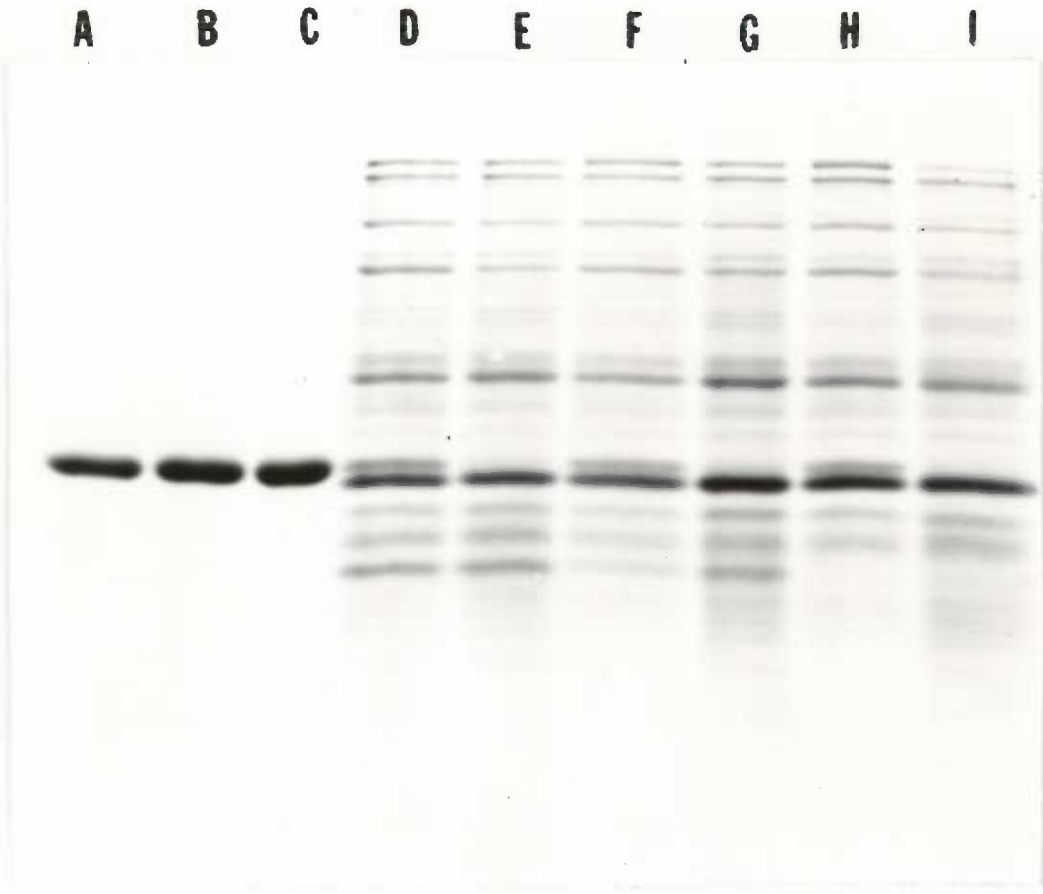

A
B
C
D
E
F
G
H
I
-94K
-67K
-43K
←37K
-30K

FIG. 2. Coomassie blue-stained SDS-PAGE gels of whole-cell lysates and purified MIRP of N. cinerea strains 33837 and 33817. Cells grown overnight on low-iron medium were used for the preparation of whole-cell lysates and for the purification of MIRP. Whole cells (40 μg) or purified MIRP (5 μg) were solubilized in sample buffer at 100°C for 10 min and separated on 10% acrylamide gel. Lanes A and B: purified MIRP from strain 33817 and 33837, respectively. Lanes C and D: whole-cell lysates of strain 33817 and 33837, respectively. Lane E: purified gonococcal MIRP.

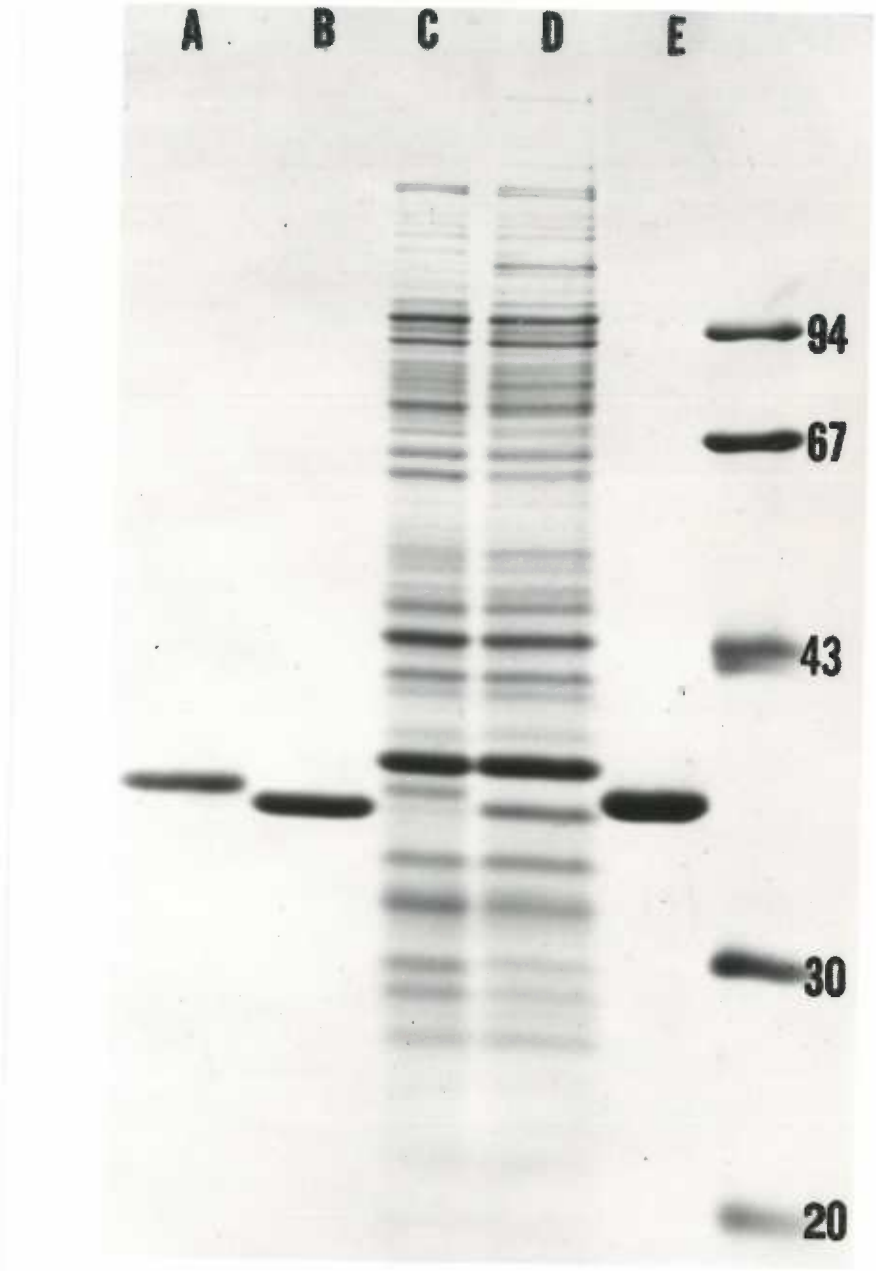

A
B
C
D
E
94
67
43
30
20

FIG. 3. Coomassie blue-stained SDS-PAGE gels of whole-cell lysates and purified MIRP of N. meningitidis strains FAM2, FAM11, FAM53, FAM58, N. subflava strain B886, and N. lactamica strain 1519. Purified MIRP (A) and whole-cell lysates (B) were prepared as previously described and separated on a 12% acrylamide gel. Lanes 1 and 7: N. subflava strain B886. Lanes 2 and 8: N. lactamica strain 1519. Lanes 3-6 and 9-12: N. meningitidis strains FAM58, FAM53, FAM11, and FAM2, respectively.

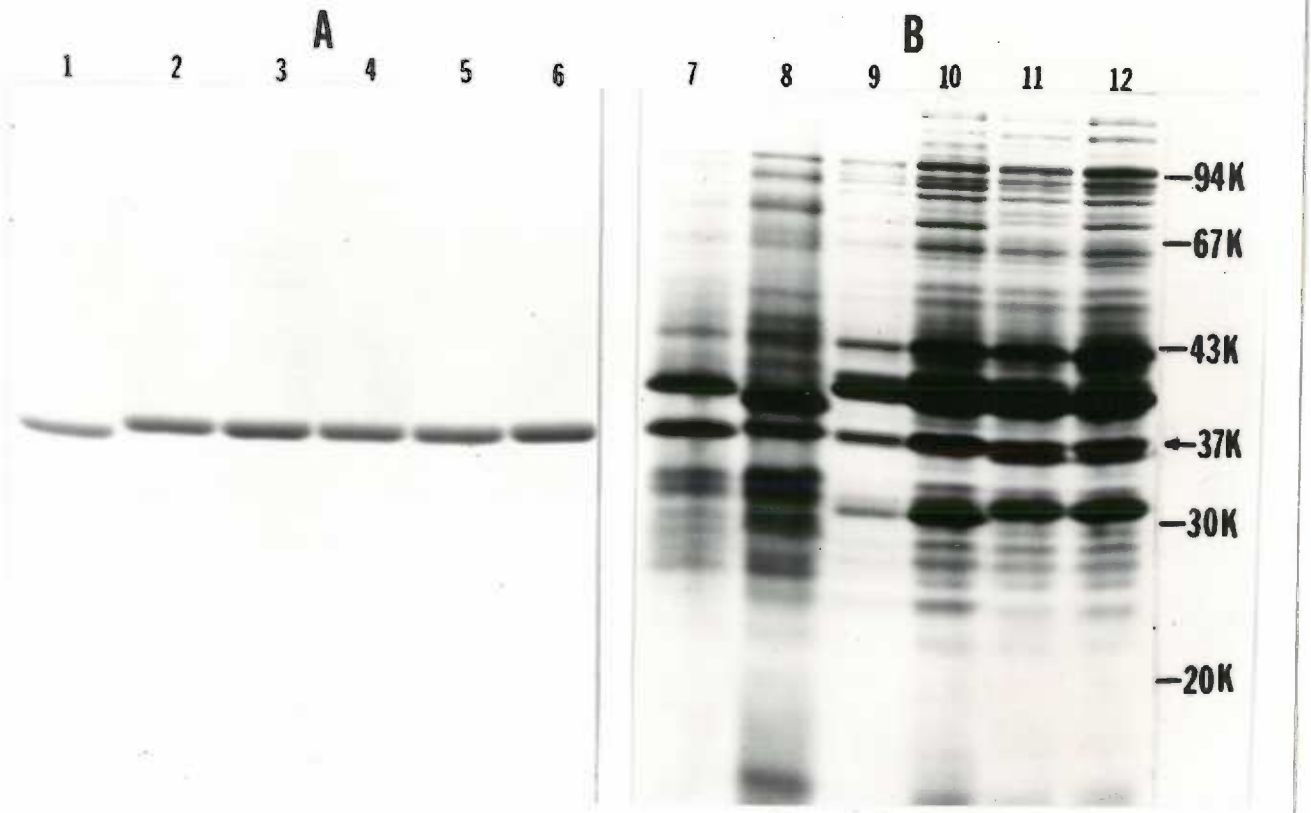

A
1
2
3
4
5
6
B
7
8
9
10
11
12
94K
67K
43K
37K
30K
20K

FIG. 4. Tryptic ^{125}I-peptide maps of purified MIRP from several species of Neisseria. A and B: N. gonorrhoeae strains FA19 and F62. C, D, and E: N. meningitidis strains FAM2, FAM11, and FAM53, respectively. F: N. subflava strain B886. G: N. cinerea strain 33837. H: N. lactamica strain 1519. TLC: thin-layer chromatography; TLE: thin-layer electrophoresis.

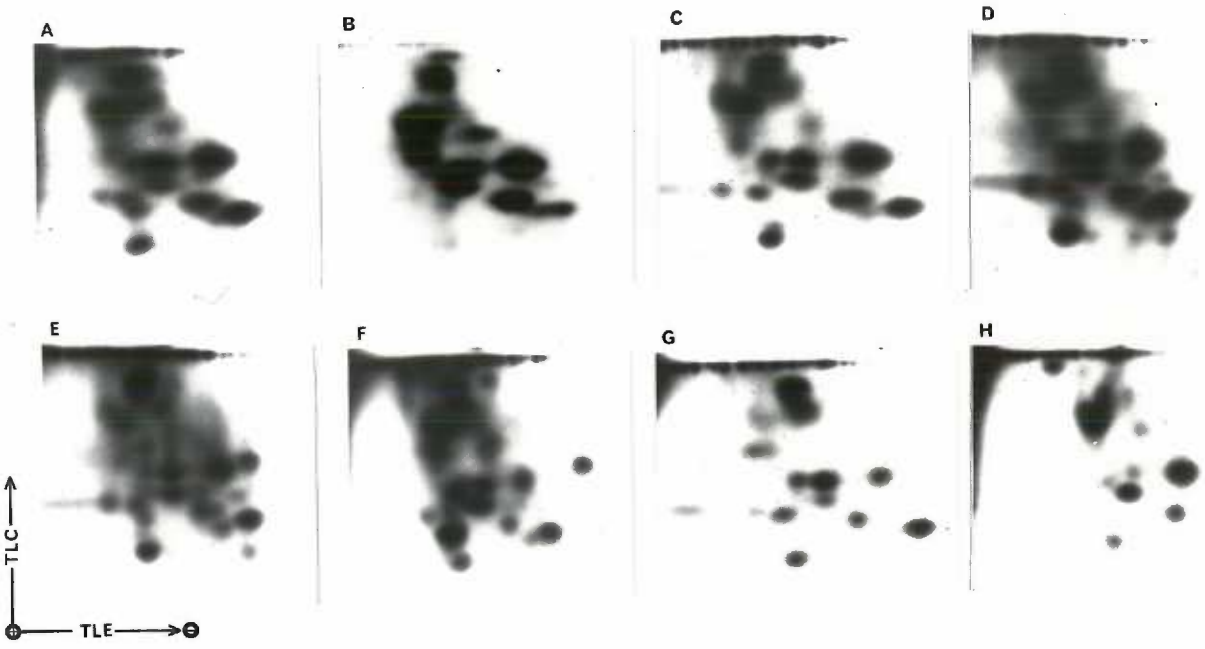
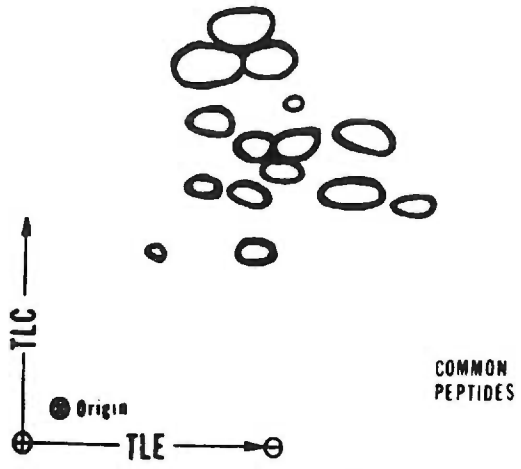

A
B
C
D
E
F
G
H
TLC
TLE

FIG. 5. Composite tryptic ^{125}I-peptide maps from purified MIRP of Neisseria spp. A: Peptides spots common to all MIRPs; B: Peptides spots that are unique, or common to two or three MIRPs. s: N. subflava; c: N. cinerea; g: N. gonorrhoeae; m: N. meningitidis; l: N. lactamica.

A.


TLC
Origin
TLE
COMMON
PEPTIDES

B.

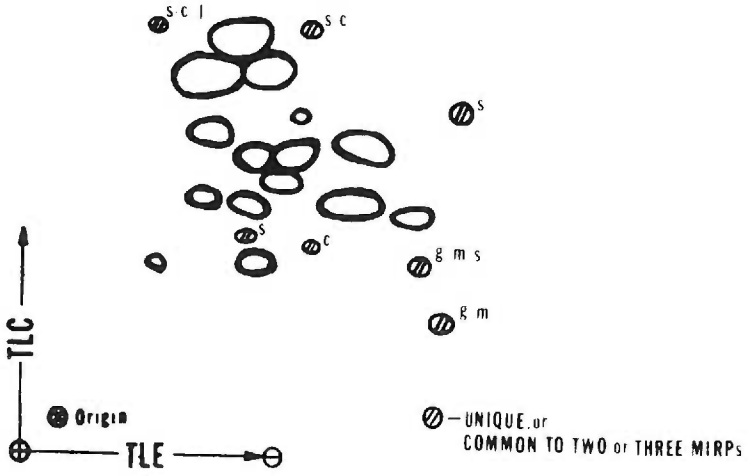

s c j
s c
s
s
c
g m s
g m
TLC
Origin
TLE
UNIQUE or
COMMON TO TWO or THREE MIRPs

FIG. 6. N-terminal amino acid sequence of the MIRP from five different Neisseria species. Abbreviations: gc: N. gonorrhoeae strain FA19; mgc: N. meningitidis strain FAM2; lac: N. lactamica strain 1519; sub: N. subflava strain B886; cin: N. cinerea strain 33837.

N-TERMINAL AMINO ACID SEQUENCE

	1								10						
gcMIRP:	Asp	Ile	Thr	Val	Tyr	Asn	Gly	Gln	His	Lys	Glu	Ala	Ala	Gln	Ala	Val	Ala
mgcMIRP:	Asp	Ile	Thr	Val	Tyr	Asn	Gly	Gln	His	Lys	Glu	Ala	Ala	Gln	Ala	Val	Ala
lacMIRP:	Asp	Ile	Thr	Val	Tyr	Asn	Gly	Gln	---	Lys	Glu	Ala	Ala	Gln	Ala	Val	Ala
subMIRP:	Asp	Ile	Thr	Val	Tyr	Asn	Gly	Gln	His	Lys	Glu	Ala	Thr	Gln	Ala	Leu	Ala
cinMIRP:	Asp	Ile	Thr	Val	Tyr	Asn	GLy	Gln	His	Lys	Glu	Ala	Thr	Gln	Ala	Leu	Ala

	20									30	
	Asp	Ala	Phe	Thr	Arg	Ala	Thr	Gly	Ile	Lys	Val	Lys	Leu
	Asp	Ala	Phe	Thr	Arg	Ala	Thr	Gly	Ile	Lys	Val	Lys	Leu
	Asp	Ala	Phe	Thr	---	Ala	Thr	Gly	Ile	Lys	Val	---	---
	Asp	Ala	Phe	Thr	Gln	Ala	Thr	Gly	Ile	Lys	Val	Lys	Ile
	Asp	Ala	Phe	Thr	Gln	Ala	Thr	Gly	Ile	Lys	Val	Lys	Ile

FIG. 7. One-dimensional peptide mapping and Western blotting. Whole-cell lysates of N. gonorrhoeae clinical isolates 36-431 (AHU^-), 83-033846 (UGI), 82-045993 (PID), and 12 (DGI) grown in low-iron medium were first separated by SDS-PAGE (10% acrylamide). The MIRP band was excised, transferred horizontally onto a second slab gel (15% acrylamide) and overlayed with S. aureus V8 protease (1-2 μg/ml) before electrophoresis was carried out. The gel was electroblotted onto a nitrocellulose sheet and probed with a polyclonal antiserum to gonococcal MIRP. Lane A: N. subflava B886; Lane B: N. gonorrhoeae F62; Lane C: N. cinerea 33837; Lane D to G: N. gonorrhoeae strains 36-431, 83-033846, 82-045993 and 12, respectively.

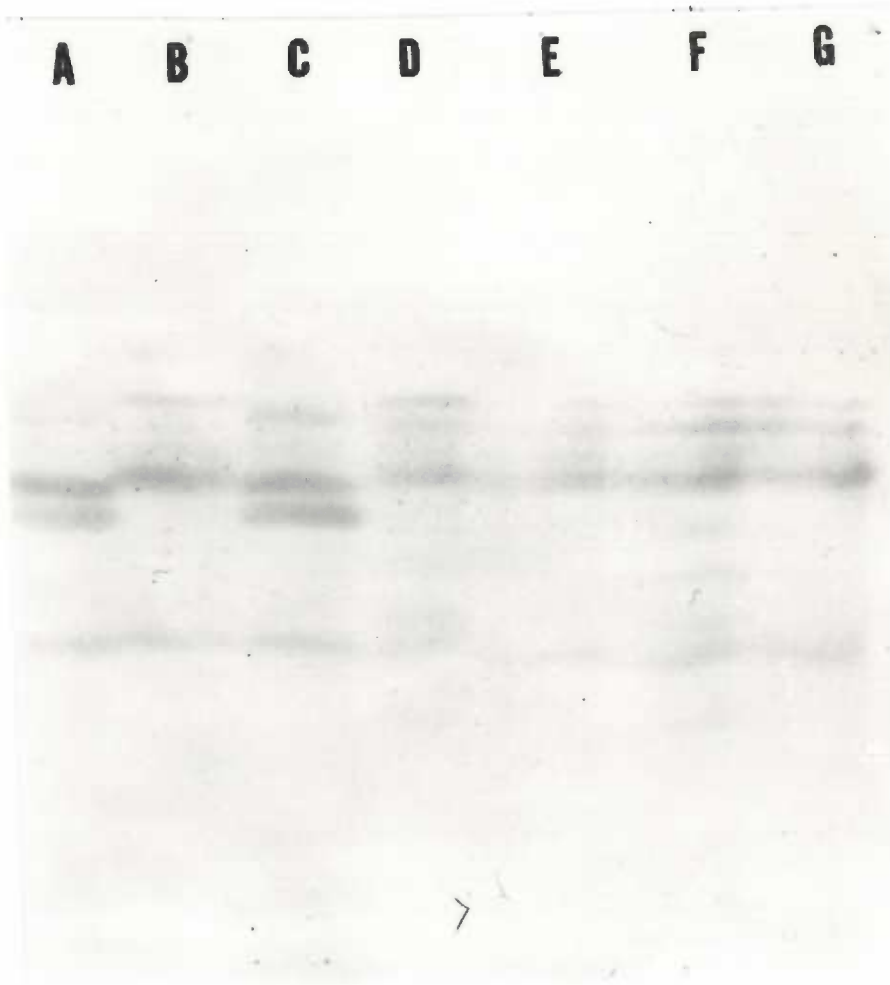

A
B
C
D
E
F
G

FIG. 8. Tryptic ^{125}I-peptide maps of purified MIRP from N. cinerea strains 33837 and 33817. A: strain 33837 (36,500 dalton MIRP); B: strain 33817 (37,000 dalton MIRP). Arrows indicate variable peptides.

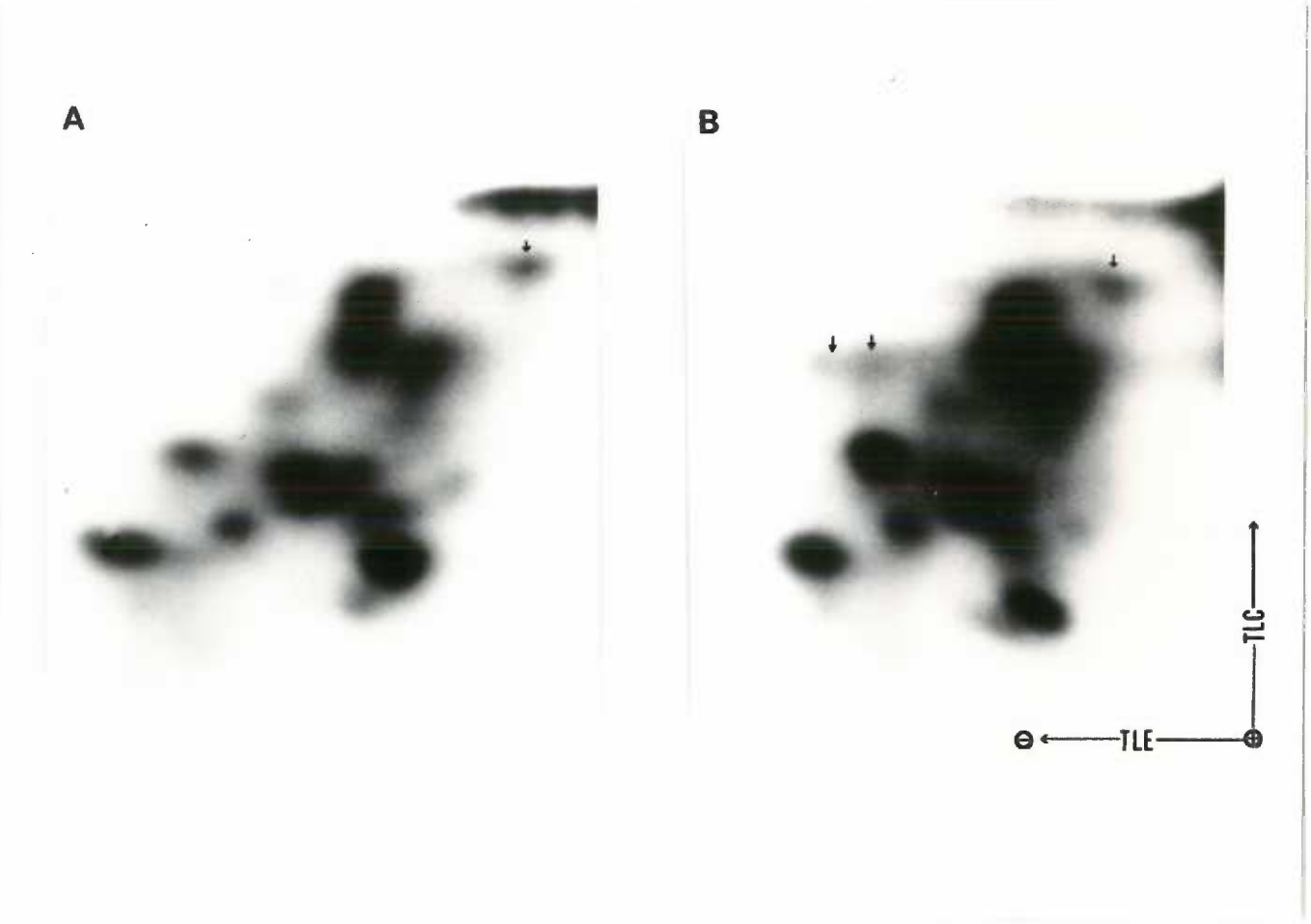

A
B
TLC
TLE
⊖
⊕

FIG. 9. Tryptic ^{125}I-peptide maps of purified MIRP form N. gonorrhoeae strains FA19 (TF^+, LF^+) (A), FA6303 (TF^-, LF^-) (B), and FA6342 (TF^-, LF^+) (C). Arrows indicate variable peptides.

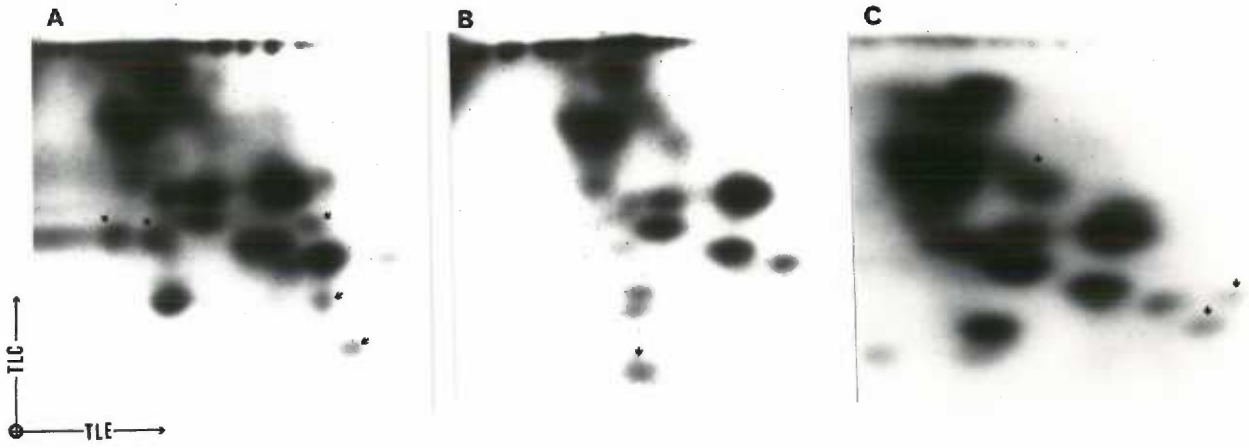
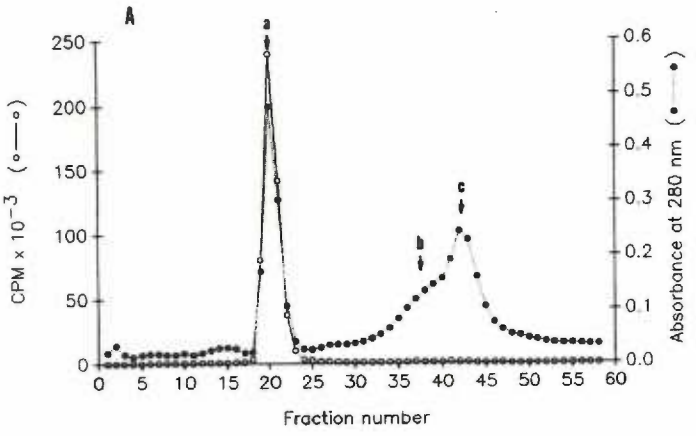

A
B
C
TLC
TLE

FIG. 10. Iron uptake from ^{55}Fe-TF by N. gonorrhoeae. N. gonorrhoeae strains FA19 (TF^+, LF^+) and FA6303 (TF^-, LF^-) were grown in liquid CDM supplemented with 25 uM Desferal. Cells were harvested during the early stationary growth phase and resuspended in fresh CDM containing ^{55}Fe-TF as the only exogenous iron source. After 30 min of incubation at 37`C, cells were collected, solubilized in CTB, and the MIRP purified by CM-Sepharose chromatography as described in Materials and Methods. Fractions (1 ml) were collected from the column and the protein concentration in each fraction was determined by absorbance at 280 nm (A_{280}). ^{55}Fe was determined by counting 0.1 ml from each fraction in a liquid scintillation counter. Panel A and B: elution profiles of strains FA19 and FA6303 from CM-Sepharose columns. Panel C: SDS-PAGE (10% acrylamide) profiles of the major protein peaks a, b, and c. Equal amounts of proteins (10 ug) were added to each lane.


A
a
b
c
CPM x 10⁻³ (o—o)
Absorbance at 280 nm (•—•)
Fraction number
B
a
b
c
CPM X 10⁻³ (o—o)
Absorbance at 280 nm (•—•)
Fraction number

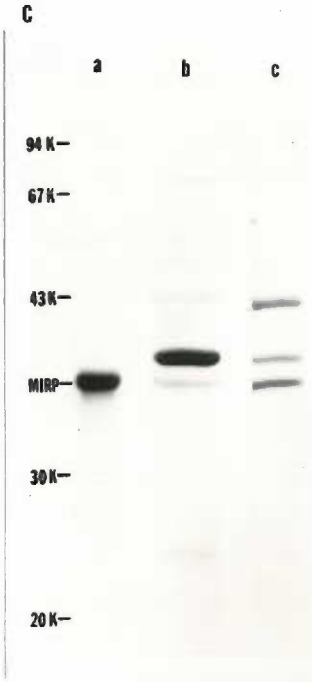

C
a
b
c
94K
67K
43K
MIRP
30K
20K

FIG. 11. Surface-exposed peptides of MIRP from N. gonorrhoeae strain FA19. Whole cells of N. gonorrhoeae strain FA19 were surface-labeled with ^{125}I. After SDS-PAGE, the MIRP band was excised, and subjected to peptide mapping as described in Materials and Methods.

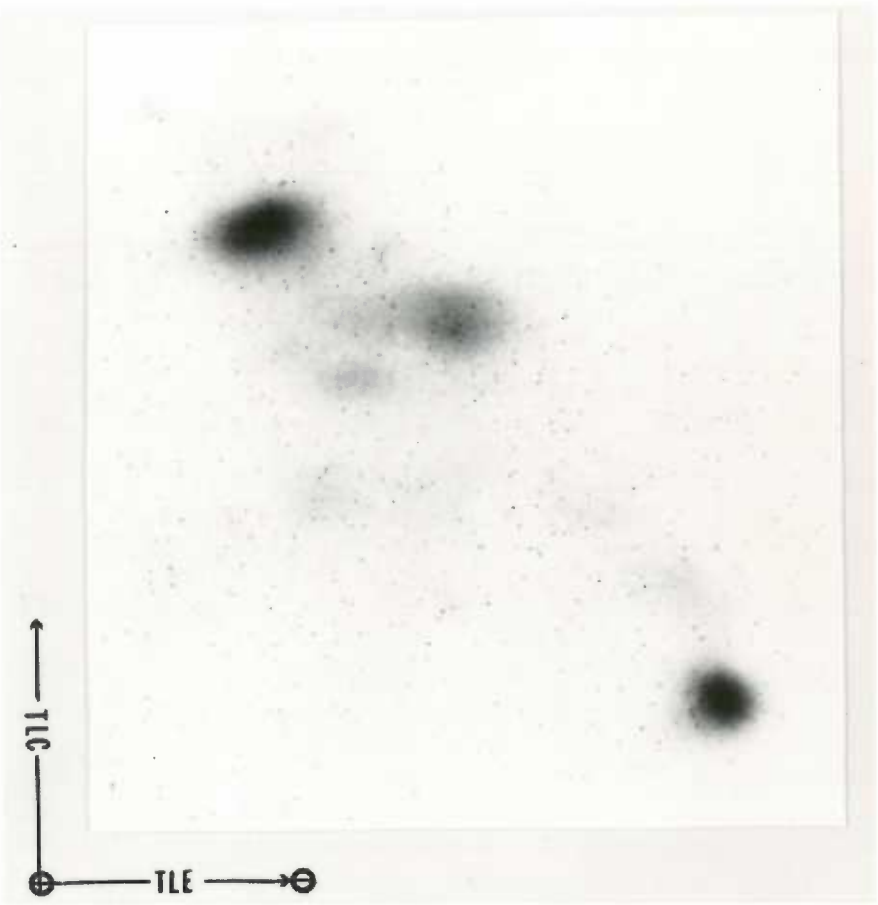
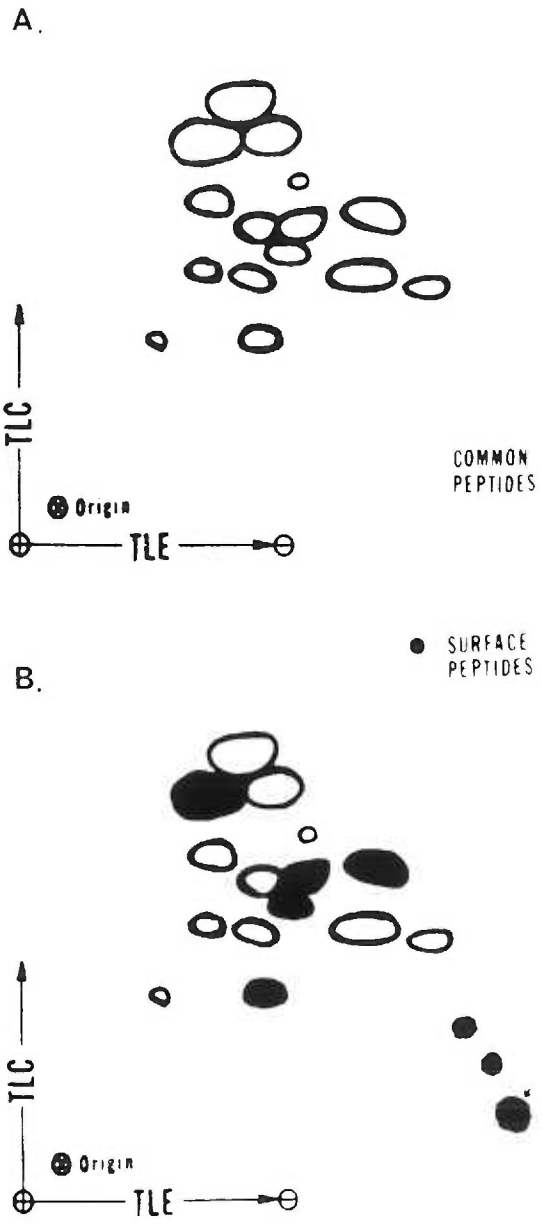

TLC
TLE

FIG. 12. Composite tryptic peptide map of MIRP of N. gonorrhoeae strain FA19. A: ○, peptides common to all Neisseria species; B: ●, surface-exposed peptides.


A.
TLC
Origin
TLE
COMMON
PEPTIDES
SURFACE
PEPTIDES
B.
TLC
Origin
TLE

CONCLUSIONS AND FUTURE PERSPECTIVES

Iron is an essential nutrient for almost all living organisms, due to its key role in electron transport, DNA synthesis, and various essential redox reactions. Since iron deprivation results in cessation of growth and eventual death of a microorganism, it follows that its availability will affect the ability of a microorganism to establish an infection in a suitable host.

The human host utilizes iron-deprivation as a form of natural defense, with most of the available iron being sequestered by the iron storage and transport proteins transferrin (TF) and lactoferrin (LF). In order to survive within the host, many bacterial pathogens have developed systems of iron acquisition involving synthesis and secretion of low molecular weight soluble iron-chelating compounds (siderophores) as well as uptake systems for the iron-siderophore complex. The latter has been shown to involve the participation of iron-regulated membrane proteins.

Pathogenic Neisseria species appear to be unique in that they do not synthesize siderophores yet still possess the capability of acquiring iron directly from several iron sources, including TF, LF, heme, and hemoglobin. Previous studies have demonstrated that the ability to acquire iron from the host has an important role in the pathogenesis of gonococcal and meningococcal infections. The mechanism of this unique iron-acquisition system has not been established to date. The identification of several iron-regulated proteins in meningococci and gonococci has been the focus of several investigations. However, the role of these various iron-regulated proteins in iron metabolism

is only beginning to be understood. Recent studies on the characterization of the specific receptor for human TF in N. meningitidis indicated that it was a 71,000 dalton iron-regulated membrane protein. A 105,000 dalton iron-regulated outer membrane protein was found to bind lactoferrin. Further studies are necessary to demonstrate whether these proteins are involved in iron uptake; nevertheless, these are initial steps towards characterizing the iron-uptake system of pathogenic Neisseria. While most of these iron-regulated proteins were heterogeneous with respect to their relative migration during SDS-PAGE and to their presence in different strains, the 37,000 dalton major iron-regulated protein (MIRP) was common to all strains of pathogenic Neisseria examined. The subsequent purification of this protein has provided an accessible yet effective tool that can be used to understand the complex process of iron-uptake.

Evidence continues to accumulate that the MIRP is an important component of the iron uptake system of pathogenic Neisseria:

1. The MIRP is expressed during iron-limited growth and in the presence of TF and LF by all strains of N. gonorrhoeae, N. meningitidis, N. cinerea, and N. lactamica examined.
2. The primary structure of these MIRPs is highly conserved as indicated by two-dimensional peptide maps, N-terminal amino acid sequences, and by reactivity with polyclonal antiserum or monoclonal antibodies prepared against gonococcal MIRP. The highly conserved nature of this protein supports the contention that it has an essential function.
3. Serum from patients with gonococcal infection contain antibodies to the MIRP. Thus, the MIRP is both expressed and antigenic in vivo.

4. The MIRP purified to homogeneity from both N. gonorrhoeae and N. meningitidis contains approximately 1 mole of Fe^{3+} per mole of protein.
5. The MIRP isolated from gonococci grown in a liquid medium containing ^{55}Fe-TF as the only source of iron was labeled with ^{55}Fe. Whether the ^{55}Fe was directly transferred to the MIRP from TF or whether there was one or more intermediates involved is not known. However, the surface exposure of the MIRP suggests that direct interaction between the MIRP and TF at the cell surface is possible.

Despite studies on iron-regulated proteins and iron-uptake systems, our knowledge of the biochemistry and mechanism of iron transport by the pathogenic Neisseria species is still in a rudimentary state. Several different approaches can be pursued to further our understanding of the mechanism(s) of iron uptake by these organisms. For example, studies to determine the mechanism(s) by which gonococci remove iron from TF or LF (reduction, chelation, protonation, or labilization of the bicarbonate ion), studies to determine the presence and location of ferric reductase, studies to purify other iron-regulated proteins, and the construction of mutants defective in iron utilization.

The studies that have been proposed to define the structure and function of the MIRP are: (1) to determine the cellular location of MIRP; (2) to analyze the ability and necessary conditions for MIRP to obtain iron from various compounds; (3) to determine the three-dimensional structure of MIRP; (4) to determine the dissociation constant of MIRP; (5) to examine whether antibodies reactive with MIRP are bactericidal; (6) to determine the potential

iron-binding site of MIRP; (7) to determine whether MIRP is a metalloprotein or an iron-binding/ transport protein; and (8) to define the surface-exposed areas of MIRP. Recently, the gene that encodes for the MIRP has been cloned and sequenced in our laboratory. This will provide the basis for examining the effects of site-specific modification on structure-function relationships. Mutants that are defective in the synthesis of MIRP will be constructed and their ability to grow in an animal model will be assessed. Future studies of the MIRP will lead to a better understanding of the pathogenesis of gonococcal infections and underscore the relationship between iron and virulence, as well as provide new strategies toward the development of safer and more effective means of preventing infection.

Appendix

The effect of Medium Composition on Iron uptake by *Neisseria gonorrhoeae*.

Samuel K. Sarafian, Cheng-Yen Chen, and Stephen A. Morse

ABSTRACT

Iron uptake by Neisseria gonorrhoeae was influenced by the composition of the incubation medium and not by the medium in which the inoculum was initially grown. Cells grown in either chemically defined (CDM) or complex (GCB) medium and incubated in CDM containing 7 μM of Fe exhibited rapid Fe uptake. Incubation in GCB containing 7 μM of Fe resulted in a slower rate of Fe uptake; the amount accumulated was 30% of the amount observed in CDM. The addition of 50 μg of chloramphenicol/ml had no effect on iron uptake, suggesting that de novo protein synthesis was not responsible for the observed differences. Significant differences were observed in the concentrations of Ca, Mg, and Zn in CDM and GCB as determined by atomic emission spectroscopy. Zn and Mg concentration did not affect the rate or extent of Fe uptake. Decreasing the Ca concentration of CDM by 80%, to that found in GCB, resulted in a 30% decrease in iron uptake. Proteose peptone no. 3 (Difco) reduced the amount of iron uptake in CDM, in a concentration-dependent fashion, to the level observed in GCB; this effect of proteose peptone no.3 could be reversed with the addition of calcium.

INTRODUCTION

Iron plays an important role in the growth of microorganisms and in host resistance to infection. Although it is essential for microbial metabolism, the acquisition of iron is difficult for bacteria invading a host where the level of free iron is typically too low to support growth (9,10). Consequently, most studies have thus far focused solely on the ability of microorganisms to obtain iron from different sources. Among the strategies for iron acquisition that have been proposed (1,4) is the production and release of siderophores (8), which are among the strongest iron chelators known. N. gonorrhoeae does not possess a siderophore-mediated iron uptake system (3); it directly removes iron from transferrin or lactoferrin with high efficiency. In addition, gonococci can utilize heme and hemoglobin as iron sources. Inspite of considerable effort , the mechanism of iron acquisition in N. gonorrhoeae is still poorly understood. In this study, we decided to take a different approach by examining the ability of various medium components to enhance or inhibit the uptake of iron by N. gonorrhoeae. A knowledge of these components will help to elucidate the process by which gonococci take up iron.

MATERIALS AND METHODS

Bacteria. N. gonorrhoeae strain FA171 that was prototrophic and serum resistant was provided by P. F. Sparling (University of North Caroline, Chapel Hill).

Media. Gonococci were maintained in the laboratory on a solid medium consisting of GC agar (Difco Laboratories, Detroit, MI), containing a growth factor supplement (6). The experiments were performed using either one of two liquid media: a chemically defined medium (CDM) (7), or a complex medium (GCB) (5), both supplemented with 0.42% $NaHCO_3$ (wt/vol). Proteose peptone no.3 (Difco) and acid-hydrolysed casein (Nutional Biochemicals Corp., Cleveland, OH) were added to CDM when appropriate. Chloramphenicol (CAM) (50 μg/ml) was used to inhibit de novo protein synthesis.

Iron uptake studies. N. gonorrhoeae strain FA171 was grown to exponential phase in a 250-ml Klett flask containing 50 ml of CDM, incubated at 37°C in a rotary shaking water bath at 150 rpm. The culture was harvested by centrifugation at 10,000 rpm for 10 min at room temperature. The cells were resuspended in CDM without supplements and were used to inoculate Klett flasks containing 30 ml of CDM or GCB with appropriate supplements. All glassware was acid-washed prior to use to remove iron. Growth was monitored by reading optical density in a Klett-Summerson colorimeter (540 nm filter). Samples (0.5 ml) were collected in duplicate, at various time intervals up to 30 min, after the addition of 2 μM $^{55}FeCl_3$ (119 CPM/pmol) (New England Nuclear Corp., Boston, MA) in 20 μM sodium citrate. Samples were passed through

cellulose acetate filters of 0.45-μM pore size (HAWP, Millipore). The bacteria trapped on each filter were washed with 4 ml of ice-cold CDM containing excess iron. The dried filters were placed in 10 ml scintillation fluid (Beckman, Ready-Solv MP) and counted in a Beckman Model LS-9800 counter using the tritium channel. The counting efficiency was 38%. Controls were run without cells to determine the association of iron and iron chelates with the filters. In the double-label uptake experiment, ^{45}Ca (357 mCi/mmol) purchased from New England Nuclear Corp., was added at the same time as ^{55}Fe.

Metal ion chelators. Ethylenediaminetetraacetate (EDTA) was purchased from Sigma Chemical Co., St. Louis, MO. Calmodulin was purchased from Calbiochem, La Jolla, CA.

Metal ion concentration estimation. Metal ion concentrations were determined by atomic emission spectroscopy.

RESULTS

The effect of the incubation medium on Fe uptake. Log-phase cells of N. gonorrhoeae strain FA171, grown in CDM, were resuspended in CDM, GCB, and CDM + CAM, all containing ^{55}Fe (Fig. 1). The rate and amount of ^{55}Fe taken up by gonococci resuspended in GCB was approximately 30% of the amount observed in cells resuspended in CDM. The same experiment was performed using gonococci initially grown to log phase in GCB (Fig. 2). The rate and amount of ^{55}Fe taken up by cells resuspended in GCB was approximately 20% of that observed in cells resuspended in CDM. The addition of CAM had no effect on the rate or extent of Fe uptake (Fig. 1 and 2).

The effect of calcium, magnesium, and zinc on Fe uptake. The concentration of various inorganic ions in CDM and GCB was determined by atomic emission spectroscopy. Major differences in calcium, magnesium, and zinc concentrations were observed (Table 1). Increasing the concentration of zinc or reducing the concentration of magnesium in CDM, to that found in GCB, had no effect on iron uptake. In contrast, when the concentration of calcium in CDM was reduced 4-fold to that of GCB, a 25% decrease in ^{55}Fe uptake was observed (data not shown). No significant differences in the concentration of copper, sodium, phosphorus, and iron was observed.

The effect of proteose peptone no.3 (PP) on Fe uptake in CDM. The rate of ^{55}Fe uptake in CDM supplemented with PP (15 g/l) was very similar to that observed with cells suspended in GCB. Log-phase gonococci were resuspended in CDM supplemented with 3.75 or 15 g PP/l. The decrease in ^{55}Fe uptake was

proportional to the concentration of PP in CDM (Fig. 3). The rate and amount of ^{55}Fe taken up decreased by as much as 70%.

The effect of calcium on Fe uptake in CDM supplemented with PP. Gonococci were resuspended in CDM in the presence of PP (15 g/l) and three different concentrations of calcium (0.02, 0.5, and 2.5 mM). The uptake of ^{55}Fe increased with increasing calcium concentration. Furthermore, a calcium concentration of 2.5 mM reversed entirely the inhibitory effect of PP on ^{55}Fe uptake (Fig. 4).

The effect of casein hydrolysate on Fe uptake. In order to investigate whether peptides present in PP were responsible for the observed inhibition, log-phase gonococci were resuspended in CDM supplemented with 3.75 or 15 g acid-hydrolyzed casein/l. The addition of the acid-hydrolysed casein to CDM inhibited ^{55}Fe uptake in a manner similar to that observed with PP (Fig. 5).

The effect of calcium chelators on Fe uptake. The final concentration of EDTA or calmodulin added to CDM (0.02 mM calcium) was 20 and 0.03 μM, respectively. Ten minutes after the addition of the two chelators, log-phase gonococci were resuspended in both media, as well as in CDM without chelators. Calmodulin and EDTA respectively resulted in 42.4 and 40% reduction of ^{55}Fe uptake after 10-min incubation.

The effect of calcium concentration on ^{45}Ca and ^{55}Fe uptake. Increasing the exogenous calcium concentration 10-fold resulted in a 100-fold increase in ^{45}Ca uptake (Fig. 6A), and a 2-fold increase in ^{55}Fe uptake (Fig. 6B). PP (15 g/l) did not inhibit the uptake of ^{45}Ca.

DISCUSSION

We examined the uptake of Fe by N. gonorrhoeae strain FA171 grown either in CDM or in GCB and resuspended in medium containing ^{55}Fe. The uptake of Fe was influenced by the suspension medium and not the medium in which cells were initially grown to exponential phase. The rate and amount of Fe taken up by gonococci grown in GCB was approximately 25% of that observed in CDM. De novo protein synthesis was not responsible for the observed differences in Fe uptake.

In order to identify the component(s) in GCB that was inhibiting Fe uptake, we examined GCB and CDM for major differences in composition. The major nitrogen source in GCB is proteose peptone no.3, whereas amino acids are the major nitrogen source in CDM. The addition of proteose peptone no.3 to CDM, at a concentration equivalent to that in GCB, reduced the rate and amount of Fe taken up to that observed with cells suspended in GCB. The contribution of unlabelled Fe to the suspension medium by proteose peptone no.3 did not account for the reduction in ^{55}Fe taken up by N. gonorrhoeae strain FA171. In order to determine whether the inhibition of Fe uptake was specific to proteose peptone no.3 or due to the peptides present therein, another complex nitrogen source was examined for its effect on Fe uptake. Various concentrations of acid-hydrolyzed casein were added to CDM. This resulted in an inhibition of Fe uptake similar to that observed with proteose peptone no.3.

Further differences were found between CDM and GCB in the concentrations of three inorganic ions. While the concentration of magnesium or zinc had no

effect on Fe uptake, reducing the concentration of calcium in CDM to that found in GCB resulted in a 25% decrease in Fe uptake. Furthermore, increasing concentrations of exogenous calcium appeared to stimulate Fe uptake. To confirm the involvment of calcium, we examined the ability of calcium chelators to reduce Fe uptake. The calcium-binding protein, calmodulin (final concentration of 0.03 μM) and the metal-ion chelator, EDTA (final concentration of 20 μM), inhibited Fe uptake to the same extent, even though the concentration of calmodulin was approximately 700 times less than that of EDTA. This and the ability of calcium to reverse the inhibitory effect of proteose peptone no.3 on Fe uptake further substantiated the importance of calcium.

Results from dual-label (^{45}Ca and ^{55}Fe) experiments indicated that calcium and iron are not co-transported. Based on these data, we postulate that a calcium-dependent event may be one factor involved in the uptake of iron by gonococci. Calcium has previously been shown to disrupt metal-ligand complexes thus allowing the metal to go into solution (2). This would provide a possible explanation for the role of calcium in promoting the availability of iron for N. gonorrhoeae.

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TABLE 1. Major differences in composition between CDM and GCB.

	GCB	CDM	CDM without Ca
Inorganic ions[a]			
Ca	0.061 mM	0.255 mM	0.02 mM
Mg	0.070 mM	1.130 mM	1.11 mM
Fe	0.007 mM	0.007 mM	0.007 mM
Zn	0.023 mM	0.003 mM	0.003 mM
Nitrogen source			
	Proteose peptone no.3 (15 g/l)	Amino acids	

[a] As determined by atomic emission spectroscopy

FIG. 1. Effect of the incubation medium on ^{55}Fe uptake. N. gonorrhoeae grown in CDM was resuspended in: CDM (○); GCB (●); CDM + CAM (△).

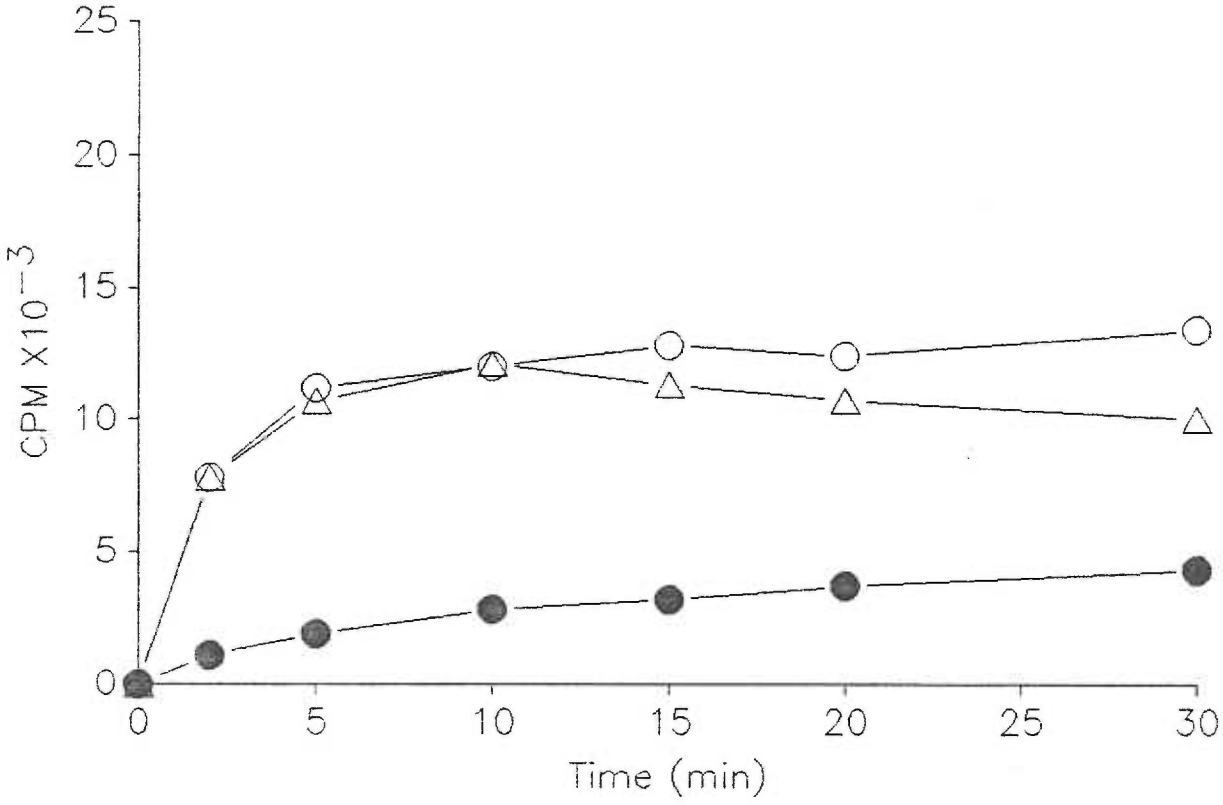

25
20
15
10
5
0
CPM X10^{-3}
0
5
10
15
20
25
30
Time (min)

FIG. 2. Effect of the incubation medium on ^{55}Fe uptake. N. gonorrhoeae grown in GCB was resuspended in: CDM (○); GCB (●); CDM + CAM (△).

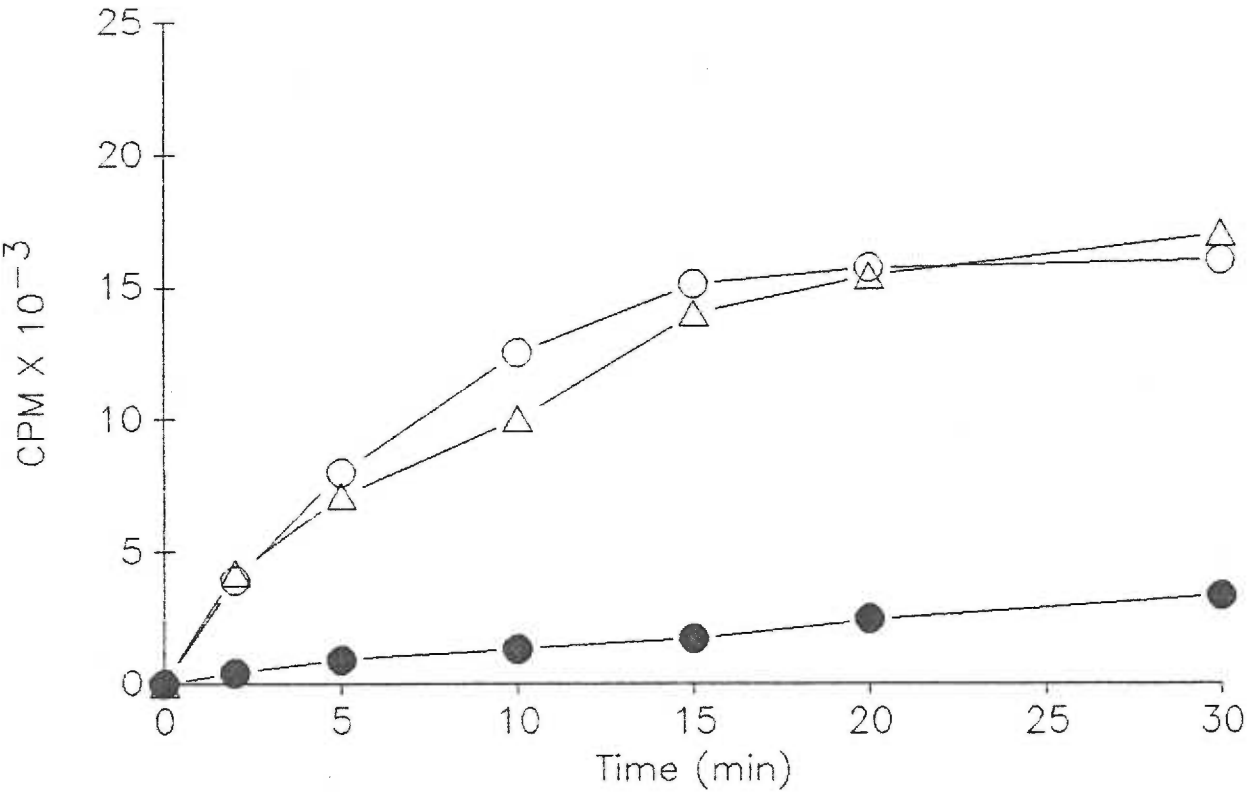

25
20
15
10
5
0
CPM X 10^{-3}
0
5
10
15
20
25
30
Time (min)

FIG. 3. Effect of proteose peptone (PP) no.3 on ^{55}Fe uptake in CDM.

CDM (○); CDM + 3.75 g PP no.3/1 (△); CDM + 15 g PP no.3/1 (●).

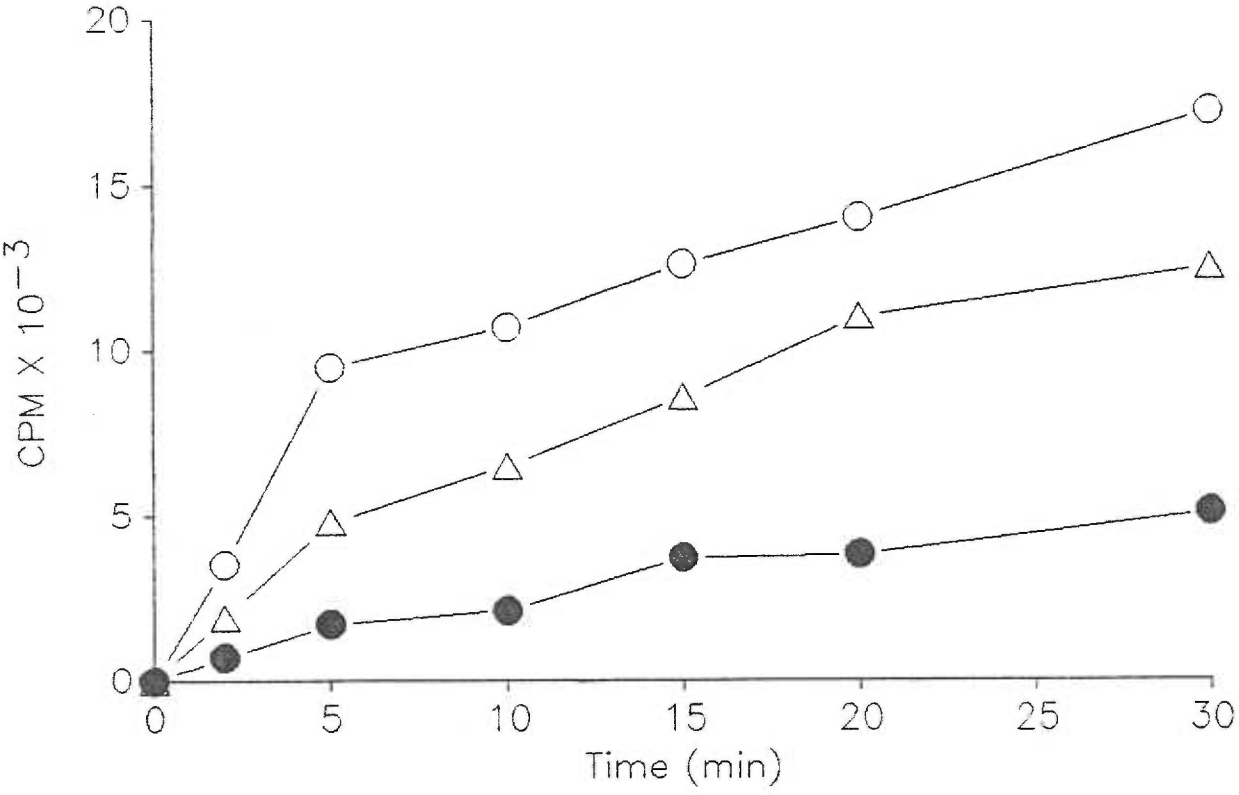

20
15
10
5
0
CPM X 10^{-3}
0
5
10
15
20
25
30
Time (min)

FIG. 4. Effect of calcium on ^{55}Fe uptake in CDM supplemented with 15 g of PP no.3/1. Calcium concentration of: 0.02 mM (○); 0.5 mM (●); 2.5 mM (△).

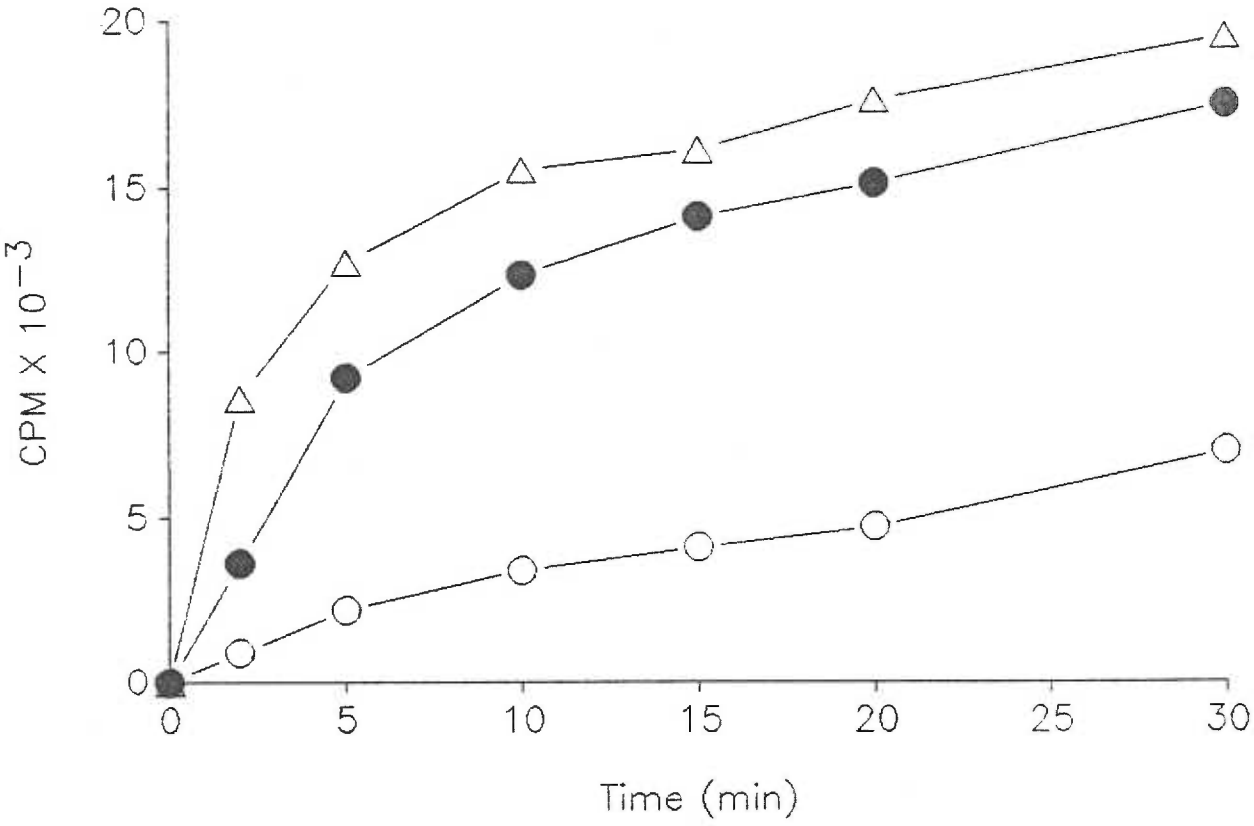

20
15
10
5
0
CPM X 10^{-3}
0
5
10
15
20
25
30
Time (min)

FIG. 5. Effect of casein hydrolysate on ^{55}Fe uptake. CDM (○); CDM with 3.75 g (△) or 15 g (●) casein hydrolysate/l.

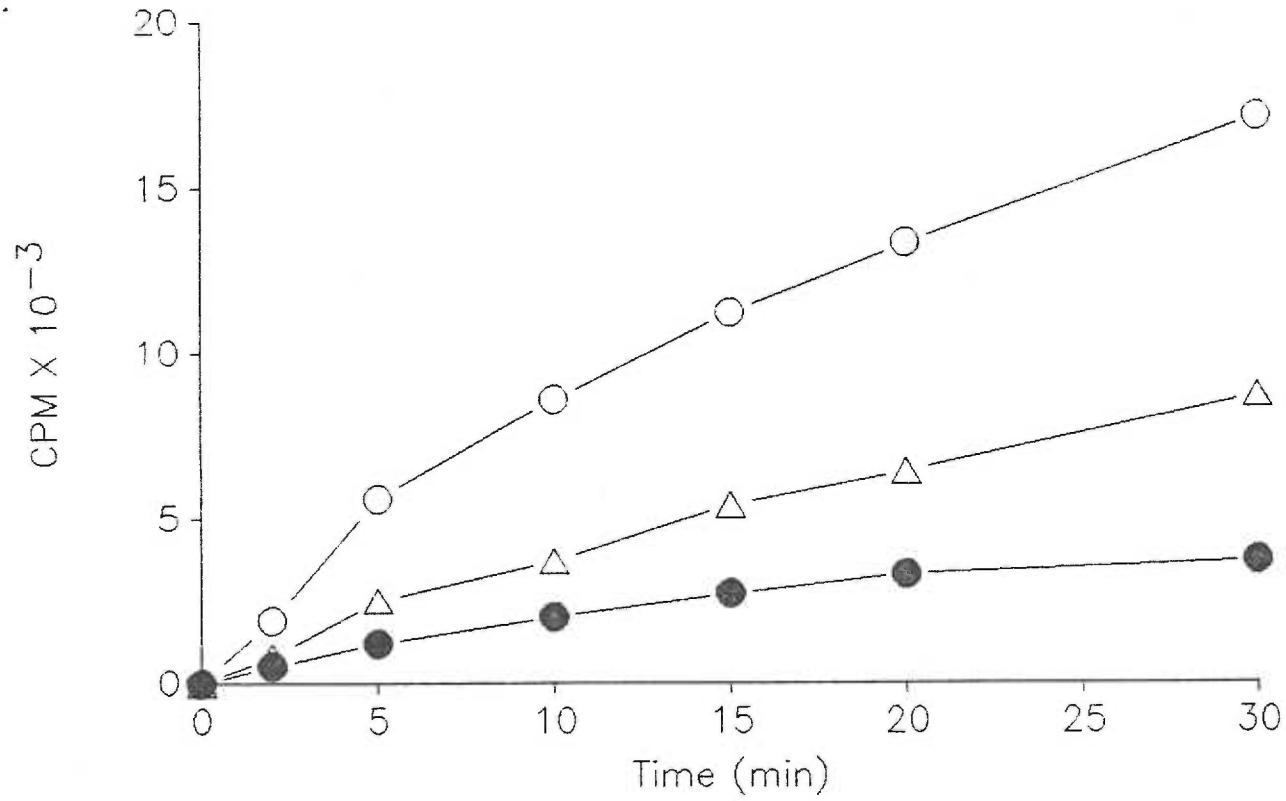
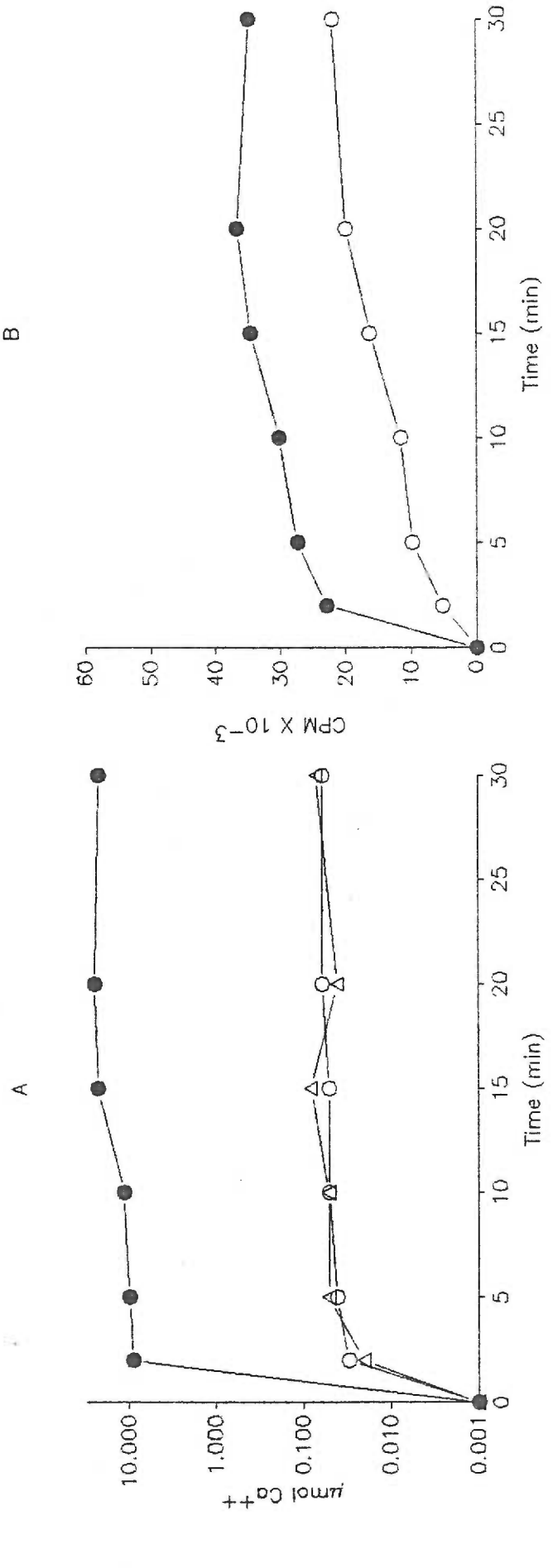

20
15
10
5
0
CPM X 10⁻³
0
5
10
15
20
25
30
Time (min)

FIG. 6. Effect of calcium concentration on ^{45}Ca (A) and ^{55}Fe (B) uptake. A, CDM containing: 0.25 mM Ca (○); 2.5 mM Ca (●); 0.25 mM Ca and PP (15g/1) (△). B, CDM containing: 0.25 mM Ca (○); 2.5 mM Ca (●).


A
B
μmol Ca++
CPM X 10⁻³
Time (min)
Time (min)
10.000
1.000
0.100
0.010
0.001
60
50
40
30
20
10
0
0
5
10
15
20
25
30