

PHYSIOLOGY OF SIALIC ACID CAPSULAR POLYSACCHARIDE SYNTHESIS IN
SEROGROUP B NEISSERIA MENINGITIDIS

by

Luke Masson



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Department of Microbiology and Immunology

McGill University

Montreal, Quebec, Canada

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This thesis is dedicated to my father, the late Joseph Earl Masson, who had desired to see me continue in post-graduate studies - but could not, and to my mother, Marie-Ange Masson - who could.

ABSTRACT

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Luke Masson

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Physiology of capsular polysaccharide synthesis in Neisseria meningitidis serogroup B.

The synthesis and regulation of the capsular polysaccharide (CP) of N. meningitidis and its relationship to the virulence of this organism for mice were examined. Two spontaneous, isogenic mutants (PRM101 and PRM102) of the wildtype (Wt) strain M986, unable to synthesize and release CP, were isolated; both exhibiting a dramatic loss of virulence and a change of colony phenotype from iridescent to opaque. Wt virulence was regained upon reversion to the Wt phenotype. Strain PRM102 possessed only 25% of Wt sialyltransferase activity but had Wt levels of the N-acetylneuraminic acid (NANA) condensing enzyme and a cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NANA) synthetase. A new meningococcal enzyme, a CMP-NANA hydrolase, was found at similar levels in both strain PRM102 and strain M986. This enzyme hydrolyzes CMP-NANA to CMP and NANA, was completely inhibited by 45.3 μ M CMP and possessed a higher affinity for CMP-NANA than the sialyltransferase, which uses CMP-NANA as a substrate and is insensitive to CMP addition. The sialyltransferase and CMP-NANA hydrolase activities were localized to the cytoplasmic membrane while the NANA condensing enzyme and the CMP-NANA synthetase were cytosolic. Sialyltransferase activity in particulate membranes or with delipidated membrane proteins, was specifically stimulated or reconstituted by undecaprenyl phosphate (UP) respectively. A transient, lipid-linked intermediate was extracted from particulate membranes. The extracted NANA was

in polymeric form with an average degree of polymerization of 4-5 NANA residues. Peptidoglycan (PG) was synthesized in vitro by particulate membranes, and was bacitracin and lysozyme sensitive. Cross-competition experiments between PG and CP synthesis revealed a competitive effect for a common component believed to be UP.

Possession of the sialic acid CP confers resistance to the serum bactericidal reaction. Strain M986, when grown under conditions of low pH or nutrient limitation, synthesized increased levels of CP which correlated with an increase in cell-surface hydrophilicity, and virulence for mice. The increase in CP was partly explained by a decrease in the specific activity of a membrane-bound CMP-NANA hydrolase. The results suggest that conditions of nutrient limitation and low pH exert profound effects on the physicochemical nature of the meningococcus cumulating in enhanced virulence of this organism for mice. Working models for the biosynthesis and regulation of the serogroup B CP are proposed and discussed.

RESUME

Ph.D.

Luke Masson

Microbiology

Physiologie de la synthèse du polysaccharide capsulaire
de *Neisseria meningitidis* du groupe sérologique B.

Tenant compte de la synthèse et de la régulation du polysaccharide capsulaire (PC) de *N. meningitidis*, la virulence de cet organisme chez la souris a été examinée. Deux mutants isogénique (PRM101 et PRM102), provenant de mutations spontanées de la souche de type sauvage M986, mais incapables de synthétiser et de libérer le PC, ont été isolés. Ces deux mutants accusèrent une diminution considérable de leur virulence et une modification de la morphologie phénotypique des colonies, passent d'un aspect iridescent à un aspect opaque. Une réversion au phénotype sauvage a entraîné la réapparition de la virulence. La souche PRM102 présentait seulement 25% de l'activité de l'enzyme sialyltransférase de type sauvage, mais possédait la même activité que la souche sauvage en ce qui concerne l'enzyme responsable de la condensation de l'acide N-acétyl-neuraminique ("NANA") et la synthétase de la cytidine 5'-mono-phosphate et de l'acide N-acétyl-neuraminique ("CMP-NANA"). Une autre enzyme du méningocoque, une hydrolase de CMP-NANA a été identifiée dans les deux souches PRM102 et M986 en quantités comparables. Cette enzyme capable d'hydrolyser le complexe CMP-NANA en CMP et NANA, a été inhibée par 45.3 uM de CMP. De plus, elle possède une plus grande affinité pour le complexe CMP-NANA que la sialyltransférase qui utilise le complexe CMP-NANA comme un substrat, et qui n'est pas inhibée par l'addition de CMP. L'activité de la sialyltransférase ainsi que celle de l'hydrolase de CMP-NANA ont été localisées au niveau de

la membrane cytoplasmique tandis que l'activité à la fois de l'enzyme qui condense NANA et de la synthétase de CMP-NANA a été localisée dans le cytosol. L'activité de la sialyltransférase dans des extraits (bruts) de membrane ou dans les protéines des membranes dépourvues de lipides a été stimulée dans le premier cas et reconstituée dans le second par l'addition d'undecaprenyl phosphate (UP). Par ailleurs, une protéine intermédiaire précoce, liée aux lipides a été extraite des membranes. Le NANA extrait présentait une forme polymérique d'un degré de polymérisation moyen de 4 à 5 résidus (de NANA). Le peptidoglucan (PG) a été synthétisé in vitro par les extraits de membranes; celui-ci était sensible à la bacitracine et à la lysozyme. Une comparaison entre la synthèse du PG et celle du CP a permis de noter un effet de "compétition" entre ces deux substances. Cet effet pourrait être dû à un élément commun, l'UP.

L'acide sialique du PC confère une résistance à la réaction bactéricide du sérum. La souche M986, exposée à des conditions de faible pH ou à une privation d'éléments nutritifs, synthétise une grande quantité de PC; cette synthèse s'accompagne d'une augmentation d'une part du degré d'hydrophilie au niveau de la surface de la cellule et d'autre part de la virulence chez la souris. Cette augmentation du PC est peut-être due en partie à une diminution de l'activité spécifique de l'hydrolase de CMP-NANA. Ces résultats suggèrent que les conditions adverses (diminution des éléments nutritifs et abaissement du pH) ont un effet considérable sur la nature physicochimique du méningocoque, entraînant une plus grande virulence chez la souris. Plusieurs modèles montrant la biosynthèse et la régulation du PC du groupe sérologique B sont proposés et discutés.

ACKNOWLEDGMENTS

I wish to express my deep appreciation to my supervisor, Dr. B. E. Holbein, for his enthusiastic support, useful advice, and most importantly, his unfailing friendship, throughout the course of this work. I would like to thank Jane Donga for both her excellent technical assistance and help with the serum bactericidal studies, and Anna Campana for her expert assistance with the electron microscope. I wish to extend my gratitude to Dr. F. E. Ashton for providing meningococcal strains M986 and PRM101.

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Finally, I would like to thank my family for their constant encouragement, especially my wife Mieke, who was able to identify with, understand, and support me through both the successes and particularly the failures.

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Physiology of sialic acid capsular polysaccharide synthesis in serogroup B *Neisseria meningitidis*.
 - a) A C₅₅-polyisoprenyl lipid coenzyme, undecaprenyl phosphate, specifically stimulates CP synthesis in particulate membranes from *N. meningitidis* strain M986 and reconstitutes sialyltransferase activity in delipidated strain M986 membranes. Lipid intermediates can be extracted during CP synthesis and shown to contain polymerized NANA residues.
 - b) Four independent regulatory mechanisms regulate the level of capsular polysaccharide synthesis.
 - i) A previously undiscovered enzyme, CMP-NANA hydrolase, is associated with the cytoplasmic membrane in *N. meningitidis* but is absent in nonpathogenic neisseriae. This enzyme breaks down in vitro synthesized dual-labeled [³H]CMP-[¹⁴C]NANA into free [³H]CMP and [¹⁴C]NANA. The hydrolase possesses a higher affinity (K_m = 0.88 μM; V_{max} = 10.75 nMol of NANA produced/ h /mg protein) for the common substrate CMP-NANA than the sialyltransferase (K_m = 2.0 μM; V_{max} = 3.45 nMol NANA incorporated/ h/ mg protein).
 - ii) CMP, a product of both the cytoplasmic membrane-associated sialyltransferase and CMP-NANA hydrolase, can negatively

modulate the activity of CMP-NANA hydrolase in particulate membranes of strain M986 or PRM102. CMP does not affect sialyltransferase activity.

- iii) Undecaprenyl phosphate is shared between CP and peptidoglycan synthesis. Addition of undecaprenyl phosphate to particulate membranes revealed that this lipid is present in less than saturating levels thus implicating lipid availability as a means of determining the levels of the appropriate cell envelope polymers.
- iv) Adverse in vitro growth conditions such as nutrient limitation (iron and nitrogen) or low pH cause an increase in the level of CP synthesized by strain M986.
- c) A technique to separate inner and outer membranes of strain M986 was developed. Sialyltransferase and CMP-NANA activities are localized to the cytoplasmic membrane whereas the NANA condensing enzyme and CMP-NANA synthetase are found exclusively in the cytosol. This technique further revealed that TMPD-oxidase is a cytoplasmic membrane-associated enzyme rather than outer membrane as previously thought.
- d) Molecular models are proposed for the production and regulation of the serogroup B CP.

2. Role of the capsular polysaccharide in virulence.

- a) Production of the sialic acid CP was clearly linked to virulence of strain M986 for mice. Spontaneous, isogenic mutants of strain M986 termed PRM101 and PRM102, are unable to elaborate CP as confirmed by a combination of electron microscopy, chemical and immunochemical techniques. Both mutants are rendered essentially avirulent for mice. Colony phenotype also changes from iridescent (wildtype) to opaque. Virulence is regained upon reversion to the wildtype phenotype.
- b) Possession of the sialic acid capsular polysaccharide confers resistance to the serum bactericidal reaction.
- c) The increased CP levels observed during growth of strain M986 under low pH or nutrient limiting conditions correlates with an increase in virulence for mice. This increase in CP also correlates with a decrease in cell-surface hydrophobicity.

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THESIS RATIONALE

Despite numerous advances in the development of antimicrobial chemotherapy, polysaccharide vaccines, and our current knowledge of infectious diseases, bacterial meningitis still remains a serious infectious disease with significant morbidity and mortality. The case/fatality rate was approximately 10% among patients with true meningitis but is as high as 70% among those with meningococemia (Petola, 1983).

The pathogenesis of meningococcal meningitis, in the absence of predisposing infections, has not been fully explained. In fact, recent experimental evidence questions the concept that meningococcal virulence is a "fixed" property of the organism and that susceptibility to meningococcal disease is solely related to lack of immunity to the invading organism (Brener et al., 1981). Bacteria, in general, appear quite adept at altering their metabolism and physicochemical surface properties in accordance with the surrounding environmental conditions (Magnusson et al., 1979a, 1979b; Brener et al., 1981; Simonson et al., 1982).

Physiological differences between carrier strains and disease isolates also remain largely undefined although differences in virulence have been determined (Holbein, 1981). A common denominator of all the bacterial species capable of causing meningitis is the presence of a capsular polysaccharide ostensibly implicating an essential role for this organelle in virulence. However, the role of meningococcal capsular polysaccharides including the mechanisms governing their production and regulation are virtually unknown. The aim of this thesis was two-fold. First, I sought to clarify the biosynthetic pathway of serogroup B sialic acid capsular polysaccharide synthesis including the regulatory mechanisms governing its production.

Secondly, I examined the relationship between possession of the capsular polysaccharide and meningococcal pathogenesis and virulence. The latter included the examination of phenotypic alterations during meningococcal growth under adverse environmental conditions, which approximated suspected prevailing environmental conditions within the host.

It is hoped that the determination of how the capsular polysaccharide is produced and regulated as well as the role this polysaccharide plays in virulence will provide a clearer understanding of the pathogenic mechanisms of invasive meningococci. In addition, since there is no effective vaccine as of yet for serogroup B meningococci, an understanding of the regulatory mechanisms governing capsule synthesis may assist in optimizing the polysaccharide levels harvested for research in the promising field of protein-polysaccharide vaccine conjugates.

Manuscripts and Authorships

This dissertation encompasses experimental results which includes two published manuscripts and an additional two manuscripts submitted for publication.

1. Masson, L., B. E. Holbein, and F. E. Ashton. 1982. Virulence linked to polysaccharide production in serogroup B Neisseria meningitidis. FEMS (Fed. Eur. Microbiol. Soc.) Lett. 13:187-190.
2. Masson, L., and B. E. Holbein. 1983. Physiology of sialic acid capsular polysaccharide synthesis in serogroup B Neisseria meningitidis. J. Bacteriol. 154:728-736.
3. Masson, L., and B. E. Holbein. 1984. The role of lipid intermediate(s) in the synthesis of serogroup B Neisseria meningitidis capsular polysaccharide. J. Bacteriol. submitted for publication.
4. Masson, L., and B. E. Holbein. 1984. Influence of nutrient limitation and low pH on serogroup B Neisseria meningitidis capsular polysaccharide levels: correlation with virulence for mice. Infect. Immun. submitted for publication.

All work presented here was conducted independently by the author, with the exception of assistance in performing the complement assays and the electron micrographs, and was supervised by Dr. B.E. Holbein. Dr. F. E. Ashton contributed to the studies on polysaccharide synthesis and release by providing the serogroup B prototype strain and the horse serogroup B antiserum.

LITERATURE REVIEW

LITERATURE REVIEW

I. The Bacterium.

i. Introduction.

One of the earliest recorded descriptions of meningococcal disease appeared at the turn of the nineteenth century (Vieusseux, 1805). The first American account of cerebrospinal meningitis occurred one year later in Medfield, Massachusetts (Danielson and Mann, 1806). However, eighty-two years would pass before the meningococcus was to be isolated from six cases of primary cerebrospinal meningitis in Vienna and described in detail under the original name of Diplokokkus intracellularis meningitidis (Weichselbaum, 1887). In 1898, the meningococcus was finally established as the etiological agent of epidemic cerebrospinal meningitis (Councilman et al., 1898). The existence of different antigenic types of meningococci was first noted by Dopter, (1909); a few years later, four distinct serogroups were identified (Gordon and Murray, 1915). Dopter, (1909) renamed these different types Parameningococci in order to differentiate meningeal strains from the indeterminate cocci found in the nasopharynx. Today, the genus Neisseria contains six species of which two are pathogenic for humans: N. meningitidis, and N. gonorrhoeae (Reyn, 1974). The morphology of the genus members appear ellipsoid when examining a single bacterium (0.8 x 0.6 um); however, they are normally found in pairs. In this diplococcus form, the opposing bacterial surfaces are flattened creating a characteristic kidney bean shape. Meningococci are typically Gram-negative,

non-motile, non-sporulating, catalase-positive, cytochrome oxidase-positive diplococci, and may be encapsulated (Goldschneider et al., 1969a), piliated (DeVoe and Gilchrist, 1975), or both (Salit and Morton, 1981). They are normally differentiated from gonococci and other non-pathogenic neisserial species on the basis of their biochemical reactions. Meningococci ferment glucose and maltose, with the production of acid, but not lactose, mannose, or sucrose (Reyn, 1974). Diagnostically, the sole biochemical reaction distinguishing gonococci from meningococci is the production of acid from maltose (Catlin, 1973). Due to the increasing problem of finding both of these organisms occasionally residing in each others natural habitat (Feldman, 1971; Taubin and Landsberg, 1971), precise identification is sometimes difficult. Identification is further complicated by the finding that gonococci are easily transformed in vitro by meningococcal DNA to become maltose fermenters (Ison et al., 1982). Interestingly, Kingsbury (1967) demonstrated, by DNA hybridization, that gonococci and meningococci are homologous for approximately 80% of their genome. Thus meningococci and gonococci belonging to one species but adapting to different environments still remains a possibility (Ison et al., 1982).

Kellogg et al., (1963) defined four gonococcal colony types on the basis of colonial size, edge morphology, granularity and color characteristics. Two of these colony types, type 1 and type 2 (T1, T2), were more virulent for human volunteers (Kellogg et al., 1968), were usually found in clinical isolates (Sparling and Yobs, 1967), and were piliated (Jephcott et al., 1971; Swanson et al., 1971). Type 3 and type 4 were less virulent, were produced by repeated in vitro subculturing, and were non-piliated. Gonococcal colonies can also be classified by colony color and optical opacity (Swanson, 1978). These

characteristics have been attributed to the level of inter-gonococcal aggregation and the presence of colony opacity-associated proteins respectively (Swanson, 1978). Meningococci have been grouped into seven different colony types (Brenner et al., 1977; Devoe and Gilchrist, 1978), but unlike gonococci, there does not appear to be a correlation between piliation and colony morphology (Devoe and Gilchrist, 1978). Furthermore, smooth (S) and rough (R) colonial morphologies have been reported for the meningococci. The S colonies were virulent, highly iridescent, transparent, blue with a yellow tinge, and nearly agranular, while R colonies appeared granular, opaque, and were relatively avirulent (Devine et al., 1972; Stephens and McGee, 1983). The authors suggested that these characteristics were caused by the presence or absence of meningococcal capsular polysaccharide.

ii. Cell-surface composition and virulence.

ii.a. Introduction.

It is important for a bacterial pathogen to possess the ability to adhere and colonize its potential host, resist or subvert host defenses, and eventually cause damage to host tissues usually through inflammation or the elaboration of toxins (Smith, 1977). The physicochemical nature of the bacterial cell surface is of crucial importance in the etiology of bacterial disease since it is this surface which interacts with the cellular and humoral immune defenses of the host and subsequently dictates the outcome of

infection (Costerton et al., 1981; Smith, 1977). It is also important to understand the molecular architecture and function of the bacterial cell surface since this surface initiates adhesion to endothelial and epithelial target cells. The cell envelope, often schematically drawn as a series of concentric layers, allows many of its constituents to be exposed on the cell surface (Peterson and Quie, 1981). These constituents may therefore be considered as part of the cell surface.

A variety of models have been used to study meningococcal virulence, which simulate, to a limited extent, various aspects of the disease (Buddingh and Polk, 1939; Arko, 1974; Craven and Frasch, 1979a). They are primarily useful for examining the protective effects of meningococcal vaccines but provide little information on meningococcal virulence determinants. A mouse model was introduced (Miller, 1933) in which hog gastric mucin was found to lower the 50% lethal dose (LD_{50}); however, the results varied among different mucin preparations (Calver et al., 1976). Calver et al., (1976) found that iron compounds could replace mucin in promoting infection. The mouse iron-dextran model of Holbein, (1979) appears ideally suited for examination of the physiological bases for meningococcal virulence since it can detect differences in virulence between carrier and disease isolates (Holbein, 1981). Furthermore, since iron plays a key role in bacterial pathogenesis (Bullen et al., 1978; Weinberg, 1978), the iron physiology of the mouse, which is comparable to man, can be monitored and manipulated in this system (Holbein, 1980).

ii.b. Pili.

Preformed monomeric protein subunits are assembled by the bacterium (Brinton, 1961) to produce pili of which seven different types have been described (Ottow, 1975). A large variety of Gram-negative organisms produce pili but, in contrast to this, relatively few cases of Gram-positive organisms elaborating pili on their surface have been detected (Yanagawa and Otsuki, 1970). Pili are thought to facilitate attachment by overcoming the electrostatic repulsion between the negatively charged surface of both the bacterium and host cell (Heckels et al., 1976). The observations that many bacteria rapidly lose their pili after primary isolation from their natural source and repeated in vitro subculture (Duguid et al., 1966; DeVoe and Gilchrist, 1975; Salit and Morton, 1981) and that virulent gonococcal types (T1 and T2) are piliated, suggested that pili may play an important colonization role in vivo. These observations assume even more importance in view of the fact that colonization of the pharyngeal mucosa is known to precede meningococcemia and meningitis (Goldschneider et al., 1969a). The secretory immune system of mucosal membranes in humans is dominated by secretion of antibody of the IgA isotype (sIgA) (Tomasi, 1972) which is believed to function by preventing bacterial adherence to relevant tissues (Williams and Gibbons, 1972; Brandtzaeg and Tolo, 1977). However, both N. meningitidis and N. gonorrhoeae produce specific sIgA proteases (Plaut et al., 1975; Mulks et al., 1980) unlike nonpathogenic neisserial species implicating the proteases as a potential virulence factor for these organisms (Kornfeld and Plaut, 1981)

The specificity of adherence to epithelial or endothelial cells may be a crucial ecological determinant affecting colonization (Salit and Morton, 1981). In a study involving carrier strains, which are primarily rough or

nongroupable, and disease isolates of meningococci, the authors concluded that there was a distinct tropism for pharyngeal rather than buccal epithelial cells and that attachment was mediated by pili since all fresh isolates adhered better than those passaged in vitro (Salit and Morton, 1981). However, rough carrier strains adhered better to pharyngeal epithelial cells than serogroupable invasive strains suggesting that possession of capsular polysaccharide interferes with adherence (Salit and Morton, 1981). Adhesion of Escherichia coli to human uroepithelial cells is also reduced when cells are grown under conditions that enhanced capsule formation (Svanborg and Hansson, 1978). Nonpathogenic neisseriae have also been shown to be piliated and capable of adhering to pharyngeal epithelial cells in significant numbers (Stephens and McGee, 1981). Therefore, it seems that although cell attachment per se appears important for colonization, it may not be a marker of virulence for the meningococcus. More importantly, these results suggest that the meningococcus possesses virulence mechanisms other than pili (Stephens and McGee, 1981). In summary, the precise role of pili in oropharyngeal carriage and in establishing systemic disease has yet to be determined (DeVoe, 1982).

ii.c. Polysaccharides.

Bacterial cell envelopes contain a variety of polysaccharides, some of which are exposed on the cell surface and consequently play an important role in virulence. The most important of these polysaccharides are teichoic and teichuronic acids produced by Gram-positive bacteria, the lipopolysaccharides produced by Gram-negative bacteria and the capsular polysaccharides which may

be produced by both Gram-negative and Gram-positive bacteria.

Teichoic acids are secondary wall polymers possessing phosphodiester groups, polyols and/or sugar residues (Ward, 1981). Some important functions of teichoic acid are cation binding and the provision of the correct cationic environment at the cell membrane. They may also participate in the activation and regulation of autolytic enzymes during cell growth (Archibald, 1974). Teichoic acids appear to play an important role as an adhesin for virulent group A streptococci; mediating adherence to pharyngeal epithelial cells (Beachey, 1981).

Meningococci can be serogrouped on the basis of their chemically and immunologically distinct capsular polysaccharides. To date there are at least nine defined serogroups (A, B, C, D, 29E, W135, X, Y, Z) (Craven et al., 1978; Craven and Frasch, 1979b) of which serogroups A, B, C, Y, W135 and 29E comprise the majority of disease isolates (Craven and Frasch, 1979b). Groups B and C are homopolymeric capsular polysaccharides possessing the nine carbon aminosugar, N-acetylneuraminic acid. Groups W135 and Y are heteropolysaccharides containing N-acetylneuraminic acid and galactose or glucose respectively (DeVoe, 1982). The two other highly pathogenic serogroups contain N-acetylmannosamine phosphate (group A) and N-acetylgalactosamine plus 3-deoxy-D-manno-octulosonic acid (group 29E). A more detailed review of the role capsular polysaccharides play in meningococcal virulence will be presented in a later chapter.

Meningococcal lipopolysaccharides appear to be of the rough "R" type in all serogroups. They possess a core polysaccharide but lack the O-antigenic side chain in spite of their smooth colonial character (Jennings et al., 1980). Meningococcal lipopolysaccharides appear to have the same components as the core structure of the Enterobacteriaceae and although

the meningococcus displays an overall similarity in composition from serogroups A, B, X, and Y, they do exhibit a high degree of serogroup specificity (Jennings et al., 1973). Meningococci have been serotyped on the basis of their immunologically distinct lipopolysaccharides (Zollinger et al., 1974; Zollinger and Mandrell, 1977). During log-phase growth, the meningococcus appears to oversynthesize its outer membrane resulting in the release of outer membrane blebs rich in lipopolysaccharide (endotoxin) (DeVoe and Gilchrist, 1973) and therefore may represent a mechanism of continued endotoxin presentation to the host (DeVoe, 1982)

ii.d. Outer membrane proteins.

The cell envelope of Gram-negative bacteria is an immunologically complex structure composed of three morphologically distinct layers, namely, the cytoplasmic membrane, a rigid peptidoglycan layer, and a second membranous structure called the outer membrane (Braun, 1973; DiRienzo et al., 1978). The neisseriae, in thin section, appear to possess a typical Gram-negative cell envelope (Cesarini et al., 1967; DeVoe and Gilchrist, 1974) as well as a few proteins predominating in their outer membranes (Johnston and Gotschlich, 1974; Schnaitman, 1970; Frasch and Gotschlich, 1974). Meningococci are divided into serotypes based on immunologically distinct outer membrane proteins and lipopolysaccharides (Frasch and Chapman, 1972; Gold and Wyle, 1970; Zollinger and Mandrell, 1977). Heckels, (1981) concluded that both the meningococcus and the gonococcus have the potential for genetic variation of their outer membrane protein composition. This is particularly important since certain gonococcal

outer membrane proteins have been associated with serum resistance and increased attachment to human epithelial cells in culture (Lambden et al., 1979). Sugawara et al., (1983) demonstrated a gonococcal nonpilus surface protein (PII) which acted as a mediator of attachment to HeLa cells. Transformation of a serum susceptible strain to serum resistant with DNA from disseminated gonococcal infection strains was accompanied by the appearance of a new principal outer membrane protein with a molecular weight (36,500 daltons) characteristic of the donor cell (Hildebrandt et al., 1978). It has been suggested that these protein components of the outer membrane confer serum resistance by virtue of the fact that they reduce membrane fluidity and subsequently exclude integration of membrane attack complexes (Taylor, 1983).

II. The Disease

i. Epidemiology.

Man is the only known reservoir of the meningococcus, its natural habitat being the mucous membrane of the nasopharynx (Griffiss and Artenstein, 1976). The term "carrier" has been applied to those who asymptotically harbor this organism and it has been estimated that the frequency of carriage among the normal population during nonepidemic periods ranges from 3 to 30% (Griffiss and Artenstein, 1976; Gold et al., 1978a). The organism is disseminated by direct contact or aerosol droplets from asymptomatic carriers (Glover, 1920; Artenstein, 1967) and is the only

etiological agent of widespread epidemics of meningitis (DeVoe, 1982). Meningococcal meningitis is primarily a disease of infancy, the peak incidence occurring between six months and two years of age. In closed populations such as those found among military personnel, a carrier rate of greater than 95% has been recorded (Gold et al., 1978a). Epidemic meningococcal disease occurs in the United States in 10 year cycles suggesting that the disease depends, for its expression, upon an immunologically virgin population (Goldschneider et al., 1969a). In other parts of the world, especially the African "meningitis belt", meningococcal disease remains a serious problem (Gotschlich, 1975; Peltola, 1983). Most outbreaks have been due to serogroups A and C but the prevalence of serogroup B is increasing (Bovre et al., 1977; Farries et al., 1975).

Serotyping of meningococci allows direct epidemiological examination of the transmission and virulence of different strains since only a single serogroup is prevalent at a given time (Frasch and Chapman, 1973). However, the occurrence of multiple serogroups during an epidemic is common at military bases (Artenstein et al., 1971). Furthermore, the serogroup of N. meningitidis has shown to be a poor predictor of epidemic occurrence of disseminated meningococcal disease (Goldschneider et al., 1969a; Makela et al., 1975). It has been noted that a single serotype, serotype 2, was found to be responsible for more than 50% of disease due to serogroup B (Frasch and Chapman, 1973) and 80% of serogroup C meningococci (Munford et al., 1975).

ii. Clinical manifestations.

A model for epidemic susceptibility to disseminating meningococcal disease was developed by Goldschneider et al., (1969a), based on strain virulence and host susceptibility factors. Colonization of the human nasopharynx precedes meningococcal disease (Goldschneider et al., 1969a) which may, depending upon both factors mentioned above, result in asymptomatic carriage and eventual clearance or alternatively, a large spectrum of disease states. Isolation of the meningococcus has been reported in cases of oropharyngitis, tonsillitis (Banks, 1948; Gotschlich, 1980), urethritis (Miller et al., 1979) and even pneumonia (Jacobs and Norton, 1974). The meningococcus can be a highly virulent organism which can kill its host with alarming rapidity (DeVoe, 1982). After invasion and the establishment of growth in the systemic circulation, various manifestations of meningococcal disease may appear. Chronic meningococcemia may occur which can spread to various joints, skin, and adrenals resulting in fulminant meningococcemia. The blood borne bacteria may also invade the cerebrospinal fluid which rapidly results in inflammation of the leptomeninges causing acute purulent meningitis (DeVoe, 1982). This may then lead to vascular collapse and shock (Ferguson and Chapman, 1948). In either meningococcemia or fulminant encephalitis, eruption of skin lesions is common and is typical of meningococcal disease (Hill and Kinney, 1947; Gotschlich, 1980).

iii. Immunity and Treatment.

Immunity to the meningococcus appears rather complex. Usually, exposure to the meningococcus results in asymptomatic nasopharyngeal carriage of the

organism for weeks usually followed by its eventual disappearance. Even during epidemic periods when the carrier rate may be near 95%, the incidence of systemic disease may be less than 1% (Goldschneider et al., 1969a, 1969b). Thus, it would appear that host factors determine, for the most part, susceptibility to the disease. Susceptibility to meningococcal disease has been correlated to a lack of protective circulating bactericidal antibodies (Goldschneider et al., 1969a). However, there is a growing body of evidence suggesting that serum complement plays an important role in resistance to neisserial infections. Lack of the complete terminal attack components has been shown to result in recurring neisserial infections (Nicholson and Lepow, 1979; Petersen et al., 1979; Lee et al., 1978).

It has been demonstrated that natural immunization against meningococcal disease occurs during childhood (between 2 to 12 years) and that neonatal immunity is associated with the transplacental passage of IgG antibodies (Goldschneider et al., 1969a, 1969b). Possession of bactericidal antibodies may occur by a variety of means. Asymptomatic colonization of the nasopharynx in adolescents and adults results in stimulation of protective immune antibodies directed not only to the homologous organism but to other meningococcal strains (Goldschneider et al., 1969b; Reller et al., 1973). Protective antibodies may also result from exposure to bacteria possessing cross reactive antigens to the meningococcus.

Escherichia coli strain 07:K1(L):NM possesses capsular polysaccharides identical to serogroup B meningococci, whereas Escherichia coli strain 048:K91:NM (BOS-12) possesses a capsular polysaccharide identical to those of both serogroup B and C meningococci (Grados and Ewing, 1970; Kasper et al., 1973a; Liu et al.,

1977). Serologic cross-reactions between other enteric bacteria, meningococci and pneumococci have also been reported (Robbins et al., 1972).

Protective antibodies may also be induced by nonpathogenic neisserial species like N. lactamica (Gold et al., 1978b).

Immunization with group A and C capsular polysaccharide vaccines elicit serum bactericidal antibodies which are highly effective in preventing disease caused by these organisms (Gotschlich et al., 1969a).

However, purified group B polysaccharide fails to elicit an immune response in humans (Wyle et al., 1972). Suggested causes of the poor immunogenicity of both serogroup B meningococci and K1 E. coli include intrinsic deficiency of the vaccines used, degradation of the polysaccharide by tissue neuraminidase, intramolecular esterification, or tolerance due to some cross-reactive tissue component (Wyle et al., 1972; Zollinger et al., 1982; Jennings and Lugowski, 1981; Kasper et al., 1973a; Lifely et al., 1981). The latter explanation may prove to be the most plausible since sialic acid-containing glycopeptides isolated from human and rat brain specifically cross-react with antisera raised against either serogroup B meningococci or K1 E. coli (Finne et al., 1983). Members of the sialic acid family of compounds can be found in a variety of tissues and body fluids, the naturally occurring sialic acids being substituted neuraminic acid derivatives (N-acetyl, N-glycolyl, N,O-diacetylneuraminic acid). These authors caution against potential adverse effects which may occur if the natural tolerance is broken down by artificial vaccines.

Noncapsular antigens have been investigated as an alternative to the group B polysaccharide (Frasch and Robbins, 1978). Serotype 2 protein vaccines are immunogenic and protect mice against group B serotype 2

meningococcemia (Craven and Frasch, 1979a); however, it is a poor immunogen in human volunteers (Zollinger et al., 1979). Combined group B-serotype 2 vaccines show dubious promise as immunogens against serogroup B meningococci. Bactericidal antibodies were not elicited against the serotype 2 antigen, although a considerable antipolysaccharide response occurred (Zollinger et al., 1979).

With regard to the classes of bactericidal antibodies produced, serotype 2 protein vaccines elicit primarily an IgG response whereas antibodies directed to the group B polysaccharide were IgM. Group B polysaccharide-serotype 2 conjugate induces only IgM production (Skevakis et al., 1984). Group B meningococci rarely cause neonatal meningitis, unlike K1 E. coli, indicating that IgG antibodies to noncapsular antigens probably protect the neonate (Skevakis et al., 1984). Since vaccine-induced IgM antibodies directed towards the polysaccharide are protective but are of short duration (Moreno et al., 1983; Zollinger et al., 1979) and of low avidity (Mandrell and Zollinger, 1982), new polysaccharide-outer membrane protein complexes are being examined. Beuvery et al., (1983), have shown that crude complexes isolated from serogroup C meningococci in which all contaminating LPS has been removed, will elicit strong IgG responses to both the serotype 2 protein and the polysaccharide in mice.

The cellular immune response to meningococcal infection has not been studied as completely as the humoral response. There is a demonstrable cellular immune response to meningococcal immunity which is antibody dependent, complement independent and is mediated by monocytes and K lymphocytes (Lowell et al., 1979, 1980). Meningococci are highly mitogenic for nonsensitized murine B lymphocytes and whether LPS and non-LPS

outer membrane components contribute to this mitogenicity and meningococcal pathogenicity is obscure (Melancon et al., 1983; Sparkes, 1983).

Wiggins et al., (1973) reported that serogroup C strains in the U. S. were showing increasing resistance to sulphadiazine. In 1966, only 17% of isolates were resistant. but by 1970 this figure had risen to 89%. Munford et al., (1974) demonstrated that the isolates from the serogroup C epidemic in Sao Paulo, Brazil, in 1972 were also sulfonamide resistant. Currently, penicillin G is the preferred antibiotic for initial treatment of meningococcal infection (Artenstein, 1975). Rifampin is given to eliminate the meningococcal carrier state since it is absorbed into body fluids to much higher concentrations than penicillin G (Conte and Barriere, 1981).

III. Bacterial capsular polysaccharides.

i. Introduction.

The bacterial capsular polysaccharide (CP), also known as the bacterial glycocalyx, is defined as being any polysaccharide containing component, of bacterial origin, residing external to the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells (Costerton and Irvin, 1981). They are subdivided into two types termed S layers and capsules. S layers are composed of regular arrays of glycoprotein subunits at the cell surface, such as that found on the surface of Spirillum serpens. Capsular polysaccharides are described as either rigid or flexible and integral or peripheral. Rigid capsules are

differentiated from flexible ones on the basis of their abilities to exclude india ink particles, whereas the designations integral or peripheral reflect the relative degrees of association of the capsule with the cell surface. Peripheral capsules have also been termed slime (Costerton and Irvin, 1981).

Bacterial capsular polysaccharides are usually highly ordered, hydrated, polymeric matrices composed of approximately 99% water. Before the advent of electron microscopy, they were structurally believed to be an amorphous mass but are now known to possess a fibrillar structure (Bayer and Thurow, 1977). Capsular polysaccharides may be simple homopolymers or very complex heteropolymers of monosaccharides, among which the neutral hexoses, 6-deoxyhexoses, polyols, uronic acids, and amino sugars, are the most common constituents. These components may be substituted to varying degrees with phosphate, formate, pyruvate, and succinate (Sutherland, 1972, 1977). The capsules of most Gram-negative bacteria are composed of hydrophilic polysaccharides carrying negative charges (Luderitz et al., 1968) and can be readily detected by immunoelectrophoresis (Orskov et al., 1977). Much of our knowledge on the composition, sequence and conformation of CP has been determined by the use of magnetic resonance spectroscopy of naturally occurring (^1H) or (^{13}C) in these structures (Jennings et al., 1977; Troy, 1979). Determination of the nature and anomeric configuration of CP has been further aided by the use of specific glycosidases (Yurewicz et al., 1971).

ii. Functions.

ii.a. General.

The capsular polysaccharides of bacteria found in natural ecosystems are involved in a wide variety of functions (Costerton and Irvin, 1981). Direct examination of cells growing in natural environments often reveal cells possessing a CP and that they usually grow in glycocalyx-enclosed microcolonies adherent to surfaces (Bae et al., 1972, Fletcher and Floodgate, 1973). Long hydrophilic extensions of the bacterial cell in the form of polysaccharide fibers (or non-polysaccharide pili) may assist in bridging the space between the bacterium and the surface thus allowing attractive forces like hydrogen bonding and ion pair formation to initiate adhesion (Corpe, 1980). Bacteria able to utilize insoluble organic compounds as a food source such as oil, cellulose, and plant cell walls, are able to adhere to these substrata by means of their glycocalyces (Akin et al., 1975; Costerton and Irvin, 1981). Streptococcus mutans and other bacteria in the human oral flora are able to produce insoluble dextrans and levans by means of an extracellular enzyme complex which subsequently forms the basis of a complex of interrelated microcolonies on the tooth surface (Mukasa and Slade, 1974; Scales et al., 1975). Other functions ascribed to CP are resistance to desiccation (Wilkinson, 1958), the provision of bacteriophage receptors (Lindberg, 1973), and blockers of bacteriophage attachment to underlying receptors (Lindberg, 1973; Radke and Siegel, 1971). Micrococcus sodonensis secretes a glucosamine containing heteropolysaccharide which appears to protect several exocellular enzymes from proteolytic degradation during their secretion (Braatz and Heath, 1974).

ii.b. Role in virulence.

The ability of capsular polysaccharides to enhance bacterial virulence has been well documented (Troy, 1979; Costerton et al., 1981). Early studies of CP indicated a correlation between possession of CP and virulence (Sjostedt, 1948); most E. coli strains isolated from human infections are found to be encapsulated (Valhne, 1945). The majority of the evidence implicating CP as a virulence factor came from epidemiological studies with bacterial strains differing in a wide range of other characteristics. Thus, the precise role of how CP, as a surface organelle, contributed to virulence remained largely obscure and complicated by the presence of other surface components like LPS and pili. Studies by Glynn and Howard, (1970) suggested that E. coli rich in capsular (K) antigens were better able to resist the complement-dependent bactericidal activity of serum and phagocytosis than K-poor strains. However, evidence to the contrary was produced by a number of investigators (McCabe et al., 1975; Kalmanson, 1975; Taylor and Robinson, 1980).

McLeod and Krauss (1950) demonstrated that the quantity of capsular polysaccharide formed in vitro was related to pneumococcal virulence. This was later confirmed for Klebsiella pneumoniae (Ehrenworth and Baer, 1956), Haemophilus influenzae type b (Sutton et al., 1982) and group B type III streptococci (Klegerman et al., 1984; Yeung and Mattingly, 1984). Furthermore, the frequency of renal infection was directly proportional to the amount of E. coli CP (Nicholson and Glynn, 1975).

Another virulence mechanism usually attributed to possession of CP was

enhanced resistance to phagocytosis (Jannsen et al., 1963; Smith, 1977; Orskov et al., 1978). Phagocytosis may occur by opsonin-independent mechanisms and it has been postulated that this mode of phagocytosis is mediated by specific bacterial surface components, primarily through their effects on surface charge and hydrophobicity (Van Oss, 1978; Ohman et al., 1982; Petersen et al., 1984). Since CP are primarily anionic, hydrophilic polymers (Troy, 1979), possession of a CP may have a profound effect on resistance to phagocytosis (Van Oss, 1978). Richardson and Sadoff (1976) provided evidence implicating gonococcal capsules in resisting phagocytosis.

The most convincing evidence for both the involvement and role of CP in microbial pathogenicity was through the utilization of isogenic mutants deficient in CP production. Unencapsulated, isogenic E. coli strains were found to be less virulent for mice than wildtype strains (Verweij-van Vught et al., 1983). In a definitive study by Horowitz and Silverstein, (1980) using isogenic noncapsular variants of E. coli, they concluded that (a) attachment by itself to polymorphonuclear leukocytes (PMN) and monocytes results in ingestion of unencapsulated but not encapsulated bacteria; (b) the bacteria are not phagocytosed or effectively killed unless complement is fixed to their surface; (c) the capsule blocks complement fixation to the bacterial surface presumably by masking surface components like LPS capable of activating the complement pathway; (d) the E. coli capsule imposes a requirement for specific antibacterial antibody for complement fixation, and (e) the complement receptor of human PMN and monocytes mediates phagocytosis of complement-coated encapsulated bacteria and is the primary mediator of phagocytosis and killing of these bacteria. Similar conclusions were drawn by Van Dijk et al., (1979),

Stevens et al., (1980), and Bortolussi et al., (1983), by demonstrating that possession of the K1 capsular antigen renders Escherichia coli resistant to opsonization by the alternate complement pathway or the antibody-independent classical pathway (Pluschke and Achtman, 1984). This resistance occurs in spite of small amounts of complement deposited on the surface of these strains (Aguero and Cabello, 1983). It has been observed that as a general phenomenon, opsonophagocytosis in E. coli is inversely proportional to the amount of surface-associated capsule (Verbrugh et al., 1979; Stevens et al., 1980). One characteristic of bacteria that commonly cause meningitis is the presence of a capsule (Tofte et al., 1979). Normal human cerebrospinal fluid was shown to be opsonic for unencapsulated Staphylococcus aureus but not for the encapsulated bacterial species, Streptococcus pneumoniae, Neisseria meningitidis, Escherichia coli, and Haemophilus influenzae. The latter organisms commonly cause meningitis (Tofte et al., 1979).

It was previously believed that resistance to serum bactericidal activity was related to the presence of a complete O-antigenic side chain in the LPS. Strains lacking this O-antigen were considered serum sensitive and relatively avirulent (Feingold, 1967; Taylor, 1983). Genski et al., (1980), and Cross et al., (1984) demonstrated that of all the serologically defined capsules of E. coli, only K1 CP is capable of protecting organisms lacking the O-antigenic side chain of LPS from the bactericidal effects of serum.

Sialic acid is a frequent component of the capsule of invasive bacterial pathogens (Liu et al., 1971b; Robbins et al., 1974; Yeung and Mattingly, 1983) suggesting that it contributes specifically to the virulence

promoted by surface polysaccharides. Although the genus Escherichia presents over 250 different somatic and capsular antigens, only the K1 capsular antigen has been associated with approximately 80% of strains isolated from the cerebrospinal fluid of neonates with meningitis (Robbins et al., 1974). In fact, loss of the sialic acid residues, which make up 25% of the type-specific antigen in type III group B streptococci, by chemical removal or mutation, results in loss of virulence of this organism for mice and its enhanced phagocytic uptake by peritoneal macrophages (Yeung and Mattingly, 1983). Sialic acid has been known to play a wide variety of biological roles. This sugar usually masks the penultimate galactosyl residue on plasma glycoproteins. Removal of the sialic acid by neuraminidase causes prompt removal of the asialoglycoproteins by hepatocytes which recognize the galactosyl residue (Morell et al., 1971). Furthermore, the presence of sialic acid on the plasma membrane receptor site is essential for the initial binding process (Pricer and Ashwell, 1971). Removal of the sialomucoid layer of tumor cells results in the increased susceptibility to cytotoxic killing by serum immunoglobulins (Hughes et al., 1972). Recently, it has been shown that influenza viruses lacking sialic acid are better activators of the alternate complement pathway (McSharry et al., 1981). This finding agrees with the findings of Kazatchkine et al., (1979) who showed that sialic acid on sheep erythrocytes is a key component in modulating activation of C3 via the alternate complement pathway. It has been suggested that sialic acid CP may play an essential role in masking recognition sites thereby protecting the pathogen from the host defense armamentarium (Kasper et al., 1973a; Finne et al., 1983; Yeung and Mattingly, 1983).

Capsular polysaccharides have also been assigned other roles in

virulence. The human lungs possess surfactants with strong antibacterial activity (Colacicchio et al., 1973); however, CP has been shown to increase bacterial resistance to this important pulmonary defense (Govan, 1975). The purified CP of Bacteroides fragilis can directly potentiate abscess formation in a rat intraabdominal sepsis model (Onderdonk, 1977). CP may promote selective adherence to cultured human cells and mucoid epithelial cell layers (Orskov et al., 1977; Smith, 1977; Swanson, 1977). Host clearance mechanisms like the accelerated sloughing of bladder epithelial cells (Mooney et al., 1976), or the mucocilliary escalator of the pulmonary system (Sturgess, 1977) do not function well in the clearance of adherent bacterial microcolonies (Costerton and Irvin, 1981). Furthermore, since these polysaccharides are usually negatively charged due to their numerous carboxylic groups in uronic acids and ketals, they may act in the trapping and concentrating of important nutrients and cations at the cell surface, while possibly impeding antibiotic penetration (Costerton and Irvin, 1982).

iii. Synthesis and assembly.

It would appear that in many cases, but by no means all, synthesis of various polysaccharides by both procaryotes and eucaryotes may follow a general pathway in which nucleotide sugars are initially formed in the bacterial cytoplasm. These are then transferred to lipid-linked sugar intermediates by membrane bound glycosyltransferases and ultimately to a growing saccharide chain (Ginsburg, 1964; Sutherland, 1977, 1982; Parodi and Leloir, 1979). In the meningococcus, the biosynthetic pathway responsible for synthesis of the sugar nucleotide involved in sialic acid capsular

polysaccharide synthesis in serogroup C N. meningitidis was unravelled by Warren and Blacklow, (1962). They initially demonstrated that the amino sugar N-acetylneuraminic acid (NANA) was formed by a condensation reaction between N-acetylmannosamine and phosphoenolpyruvate. This is dissimilar from the mammalian system which condenses N-acetylmannosamine 6-phosphate and phosphoenolpyruvate to form a phosphorylated N-acetylneuraminic acid (Blacklow and Warren, 1962; Warren and Felsenfeld, 1962) which is eventually dephosphorylated by a specific phosphatase to produce free NANA (Blacklow and Warren, 1962). Afterwards, free NANA condenses irreversibly with cytidine triphosphate catalyzed by the enzyme CMP-NANA synthetase, to form cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NANA). This step is similar in both the procaryotic and mammalian cells (Roseman, 1962). This nucleotide has been isolated from E. coli K-235 (Comb et al., 1966) which elaborates a sialyl polymer called colominic acid (Barry and Goebel, 1957). CMP-NANA is unique in that it possesses a single phosphate group whereas other sugar nucleotides normally possess a pyrophosphate group (Ginsburg, 1964). In contrast to other known, natural, sialidase-sensitive sialic acid glycosides, the isolated CMP-NANA was shown to be in a β -anomeric form and was insensitive to snake venom 5'-nucleotidase, E. coli alkaline phosphatase, NANA aldolase, and phosphodiesterase (Comb et al., 1966).

The involvement of a unique membrane lipid acting as an intermediate carrier of glycosyl residues has been shown for LPS (Weiner et al., 1965; Wright et al., 1967), peptidoglycan (Anderson et al., 1967), teichoic acid (Brooks and Baddiley, 1969), and mannan synthesis (Lahav et al., 1969). Wright et al., (1967) determined that O-antigen repeating oligosaccharide subunits of LPS were covalently bound by

a pyrophosphate linkage to the bacterial lipid intermediate which was a C₅₅-polyisoprenyl monophosphate (undecaprenyl phosphate). Bray and Robbins, (1967) demonstrated that these O-antigenic subunits were polymerized on the lipid intermediate.

The involvement of lipid-linked intermediates in the production of bacterial polysaccharides was first discovered in Aerobacter aerogenes (Troy et al., 1971). This capsular heteropolysaccharide, composed of regularly repeating subunits, was synthesized in a manner analogous to O-antigen synthesis (ie. polymerization occurring at the non-reducing terminus) (Troy et al., 1971). Troy et al., (1975) further demonstrated a similar involvement of undecaprenyl phosphate in the synthesis of the sialic acid capsular polysaccharide of Escherichia coli K-235, thus clearly establishing the central role of polyisoprenyl compounds as carrier lipids in the biosynthesis of complex bacterial capsular polysaccharides. However, Vann et al., (1978) found no evidence for the existence of a lipid-soluble intermediate participating in the synthesis of group C meningococcal CP. Apparent exceptions to the general use of lipid intermediates for the transfer of glycosyl residues to growing oligosaccharide chains have been noted in both teichoic acid (Fiedler and Glaser, 1974) and O-antigen synthesis in E. coli 09:K29- (Kopmann and Jann, 1975).

Bayer and Thurow, (1977) proposed 200-400 "export" sites for the transport of the K29 capsular antigen to the outer membrane of E. coli. They further proposed that these sites were located at sites of adhesion between inner and outer membranes. These sites are also the export channels for LPS and outer membrane proteins (Smit and Nikaido, 1978; Lin and Wu, 1980). It has been estimated that synthesis and export times for CP is

between 10 to 15 min (Bayer and Thurow, 1977; Whitfield et al., 1984) which is longer than 1 to 2 min for outer membrane proteins (Lin and Wu, 1980) and 1.5 min for lipopolysaccharide (Muhlradt et al., 1973). The molecular mechanism of how these polymers are transported through these localized sites, and once transported how they are anchored to the outer membrane, remains uncertain. Identification of the ultimate acceptor present in the cell membrane, to which the completed polysaccharide or individual glycosyl residues or oligosaccharide units are transferred, also remains obscure. Troy and McCloskey, (1979) were able to uncouple the synthesis of polysialic acid from synthesis of the sialyl lipid-linked intermediate by culturing E. coli K-235 at low temperatures. These authors suggested that a membrane protein may be responsible for accepting completed sialyl polymers. They based their hypothesis on the absence of various membrane proteins during growth at low temperature and an altered chromatographic profile of purified CP by ion-exchange chromatography after treatment with pronase. Furthermore, examination of a neuraminidase-resistant core of purified CP failed to provide any evidence of associated protein. In a study examining the reducing termini of purified group A, B, and C meningococcal CP, Gotschlich et al., (1981) detected the presence of two different 1,2-diacylglycerols, thus suggesting a mechanism whereby the sialyl polymers are anchored in the bacterial membrane.

iv. Regulation of CP synthesis.

There exists a paucity of information on regulatory control mechanisms governing the synthesis of bacterial capsular polysaccharides. However, three

major factors are immediately recognizable: nutritional and environmental factors, genetic and enzyme regulation, and the availability of lipid intermediates.

The initial site at which control mechanisms may influence CP production is nutrient uptake. In strains of E. coli and Enterobacter aerogenes, polysaccharide production increased under conditions where growth was limited by the nitrogen, phosphorus, sulphur, or potassium concentrations of the growth media (Sutherland, 1982). However, Pseudomonas NCIB 11264 possessed a reduced rate of exopolysaccharide synthesis under phosphorus limitation (Williams and Wimpenny, 1980). Phosphate limitation has also been shown to inhibit teichoic acid synthesis, which is replaced by teichuronic acids under these conditions (Ellwood and Tempest, 1972). An increase in gonococcal LPS serotype antigen with increasing glucose concentrations in a continuous culture system was demonstrated by Morse et al., (1983).

Environmental conditions profoundly influence polysaccharide production. Increased aeration of broth medium resulted in a significant increase in lipopolysaccharide synthesis by serogroup B meningococci (Frasch et al., 1976). Early studies showed that growth at low temperatures of Salmonella, Aerobacter, and Escherichia species resulted in enhanced CP production for most of the strains (Duguid, and Wilkinson, 1953). The relationship between temperature and CP production in K235 or K1 E. coli was studied by various investigators (Bortolussi et al., 1983; Whitfield et al., 1984). In contrast to earlier studies with other bacterial groups, these authors demonstrated a lack of detectable sialic acid CP when the cells were grown at 22°C. This phenomenon, shown previously by Troy and McClosky, (1979) with E. coli K-235, was

attributed to a failure by the bacteria in synthesizing or assembling components of the sialyltransferase complexes, presumably a functional endogenous acceptor. Furthermore, protein synthesis has been shown to be essential for the initiation but not the subsequent elongation of polysialic acid chains (Whitfield et al., 1984). Acidic pH levels have been implicated in the increased production of gonococcal capsules (Richardson and Sadoff, 1976; Morse and Hebeler, 1978).

Genetic studies on polysaccharide regulation have mainly been confined to Streptococcus pneumoniae and enterobacteria (Sutherland, 1982).

The CP of E. coli K12, colanic acid, is a heteropolysaccharide composed of glucose, galactose, fucose and glucuronic acid (1:2:2:1). Synthesis of colanic acid is controlled by three regulator genes, CapR, CapS, and CapT which map at different loci on the E. coli chromosome. Mutation at any one of these genes resulted in overproduction of CP producing a mucoid phenotype (Leiberman and Markovitz, 1970). The CapR locus is responsible for the control of 10 enzymes involved in colanic acid synthesis, which are found in different operons within the genome. The CapR gene product represses four spacially distinct operons in wildtype cells. It also exerts control over such diverse functions as cell division and the appearance of a surface protein (Gayda et al., 1976; Gayda et al., 1979). The precise relationships among the different CapR functions remain obscure. It also remains to be established whether other CP synthesizing systems are under such complex regulatory mechanisms. Echarti et al., (1983) demonstrated that a minimum of nine Kb of DNA split into two gene blocks was involved in synthesis and assembly of the K1 CP of E. coli. One of the blocks was responsible for CP biosynthesis and the other for extracellular appearance of the polysaccharide. Although K1 E. coli

and serogroup B meningococci produce identical CP (Kasper et al., 1973a), there appeared to be no homology between K1 genes and group B meningococcal DNA.

The enzymatic basis for cessation of teichoic acid production in Bacillus subtilis during phosphate limitation is caused by a two-step process. There is an initial reversible inhibition of teichoic acid synthesis, followed by the induction of a protein or proteins which results in irreversible inhibition of the initial enzyme involved in teichoic acid synthesis (Glaser and Loewy, 1979). Feedback inhibition plays an important role in regulating various biosynthetic pathways. In mammalian cells, CMP-NANA inhibits the first enzyme of the pathway unique to its biosynthesis (Kornfeld et al., 1964) thus regulating the intracellular concentration of this sugar. However, some lines of evidence indicate that regulation of intracellular levels of nucleotide sugars may occur through the use of various nucleotide sugar hydrolases. Glaser (1965) demonstrated the existence of a CDP-glycerol pyrophosphatase in Bacillus subtilis. He proposed that this enzyme prevents accumulation of CDP-glycerol under conditions where the rate of teichoic acid synthesis was decreased. Glaser et al., (1967) discovered the presence of two specific membrane-associated nucleotide diphosphate sugar hydrolases in E. coli. These hydrolases were found to be inhibited by a purified protein inhibitor isolated from the same cells. The authors suggested that these enzymes may cause a rapid turnover of the intracellular pool of the sugar nucleotides which could be modified to varying degrees by the inhibitor. Kean and Bighouse, (1974) detected the presence of CMP-NANA hydrolase activity in the plasma membrane of rat liver cells but found little activity in other tissues. In view of the role that sialic acid plays in the hepatocyte surface

receptor for the binding of asialyloglycoproteins, it was proposed that the CMP-NANA hydrolase may be one of the factors regulating the intracellular concentration of CMP-NANA available to the membrane sialyltransferases.

A system of polysaccharide regulation through availability of the lipid carrier has been postulated (Anderson et al., 1972; Sutherland, 1977, 1982). In Gram-negative bacteria, undecaprenyl phosphate is employed in peptidoglycan, LPS, and presumably CP synthesis. In Gram-positive bacteria, a similar situation exists except teichoic acid is synthesized instead of LPS. The discovery of both a polyisoprenyl phosphatase (Willoughby et al., 1972) and an ATP-phosphokinase (Higashi et al., 1970) led to the proposition that availability of functional undecaprenol (undecaprenyl phosphate) could regulate polymer synthesis (Willoughby et al., 1972). It has been demonstrated that, in Staphylococcus lactis I3 (Watkinson et al., 1971), Bacillus subtilis and Bacillus licheniformis (Anderson et al., 1972), nucleotide precursors of the teichoic acid biosynthetic pathway can inhibit peptidoglycan synthesis. The same occurs in the reverse case thus demonstrating that undecaprenyl phosphate was a common component of the two systems and that availability of the lipid carrier may regulate these two polymers.

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Bacterial strains.

N. meningitidis strain M986, a serogroup B, serotype 2, 7, disease isolate and a spontaneous isogenic polysaccharide release mutant (PRM) PRM101 was obtained from Dr. F. E. Ashton, Laboratory Centre for Disease Control (LCDC), Health and Welfare Canada, Ottawa, Ont., Canada. N. meningitidis strains 604-A, M1011, and 2241-C, serogroup A, B, C disease isolates respectively, were also obtained from LCDC. Neisseria flava strain M953 was obtained from the Neisseria repository, NAMRU, School of Public Health, University of California, Berkeley, Ca. Escherichia coli K12 (ATCC# 10798) and N. perflava (ATCC# 14799) were obtained from the American Type Culture Collection. E. coli 01:K1:HNM, a disease isolate, was obtained from L. Therien, Dept. of Microbiology and Immunology, McGill University, Montreal, Quebec. N. meningitidis strain PRM102 and an isogenic variant PRM101r were obtained as described below (section III. Isolation of mutants).

Purity of strains were performed according to Vedros (see appendix, table 13).

II. Culture conditions.

To assure standardized results, all cultures, with the exception of N. flava, N. perflava, and K12 E. coli, were reconstituted from lyophilized stocks, and cultured on Columbia blood agar

(CBA) plates for 18 to 24 h at 37°C in a CO₂ incubator with an atmosphere of 10% CO₂-balance air and 100% relative humidity. N. flava, N. perflava, and K12 E. coli were stored frozen (-70°C) on Mueller-Hinton (MH) slants. These cultures were thawed and streaked onto CBA plates before use. Cells harvested from CBA plates were used to inoculate 50 ml of Neisseria-defined medium (Archibald and DeVoe, 1978) containing 0.1% (wt/vol) casein hydrolysate (Sigma Chemical Corp., St. Louis, Mo.) and 200 ng of Fe per ml (NDM-C) (Masson et al., 1982). NDM-C composition is shown in table 14 (appendix). Cultures were grown at 37°C in a reciprocating water bath shaker for 12 h at 140 rev/min (New Brunswick Scientific Co., New Brunswick, NJ.) in an atmosphere of 10% CO₂-balance air.

Low pH and nutrient limited media were prepared as modifications of NDM-C. The casein hydrolysate concentration was reduced to 0.05% and this medium was subsequently referred to as Normal NDM-C. Medium, limited for iron, was made from Normal medium without added iron and to which the iron chelator, ethylenediaminediorthohydroxyphenylacetate (EDDA; Sigma) was added to a final concentration of 400 ng/ml of medium. Low pH medium was made in the same manner as Normal NDM-C but was buffered with 50 mM Tris(hydroxymethyl)aminomethane-maleate (Tris-maleate) and adjusted to pH 6.6 with NaOH. This pH is the lowest at which serogroup B meningococci are able to grow (Brener et al., 1981). Medium limited for nitrogen was prepared from Normal NDM-C lacking glutamic acid, NH₄Cl, and possessing only 0.5 ug/ml of casein hydrolysate. Limited growth in this medium was shown to be due to nitrogen starvation and not amino acid deprivation since the cells grew well in medium lacking glutamic acid and 0.05% casein but possessing normal levels of NH₄Cl. The different media were inoculated

(0.75% vol/vol) from a 12-14 h culture in Normal NDM-C and were allowed to grow for 5-6 h. Growth was monitored spectrophotometrically at 650 nm with a Bausch and Lomb, spectronic 100.

III. Mutant isolation.

Polysaccharide production in N. meningitidis strain M986 was confirmed by the ability of serogroup B rabbit antisera to agglutinate the bacterial cells, by iridescence of colonies when grown on MH or NDM-C agar (Devine et al., 1972), and by the production of halos of immunoprecipitates around the colonies when grown on MH or NDM-C agar containing serogroup B horse antiserum (Ashton et al., 1979) obtained from Dr. C. E. Frasch.

NDM-C broth cultures of N. meningitidis strain M986 were enriched for spontaneous mutations, resulting in the loss of cell-associated capsular polysaccharide, by the addition of filter-sterilized horse serogroup B antiserum to an aliquot of a mid-logarithmic phase culture. The cell suspension was mixed and allowed to agglutinate for 30 seconds followed by pelleting the agglutinated cells gently. The supernatant was added to fresh NDM-C broth and allowed to grow for 5 h. The agglutination procedure was repeated three times after which mutants deficient in polysaccharide release (PRM) were detected by their inability to produce halos when grown on NDM-C agar containing serogroup B horse antiserum and by a concomitant loss of colony iridescence. Potential mutants were passaged twice on NDM-C agar plates and finally on antibody agar in order to select a stable mutant. Two spontaneous and independent mutants, PRM101 and PRM102, were selected. A

revertant was obtained from mice pretreated with iron dextran (Holbein et al., 1979) and lethally infected with 10^8 colony-forming units (CFU) of PRM101. Blood was obtained by cardiac puncture just prior to death (24 h post injection), the blood diluted in NDM-C and plated on MH agar. Iridescent colonies were picked and subsequently plated on horse antiserum agar to confirm polysaccharide production. A positive clone was retained as PRM101r.

IV. Preparation of crude cytosolic and membrane fractions.

Bacterial cells grown in NDM-C broth culture were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C . The cells were washed in one-third the original culture volume using 0.15 M morpholinopropanesulfonic acid (MOPS) buffer, pH 7.7, containing 20mM MgCl_2 and 25 mM 2-mercaptoethanol. Washed cells were resuspended in 10 mls of the same buffer containing 50 ug of DNAase and RNAase (Sigma) per ml. The cells were then broken by two passes in a French pressure cell at $15,000 \text{ lb/in}^2$. Cellular debris was removed by two centrifugations at $10,000 \times g$ for 10 min at 4°C . The resulting supernatant fraction was separated into crude cytosolic and membrane fractions by centrifugation at $220,000 \times g$ (maximum) for 6 h at 4°C . The membrane fraction was washed once in 25 ml of 0.15 M MOPS buffer (pH 7.7) containing 20 mM MgCl_2 and stored in 3 ml of the same buffer at 4°C . There was no loss of sialyltransferase or cytidine 5'-monophosphosialic acid (CMP-NANA) hydrolase activities during storage for three weeks under these conditions.

Aliquots of membranes were delipidated according to the procedure of Troy et al., (1971). One ml of crude particulate membranes from

N. meningitidis strain M986 (17 mg protein/ml) was added to 40 volumes of acetone and stirred for five min. The contents were centrifuged at 5,000 x g for 5 min and the pellet resuspended in 20 volumes 1-butanol and the process repeated using 40 volumes of acetone and 40 volumes of diethyl ether. All solvent manipulations were kept at -20°C to reduce irreversible protein denaturation. Residual solvent was removed from the proteins under reduced pressure and these were stored at 4°C in a dessicator. Sialyltransferase activity appeared to be stable for several weeks under these conditions.

V. Membrane separation and purification.

NDM-C broth cultures of N. meningitidis strain M986 were harvested after 6 h of growth and washed once with one-third the original culture volume using 50 mM Tris-hydrochloride buffer, pH 8.0, and suspended in 15 ml of Tris buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 50 ug of DNAase and RNAase per ml. After cell disruption by two passes in a French pressure cell and the removal of cellular debris (see above), a 12 ml portion of the membrane fraction was layered onto a discontinuous sucrose density gradient consisting of six ml of 55% (wt/wt) sucrose (Sigma; grade I) in 3 mM EDTA adjusted to pH 8.0 with NaOH and nine ml of 15% (wt/wt) sucrose-EDTA, pH 8.0, all in a Beckman Ti-60 centrifuge bottle. The gradient was centrifuged at 300,000 x g (maximum) for two h at 4°C. The membrane layer, positioned at the interface of the two sucrose steps, was collected from beneath with a J-shaped Pasteur pipette and diluted in 3 mM EDTA, pH 8.0, until its sucrose concentration was approximately 30%

(wt/wt). The membranes (2.3 ml) were then applied to the top of a second discontinuous sucrose gradient consisting of three 2.9-ml steps of 45, 40, and 35% (wt/wt) sucrose in 3 mM EDTA, pH 8.0, on top of a cushion of 1.4 ml of 50% (wt/wt) sucrose in a Beckman SW-40 tube. These gradients were centrifuged for 30 to 35 h at $189,000 \times g$ (average) at 4°C . The gradients were fractionated from the bottom of the tubes after puncturing, with the collection of 15-drop fractions. The relative densities of the gradient fractions were determined with the Abbe-3L refractometer (Bausch and Lomb) and protein levels determined by the method of Lowry et al., (1951) using bovine serum albumin (BSA) as a standard (see appendix, Figure 24). The fractions were subsequently dialyzed by using a microdialyzer (Bethesda Research Laboratories, Bethesda, Md.) against 0.15 M MOPS buffer (pH 7.7) containing 20 mM MgCl_2 at 4°C for 12 h.

VI. Outer membrane protein iodination.

Outer membrane proteins of N. meningitidis strain M986 were labeled by surface iodination of whole cells using the procedure of Brener et al., (1981). Briefly, 250 ug of lactoperoxidase were added to two ml of 0.01 M phosphate-buffered saline (PBS; pH 7.4) containing PBS-washed M986 cells at an optical density at 650 nm of approximately 1.0. The solution was incubated in a water bath at 30°C . Carrier-free ^{125}I (ca. 100 uCi) was added (see appendix, Figure 25), followed by 50- μl additions of 0.01 M H_2O_2 at 2.5-min intervals until 12.5 min, at which point two ml of 5 mM cysteine was added to stop the reaction. After washing in PBS, the labeled cells were added back to unlabeled whole cells, disrupted in a French

pressure cell, and fractionated on a discontinuous sucrose gradient as described in section V.

VII. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Discontinuous polyacrylamide slab gels were prepared according to the method of Laemmli, (1970). Samples, approximately 40 ug of membrane protein each, from membranes separated and purified as described in section V., were solubilized by boiling for 5 min in 0.0625 M Tris-HCl buffer containing 2% (wt/vol) sodium dodecyl sulfate, 10% (vol/vol) glycerol, 5% (wt/vol) 2-mercaptoethanol and 0.2% (wt/vol) bromophenol blue and applied per well. A constant current of 15 mA/gel was maintained during migration of the tracking dye through the upper stacking gel. The current was then increased to 30 mA when the dye entered the separating gel until the dye reached to within 1 cm of the gel bottom. For the determination of protein molecular weights, see appendix Figure 26.

Gels were stained for 6 h in 0.4% (wt/vol) Coomassie brilliant blue dissolved in 15% (vol/vol) glacial acetic acid and 50% (vol/vol) methanol. The gels were destained in the same solvent system without added dye.

VIII. Preparation of lipids.

Undecaprenyl phosphate (UP; Calbiochem., La Jolla, Ca.), phosphatidylethanolamine (PLE; Sigma), and phosphatidylglycerol (PLG; Sigma), were prepared by removing the solvent under a stream of nitrogen gas followed by the addition of 0.1% Triton-X-100 in 0.15 M MOPS buffer (pH 7.7)

containing 20 mM MgCl_2 . The lipids were resuspended (in solution) by mild sonication (Bronson Sonic Power Co.). Lipid concentrations were determined by measuring inorganic phosphorus by the method of Chen et al., (1956) (see appendix, Figure 27).

IX. Extraction of lipid-linked intermediates.

Incorporation of [^{14}C]-NANA into lipid-linked intermediates was measured by the addition of 0.21 ml samples of reaction mixtures containing 10.0 mg of particulate membrane protein, 20 mM MgCl_2 , 4.0 uCi CMP-[^{14}C]NANA (319 mCi/mMol), and 5.6 uMol undecaprenyl phosphate (0.025% final Triton-X-100 concentration), and 0.15 M MOPS buffer (pH 7.7) in a final volume of 1.4 ml, to 4 ml of chloroform:methanol (C:M) (2:1) according to the method of Osborn, (1972). After 15 min the organic phase was washed four times with pure solvent upper phase produced by the addition of 1.5 ml of chloroform and 24 ml of methanol to a solution containing 0.183 g of KCl in 23.5 ml of glass-distilled water (Folch et al., 1957). Samples of the washed organic phase were removed and the solvent allowed to evaporate overnight in liquid scintillation vials. The level of radioactivity was determined by liquid scintillation after the addition of 0.5 ml of distilled water to the dried residue followed by 10 ml of Aquasol. To determine the degree of polymerization of extracted NANA residues, reaction mixtures (above) were extracted by C:M (2:1) at 3.75 min post label addition. The washed organic phase was spotted onto thin layer silica gel G plates and developed in C:M:water (60:25:4). The origin was scraped and the dried gel was extracted twice by either 0.1% Triton-X-114 (Sigma), C:M (2:1), or

methanol:acetic acid (95:5). The non-detergent extractions were subsequently dried under a stream of nitrogen gas and resuspended, with mild sonication, in 0.1% Triton-X-114. These solutions were subjected to sequential oxidation and reduction by NaIO_4 and KBH_4 followed by digestion with 1.0 unit of neuraminidase for 24 hours according to the procedure of Van Lenten and Ashwell, (1971) as modified by Rohr and Troy, (1980). NANA and 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid (sialitol; $R_{\text{NANA}} = 1.7$) levels were determined after spotting the reaction mixtures onto Whatman 3MM paper and developed by descending chromatography (as described in section XII). As an internal control, 0.5 μCi of $\text{CMP}-[^{14}\text{C}]\text{NANA}$ (319 mCi/mMol) was used for determining the completeness of oxidation. Both $\text{CMP}-[^{14}\text{C}]\text{NANA}$ and $\text{CMP}-[^{14}\text{C}]\text{sialitol}$ were acid hydrolyzed in 50mM KCl-HCl buffer (pH 2.0) for one h at 37°C and were subsequently used as markers. Since the CMP-NANA employed in this study was labeled at carbon atoms 4 through 9, the level of radioactivity recovered as sialitol was corrected for the loss of C8 and C9.

X. Enzyme assays.

The determination of NANA condensing enzyme, CMP-NANA synthetase, sialyltransferase, and CMP-NANA hydrolase activities were as described elsewhere (Masson and Holbein, 1983). Cytosolic and purified membrane protein levels were determined by the method of Lowry *et al.*, (1951). Membrane protein levels were adjusted to approximately 8.0 mg protein/ml.

NANA condensing enzyme activity was measured by the condensation of $[^3\text{H}]\text{N-acetylmannosamine}$ (NAM) with phosphoenolpyruvate (PEP) by the

method of Blacklow and Warren, (1962). Assay mixtures included 200 ug of crude cytosolic protein, 1 uMol of PEP, 1 uMol of [^3H]NAM (3 uCi/uMol), and 25 mM 2-mercaptoethanol, 0.15 M MOPS buffer (pH 7.7), 20 mM MgCl_2 in a final volume of 0.21 ml. The reaction mixture was incubated at 37°C, and 1 unit of enzyme was defined as the amount producing 1 uMol of [^3H]NANA per h at 37°C. CMP-NANA synthetase activity (Warren and Blacklow, 1962), was assayed by measuring the appearance of CMP-[^{14}C]NANA in reaction mixtures containing 200 ug of crude enzyme protein, 1 uMol of cytidine triphosphate (CTP), 1 uMol of [^{14}C]NANA (0.1 mCi/mMol), 0.15 M MOPS buffer (pH 7.7), 20 mM MgCl_2 and 25 mM 2-mercaptoethanol in a final volume of 0.21 ml. One unit of enzyme was defined as the amount producing 1 uMol of CMP-[^{14}C]NANA per h at 37°C. Sialyltransferase activity was measured by the incorporation of [^{14}C]-NANA from CMP-[^{14}C]NANA into high molecular weight polymer. CMP-NANA hydrolase activity was measured simultaneously by the appearance of free [^{14}C]NANA from CMP-[^{14}C]NANA (Kundig et al., 1971; Masson and Holbein, 1983). Enzyme reaction mixtures were composed of approx. 60 ug membrane protein, 20 mM MgCl_2 , 0.15 M MOPS buffer (pH 7.7) and 0.14 uCi of CMP-[^{14}C]NANA (319 mCi/mMol) in a final volume of 0.1 ml. One unit of sialyltransferase was defined as the amount of enzyme incorporating 1 nMol of [^{14}C]NANA into high molecular weight polymer per h, whereas 1 unit of CMP-NANA hydrolase was defined as the amount of enzyme releasing 1 nMol of [^{14}C]NANA from CMP-[^{14}C]NANA per h.

Sialyltransferase activities of particulate membranes in the presence or absence of undecaprenyl phosphate, phosphatidylglycerol or phosphatidylethanolamine were assayed in mixtures containing 68 ug of particulate membrane protein, 35 uM of cytidine 5'-monophosphate (CMP), 20 mM

MgCl₂, 0.14 uCi of CMP- ¹⁴C NANA (319 mCi/mmol), and 0.15 M MOPS buffer (pH 7.7) in a final volume of 110 ul. The lipids were added so that the assay mixture contained a final concentration of 0.045% Triton-X-100. All reactions were preincubated for 15 min at 37°C and for a further 15 min after substrate addition. Reconstitution of sialyltransferase activity was measured in assay mixtures containing 200 ug of delipidated protein, 25 mM 2-mercaptoethanol, 100 mM (NH₄)₂SO₄, 0.14 uCi of CMP- ¹⁴C NANA (319 mCi/mmol), 20 mM MgCl₂, and 0.15 M MOPS buffer (pH 7.7) in a final volume of 130 ul. The lipids were added so that the assay mixture contained a final concentration of 0.012% Triton-X-100. The assay mixtures were preincubated at 37°C for 15 min followed by a further two hours incubation after substrate addition. NANA aldolase was assayed by the method of Brunetti et al., (1963) and neuraminidase was measured by the method of Cassidy et al., (1965). NANA condensing enzyme, CMP-NANA synthetase, sialyltransferase, CMP-NANA hydrolase, NANA aldolase, and neuraminidase substrates and reaction products were measured by paper chromatography as described in section XII.

Succinate dehydrogenase (SDH) was assayed by the method of Kasahara and Anraku, (1974). One unit of SDH was defined as the amount reducing 1 nMol of 2,6-dichlorophenol-indophenol per min at 22°C.

Tetramethylphenylenediamine (TMPD)-oxidase was measured by the method of DeVoe and Gilchrist, (1976). One unit of TMPD-oxidase was defined as the amount of enzyme producing an increase in absorbance at 520 nm of 0.1 per min at 22°C. Both SDH and TMPD-oxidase were measured with a Gilford 2000 recording spectrophotometer. Glucose-6-phosphate dehydrogenase was measured by the method of Scott and Tatum, (1971).

Peptidoglycan synthesis was measured by the incorporation of

UDP-[^{14}C]N-acetylglucosamine into high molecular weight polymer. Standard incubation mixtures contained 1.3 mg of particulate membrane protein, 0.8 mM UDP-N-acetylmuramic acid (UDP-NAMA) pentapeptide (UDP-N-acetylmuramyl-L ala-D glu-meso DAP-D ala-D ala), 0.3 uCi UDP-[^{14}C]N-acetylglucosamine (306 mCi/mMol), 100 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgCl_2 , and 0.15 M MOPS buffer (pH 7.7), in a final volume of 178 μl . The UDP-NAMA pentapeptide used in our assays was identical to that found in Neisseria gonorrhoeae (Hebeler and Young, 1976). Levels of radioactivity incorporated into peptidoglycan polymer were determined by paper chromatography (see section XII) or by the method of Anderson et al., (1972). Briefly, the reactions, in 50 μl aliquot samples, were terminated by the addition of 0.5 ml of cold 0.3 M perchloric acid and thoroughly vortexed. After 10 min at 4°C , the samples were centrifuged ($12,000 \times g$) for five min in an Eppendorf centrifuge (Brinkmann, W. Germany) and the pellet washed three times with 0.25 ml cold 0.3 M perchloric acid. The pellet was finally resuspended in 0.5 ml of distilled water, added to 10 mls of Aquasol (New England Nuclear, Lachine, Quebec) and counted in a Beckman LS 8000 scintillation spectrometer.

XI. Synthesis and purification of dual-labeled meningococcal CMP-NANA.

Dual labeled [^3H]CMP-[^{14}C]NANA was synthesized in reaction mixtures containing 1.5 mg of crude cytosolic protein, 2 μMol [^{14}C]NANA (1 mCi/mMol), 2 μMol [^3H]CTP (9 mCi/mMol), 0.15 M MOPS buffer (pH 7.7) containing 20 mM MgCl_2 and 25 mM 2-mercaptoethanol in a final volume of 1.22 ml. After incubation at 37°C for 80 min, 14 ml of glass-distilled

water was added.

A diethylaminoethyl(DEAE)-cellulose column (1 cm x 15 cm) was prepared and washed with 200 ml of 5 mM MOPS buffer (pH 7.7). After the sample was added, the column was washed with 150 ml of 10 mM MOPS buffer (pH 7.7) to remove unreacted CTP and NANA. The $[^3\text{H}]\text{CMP}-[^{14}\text{C}]\text{NANA}$ was eluted from the column by a linear gradient of LiCl using 200 ml of 0.2 M LiCl in 10 mM Mops (pH 7.7), and 200 ml of 10 mM MOPS (pH 7.7). Five ml fractions were collected and counted in 10 ml of Aquasol (New England Nuclear).

XII. Chromatography.

Samples of the reaction mixtures (20 μl) for the determination of NANA condensing enzyme, CMP-NANA synthetase, sialyltransferase, CMP-NANA hydrolase, NANA aldolase, and neuraminidase as well as peptidoglycan polymer (50 μl) were spotted directly onto Whatman 3MM chromatography paper. The products were separated from the reactants by descending chromatography with ethanol-1.0 M ammonium acetate (7:3) pH 7.5. After the solvent front had migrated 36 cm, the chromatograms were removed and air-dried. To determine the levels of the various reactants or products of the enzyme reactions (see appendix, table 15), radioactive spots were located and cut out, added to 10 ml of Econofluor (New England Nuclear) and the level of radioactivity measured. To determine NANA and sialitol levels, the paper chromatograms were developed in butyl acetate:acetic acid:water (3:2:0.5) for 48 h. The paper was air-dried, cut into 2.5 cm strips and counted in 10 mls of Scintiverse (Fisher Scientific Co.). Counting efficiencies for ^{14}C and ^3H on paper chromatograms were 90% and 10% respectively.

Thin layer chromatography of C:M (2:1) fractions was performed on Redi-Plates (silica gel, 0.25 mm thickness, Fisher Scientific Co.) and developed using C:M:water (65:35:8), C:M:water (60:25:4) or heptane:ethyl acetate (85:15). The developed plates were sprayed with En³Hance (New England Nuclear) and autoradiographed with Kodak X-OMAT film.

XIII. Serum bactericidal assays.

N. meningitidis strains M986 and PRM102 were grown to mid-logarithmic phase and harvested by centrifugation at 10,000 x g for 5 min at 4°C. The cell pellets were washed twice in 0.1 M PBS pH 7.2, and finally resuspended in PBS supplemented with 0.01% bovine serum albumin. The optical density at 650nm was adjusted to 0.20. This cell suspension was equivalent to approximately 50-70 colony forming units per 25 ul. Reaction mixtures consisted of 25 ul of cell suspension, 25 ul of PBS containing 5 mM MgCl₂ and 1 mM ethyleneglycol-bis-(2-aminoethyl ether) N,N'-tetraacetic acid (EGTA; Sigma), and 25 ul of either normal rabbit serum (NRS), PBS, or heat-inactivated (56°C, 30 min) normal rabbit serum (H-NRS). To examine bacterial killing by the classical complement pathway, 25 ul of cells were added to 25 ul of PBS containing 5 mM MgCl₂, 1 mM CaCl₂, horse serogroup B antiserum (1:128 dilution), and 25 ul of either NRS, PBS, or H-NRS. All assays, performed in triplicate, were incubated at 37°C in an atmosphere of 5% CO₂-balance air for 30 min after which each reaction mixture was plated separately on MH agar. Surviving bacteria were enumerated on MH agar plates after 24 h of growth and % killing was determined with respect to control samples in PBS alone.

XIV. Virulence studies.

LD₅₀ determinations were performed using C-57/6 black mice as described previously (Holbein et al., 1979). Mice were obtained at four weeks of age from the University of Calgary Medical Vivarium, Calgary, Alta. Mice obtained from Jackson Laboratories were used in the low pH or nutrient limitation experiments. The mice were fed standard mouse chow and watered ad libitum and were used when the average weight reached approximately 18 to 20 grams. Determinations were made at least three separate times for each strain. Experiments examining enhancement of virulence by low pH and nutrient limitation were performed at least three times for each culture type after 5 h of growth, using five mice per group. The mice were pretreated by the injection of 0.5 ml of iron dextran intraperitoneally (250 mg iron/Kg body weight; Dextran Products Ltd., Scarborough, Ont.). Control mice were given 0.5 ml of sterile 0.9% saline or iron dextran only. Cumulative mortality data at 72 h post injection were used to measure the 50% lethal dose as determined by the method of Reed and Muench, (1938).

XV. Quantitation of sialyl polymer content.

One ml samples of broth cultures were removed after 5-6 hours of growth, centrifuged at 10,000 x g and the supernatant fluids removed. Both the cell pellet and the supernatant fractions were stored at -20°C.

Aliquots of the broth cultures were removed and fixed in 1% formaldehyde. Direct counts of the bacteria were performed in duplicate using a Petroff-Hausser bacterial counting chamber (Blue Bell, Pa.). The cell pellet and supernatant fractions were prepared for rocket immunoelectrophoresis by precipitation of the thawed fractions with cold absolute ethanol. After leaving the samples sit for 15 min at 4°C, they were subsequently centrifuged at 12,000 x g for 10 min, the ethanol wash was removed, and the pellets were resuspended in 0.1 M Barbitol buffer pH 8.6. Samples prepared in this fashion gave highly reproducible results and were far superior to samples prepared from cells disrupted by repeated freeze-thawing.

Monospecific antiserum directed to the serogroup B capsular polysaccharide was prepared in the following manner. A broth culture of PRM102 was grown in Normal NDM-C medium. After 6 h of growth, the bacteria were harvested, washed twice in PBS and the pellet was resuspended in PBS containing 1.0 mM mercuric chloride. These cells were then incubated for one h and then washed twice in 0.15 M MOPS buffer (pH 7.7) containing 20 mM $MgCl_2$. This treatment prevented autolysis of this organism, as confirmed by phase contrast microscopy, for at least one week (Peetz and Kenney, 1978). Any surface polysaccharide that might have been present was removed by the addition of 2.0 units of neuraminidase (type V; Sigma). After neuraminidase treatment at 37°C for two days, the cells were washed twice in PBS to remove the neuraminidase and the harvested cells were used to absorb serogroup B horse antiserum according to the method of Devine and Hagerman, (1970). The antiserum was then filter-sterilized and kept frozen at -20°C until required. The sialic acid capsular polysaccharide was quantitated by rocket immunoelectrophoresis using the technique of Weeke, (1973). The antisera was added to 1.0% (wt/vol) agarose (Sea Kem LE agarose) at a final

concentration of 11%. Samples (20 μ l) were added to wells (four mm diameter) and electrophoresed at eight volts/cm in the gel for four h. Coomassie brilliant blue (0.2% wt/vol), dissolved in barbital buffer, was used as a tracking dye. Purified serogroup B polysaccharide (provided by Dr. H. J. Jennings) was used as a standard (see appendix, figure 28).

XVI. Capsular polysaccharide production and release.

N. meningitidis strains M986, PRM101, PRM102, and PRM101r were grown in NDM-C and at intervals, four ml samples of the cultures were removed and harvested by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant fractions were dialyzed against glass-distilled water at 4°C for 12 h and then assayed for their cell-free sialic acid content after correcting for volume increases due to dialysis. The bacterial cells were washed once in PBS and finally resuspended in four ml of water. Washing was found not to remove any detectable cell-associated sialic acid. Dry cell weights were determined by removing one ml washed cell samples and drying them overnight at 50°C followed by three days under reduced pressure at 70°C until a constant weight reading was obtained. Cell-associated and cell-free sialic acid levels were determined using the method of Svennerholm, (1957) (see appendix, figure 29).

XVII. Ferritin-tagged antibodies and electron microscopy.

The globulin fraction of monospecific antibodies prepared as outlined in section XV was tagged with ferritin (Polysciences, Warrington, Pa.), using

xylylene metaisocyanate (Polysciences) as a linking agent, by the method of Breeze and Hsu, (1971). N. meningitidis strains M986 and PRM102 were grown to mid-log phase in NDM-C, washed in PBS and resuspended to an optical density at 650 nm of 1.0 in 0.15 M Mops buffer (pH 7.7). Functional conjugated antibody was determined by the ability to agglutinate N. meningitidis strain M986 cells. The antibody conjugate (50 ul) was added to 200 ul of bacterial cells and incubated for 10 min at 37°C. The cells were then harvested, washed twice in MOPS buffer (pH 7.7), and fixed in 3% glutaraldehyde for one h, and then post-fixed in Veronal buffer (pH 7.4) containing 1% (wt/vol) osmium tetroxide (Palade fixative). After acetone dehydration, the cells were impregnated in Epon 8-12 epoxy resin and thin sections cut using a Sorvall-Dupont ultramicrotome. The sections were stained with 2% (wt/vol) uranyl acetate in 0.05 M sodium maleate buffer (pH 6.0) and examined using a Philips 300 electron microscope.

XVIII. Determination of cell-surface hydrophobicity.

Adherence of cells to hexadecane was measured by the method of Rosenberg et al., (1980). Briefly, harvested bacteria were washed once in PUM buffer (22.2 g K_2HPO_4 , 7.26 g KH_2PO_4 , 1.8 g Urea, 0.2 g $MgSO_4 \cdot 7H_2O$ diluted to 1 litre with distilled water) and resuspended to an optical density (550 nm) of 0.85 in the same buffer. To three ml of the suspension, 0.4 ml of hexadecane was added and vigorously vortexed for two 30 second periods. The optical density 550 nm of the aqueous phase was measured after the phases had been separated to determine the % adsorbance to

hexadecane.

XIX. Chemicals.

All chemicals used were either analytical or reagent grade.

UDP-N-acetylmuramic acid pentapeptide was provided by Dr. E. Ishiguro, University of Victoria, Victoria, B. C. Horse group B antiserum was provided by Dr. C. E. Frasch, Food and Drug Administration, Bethesda, Md.

RESULTS

RESULTS

Chapter I.

i. Introduction.

Bacterial capsular polysaccharides play a variety of important roles in pathogenic bacterial cell-environment interactions (Troy, 1979). Rough and smooth variants from strains of N. meningitidis have been observed (Devine et al., 1972; Stephens and McGee, 1983), and it has been suggested that capsular polysaccharide may be responsible for this phenotype. It has also been suggested by these investigators that the presence of CP on smooth variants could be associated with virulence. A complete understanding of how the sialic acid polysaccharide of serogroup B N. meningitidis functions as a virulence factor necessitates an understanding of not only the structure of the polymer, but also how its precursors are synthesized, the regulatory mechanisms involved in its production, and its mechanism of assembly. The overall mechanism for the synthesis and regulation of polysaccharide production in the meningococcus has not been defined.

This chapter deals primarily with the physiology of CP production at the cellular and the molecular level. One of the approaches used in probing this area was through the isolation and use of naturally occurring, isogenic mutants deficient in their ability to produce the serogroup B specific $\alpha 2 \rightarrow 8$ linked homopolymer of sialic acid. Experiments are presented in the first part of this chapter demonstrating the responsible enzymes involved in the biosynthetic pathway of CP production, their locations as well as the detection and characterization of a previously undiscovered CMP-NANA

hydrolase enzyme.

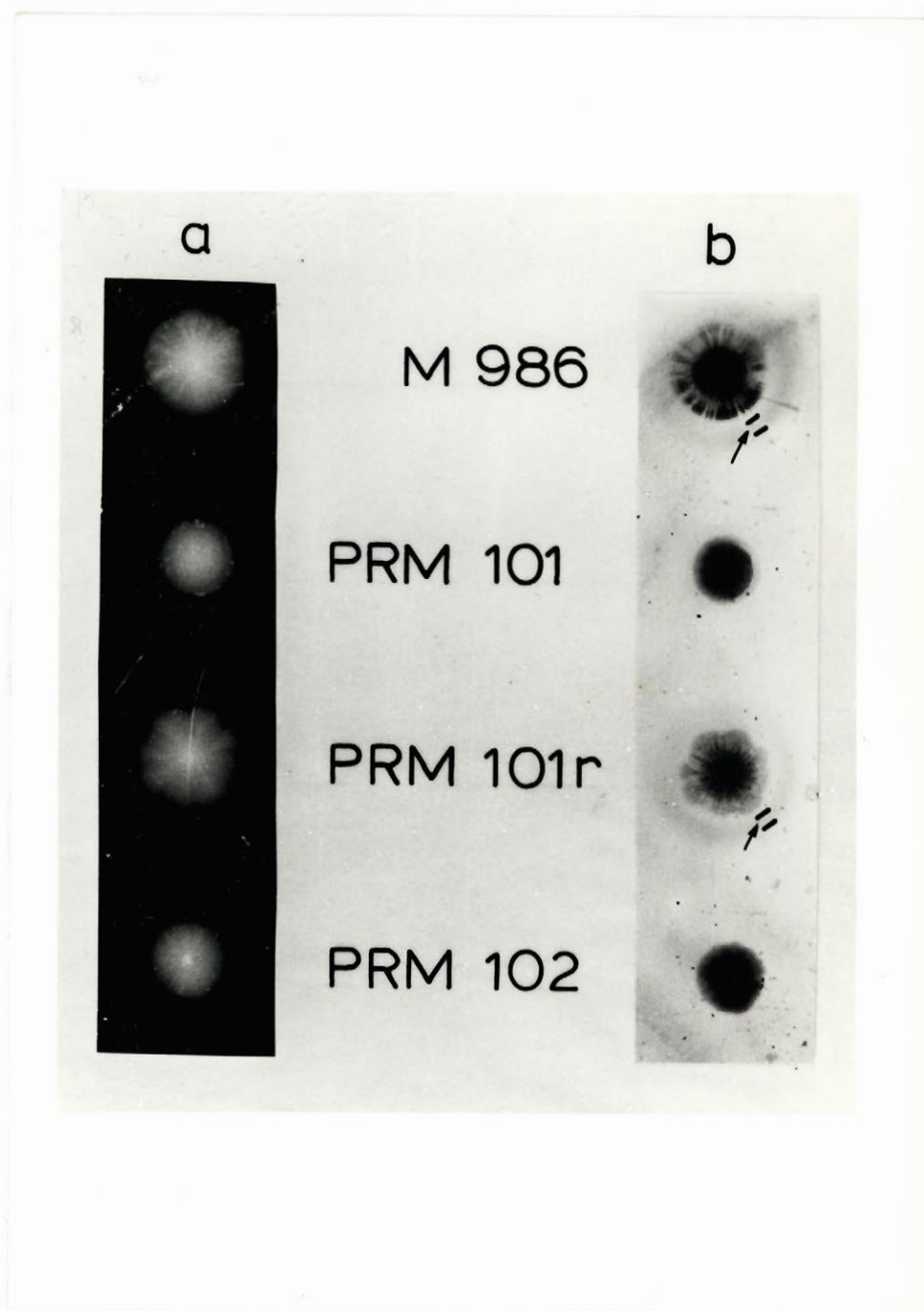
A variety of membrane-bound glycosyltransferases utilize lipid intermediates (Weiner et al., 1965; Sutherland, 1977, 1982). It has been postulated that these lipids may play an important regulatory role in the synthesis of macromolecular cell envelope components (Anderson et al., 1972; Sutherland, 1982). Previous attempts in determining the involvement of lipid intermediates in meningococcal CP synthesis produced negative results (Vann et al., 1978). The results presented in the latter part of this chapter demonstrate the participation of a lipid carrier involved in CP production and its role in synthesis, assembly and regulation of serogroup B N. meningitidis sialic acid CP.

ii. Isolation of noncapsular mutants.

The ability of encapsulated serogroup B meningococci to produce diffuse immunoprecipitates (halos) when grown on agar containing horse antiserum was exploited to selectively isolate spontaneous, isogenic, polysaccharide release mutants (PRM). Two such mutants, PRM101 and PRM102, were isolated from the encapsulated wildtype strain M986 (Figure 1). PRM mutants were detected by their inability to produce halos and by a concomitant loss of colony iridescence. Both strains were stable for these characteristics after repeated subculturing. A revertant strain PRM101r was obtained from mice pretreated with iron dextran and lethally infected with 10^8 colony forming units (cfu) of PRM101. Iridescent colonies isolated from infected blood, taken prior to death, regained the ability to produce CP as evidenced by formation of halos on antibody-agar.

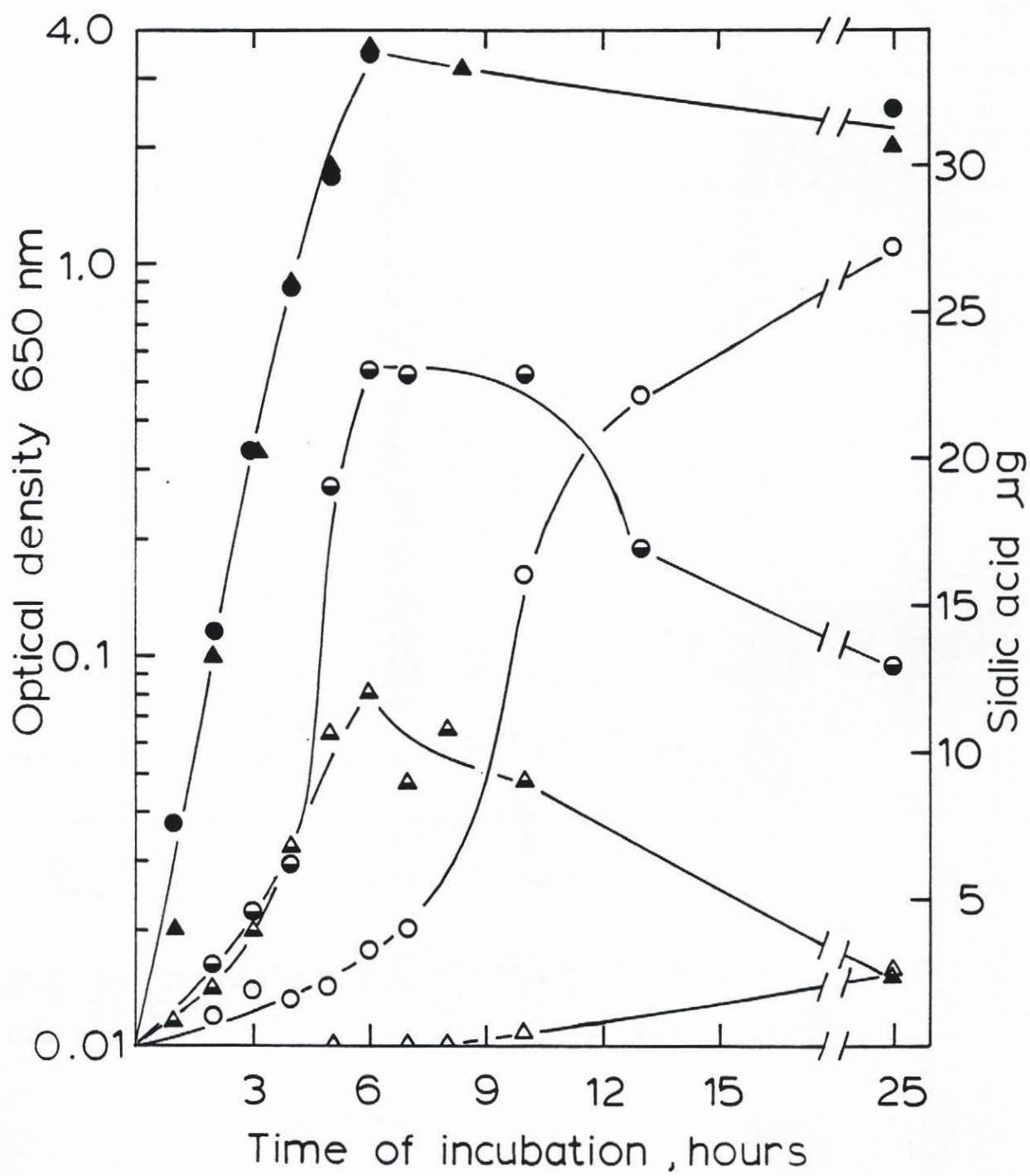
Cell-free and cell-associated sialic acids were further characterized by

Figure 1. Colony morphology and halo production of bacterial strains. Individual colonies of N. meningitidis strains M986, PRM101, PRM102, and PRM101r were inoculated onto NDM-C agar containing 15% horse serogroup B antiserum and incubated for 18 h (column a). The colonies were washed off and the agar was stained (column b) with 0.4% (wt/vol) Coomassie brilliant blue dissolved in 15% (vol/vol) glacial acetic acid, 50% (vol/vol) methanol and 34.6% water for four h and destained for two days in the same solvent system without dye to visualize the halos (bars).



the growth of strains M986 and PRM102 in batch cultures of NDM-C medium. It is important to note that although there have been several reports in the literature concerning interference of 2-keto-3-deoxyoctonate (KDO) with assays developed for the determination of sialic acid (Kuwahara and Snetting, 1979; Kuwahara, 1980), KDO did not interfere with the determination of NANA by the HCl-resorcinol method (see appendix, Figure 29). A comparative analysis of the synthesis and release kinetics of sialic acid during growth revealed that sialic acid was produced during the exponential phase of growth of strain M986 and significant release of the polysaccharide to the surrounding medium occurred only when the culture has entered the stationary phase of growth (Figure 2). There was no net synthesis of sialic acid after cessation of growth and the appearance of sialic acid in the culture medium corresponded with a loss of cell-associated sialic acid. Released polysaccharide was non-dialyzable and therefore had a $M_r > 12000$. Glucose-6-phosphate dehydrogenase was also released during the stationary phase suggesting that the release of polysaccharide may have been related to cellular autolysis (data not shown). A similar pattern of growth, sialic acid synthesis and sialic acid release was obtained with strain PRM101r. All the sialic acid which was cell-associated after seven hours of growth of strain M986 was released by sonication of the bacterial cells and this material was soluble at $220000 \times g$ and was non-dialyzable. This evidence indicated that the cell-associated sialic acid was easily sheared from the cells as free high M_r polysaccharide. Strains PRM102 and PRM101 exhibited similar patterns of growth and polysaccharide synthesis and release, therefore only the results obtained with PRM102 are shown in figure 2. It is important to note that CP production appears unessential for growth as evidenced by the similar rates of growth between strains PRM102 and M986 in NDM-C. There was evidence for synthesis of cell-associated sialic acid by PRM102 during the

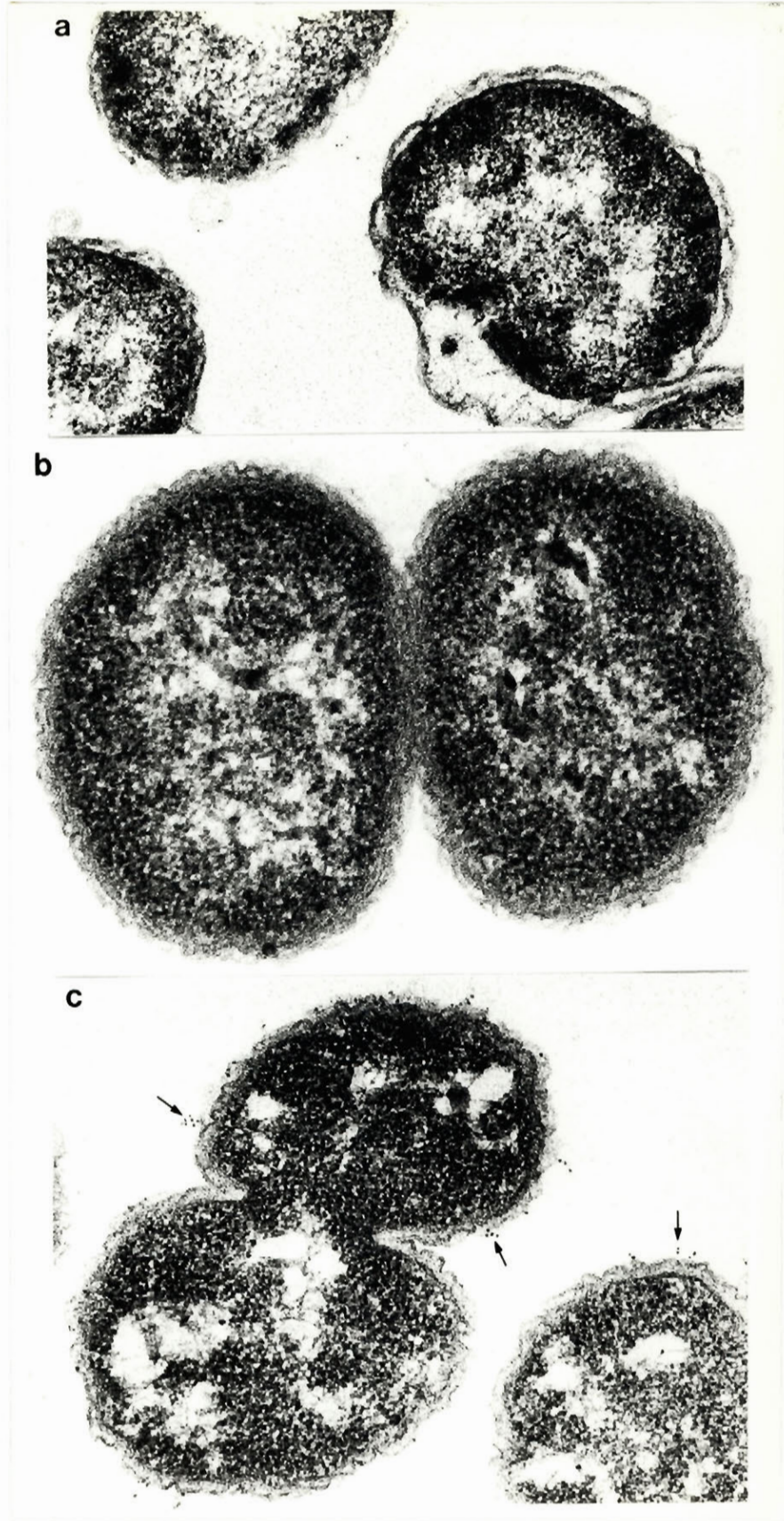
Figure 2. Synthesis and release of sialic acid during growth of N. meningitidis. N. meningitidis strain M986 was inoculated into NDM-C medium and at intervals was examined for growth (●), cell-associated sialic acid (◐) and cell-free sialic acid (○). Similarly, N. meningitidis strain PRM102, an isogenic mutant of strain M986, deficient in polysaccharide release, was examined for growth (▲), cell-associated sialic acid (◑), and cell-free sialic acid (△).



exponential phase of growth. Synthesis ceased when growth ceased but only small amounts were released by the cells even at 25 h of growth (Figure 2). However, cell-associated sialic acid did diminish. Thus, there was little cell-associated or released sialic acid in the cultures after 25 h of growth. This suggested that the cell-associated sialic acid of strain PRM102 which was observed during the exponential phase of growth was not high M_r polysaccharide. In fact, approximately 50% of the cell-associated sialic acid of strain PRM102 at 7 h of growth was dialyzable from the supernatant fractions of sonicated cells. Therefore a large portion of the sialic acid of strain PRM102 was of low M_r and had not been incorporated into high M_r polysaccharide suggesting the presence of pools of unincorporated sialic acid in the mutant strains.

Additional evidence indicating the lack of cell-surface CP on strain PRM102 is shown in the electron micrographs in figure 3 as well as by rocket immunoelectrophoresis using serogroup B anticapsular antibodies (Chapter, II section iii). The presence of sialic acid CP on the outer surface of strain M986 but not PRM102 was visualized by the use of ferritin-tagged anticapsular antibodies. The spotted appearance of the ferritin grains surrounding the thin sections seen in panel (c) suggested that sialic acid CP does not completely mask the meningococcal outer membrane. This contention is also supported by the ability to induce protective IgG serotype antibodies directed to the outer membrane proteins of encapsulated organisms during meningococcal disease (Munford et al., 1975). Mercuric ion and neuraminidase-treated strain PRM102 was included as an additional control and as shown in panel (a), ferritin-tagged anticapsular antibodies were absent. Interestingly, these organisms retain an intact outer membrane although it tends to attach loosely to the cell at various loci, presumably at junctions of inner and outer membranes.

Figure 3. Detection of the sialic acid capsular polysaccharide of N. meningitidis with ferritin-tagged anticapsular antibodies. Electron micrographs of thin sections of N. meningitidis strains: mercury-neuraminidase treated PRM102 (a), PRM102 (b), and M986 (c). Arrows demonstrate the presence of ferritin-tagged anticapsular antibodies.



iii. Fractionation and localization of CP biosynthetic enzymes.

- a) Localization of the lesion in capsular polysaccharide synthesis in N. meningitidis strain PRM102.

To determine the nature of the lesion in the sialic acid biosynthetic pathway of strain PRM102, it was necessary to examine for the presence of a NANA condensing enzyme, a CMP-NANA synthetase, and a terminal sialyltransferase. Strains M986 and PRM102 were grown in NDM-C. The cells were disrupted and the cytoplasmic and membrane fractions separated by ultracentrifugation. The NANA condensing enzyme and CMP-NANA synthetase were detected and were located exclusively in the cytoplasmic fraction, whereas the sialyltransferase was associated only with the membrane fraction. The CMP-NANA synthetase was highly labile and lost activity rapidly when stored at 4°C or when dialyzed against MOPS buffer containing 25 mM 2-mercaptoethanol at 4°C. Therefore all cytosolic preparations were processed and assayed immediately. The NANA condensing enzyme, although stable, was treated in a similar fashion. These enzymes were present at similar levels and specific activities in both strain M986 and strain PRM102 (Table 1).

Sialyltransferase activity was monitored by incubation of purified meningococcal particulate membranes with CMP-[¹⁴C]NANA. Since polymerized NANA residues, unlike CMP-NANA or NANA (see Table 15, appendix), do not migrate during paper chromatography developed in ethanol-1 M ammonium acetate (7:3), the measurement of labeled NANA remaining at the origin was a convenient way of determining sialyltransferase activity. To verify that

polymerized NANA was the component remaining at the origin, a sialyltransferase reaction mixture was incubated with CMP-[^{14}C]NANA. After one h, the reaction was stopped and neuraminidase was added. As demonstrated in figure 4, there was a progressive loss of label resolved at the origin after neuraminidase treatment indicating that the label remaining at the origin was indeed in polymeric form.

The sialyltransferase activities of crude membrane preparations differed markedly between strains M986 and PRM102 (Table 1). Strain PRM102 possessed approximately 25% of the sialyltransferase activity that was found in strain M986. This may have accounted for the lack of polysaccharide synthesis in PRM102 but an examination of the sialyltransferase activity of K1 E. coli, which produces an immunologically and chemically identical CP to that of serogroup B meningococci, revealed much less activity than that found in strain PRM102 (14% of the activity found in strain PRM102, Table 1). It is important to note that this strain of E. coli elaborated surface sialic acid CP as determined by slide agglutination using horse serogroup B antiserum. Thus, it appeared that the reduced level of sialyltransferase activity of N. meningitidis strain PRM102 could not account completely for the inability of this strain to elaborate polysaccharide.

On the premise that NANA or CMP-NANA synthesis was suppressed in strain PRM102, consequently providing insufficient substrate for the sialyltransferase, both the cytosol and membrane fractions were assayed for neuraminidase and NANA aldolase activities. Neither enzyme was detected in these preparations. Therefore strain PRM102 differed from strain M986 only with respect to sialyltransferase content.

Problems were encountered initially in the sialyltransferase assays when using 50 mM Tris-hydrochloride (pH 7.5). The CMP-NANA substrate, a labile sugar nucleotide (Comb et al., 1966), underwent substantial

Figure 4. Neuraminidase digestion of [^{14}C]-labeled sialyl polymer synthesized by particulate membranes of N. meningitidis strain M986. A standard reaction mixture of particulate membranes from strain M986 was allowed to react with substrate for one hour after which the mixture was placed in a boiling water-bath for three minutes, cooled to 37°C , followed by the addition of one unit of neuraminidase. Samples (10 μl) were removed and directly spotted on 3MM Whatman chromatography paper and analyzed for loss of label at the origin. Averaged data from two experiments are shown.

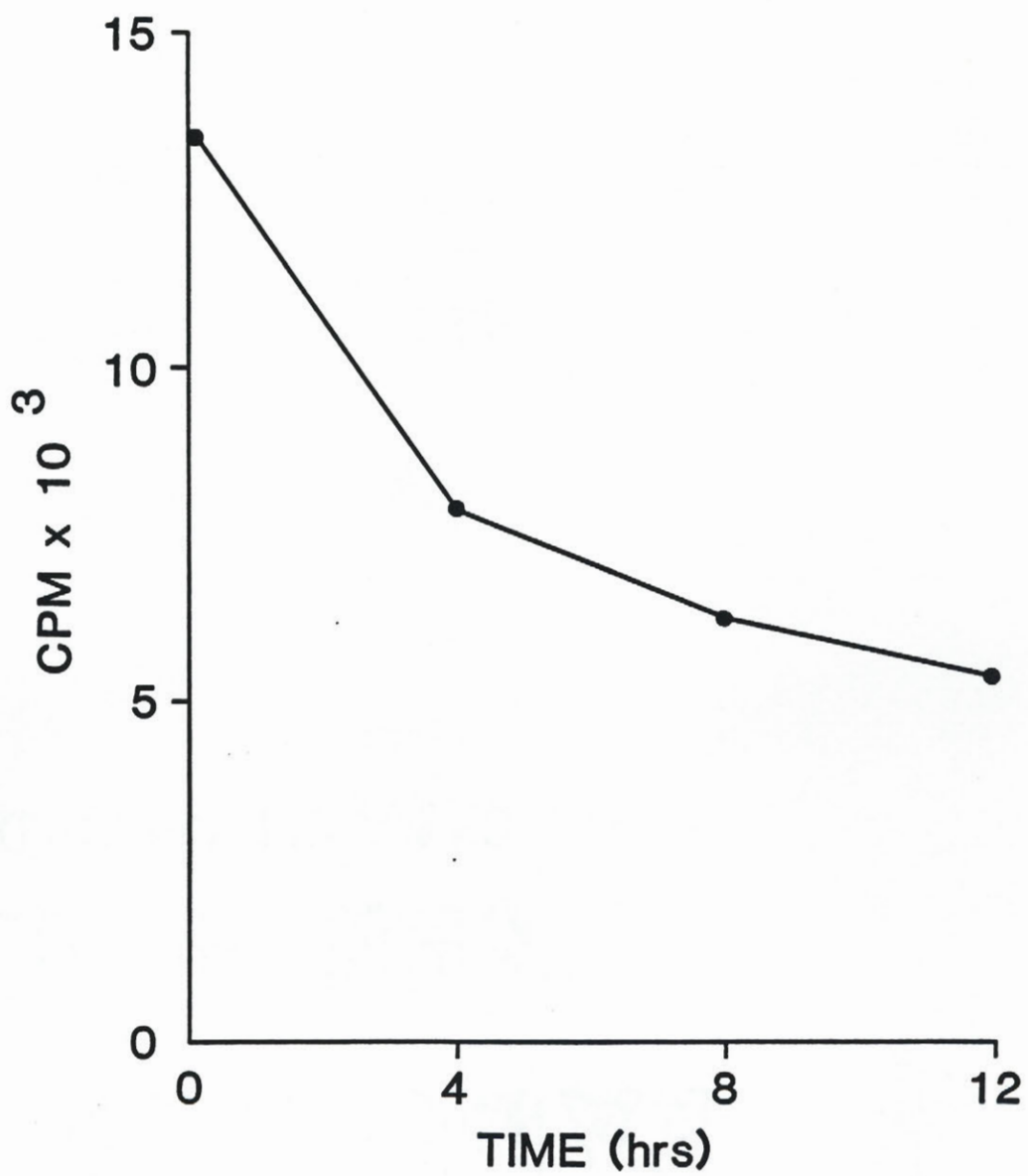


TABLE 1. Comparison of the amounts of various enzymes involved in sialic acid synthesis in bacterial strains

Strain	Sp act (U/mg of protein) ^a			
	NANA condensing enzyme	CMP-NANA synthetase	Sialyl- transferase	CMP-NANA hydrolase
<u>N. meningitidis</u> M986	0.21	1.22	3.53	10.79
<u>N. meningitidis</u> PRM 102	0.19	1.11	0.96	8.95
<u>E. coli</u> 01:K1:HNM	ND ^b	ND	0.13	0.00

^a Each value is the average of three separate experiments.

^b ND, Not determined.

nonenzymatic hydrolysis as encountered with heat-denatured (100°C, 4 min) control enzyme preparations. CMP-NANA was found to be stable in 0.15 M MOPS buffer (pH 7.7). However, substantial hydrolysis of CMP-NANA still occurred when active membrane preparations were added, suggesting the presence of a hydrolytic enzyme in the membrane fractions (Table 1). CMP-NANA is a unique type of sugar nucleotide; it possesses only one phosphate group and it is present in the β -anomeric form, unlike other naturally occurring, sialidase-sensitive sialic acid glycosides (Comb et al., 1966). It is not degraded by neuraminidase or NANA aldolase or by digestion with E. coli alkaline phosphatase, venom phosphodiesterase, or 5'-nucleotidases (Comb et al., 1966). As demonstrated in figure 5, the hydrolysis of CMP-NANA, as well as the incorporation of NANA into high molecular weight polymer, increased proportionally with increasing amounts of membrane protein from N. meningitidis strain M986. Thus, the evidence suggested the presence of a specific membrane-associated CMP-NANA hydrolase. Kean and Bighouse, (1974) have demonstrated CMP-NANA hydrolase activity associated with the plasma membrane of rat liver cells. However, the present appears to be the first evidence for a procaryotic CMP-NANA hydrolase.

CMP-[¹⁴C]NANA incubated with crude membrane preparations from strain M986 was rapidly hydrolyzed with the appearance of free [¹⁴C]NANA and high molecular weight polymer (Figure 6). These results show the interrelationships between the membrane-associated sialyltransferase and hydrolase activities. CMP-NANA hydrolysis occurred at a higher rate than polymer formation. Both strain M986 and strain PRM102 possessed similar levels of the CMP-NANA hydrolase (Table 1). No activity was evident in the cytoplasmic fractions of either strain or in the membrane or cytoplasmic fractions of K1 E. coli. Vijay and Troy, (1975) were unable to find CMP-NANA hydrolase activity in K-235 E. coli which also produces a

Figure 5. Effect of increasing membrane protein concentration on sialyltransferase and CMP-NANA hydrolase activity. Reaction mixtures were set up containing increasing amounts of N. meningitidis strain M986 particulate membrane protein in 0.15 M MOPS buffer (pH 7.7), 20 mM MgCl_2 , and 0.2 uCi CMP- ^{14}C NANA (247 mCi/mMol) in a final volume of 125 μl . Sialyltransferase and CMP-NANA hydrolase initial reaction velocities were measured over a 20 min period by spotting 20 μl aliquots on Whatman chromatography paper and determining the level of sialyl polymer synthesis (●) and the appearance of free NANA (○).

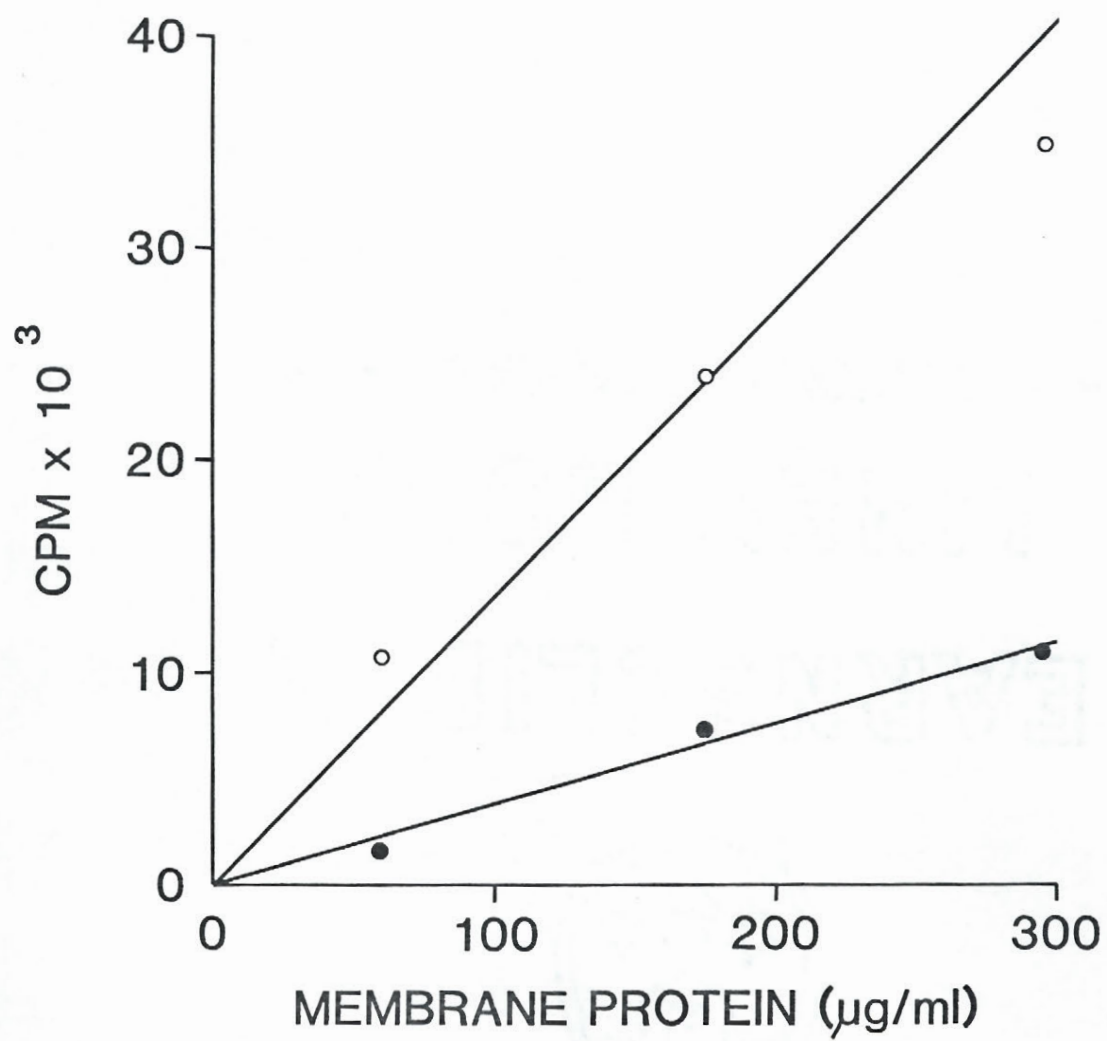
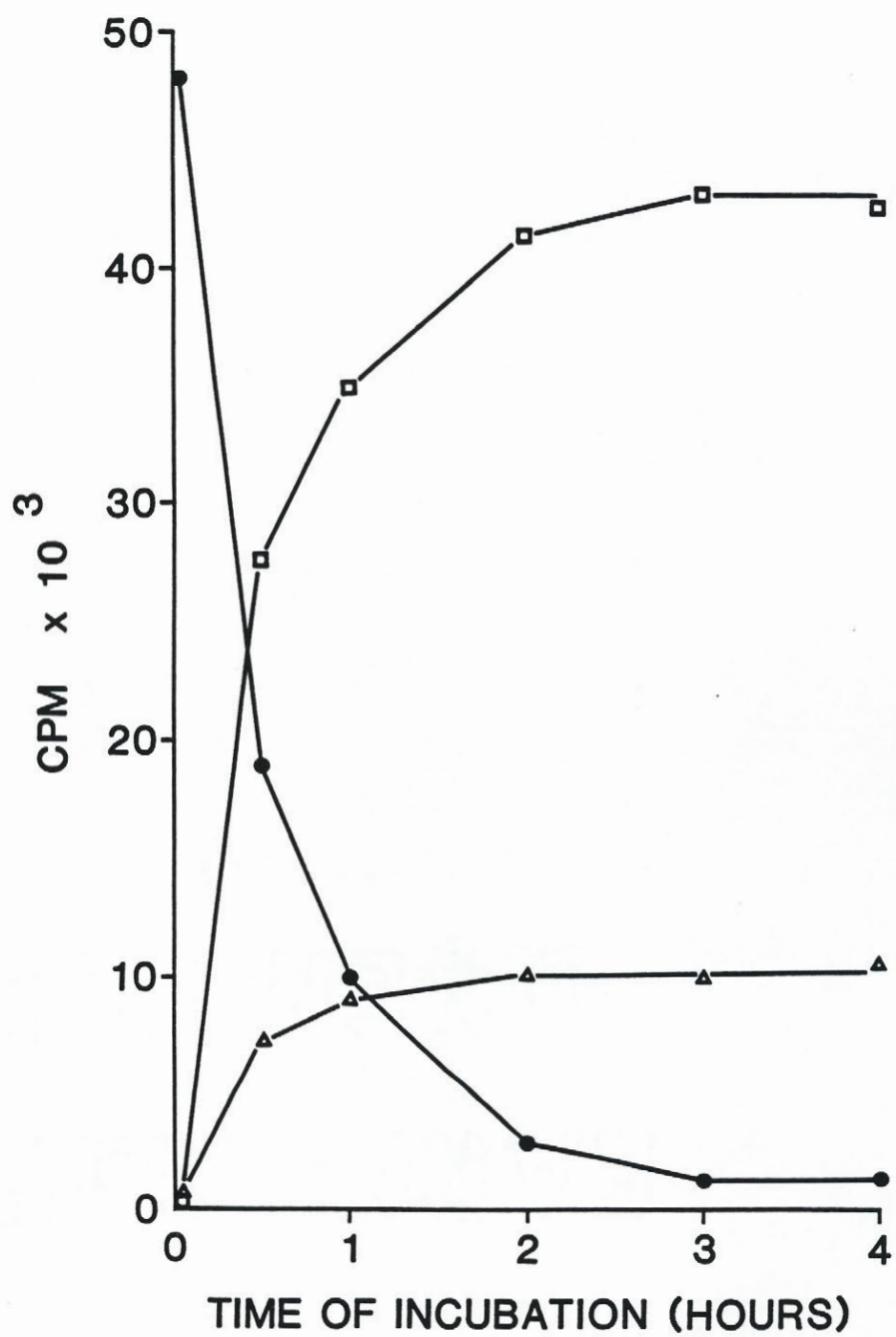


Figure 6. Activities of sialyltransferase and CMP-NANA hydrolase in crude membranes of N. meningitidis strain M986. Crude membranes were incubated with CMP- ^{14}C NANA. At intervals, samples of the reaction mixtures were removed and chromatographed to determine radioactivity in CMP-NANA (●), NANA (□), and high molecular weight sialic acid capsular polysaccharide (Δ).



sialic acid CP. Thus, membrane preparations from both strain M986 and strain PRM102 possessed sialyltransferase and CMP-NANA hydrolase activities, both of which used CMP-NANA as a substrate. This suggested that the presence of wild-type levels of CMP-NANA hydrolase and reduced levels of sialyltransferase might be related to the inability of strain PRM102 to elaborate polysaccharide.

b) Membrane fractionation and enzyme localization.

The meningococcus was not amenable to the usual techniques established previously for the separation of the inner and outer membranes of other Gram-negative bacteria (Osborn et al., 1972; Schnaitman, 1970).

MacLeod and DeVoe, (1981) employed both high-pressure extrusion and EDTA pretreatment with serogroup B meningococci, followed by sucrose density gradient centrifugation. Fractions enriched with inner and outer membranes were obtained but these remained heavily cross-contaminated (MacLeod and DeVoe, 1981). Loeb et al., (1981) suggested that sedimentation of the cell envelope into pellets after high-pressure extrusion was detrimental to the subsequent fractionation of inner and outer membranes of Haemophilus influenzae. Thus, they employed a prepurification step where the disrupted membranes were first layered onto a sucrose cushion.

Inner and outer membranes of N. meningitidis strain M986 were successfully separated by the following fractionation procedure. The disrupted envelopes of strain M986 were first centrifuged through a 15% (wt/wt) sucrose layer containing EDTA onto a 55% (wt/wt) sucrose cushion containing EDTA. When such prepurification gradients were not used, the washed membranes could be subsequently fractionated with the appearance of four discrete membrane bands, but high levels of succinate dehydrogenase

(SDH) were found associated with putative outer membrane. This may have been due to the nonspecific reassociation of released SDH with the outer membrane during preparation. Washing of the membranes in a buffer of high ionic strength (50 mM Tris-hydrochloride, pH 8.0, containing 0.5 M NaCl) did not alleviate this problem. Maintenance of a low ionic strength with EDTA during cell disruption aids in destabilizing the outer membrane and facilitates the separation of the cytoplasmic membrane from the murein layer in E. coli (Schnaitman, 1970). Incorporation of EDTA into the density gradients also has been reported to be essential for satisfactory resolution of the various membranes of Salmonella typhimurium (Osborn et al., 1972). EDTA was therefore incorporated into both our prepurification and separation gradients.

Centrifugation of meningococcal membranes on sucrose density separation gradients yielded four distinct bands as shown in figure 7. After gradient fractionation the membrane distribution was similar to that observed by Osborn et al., (1972) for S. typhimurium, therefore, the membrane bands were termed H (heavy), M (middle), and L2 and L1 (light) in order of their decreasing buoyant densities (Figure 8). The densities of the membrane fractions as well as the distribution of the various enzymes in these membrane fractions are shown in table 2. Both the sialyltransferase and the CMP-NANA hydrolase activities were found predominantly in the cytoplasmic membrane fractions, L1 and L2, identified by the high levels of SDH activity present in these fractions. Higher levels of sialyltransferase activity were found in the L1 fraction, whereas higher CMP-NANA hydrolase activities were found in the L2 fraction (Table 2). The significance of this observation is obscure. Both the sialyltransferase and the CMP-NANA hydrolase activities were found similarly distributed between the L1 and L2 fractions from the mutant strain PRM102.

Figure 7. Distribution of membrane bands from N. meningitidis strain M986 after discontinuous isopycnic sucrose density gradient centrifugation.

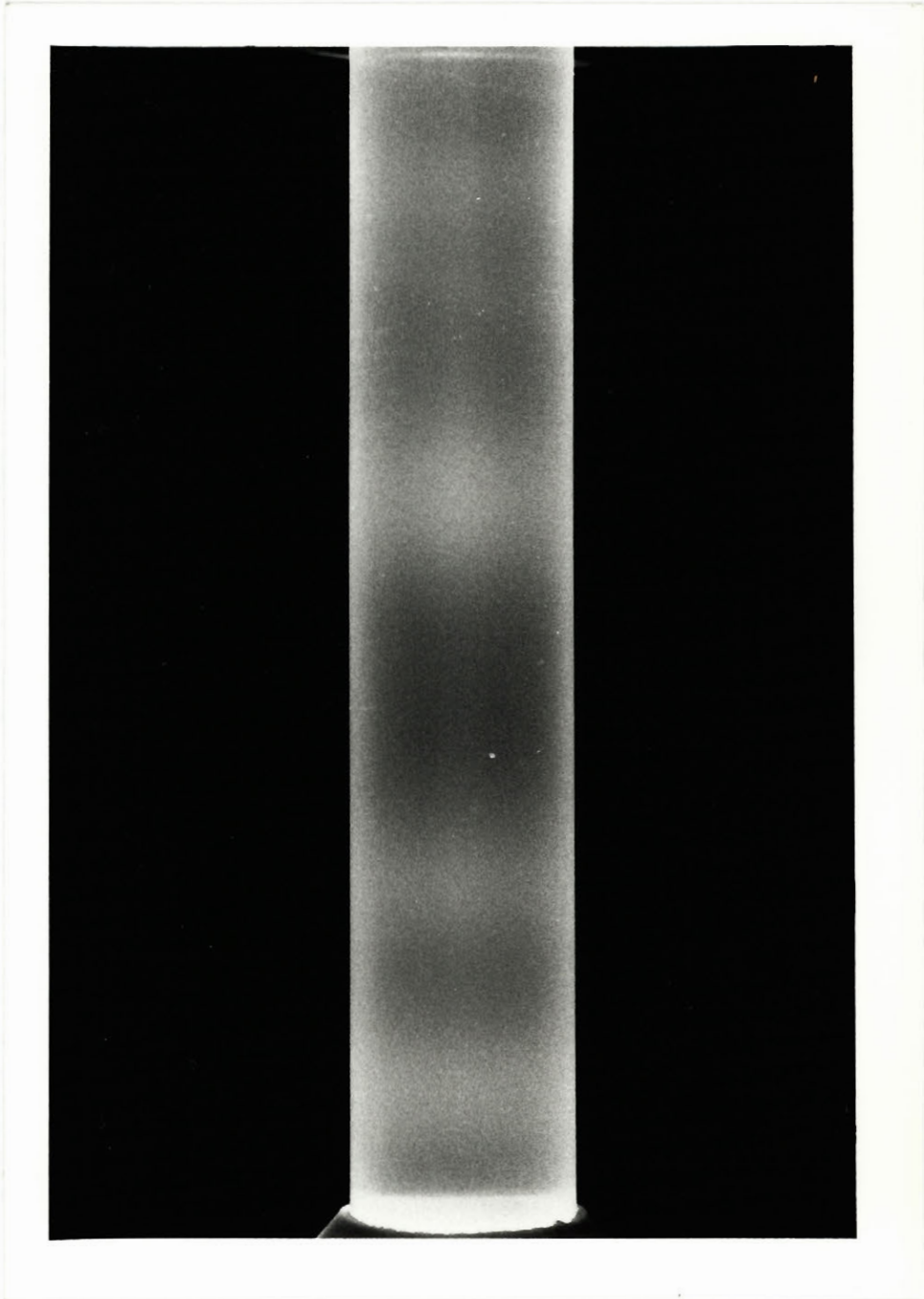


Figure 8. Fractionation of membranes from N. meningitidis strain M986 on discontinuous sucrose density gradients. Crude membranes from N. meningitidis, prepurified on a preparative discontinuous gradient, were applied to a second discontinuous gradient and centrifuged for 35 h at 189,000 x g (average) at 4°C. The gradients were fractionated from the bottom and collected into 15-drop fractions. Averaged data from two experiments are shown. Fractions were assayed for their contents of protein (O) and SDH (□) as well as their densities (■). In a separate experiment, whole cells were first surface labeled with ^{125}I before disruption and fractionation, and the resulting fractions were measured for ^{125}I content (●) to reveal the location of the outer membranes.

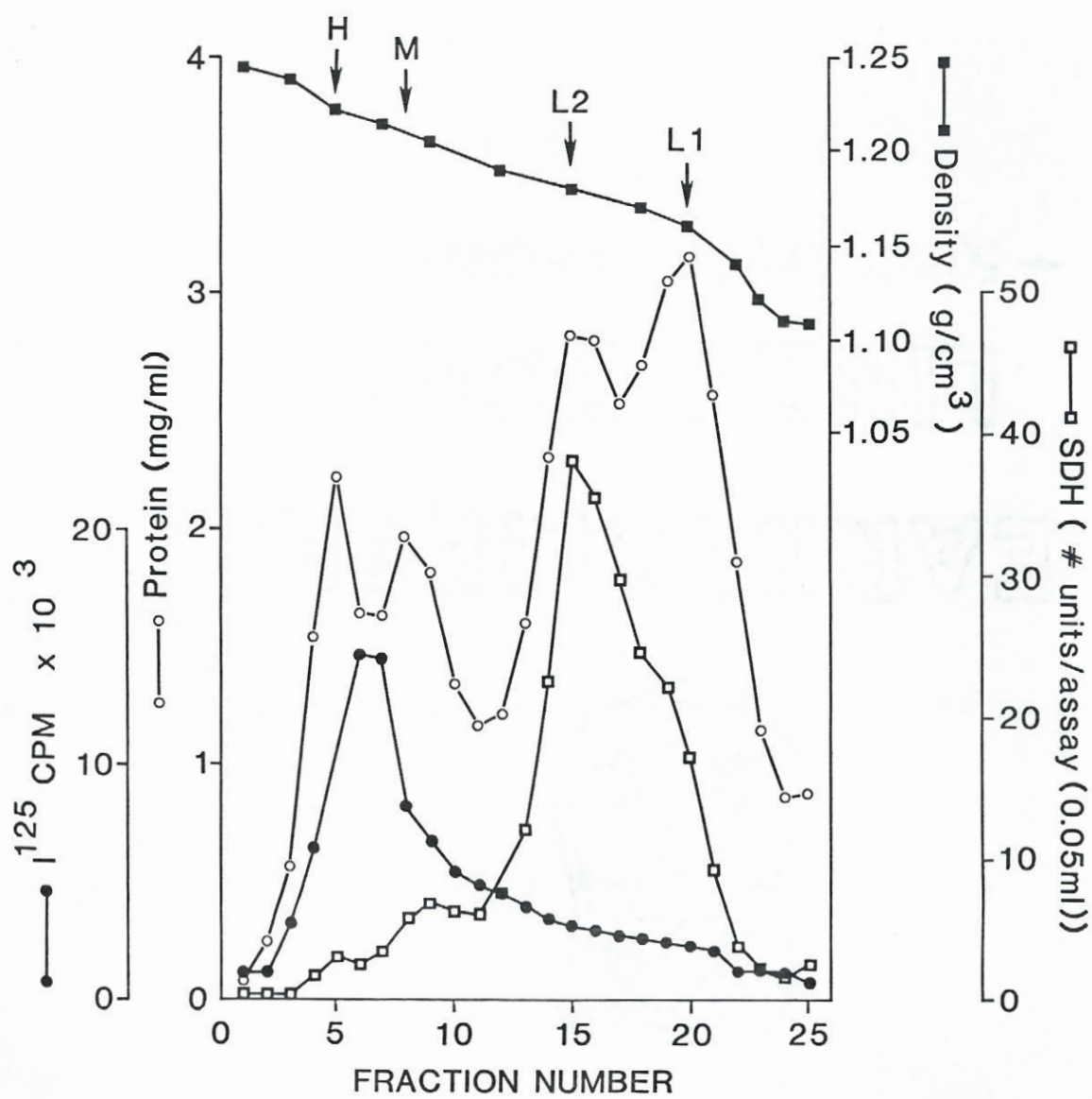


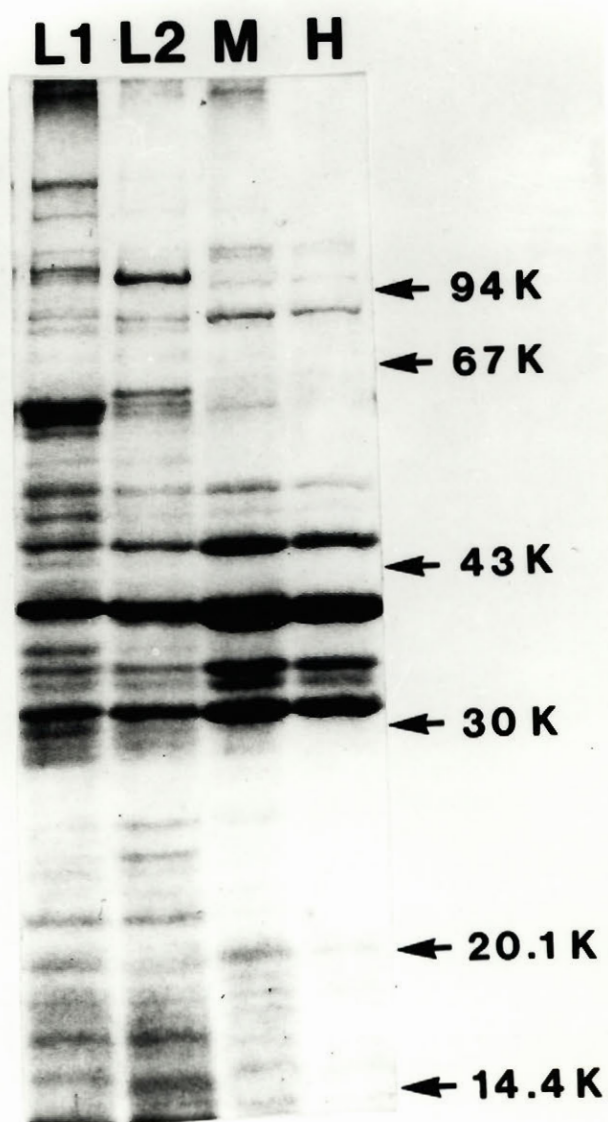
TABLE 2. Enzyme distributions in purified membrane fractions from N. meningitidis strain M986

Membrane fraction	Density (gm/cm ³)	Sp act (U/mg of protein)			
		Sialyl-transferase	CMP-NANA hydrolase	SDH	TMPD-oxidase
L1	1.16	3.04	8.93	140	11.55
L2	1.18	1.67	14.03	260	16.20
M	1.21	2.20	4.20	70	4.21
H	1.22	0.78	1.56	20	0.42

^a Pooled membrane fractions were prepared on sucrose density gradients (Fig. 6) and pooled as follows: H, tube fractions 4 and 5; M, tube fractions 8 and 9; L2, tube fractions 15 and 16; L1, tube fractions 19 and 20.

Sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis of the membrane fractions revealed different protein compositions in the various fractions (Figure 9). The L1 and L2 possessed many more proteins than both the M and H fractions. However, differences were noted between the L1 and L2 fractions, especially for proteins greater than 50 kilodaltons in size. The M and H fractions displayed similar protein compositions, but the M fraction did appear to possess at least one high molecular weight protein not observed in the H fraction and substantially more proteins of less than 20 kilodaltons. These smaller proteins were observed in the L1 and L2 fractions. The H fraction possessed the smallest number of proteins and displayed an array similar to that previously shown for CaCl_2 -extracted outer membrane complexes of strain M986 (Frasch et al., 1976). Therefore, the distribution of SDH and the electrophoresis results indicated that fractions L1 and L2 were of cytoplasmic membrane origin and suggested that the M fraction contained significant amounts of cytoplasmic membrane. The H fraction was taken to represent highly purified outer membrane. This was confirmed by independent physical means employing surface iodination of outer membranes on intact cells. Most of the ^{125}I -labeled membranes copurified with the H fraction, with significant amounts also being found in the M fraction (Figure 8). These results provided additional evidence confirming the association of sialyltransferase and CMP-NANA hydrolase activities with the cytoplasmic membrane. In addition, it was discovered that TMPD-oxidase was associated with the cytoplasmic membrane in N. meningitidis strain M986 (Table 2) and not outer membrane associated as reported previously for N. meningitidis SDIC outer membrane blebs (DeVoe and Gilchrist, 1976). These results further suggest that meningococcal blebs might also contain cytoplasmic membrane.

Figure 9. Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis of membrane fractions from N. meningitidis strain M986. Pooled membrane fractions (Table 2) were prepared and electrophoresed. Similar amounts of protein (40 ug) were applied for each preparation. The arrows show the location of protein standards from adjacent lanes.



iv. Characterization of sialyltransferase and CMP-NANA hydrolase activities.

The products resulting from the action of CMP-NANA hydrolase on the substrate CMP-NANA were confirmed by examination of the products after hydrolase reaction with dual-labeled CMP-NANA. [^3H]CMP-[^{14}C]NANA was synthesized by the addition of [^3H]CTP and [^{14}C]NANA to a crude cytoplasmic extract of N. meningitidis strain M986. The in vitro synthesized dual-labeled CMP-NANA was purified by ion-exchange chromatography (Figure 10) and fractions 12 to 20 were pooled. Pure dual-labeled CMP-NANA, confirmed by paper chromatography of the pooled fractions, was incubated with crude membranes of strain PRM102. The products were separated from unused substrate by paper chromatography and were found to be free CMP and NANA in approximately equimolar concentrations.

Pronase preincubation of membrane preparations of strain M986 destroyed both sialyltransferase and CMP-NANA hydrolase activities (Table 3). Both the sialyltransferase and CMP-NANA hydrolase appeared to display characteristics different from those reported in the literature for similar enzymes (Kundig et al., 1971; Kean and Bighouse, 1974), with the exception of serogroup C meningococcal sialyltransferase (Vann et al., 1978). The sialyltransferase did not display a requirement for disulfide reducing agents for in vitro activity nor did the CMP-NANA hydrolase appear to be sensitive to 2-mercaptoethanol whether metal ions were present or absent (Table 3), unlike the situation found with rat liver CMP-NANA hydrolase (Kean and Bighouse, 1974). Metal ions were not essential for either sialyltransferase or CMP-NANA hydrolase activities, although both were stimulated by Mg^{2+} , 20 mM MgCl_2 being optimal for both (data not shown). The sialyltransferase and the CMP-NANA reactions also appeared to be

Figure 10. Purification of in vitro synthesized dual-labeled meningococcal CMP-NANA. In vitro synthesized meningococcal [^3H]CMP-[^{14}C]NANA was purified on DEAE-cellulose. The column was washed with 10 mM MOPS buffer (pH 7.7) to remove unreacted substrate and the [^3H]CMP-[^{14}C]NANA was eluted by a linear gradient of 0-0.1 M LiCl in 10 mM MOPS buffer (pH 7.7). Aliquots (200 μl) of the fractions were analyzed for ^3H (●) and ^{14}C (○) levels.

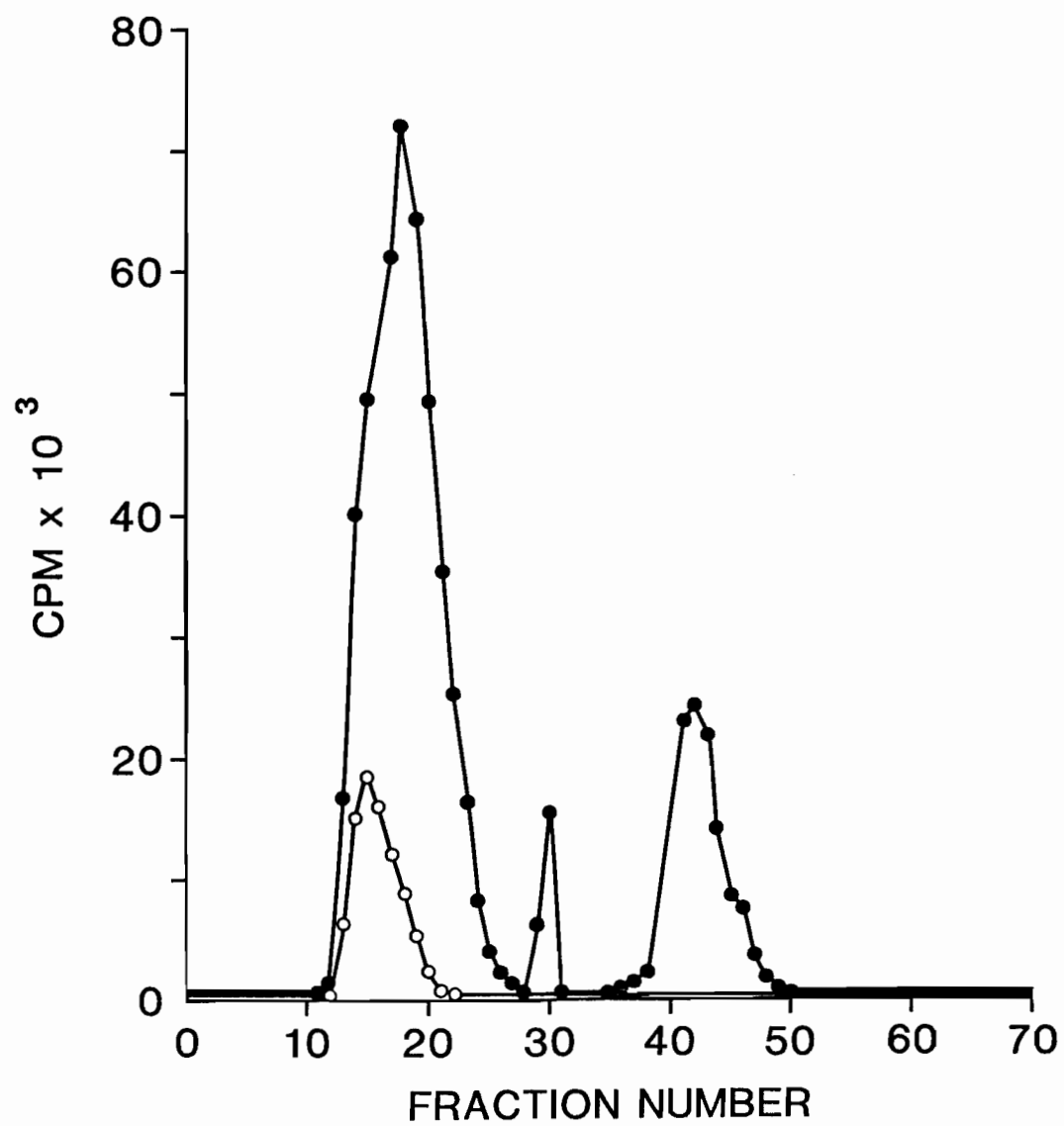


TABLE 3. Properties of sialyltransferase and CMP-NANA hydrolase from *N. meningitidis* strain M986

Treatment	% Control Activity ^a	
	Sialyl transferase	CMP-NANA hydrolase
None (control) ^b	100	100
Pronase (100 ug/ml) ^c	0	0
2-Mercaptoethanol (25 mM)	100	100
EDTA (20 mM), no Mg ²⁺	51	73
EDTA (20 mM), no Mg ²⁺ , + 2-mercaptoethanol (25 mM)	52	78
NAM (75.5 uM)	84	103
UDP-N-acetylglucosamine (11.5 uM)	100	100
NANA (75.5 uM)	99	100
CMP (15.1 uM)	133	43
CMP (30.2 uM)	122	29
CMP (45.3 uM)	107	0
CTP (75.5 uM)	93	96

^a Data shown are for a single experiment conducted with the same enzyme preparations but are typical of the results obtained in three separate experiments.

^b The CMP-NANA concentration in the standard reaction mixture was 7.6 uM.

^c There was a 20-min pretreatment of the membranes in the reaction mixture.

irreversible (data not shown). The CMP-NANA hydrolase had an optimal activity at pH 7.7 in 0.15 M MOPS buffer (Figure 11). Rat CMP-NANA hydrolase was found to have optimal activity at pH 9.0 and was inhibited by UDP-N-acetylglucosamine (Kean and Bighouse, 1974). The meningococcal CMP-NANA hydrolase was insensitive to UDP-N-acetylglucosamine (Table 3). One other major difference noted was the inhibition of enzyme activity by cytidine nucleotides (Table 3). Although both the rat liver (Kean and Bighouse, 1974) and the meningococcal hydrolases appeared sensitive to CMP, CTP did not inhibit the meningococcal hydrolase, even when present at a concentration 10-fold in excess of CMP-NANA (Table 3). In the case of the rat liver hydrolase, there was approximately 80% inhibition when CTP was present at a concentration fourfold less than the CMP-NANA concentration (Kean and Bighouse, 1974). Thus the differential sensitivities of sialyltransferase and CMP-NANA hydrolase to CMP (Table 3) may have important implications in the overall regulation of polysaccharide synthesis in meningococci.

Kinetic analysis using Eadie-Hofstee plots revealed apparent K_m values of 2.0 μM for the sialyltransferase (Figure 12) and 0.88 μM for the CMP-NANA hydrolase (Figure 13) with maximal velocities of 3.45 nMol of NANA incorporated into sialyl polymer per h per mg protein for the sialyltransferase and 10.75 nMol of NANA produced per h per mg protein for the CMP-NANA hydrolase. Thus it appeared that the CMP-NANA hydrolase possessed a higher affinity for CMP-NANA than the sialyltransferase. Furthermore, the K_m value for the hydrolase was much lower than the 0.4 mM reported for rat liver CMP-NANA hydrolase (Kean and Bighouse, 1974). The pH optimum and kinetic analyses of CMP-NANA hydrolase were performed with strain PRM102, thus minimizing interference from the membrane-associated sialyltransferase activity. Kinetic analyses of sialyltransferase was performed with strain M986 in the presence of CMP to minimize interference by

Figure 11. Influence of pH on the activity of meningococcal CMP-NANA hydrolase. Identical samples of a crude membrane preparation from N. meningitidis strain PRM102 were assayed in 0.15 M MOPS buffer at the various pH values shown. Activities were expressed as a percentage of the maximum CMP-NANA hydrolase activity to reveal the pH optimum and the influence of pH on enzyme activity.

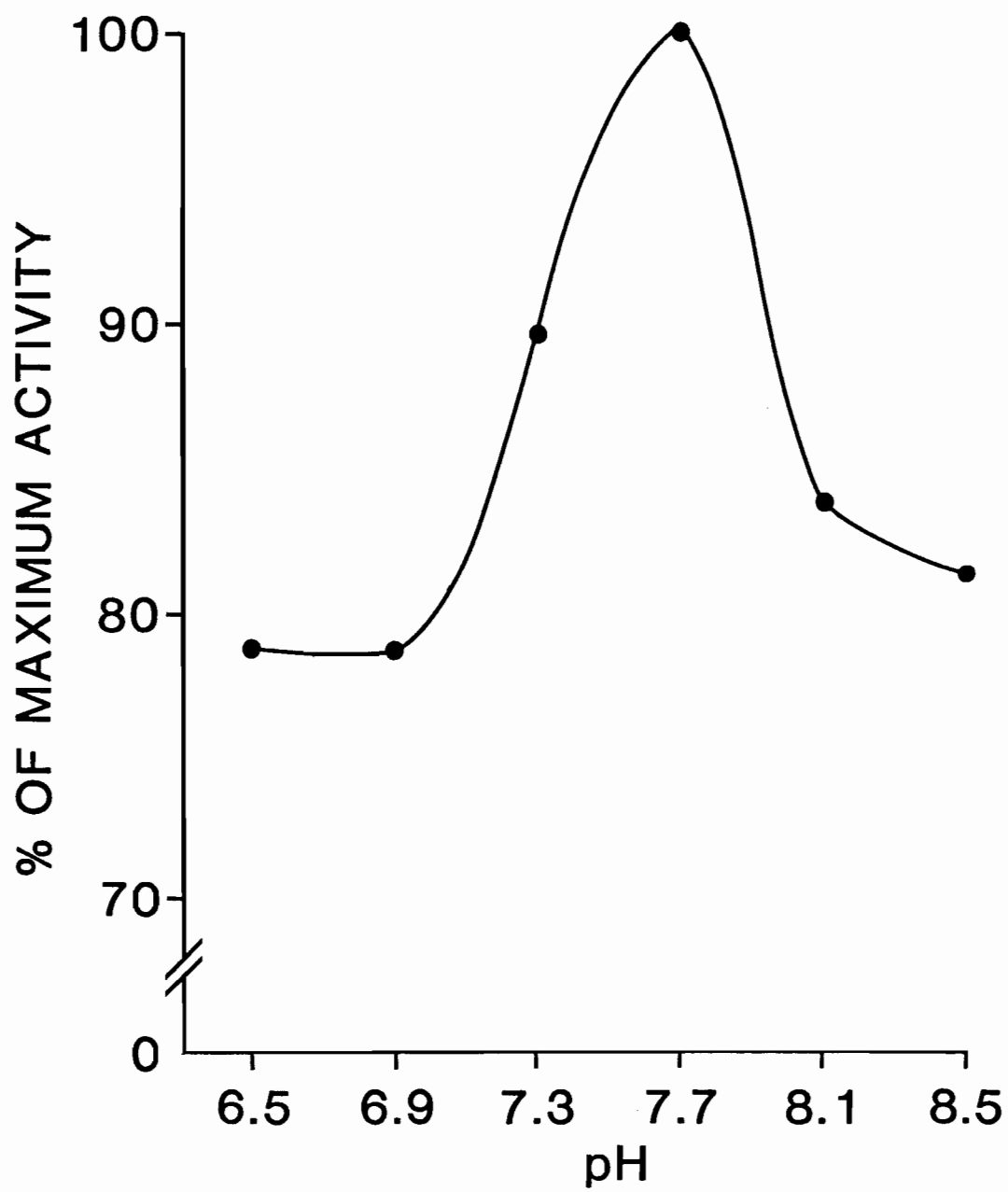


Figure 12. Effect of CMP-NANA concentration on the initial reaction velocity for sialyltransferase in particulate membranes of N. meningitidis strain M986. Standard reaction mixtures were prepared containing 35 μ M CMP and 0.26 μ Ci CMP- 14 C]NANA at the final concentrations indicated. The initial reaction velocities (V_o) were calculated, during a 15 min incubation period, by spotting 20 μ l aliquots on Whatman 3MM chromatography paper and determining the level of sialyl polymer synthesis at the origin. Kinetic data is presented in an Eadie-Hofstee plot and represents the average of three separate experiments.

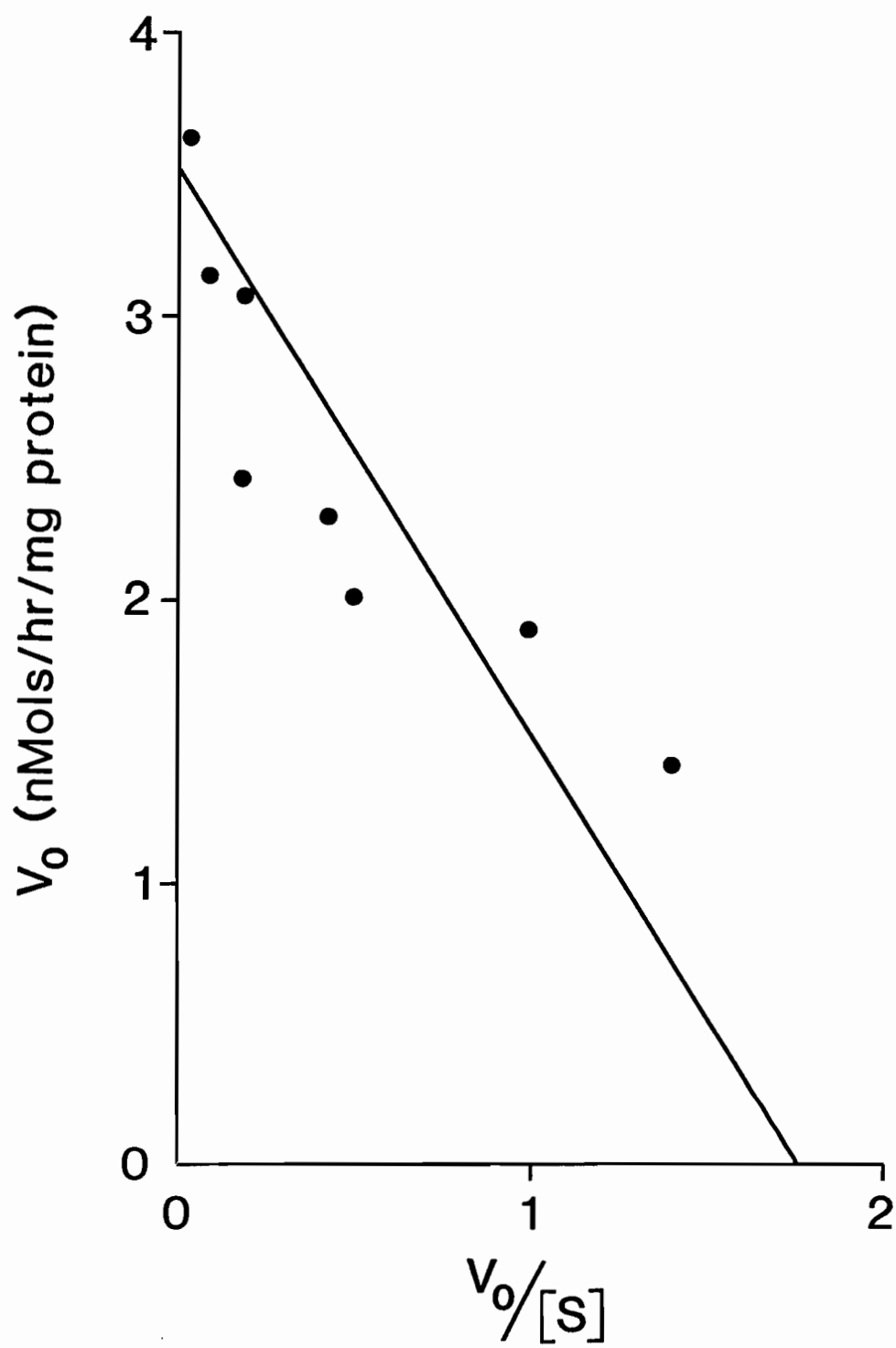
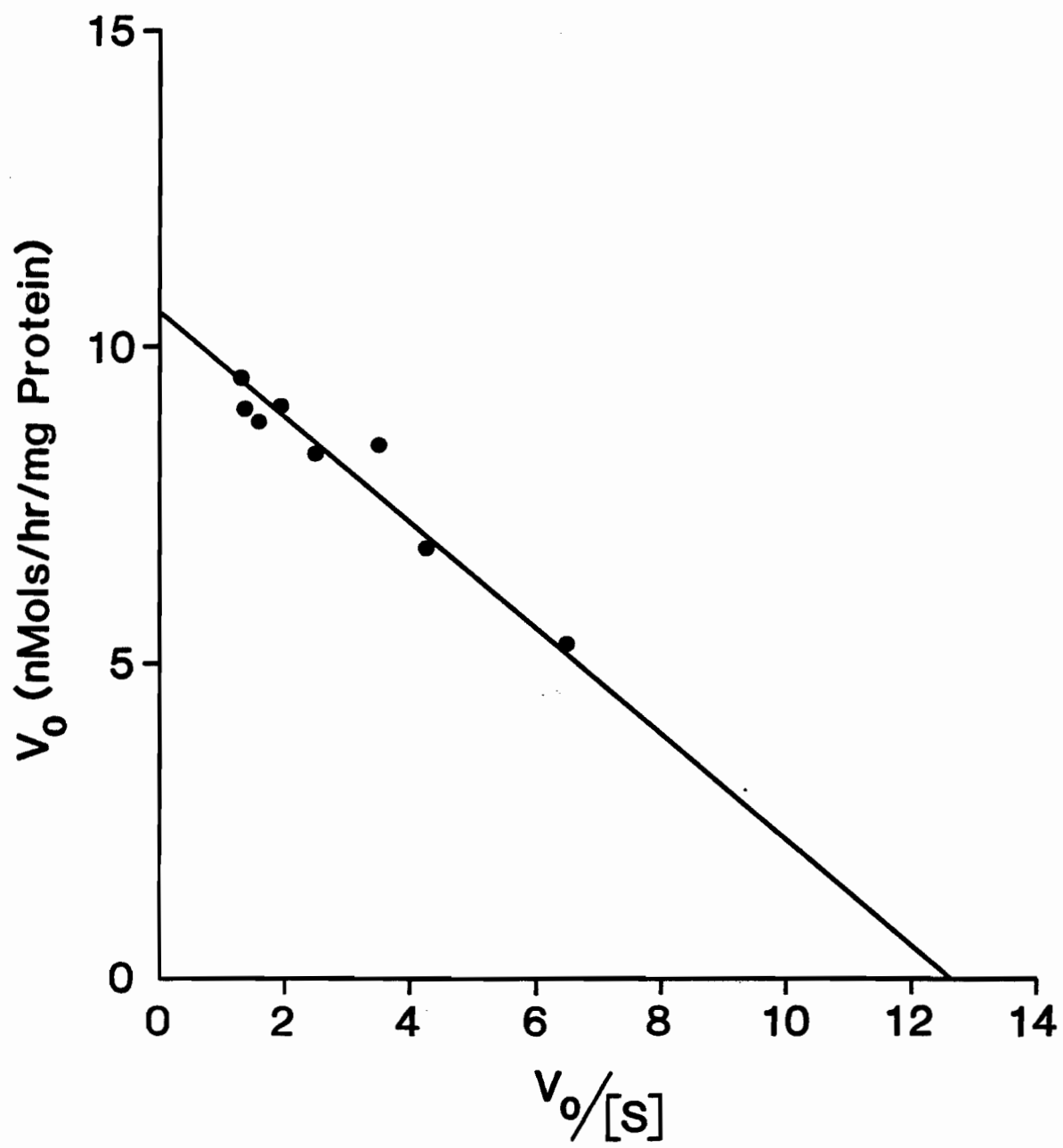


Figure 13. Effect of CMP-NANA concentration on the initial reaction velocity for CMP-NANA hydrolase in particulate membranes of N. meningitidis strain PRM102. Standard reaction mixtures were prepared containing various concentrations of CMP- ^{14}C NANA (302 mCi/mMol) as indicated. The initial reaction velocity (V_o) was determined for a 15 min period by spotting 20 μl aliquots on Whatman 3MM chromatography paper and determining the level of free NANA. Kinetic data is presented in an Eadie-Hofstee plot and represents the average of three separate experiments.



the CMP-NANA hydrolase. Attempts to partially purify and separate the two enzymes were unsuccessful.

The intriguing appearance of the CMP-NANA hydrolase prompted a brief survey of this enzyme in other bacterial strains (Table 4). This enzyme was not found in two unencapsulated, nonpathogenic neisseriae species, N. perflava and N. flava and two E. coli strains. CMP-NANA hydrolase was found in serogroup C meningococci, which also produces a sialic acid CP. Surprisingly, CMP-NANA hydrolase, but not sialyltransferase activity, was found in a serogroup A meningococcal strain. Serogroup A meningococci elaborates a CP composed of repeating subunits of N-acetylmannosamine (Liu et al., 1971a; Bundle et al., 1974). This result suggests that serogroup A organisms may have evolved from serogroup B or C organisms. Alternatively, the CMP-NANA hydrolase may possess an additional function(s) in the bacterial cell although the absence of this enzyme in nonpathogenic neisseriae species and the resistance of CMP-NANA to enzymatic degradation by phosphatases, 5'-nucleotidases, and phosphodiesterases i.e. enzyme specificity (Comb et al., 1966), argues against this possibility. The hydrolase was found in serogroup B N. meningitidis Dres 03, an avirulent carrier strain (Holbein, 1981), but like strain PRM102, it possessed low sialyltransferase activity suggesting that it may possess a low level of CP. This possibility was not pursued.

v. Involvement of lipid intermediates.

The biosynthesis of a variety of complex microbial cell envelope polysaccharides employs a surprisingly similar mechanism whereby sugar

TABLE 4. Distribution of sialyltransferase and CMP-NANA hydrolase activities among various strains

Organism	Strain ^a	Sialyl- transferase	CMP-NANA hydrolase
<u>Neisseria meningitidis</u>	604-A (A)	-	+
<u>Neisseria meningitidis</u>	M986 (B)	+	+
<u>Neisseria meningitidis</u>	M1011 (B)	+	+
<u>Neisseria meningitidis</u>	M990 (B)	+	+
<u>Neisseria meningitidis</u>	Dres 03 (B)	+/-	+
<u>Neisseria meningitidis</u>	2241-C (C)	+	+
<u>Neisseria meningitidis</u>	PRM102 (B)	+/-	+
<u>Neisseria flava</u>	M953 (NG)	-	-
<u>Neisseria perflava</u>	ATCC #14799 (NG)	-	-
<u>Escherichia coli</u>	O1:K1:HNM (B)	+/-	-
<u>Escherichia coli</u>	ATCC #10798 (NG)	-	-

^a Letters in parentheses indicate serogroup as determined by slide agglutination using serogroup A,B or C rabbit antisera (NG = nongroupable).

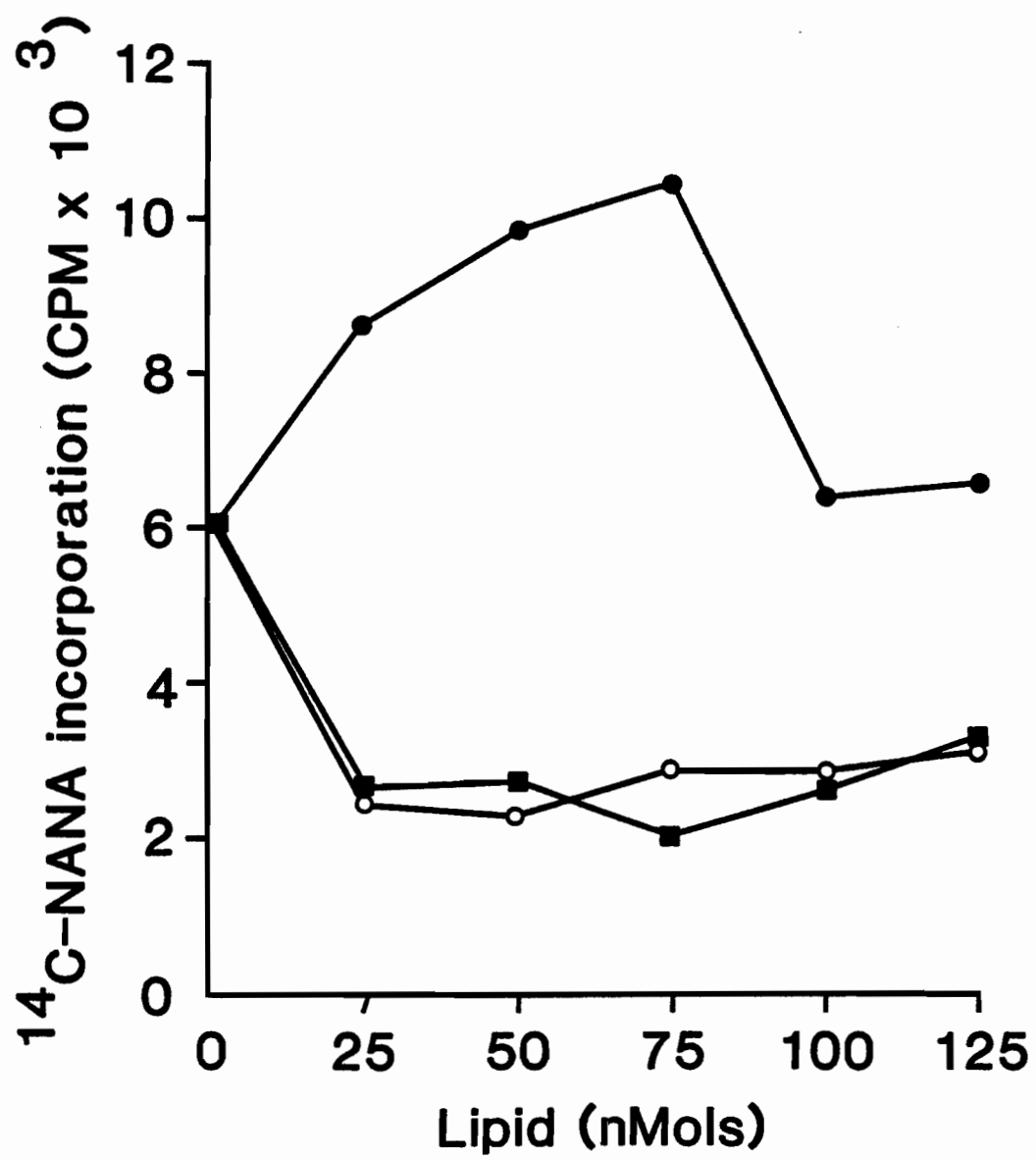
^b - = no detectible activity.
 +/- = low specific activity relative to Neisseria meningitidis strain M986.
 + = specific activity similar to Neisseria meningitidis strain M986.

residues are transferred from their sugar-nucleotide precursors to a membrane lipid carrier (Troy, 1979). In peptidoglycan (Anderson et al., 1967), lipopolysaccharide (Osborn, 1969; Weiner et al., 1965) and teichoic acid synthesis (Brooks and Baddiley, 1969), monosaccharide transfer is mediated by membrane-bound glycosyltransferases which subsequently form pre-assembled oligosaccharide subunits on undecaprenyl phosphate (UP), a C_{55} -polyisoprenyl phosphate coenzyme carrier. These subunits are attached, in turn, to growing nascent oligosaccharide chains. However, not all glycosyltransferases transfer sugar residues to a lipid carrier; some appear to transfer directly from the sugar-nucleotide to the growing polymer (Fiedler and Glaser, 1974; Kopmann and Jann, 1975).

Although studies on capsular polysaccharide synthesis in Aerobacter aerogenes (Troy et al., 1971), E. coli (Troy et al., 1975) and Micrococcus lysodeikticus (Lehav et al., 1969) have now included capsular polysaccharide synthesis in the group which utilize lipid intermediates, previous investigators have failed to detect the involvement of radioactively labeled sugar-linked lipid intermediates in particulate membrane preparations of serogroup C meningococci (Vann et al., 1978).

In order to determine if undecaprenyl phosphate was involved in the synthesis of serogroup B capsular polysaccharide, the effect of exogenous UP on sialyltransferase activity in particulate membranes was initially examined in the presence of detergent. The purpose of the detergent was two-fold. It was required for initial solubilization of UP and to enhance insertion of UP into the particulate membrane vesicles. As shown in Figure 14, addition of exogenous UP stimulated sialyl polymer production up to a concentration of 75 nMols of lipid. Addition of UP beyond this level resulted in loss of sialyltransferase stimulation. Inhibition of polymer synthesis at higher

Figure 14. Effect of exogenous undecaprenyl phosphate or phospholipid addition on capsular polysaccharide synthesis in particulate membranes of N. meningitidis strain M986. Particulate membranes were incubated with CMP- $[^{14}\text{C}]$ NANA in standard incubation mixtures containing increasing concentrations of undecaprenyl phosphate (●), phosphatidylethanolamine (■), or phosphatidylglycerol (○). Samples were removed and chromatographed to determine the level of $[^{14}\text{C}]$ NANA incorporated into high molecular weight polysaccharide. Averaged data from duplicate experiments are shown.

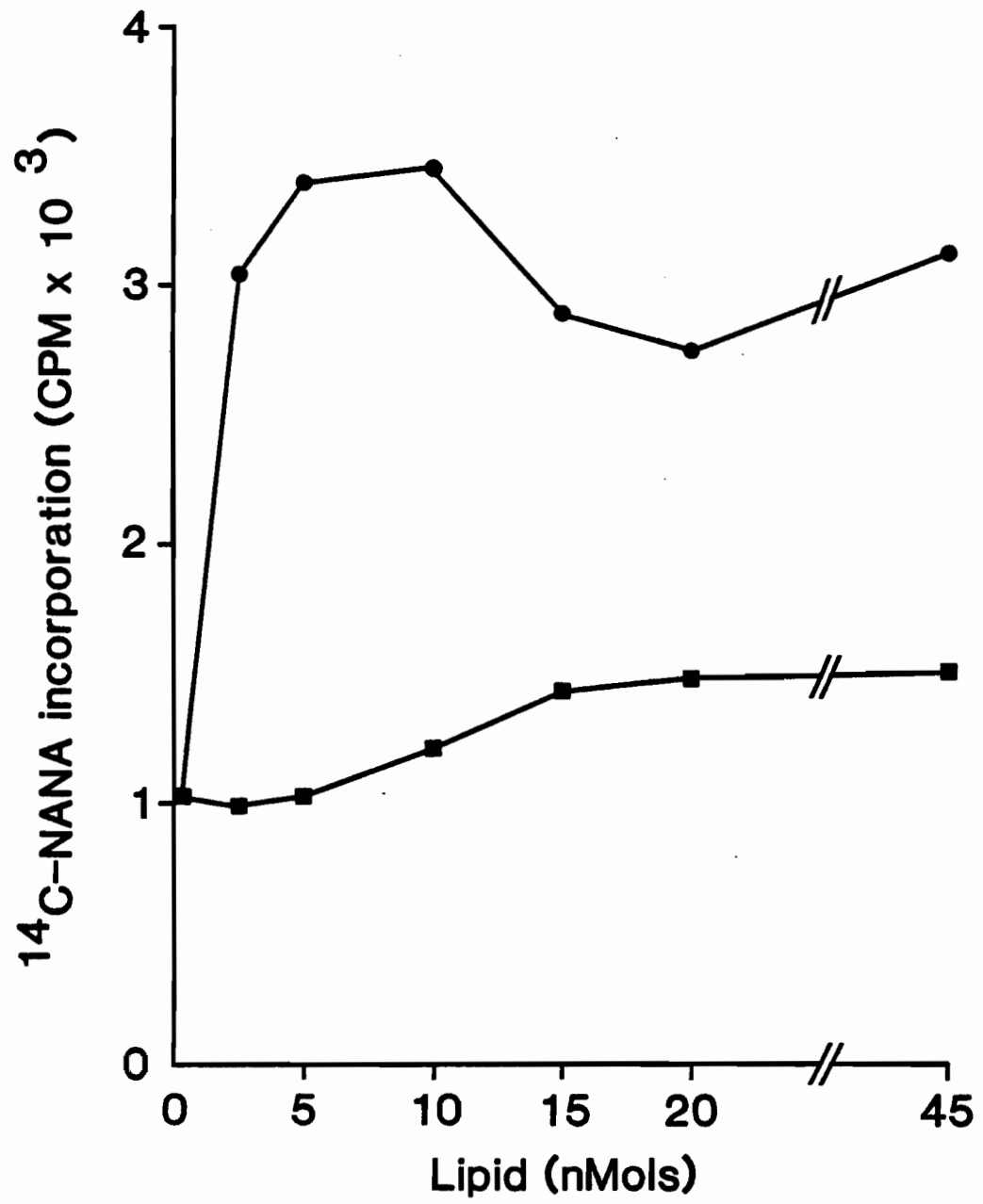


lipid concentrations has been observed by other investigators with E. coli and M. lysodeikticus and is thought to be due to the ratio of surfactant to UP (Lehav et al., 1969; Troy et al., 1975).

When the carrier lipid is present at a concentration three-fold higher than the surfactant (optimal ratio), inhibition of glycosyltransferases may occur (Lehav et al., 1969; Troy et al., 1975). Stimulation of sialyltransferase activity was specific for UP since the addition of similar levels of either PLE or PLG did not enhance polymer production but rather resulted in inhibition (Figure 14). The reasons for the observed inhibition were unclear although the phenomenon of phospholipid inhibition has been observed previously during synthesis of the O-antigen of Salmonella typhimurium (Osborn, 1969, 1972). Due to the presence of a CMP-NANA hydrolase in strain M986 membranes (Masson and Holbein, 1983), CMP (35 μ M final concentration) was added to the reaction mixtures to inhibit any hydrolytic activity. However, the presence of CMP did not alter the effects of UP, PLE or PLG on the production of sialyl polymer (data not shown).

To obtain direct evidence of the requirements of sialyl polymer production for UP, membrane proteins were delipidated with cold organic solvents and reconstituted with either UP or PLG. The results in figure 15 demonstrate that only UP was able to reconstitute sialyltransferase activity indicating the specific dependence of sialyl polymer production on the presence of UP. Reconstitution of CMP-NANA hydrolase activity, after the delipidation procedure, was unsuccessful. The production of sialyl polymer occurred maximally at 5 nMols of UP. Surprisingly, increasing the lipid concentrations did not inhibit the reaction to as great an extent as seen with the particulate membranes (Figure 14) or with that reported for the delipidated mannosyltransferases of M. lysodeikticus (Lehav et al., 1969), but was comparable to the situation found in E. coli

Figure 15. Reconstitution of delipidated membrane proteins with exogenously added lipids. Standard reaction mixtures were examined for polysaccharide production in the presence of undecaprenyl phosphate (●) or phosphatidylglycerol (■). Averaged data from duplicate experiments are shown.



(Troy et al., 1975). Triton-X-100, at the concentration used (0.012 %), had no effect on the reaction. It was interesting to note that the sialyltransferase, a membrane-bound enzyme, exhibited different properties in vitro when removed from its normal lipid milieu (Table 5). In particulate membranes, sialyltransferase activity displayed no apparent dependence on sulfhydryl reducing agents like 2-mercaptoethanol and a slight but not absolute dependence on Mg^{2+} ions (Masson and Holbein, 1983). However, when the lipid environment was removed, sialyltransferase activity demonstrated only 34% of control activity in the absence of 2-mercaptoethanol and only 18% if N-ethylmaleimide was added. N-ethylmaleimide exerted no effect on sialyltransferase activities of particulate membranes (data not shown). The presence of $MgCl_2$ appeared to have an inhibitory effect; the complete removal of which resulted in a 2.5-fold stimulation in sialyl polymer production. The delipidated enzyme also had a high dependence on the presence of ammonium sulfate for activity, losing approximately 66% of activity in the absence of this salt. A similar dependence has been shown for E. coli (Vijay and Troy, 1975).

Further evidence suggesting the involvement of lipid-linked glycosyl intermediates was obtained after the extraction of samples from the in vitro particulate sialyltransferase reaction with C:M (2:1) (Figure 16). Solvent extractable radioactivity was absent at time zero either in the presence or absence of UP indicating that the washing procedure was effective in separating the CMP-NANA substrate from the lipid soluble intermediates. Extractable intermediates were evident by 2.5 min and increased until 5 min after which the level decreased to a steady state. This decline was not due to substrate depletion as evidenced by the linear increase in sialyl polymer throughout the reaction. Furthermore, it was determined that approximately 65% of the initial substrate remained at the completion of this reaction. The

TABLE 5.

**Properties of delipidated sialyltransferase from
N. meningitidis M986**

<u>TREATMENT</u> ^a	<u>% CONTROL ACTIVITY</u> ^d
complete ^b	100
- mercaptoethanol ^c	51.0
- ammonium sulfate	34.0
- mercaptoethanol and ammonium sulfate	9.7
- mercaptoethanol + N-ethylmaleimide (3mM)	18.0
- Mg ²⁺ + EDTA (25mM)	250.2

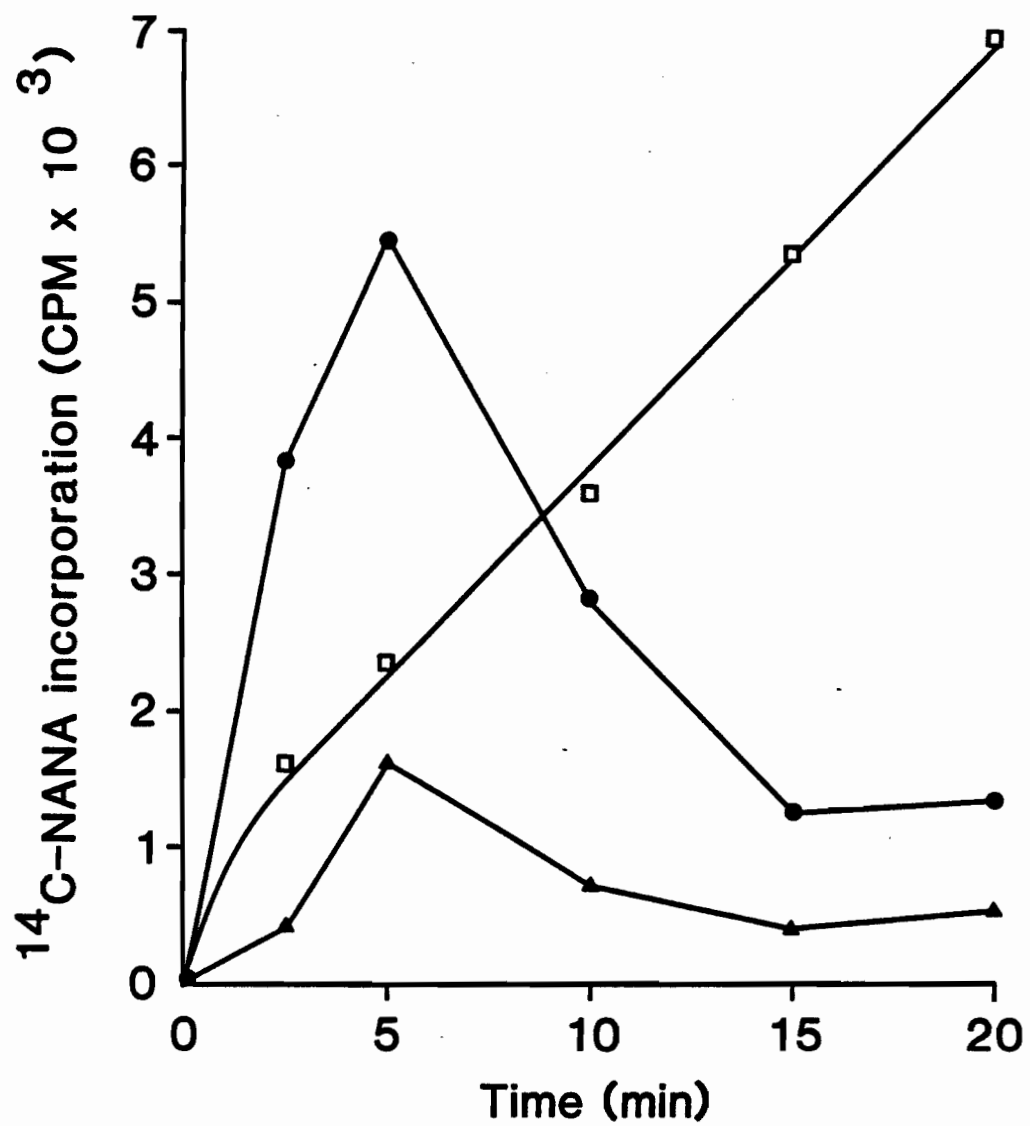
a) the concentrations employed in the delipidated enzyme assays are as described in materials and methods. Concentrations of additional components are indicated in the table.

b) rate of polymer synthesis in the complete reaction mixture was considered to be the control reaction.

c) +/- signs indicate addition or deletion of the indicated component.

d) averaged data from the minimum of two experiments are shown.

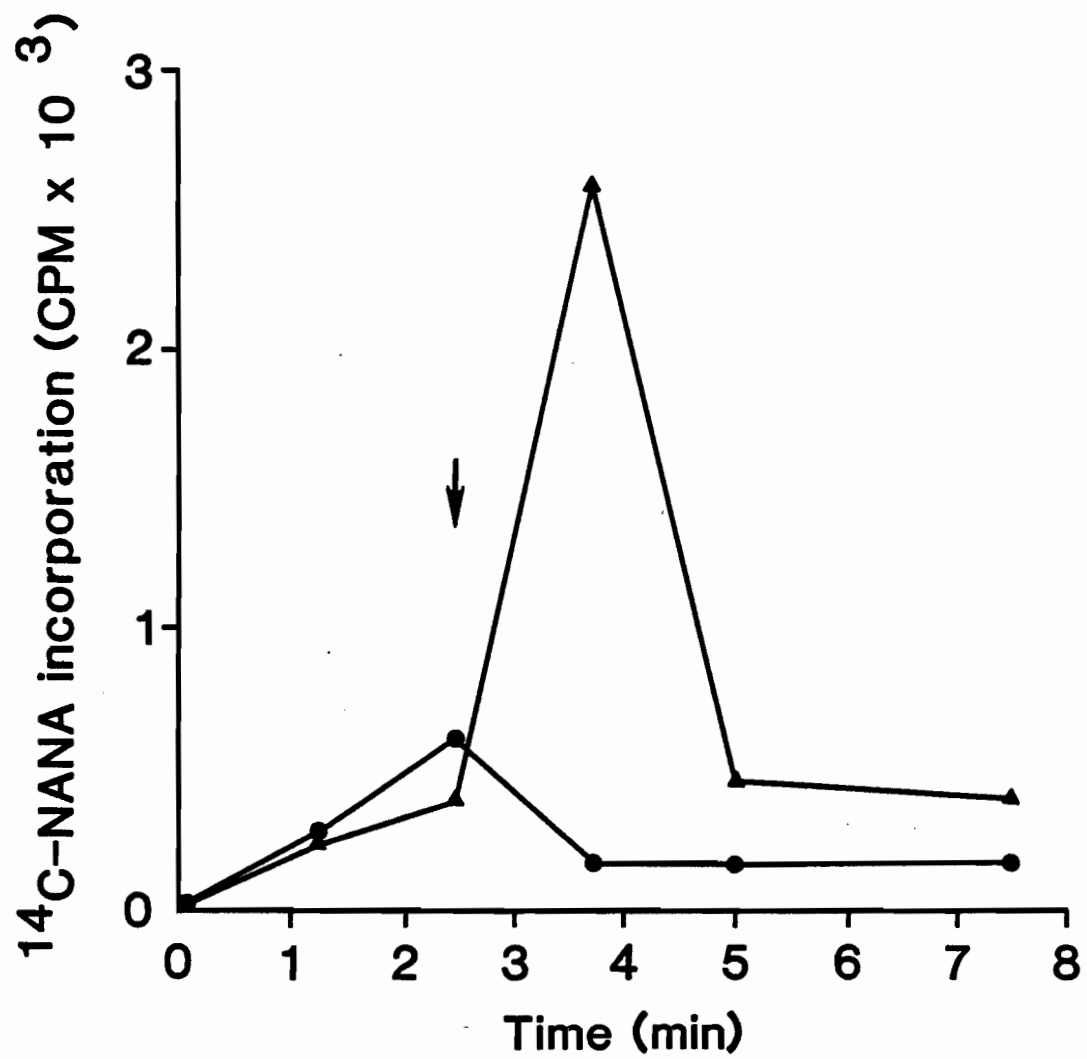
Figure 16. Chloroform:methanol (2:1) extraction of [^{14}C]-labeled lipid intermediates. Aliquots of particulate membrane reaction mixtures containing CMP- ^{14}C NANA in the presence (●) or absence (▲) of exogenous UP were extracted, washed and assayed for lipid-soluble radioactivity. Typical results from a single experiment are shown. Capsular polysaccharide (□) was measured by adding two ul of the above reaction mixtures into 30 ul of absolute ethanol to terminate polymer synthesis and then spotting the whole mixture directly onto Whatman 3MM chromatography paper which was then developed in ethanol: 1 M ammonium acetate (7:3).



reason for the decline was uncertain but may reflect the transfer of NANA residues from the lipid fraction into the sialyl polymer. However, direct evidence of such a transfer would be difficult to obtain. On the other hand, considering the negative charge of NANA residues, the decline may be occurring if sialic acid residues were polymerizing on UP. Thus, the polymer could become increasingly hydrophilic and consequently rendered unextractable by organic solvents. One can also see from figure 16 that addition of UP to particulate membranes enhanced the level of radioactivity 2.5-fold in the C:M lipid soluble fractions at 5 min presumably due to increased UP availability for the sialyltransferase. PLG, when substituted for UP, resulted in nearly a complete loss of C:M (2:1) extractable radioactivity (data not shown).

The exact mechanism of NANA transfer effected by the lipid is unclear. It could be a direct transfer to an acceptor or alternatively, polymerization of NANA residues may occur directly on UP itself. Evidence supporting the latter hypothesis can be seen in figure 17. CMP-[¹⁴C]NANA pulsed particulate membranes were chased with a 1000-fold excess of unlabeled CMP-NANA after 2.5 min of incubation. The results show an immediate loss of radioactivity in the lipid fraction while the amount of label in the unchased reaction continued to increase. This rapid decline suggested that a transient pool of sialyl-linked UP was present. However, the label was not completely chased from the lipid fraction suggesting that some polymer elongation may have been occurring in association with UP. In agreement with this hypothesis, autoradiograms of chromatographed C:M fractions such as those from figure 16, demonstrated most of the radioactivity at the origins. This suggested that the lipid-linked sialic acid could be in an oligomeric form since hydrophilic molecules like NANA do not migrate in C:M:water or heptane:ethyl acetate solvents. One would expect that if UP was linked to a single residue of sialic acid, the extracted radioactivity would exhibit

Figure 17. Pulse chase study of the synthesis of [^{14}C]-labeled lipid intermediates during polysaccharide synthesis. Standard reaction mixtures containing 5.6 μMol of exogenously added UP were divided into two equal fractions. After addition of two μCi of $\text{CMP-}[^{14}\text{C}]\text{NANA}$ (319 mCi/mMol), 0.1 ml aliquots were removed and extracted with two ml of C:M (2:1). At 2.5 min. (arrow), the label was chased with a 1000-fold excess of unlabeled CMP-NANA which was added in a 20 μl volume of 0.15 M Mops buffer pH 7.7 (●) or just buffer alone (▲). Averaged data from duplicate experiments are shown.



amphipathic properties and would migrate in the developing solvent used. However, some of the autoradiograms possessed extremely faint bands with Rf's. of 0.33 at 2.5 and 3.75 minutes (data not shown). The reasons for the virtual absence of migrating amphipathic lipid intermediates in the extracts, but present in chromatographed C:M extracts of E. coli (Troy et al., 1975), could be explained by a rapid turnover of migrating monosialic-monophosphorylundecaprenol into a non-migrating oligosaccharide-linked UP. To directly determine if the extracted NANA was present in a polymeric form, the C:M (2:1) extracts were subjected to sequential periodate oxidation and borohydride reduction followed by digestion with neuraminidase. The results in table 6 show that NANA residues were present as small oligosaccharides with an average degree of polymerization of approximately 4 to 5 residues per chain. Furthermore, all three procedures employed for extraction of the residue at the silica gel origin produced relatively similar results.

In Gram-negative bacteria, UP is employed in a number of major cell envelope biosynthetic pathways such as peptidoglycan (PG), lipopolysaccharide (LPS), and in some cases, CP synthesis. In the case of Gram-positive bacteria, aside from peptidoglycan synthesis, UP is also involved in teichoic acid synthesis. Therefore, the necessary complexes of membrane enzymes involved in these biosynthetic pathways may either utilize individual pools of UP, solely for their own purposes, or they may share UP from a common pool. Inhibition experiments with Bacillus licheniformis revealed that UP molecules participating in the synthesis of peptidoglycan were shared with the system that synthesized teichoic acids (Anderson et al., 1972). Furthermore, it was concluded that UP was returned to a common pool only after the completion of a polymer chain on the basis that preincubation of the precursors from one pathway was able to cause inhibition of the other

TABLE 6. Degree of polymerization of C:M (2:1)
extracted lipid-linked intermediates

Solvent ^a	Components released by ^b Neuraminidase treatment (pMol)		Average chain length
	Sialitol	NANA	
C:M (2:1)	0.14	0.50	4.6
Triton-x-114	0.26	0.71	3.7
Methanol/acetic acid	0.20	0.82	5.1

^a Solvent used to extract [¹⁴C]-labelled sugars from silica gel.

^b As per Materials and Methods.

whereas no preincubation resulted in no inhibition.

The results provided by the particulate membrane experiments and the results of previous investigators (Lehav et al., 1969 , Troy et al., 1975, Vijay and Troy, 1975) suggest that endogenous UP is present in less than saturating levels. It would seem probable, therefore, that UP may play a pivotal role in the regulation of cell envelope polymers. Since, N. meningitidis does not possess an O-antigenic side chain (Jennings et al., 1980), this would leave UP being involved primarily in peptidoglycan and now CP synthesis. Thus, inhibition of one pathway by the precursors of the other would provide evidence supporting this contention and indirectly provide further proof of the involvement of UP in the synthesis of the sialic acid CP.

Bacitracin inhibitable, acid precipitable material was synthesized by particulate membranes in the presence of UDP- $[^{14}\text{C}]$ N-acetylglucosamine and UDP-NAMA pentapeptide (Figure 18). Addition of bacitracin, at a concentration of 80 ug/ml, inhibited PG synthesis by approximately 50%. PG synthesis was found to be optimal at room temperature in the presence of 100 mM $(\text{NH}_4)_2\text{SO}_4$. To conclusively prove that the high molecular weight polymer found at the origins were indeed chains of peptidoglycan, crude membranes from strain M986 were incubated for two hours in the presence of UDP-NAMA pentapeptide and UDP- $[^{14}\text{C}]$ N-acetylglucosamine. The radioactive, acid insoluble material was precipitated, washed and finally resuspended in buffer in the presence of lysozyme. The gradual decline in the amount of label initially present at time zero, as shown in figure 19, suggests that the labeled material at the origin represented chains of peptidoglycan.

To determine if UP was shared between PG and CP biosynthetic pathways, cross-competition reactions were devised (Table 7a and 7b). When unlabeled CMP-NANA was incubated with UDP-N-acetylmuramic acid pentapeptide and

Figure 18. Synthesis of [^{14}C]-labeled meningococcal peptidoglycan.

Samples (20 μl) were removed from incubation mixtures containing 1.3 mg of particulate membrane protein from strain M986, 0.8 mM UDP-N-acetylmuramic acid (UDP-NAMA) pentapeptide, 0.3 μCi UDP- ^{14}C -N-acetylglucosamine (306 mCi/mMol), 100 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgCl_2 , and 0.15 M MOPS buffer (pH 7.7) in a final volume of 178 μl in the presence (●) or absence (○) of bacitracin (80 $\mu\text{g/ml}$). The samples were analyzed by paper chromatography developed in ethanol:1 M ammonium acetate (7:3). Averaged data from duplicate experiments are shown.

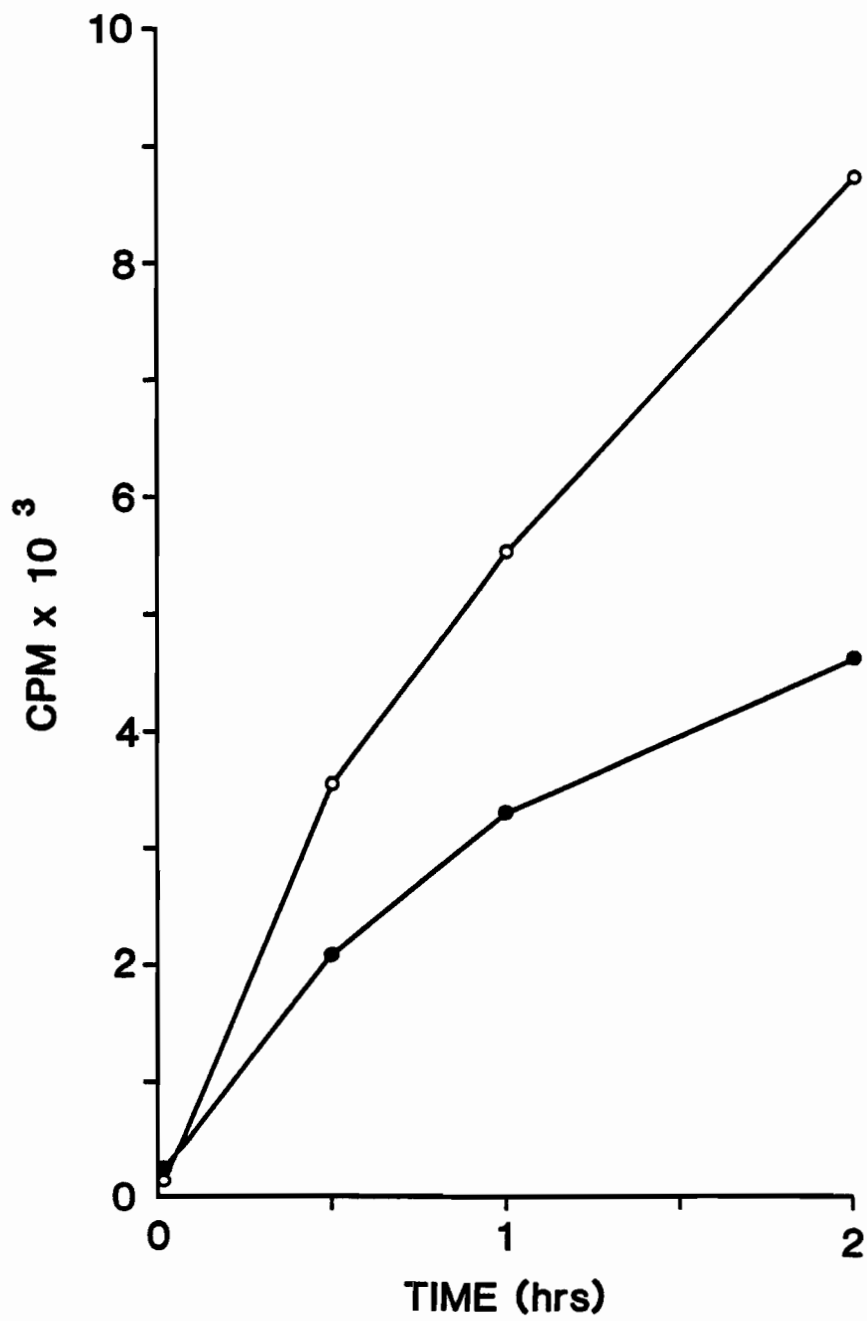


Figure 19. Digestion of [^{14}C]-labeled meningococcal peptidoglycan by lysozyme. [^{14}C]-labeled meningococcal peptidoglycan was synthesized for two h in standard reaction mixtures followed by precipitation with 0.3 M perchloric acid. The precipitate was resuspended in 165 μl of 0.15 M MOPS buffer (pH 7.7) containing 20 mM MgCl_2 . Lysozyme (8000 units) was added at time zero, and at intervals, 20 μl aliquots were spotted on Whatmann 3MM chromatography paper and developed in ethanol: 1 M ammonium acetate (7:3). The chromatograms were analyzed for loss of label at the origin. Averaged data from two experiments are shown.

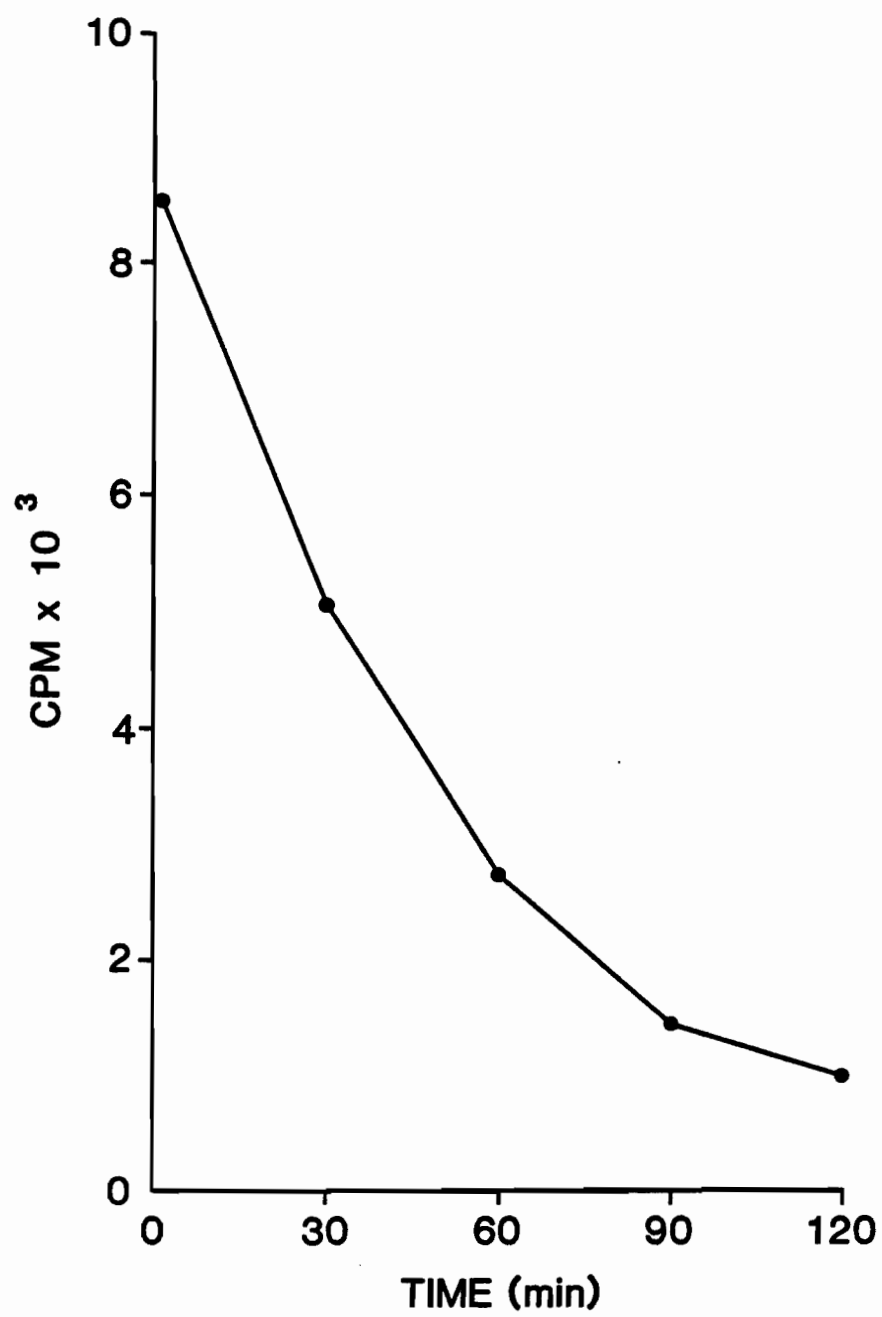


TABLE 7.

**A. Inhibition of peptidoglycan synthesis by
sialyl polymer precursor**

<u>ADDITION</u> ^a	<u>% INHIBITION OF</u> ^b <u>PEPTIDOGLYCAN SYNTHESIS</u>
none (control)	0
bacitracin (80µg/ml)	46.8
CMP (120µM)	0.1
CMPNANA (3.3mM)	29.3

**B. Inhibition of sialyl polymer synthesis by a
peptidoglycan precursor**

<u>ADDITION</u> ^c	<u>% INHIBITION OF</u> ^d <u>SIALYL POLYMER</u>
none (control)	0
bacitracin (80µg/ml)	0
UMP (0.4mM)	3.1
UDP-NAMA pentapeptide (0.8mM) and Bacitracin (80µg/ml)	27.9
UDP-NAMA pentapeptide (0.8mM)	25.2

a) reaction mixtures were composed of 1.3mg membrane protein, 0.8mM UDP-NAMA pentapeptide, 0.5µCi UDP-[¹⁴C] N-acetylglucosamine (306 mCi/mMol), 100mM (NH₄)₂SO₄, 0.15M MOPS (pH 7.7) in a final vol. of 178µl. Mixtures were incubated at 22°C for two hours after which 50µl was spotted and chromatographed on Whatman 3MM paper.

b,d) results are average values from three separate experiments.

c) reaction mixtures were composed of 84µg membrane protein, 100mM (NH₄)₂SO₄, 35µM CMP, 0.15M MOPS (pH 7.7) in a final vol. of 0.1ml. The mixtures were preincubated for 30min at 22°C in the presence or absence of UDP-NAMA pentapeptide as indicated. Mixtures were warmed to 37°C for five min after which 0.14µCi CMP-[¹⁴C]NANA was added. After 15min, 20µl was spotted and chromatographed on Whatman 3MM paper

UDP-[¹⁴C]N-acetylglucosamine, there was approximately a 30% inhibition in the production of PG polymer suggesting that the unlabeled CMP-NANA was indeed competing with the peptidoglycan enzyme complex at the level of UP. The reverse situation, i.e. incubation of UDP-N-acetylmuramic acid with CMP-[¹⁴C]NANA, resulted in inhibition of CP synthesis. During studies of CP synthesis inhibition by PG precursors, it was necessary to inactivate the lipid in order to see inhibition. This was accomplished by the addition of UDP-NAMA pentapeptide in the absence of UDP-N-acetylglucosamine. The lack of UDP-N-acetylglucosamine results in the inability of undecaprenyl-pyrophosphoryl-N-acetylmuramic acid pentapeptide to recycle the lipid carrier thus rendering it unavailable for further use (Anderson et al., 1972). If UDP-N-acetylglucosamine was present, addition of bacitracin also would have inactivated UP. Bacitracin inhibits the synthesis of both peptidoglycan and the O-antigenic side chains of lipopolysaccharides by inhibiting the monodephosphorylation of undecaprenyl pyrophosphate (Siewert and Strominger, 1967). Thus, the functional monophosphoryl-UP molecule is not recycled and consequently, is rendered unavailable for further synthesis. Surprisingly, when bacitracin was added to the incubation mixture in the absence of UDP-N-acetylglucosamine, it enhanced the inhibition of sialyl polymer synthesis by UDP-NAMA slightly. However, bacitracin alone had no effect on CP synthesis. It was unclear why bacitracin caused the slight increase in inhibition in the absence of UDP-N-acetylglucosamine although it may have been due to the presence of endogenous UP-disaccharide-pentapeptide in the particulate membrane preparations. These results are in accord with those for sialic acid CP synthesis in E. coli (Troy et al., 1975) and in serogroup C meningococci (Vann et al., 1978) which are unaffected by bacitracin since a pyrophosphate linkage between the sialic acid molecule and UP does not occur.

It was interesting to note that the inhibition of PG by CMP-NANA occurred without any interference with UP, as was necessary for the inhibition of CP synthesis. This may reflect either a higher affinity of the CP enzyme complex for UP or, alternatively, it may be due to the polymerization of NANA residues directly on UP thus delaying the recycling of UP back into the common pool. The data does not discriminate between these two possibilities.

vi. Summary.

Two spontaneous, isogenic, noncapsular mutants of N. meningitidis strain M986 were isolated and named PRM101 and PRM102. An encapsulated revertant, PRM101r, was obtained from PRM101. Strain M986 and PRM101r synthesized and released CP, unlike strains PRM101 and PRM102 as verified by chemical analysis and electron microscopy. Similar levels of a NANA condensing enzyme and a CMP-NANA synthetase were found in strains PRM102 and M986 and were localized exclusively to the cytosol. A reduced level of sialyltransferase was found in strain PRM102 along with appearance of a novel enzyme, a CMP-NANA hydrolase. The hydrolase was present at similar levels in both strains but was not found in the nonpathogenic neisserial species surveyed. The reaction products of the CMP-NANA hydrolase were CMP and NANA. The hydrolase, but not the sialyltransferase, was inhibited by CMP. Both the sialyltransferase and CMP-NANA hydrolase were found associated solely with crude membranes. Fractionation of crude membranes was achieved and produced four distinct fractions. Sialyltransferase, CMP-NANA hydrolase and TMPD-oxidase activities were localized to the cytoplasmic membrane.

Undecaprenyl phosphate was found to play a specific role in CP

biosynthesis. NANA-linked lipid intermediates were extractable in organic solvents and examination of these extracted intermediates indicated that polymerization of NANA residues occurred on UP. CP precursors inhibited PG synthesis as well as the reverse case, suggesting the sharing of a common component, presumably UP.

CHAPTER II

i. Introduction.

To date, it remains unexplained as to how the meningococcus can be carried, without overt clinical symptoms in the nasopharynx by 5 to 30% of the normal population, in spite of the fact that this organism is capable of causing either sporadic or large scale epidemics of invasive disease (DeVoe, 1982). Immunity to meningococcal disease is dependent on the presence of circulating bactericidal antibodies directed towards the capsular and non-capsular surface antigens (Goldschneider et al., 1969a, 1969b; Frasch, 1979), and the presence of the complete terminal attack sequence of complement (Nicholson and Lepow, 1979; Petersen et al., 1979).

In recent years, there has been a growing body of evidence implicating bacterial capsular polysaccharides as important virulence factors and defining their role in the enhancement of pathogenesis (Gemski et al., 1980; Horowitz and Silverstein; 1980, Taylor, 1983; Bortolussi et al., 1983). With some organisms, the degree of virulence is related to the level of CP produced (Nicholson and Glynn, 1975; Craven et al., 1980; Klegerman et al., 1984). In the case of meningococci, Craven et al., (1980) noted that encapsulated strains from disease isolates possessed a higher level of capsular polysaccharide than encapsulated strains from carrier isolates.

The natural habitat of the meningococcus, the human nasopharynx, is where this organism encounters a variety of environmental and nutritional conditions, many of which would exert a pronounced effect on determining the expression of various virulence factors important to the invasiveness of this

organism (Van Snick et al., 1978; Magnusson et al., 1979a, 1979b; Morse et al., 1983; Brener et al., 1981). In the meningococcus, in vitro growth under low pH/iron limiting conditions greatly enhanced the virulence of this organism for mice (Brener et al., 1981). These conditions were thought to mimic the in vivo conditions of heavy nasopharyngeal carriage of meningococci or the infected abdominal cavity of mice (Brener et al., 1981; DeVoe, 1982). However, the enhanced virulence of this organism was attributed, only in part, to enhanced iron uptake ability (Brener et al., 1981; Simonson et al., 1982). Since bacteria appear quite adept at altering their surface components in relation to their surroundings (Ellwood and Tempest, 1972; Magnusson, 1979a, 1979b; Brener et al., 1981), examination of the meningococcus under conditions of nutrient limitation and conditions of low pH would be relevant to a better understanding of its pathogenicity.

In this chapter, the importance of the sialic acid CP of serogroup B N. meningitidis as a virulence factor for this organism was determined. The effect CP has on resistance to the serum bactericidal reaction was examined. Furthermore, experiments examining the effects of nutrient limitation and low pH conditions on the production of the serogroup B capsular polysaccharide as well as their effect on surface hydrophobicity and virulence for mice are presented.

ii. Characterization of bacterial strains.

To determine the role of the sialic acid CP of serogroup B N. meningitidis, isogenic noncapsular mutants PRM101 and PRM102, as well as an encapsulated revertant PRM101r, were isolated (Chapter I, section ii). The

maximum sialic acid produced/mg cell dry weight of each strain was determined and compared to virulence for C57BL/6 mice (Table 8). The results revealed that the unencapsulated mutant strains, which were unable to produce halos on agar containing horse serogroup B antiserum, possessed approximately 25% of the total sialic acid produced by the wildtype strain. This loss of polysaccharide biosynthetic capability resulted in a dramatic increase (approximately 20,000-fold) in the 50% lethal dose for mice, i.e. these strains were essentially avirulent. Virulence could be regained upon reversion to the wildtype phenotype as demonstrated by PRM101r (Table 8).

A variety of components associated with the outer membrane of Gram-negative bacteria are known to be involved in resistance to serum complement (Taylor, 1983). It has been reported, for N. meningitidis, that cells from transparent colonies are more resistant to killing by normal serum than those from opaque colonies (Stephens and McGee, 1983). Since it has been shown elsewhere in this dissertation (Chapter I, section ii.) that these colony phenotypes were due primarily to the presence or absence of the sialic acid capsular polysaccharide, the effects of normal serum on N. meningitidis strain M986 and its isogenic noncapsular variant PRM102 were examined. These isogenic strains afforded the ability to determine conclusively if the production of CP confers serum resistance.

N. meningitidis strain M986, possessing CP, demonstrated complete resistance to serum killing (98% survival), as shown in table 9. This resistance occurred in the presence of EGTA-chelated normal rat serum (NRS) which inhibits activation of the classical complement pathway. However, N. meningitidis strain PRM102 which lacks surface CP, was completely sensitive. The serum component(s) responsible for the bacterial killing was heat labile as shown by replacing normal rabbit serum for heat-inactivated serum (H-NRS). E. coli K12 was used as an internal control for the

TABLE 8. Characteristics of bacterial strains

Strain	Halo ^a production	Total sialic acid ^b ug/mg cells	LD ₅₀ ^c (CFU)
M986	+	39.4	3.6×10^3
PRM101	-	12.8	7.4×10^7
PRM101r	+	43.8	9.3×10^3
PRM102	-	11.9	2.0×10^8

^a As detected on agar containing horse antiserum to serogroup B.

^b Maximum sialic acid/mg cell dry weight determined just prior to stationary phase after 6-7 h growth in NDM-C.

^c 50% lethal dose in CFU for C-57 black mice, average result of three experiments each.

TABLE 9. Sensitivity of N. meningitidis to killing by complement.

Bacteria	Addition ^a	% kill after 30 min ^b
<u>E. coli</u> ^c K12	NRS	91.67 ± 6.89
	PBS	2.89 ± 2.89
	H-NRS	0 ± 0
<u>N. meningitidis</u> PRM102	NRS	100 ± 0
	PBS	0 ± 0
	H-NRS	0 ± 0
<u>N. meningitidis</u> ^d PRM102	NRS	100 ± 0
	PBS	0 ± 0
	H-NRS	0 ± 0
<u>N. meningitidis</u> M986	NRS	2.08 ± 1.73
	PBS	2.04 ± 1.30
	H-NRS	0 ± 0
<u>N. meningitidis</u> M986	NRS + Ab ^e	94.21 ± 2.22
	PBS + Ab	7.99 ± 4.63
	H-NRS + Ab	0 ± 0

^a Additions to standard reaction mixtures as per Materials and Methods. Addition of lysozyme to all the above reactions had no effect on killing.

^b Data shown is the result of at least four separate experiments each performed in triplicate.

^c E. coli was used as a control for the alternate complement pathway.

^d Purified serogroup B polysaccharide was added to each mixture to a final concentration of 2 mg/ml.

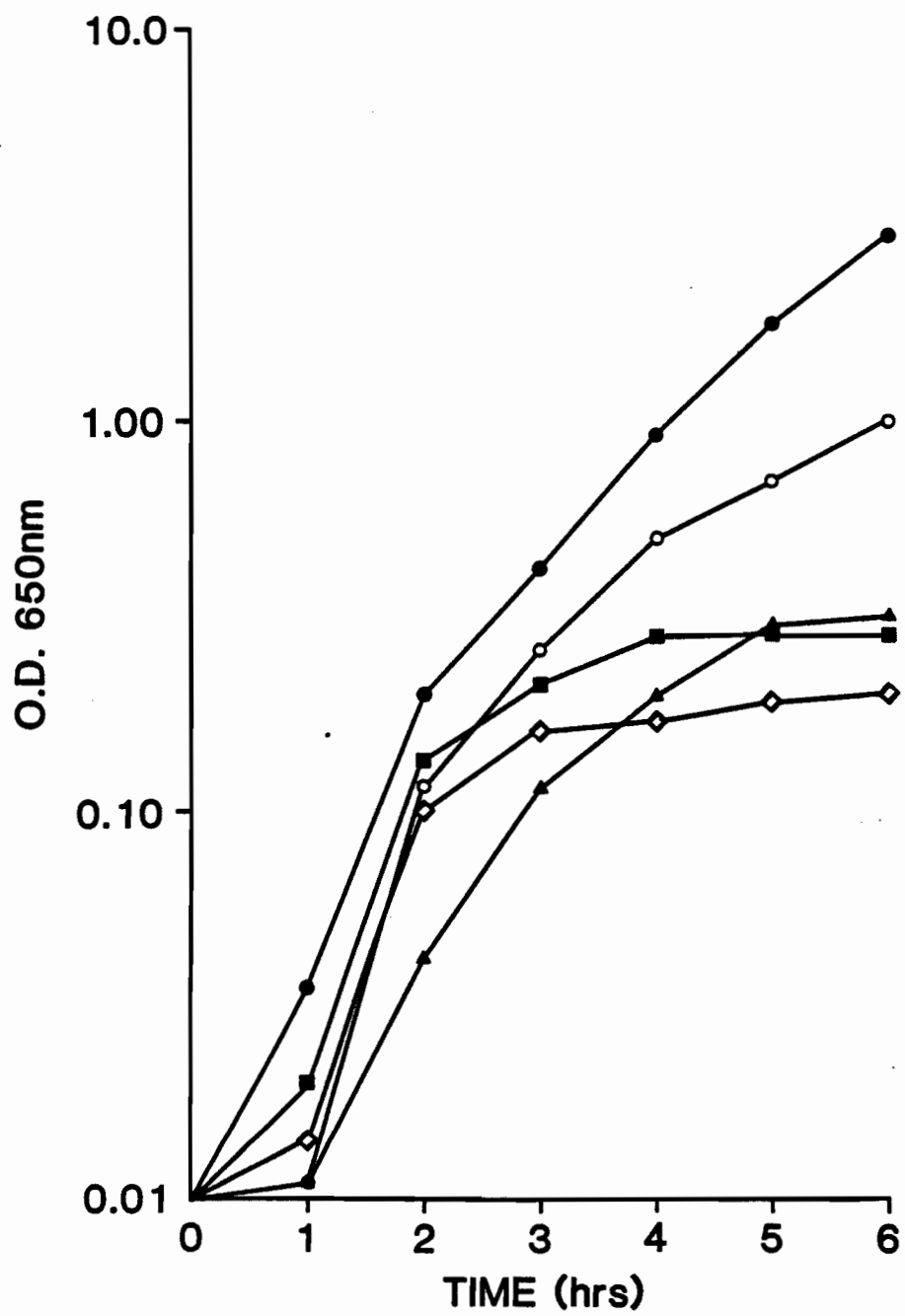
^e Sterile horse group B antiserum (1:128 dilution in PBS) was added to standard reaction mixtures as per Materials and Methods.

alternate complement pathway. Loss of serum resistance with N. meningitidis strain M986 occurred in the presence of immune serum. The data indicates, therefore, that possession of the sialic acid CP dictates the presence of immune antibodies for effective complement-mediated killing of N. meningitidis strain M986. Furthermore, cell surface-associated sialic acid was important for resistance as the addition of purified serogroup B polysaccharide did not abrogate the serum bactericidal effect observed with N. meningitidis strain PRM102. This observation has also been noted with type III serogroup B streptococci (Yeung and Mattingly, 1984). It has been shown that the competition between factor B and β 1H for binding to surface-associated C3b is influenced by bound sialic acid which increases the affinity of cell-bound C3b for β 1H but not for factor B (Fearon, 1978; Kazatchkine et al., 1979). This would result in inhibition of the amplification loop of the alternate complement pathway since the outcome of the competition between factor B and β 1H for C3b binding determines whether the cell will activate the alternate complement pathway.

iii. Effect of low pH and nutrient limiting conditions.

In order to determine the effects that nutrient limitation and conditions of low pH exerted on CP levels, their effects on the growth rate of N. meningitidis strain M986 were initially characterized. Cells were grown under conditions of nitrogen or iron limitation as well as low pH or low pH/iron limiting conditions in appropriately modified NDM-C broth. All modified cultures exhibited a prolonged lag phase (Figure 20). These cells were able to adapt to the new conditions and began to grow rapidly for

Figure 20. Growth of N. meningitidis strain M986 under conditions of low pH and nutrient limitation. Results represent the average of two separate growth experiments using (●) Normal, (■) nitrogen limited, (▲) iron limited, (○) pH 6.6, and (◇) pH 6.6/iron limited NDM-C as described in materials and methods.



approximately. 2-3 h after which growth was limited and eventually ceased in the case of nutrient limited cultures. Low pH grown cells, not subjected to nutrient limitations, continued to grow at below normal rates. Samples of the various cultures were removed at 5-6 h when net growth had ceased and the cells were subsequently assayed for both their cell-associated and cell-free sialyl polymer content by rocket immunoelectrophoresis (Table 10). Since this was a more sensitive technique for determining the presence of high molecular weight sialyl polymer than that previously employed (Chapter I, section ii.), strain PRM102, grown in Normal NDM-C, was included as a control to determine if either unabsorbed noncapsular antibodies or the presence of cell-surface polysaccharide could be detected. No immunoprecipitation was observed in the agarose gel providing further support for the contention that strain PRM102 does not possess detectable surface polysaccharide. In all other cases of low pH and nutrient limitation examined, cell-associated CP in strain M986 was present at a higher concentration than cells grown in Normal NDM-C medium. The cells grown under low pH/iron limiting conditions produced the highest concentration (4-fold higher than normal). This result is interesting in view of recent results published by Brener et al. (1981) in which meningococci grown under low pH/iron limiting conditions displayed a 1000-fold increase in their virulence for mice and that cells grown either in low pH or iron limited conditions displayed a smaller but significant increase. All culture conditions, when compared to normal, resulted in an increased level of cell-free polysaccharide. It was not determined if this was due to enhanced autolysis but it was observed that in cases where low pH conditions were employed, less polysaccharide (approximately. 50%) appeared as cell-free. It has been shown that gonococci grown in a low pH environment may be more stable and less prone to peptidoglycan hydrolysis (Wegener et al., 1977). Bortolussi et al., (1983) had observed that K1 E.

TABLE 10. Determination of cell-associated and cell-free sialic acid capsular polysaccharide levels in broth cultures of *N. meningitidis* strain M986 under low pH or nutrient-limiting conditions

Bacterial ^b strains	Culture ^c conditions	Sialic acid level ^a		
		Cell-associated PS ($\mu\text{g PS}/10^9$ cells)	Cell-free PS ($\mu\text{g PS}/10^9$ cells)	TOTAL PS ($\mu\text{g PS}/10^9$ cells)
PRM102	Normal	0.0 \pm 0.0	0.0 \pm 0.0	0
M986	Normal	2.47 \pm 0.17	0.66 \pm 0.10	3.13
M986	Iron-limited	4.83 \pm 0.19	4.54 \pm 0.56	9.37
M986	N-limited	5.34 \pm 0.32	4.57 \pm 0.19	9.91
M986	pH 6.6	5.92 \pm 0.26	2.33 \pm 0.26	8.25
M986	pH 6.6/iron-limited	9.78 \pm 0.39	2.57 \pm 0.19	12.35

^a Data shown are the results of at least 3 experiments. All data are significantly different from normal NDM-C grown strain M986 ($p < .001$).

^b Strain PRM102 was used as a control for completeness of noncapsular Ab absorption.

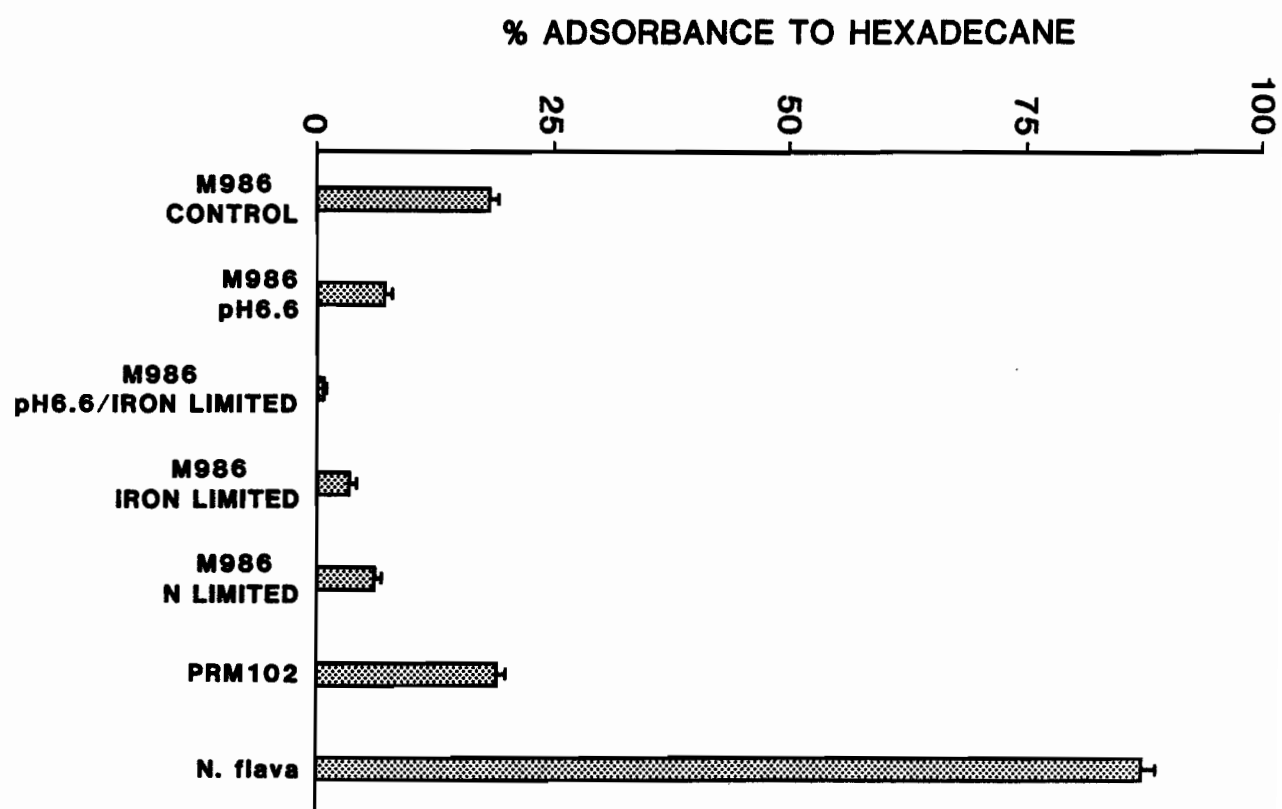
^c As defined in Materials and Methods.

coli cells lost the ability to synthesize the sialic acid CP when grown at 22°C. However, it was observed that N. meningitidis strain M986, when spotted onto antibody-agar plates and incubated at 22°C, was able to synthesize CP as observed by the appearance of halo formation in the agar.

Due to the anionic nature of sialic acid and the observed enhancement of CP production under conditions of nutrient limitation, the effect that increased CP levels had on cell-surface hydrophobicity was examined. Figure 21 shows a strong correlation between an increase in CP levels with increasing cell-surface hydrophilicity as demonstrated by reduced binding to hexadecane. Cells grown under low pH/iron limiting conditions had a greatly reduced hydrophobicity. One intriguing finding was that the noncapsular mutant N. meningitidis strain PRM102 appeared to be as hydrophilic as strain M986 grown in Normal NDM-C. In most cases, the importance of capsular polysaccharides is usually attributed to both cell-surface hydrophilicity and resistance to the bactericidal effects of serum (Horowitz and Silverstein, 1980; Nicholson and Lepow, 1979; Taylor, 1983). Thus, it would appear that the loss of virulence of strain PRM102 for mice cannot be explained simply by an increase in hydrophobicity, especially when compared to the degree of hydrophobicity exhibited by the nonpathogenic Neisseria flava. The results do lend support to the importance of the CP in resisting complement. In accordance with this observation, other investigators have suggested that the importance of the capsular polysaccharide lies in its ability to inhibit opsonophagocytosis in the absence of immune antibodies by preventing the effective activation of the alternate complement pathway (Verbrugh et al., 1979; Horowitz and Silverstein, 1980; Bortolussi et al., 1983).

One possible explanation for the increased levels of CP observed with

Figure 21. Surface hydrophobicity of cells grown under low pH or nutrient limiting conditions. Neisseria flava, and N. meningitidis strains M986 (control) and PRM102 were grown in Normal NDM-C. Strain M986 was also grown under conditions of low pH and nutrient limitation as indicated. Washed cell suspensions were vortexed in the presence of hexadecane and the percent adsorbance to hexadecane was calculated by measuring the decrease in the optical density (A_{550}) of the aqueous phase after partitioning of the two phases. The bars represent the standard error.



cells grown under nutrient limitation and low pH conditions could be an increase in the synthesis of enzymes involved in CP synthesis. To this end, the two principal enzymes, sialyltransferase and CMP-NANA hydrolase, which are involved in the both synthesis and regulation of the serogroup B polysaccharide, were examined. The data in table 11 suggest that sialyltransferase activity appears not to be inducible by the conditions employed, although cells grown under low pH/iron limiting conditions demonstrated an actual reduction in sialyltransferase activity to a level comparable with the noncapsular mutant strain PRM102. This apparently anomalous result could be partially explained by a decrease in CMP-NANA hydrolase activity. In fact, all cells producing increasingly higher levels of CP than cells grown in Normal NDM-C were found to possess a proportional decrease in CMP-NANA hydrolase activity. Although cells grown in low pH/iron limited NDM-C medium possessed a similar level of sialyltransferase as strain PRM102, the CMP-NANA hydrolase activity was much reduced. This reduction would help to explain, in part, why there was a significant increase in CP content in cells grown under conditions of nutrient limitation and low pH. The possibility that other enzyme activities involved in CP synthesis (i.e. NANA condensing enzyme, CMP-NANA synthetase) had been altered, was not investigated and cannot not be ruled out.

To ascertain whether enhanced polysaccharide production correlated with enhanced virulence of N. meningitidis strain M986 for mice, cultures were grown under conditions of nitrogen limitation, low pH and low pH/iron limitation. The data presented in table 12 revealed that cells enhanced in their ability to produce CP were more virulent for mice. Therefore our findings were consistent with those of Brener et al., (1981), in that cells grown in low pH/iron limited medium were the most virulent for mice; the decrease in LD₅₀ being almost two orders of magnitude.

TABLE 11. Effect of cultural conditions on the specific activities of sialyltransferase and CMP-NANA hydrolase in bacterial strains.

Strain	Culture ^b conditions	Specific Activity (Units/mg protein) ^a	
		Sialyltransferase ^c	CMP-NANA ^d hydrolase
M986	Normal	1.92 ± 0.04	8.03 ± 0.49
M986	pH 6.6	1.86 ± 0.25	7.08 ± 0.74
M986	N-limited	1.90 ± 0.23	4.42 ± 0.83
M986	Iron-limited	1.51 ± 0.01	6.07 ± 0.08
M986	pH 6.6/iron-limited	0.56 ± 0.10	3.98 ± 0.25
PRM102	Normal	0.55 ± 0.03	6.94 ± 0.17

^a 1 unit of enzyme = 1 nMol NANA/h as defined in Materials and Methods.

^b Enzyme reaction mixtures as described in Materials and Methods.

^c N-limited and pH 6.6 sialyltransferase activities are not significantly different from normal M986. All other values have $p < .001$.

^d All values are significantly different from Normal CMP-NANA hydrolase activity ($p < .001$).

TABLE 12. Virulence for mice of *N. meningitidis* strain M986 cultured in vitro under nutrient limitation or low pH conditions.

Culture ^a conditions	LD ₅₀ ^b (CFU)	p ^c
Normal	1.10 ± 0.31 × 10 ⁶	-
pH 6.6	1.51 ± 0.06 × 10 ⁵	<.01
N-limited	4.25 ± 1.10 × 10 ⁴	<.005
pH 6.6/iron-limited	2.22 ± 0.44 × 10 ⁴	<.005

^a Conditions are as defined in Materials and Methods.

^b Mean of three separate experiments as calculated by the method of Reed and Muench.

^c P = probability level of significance (one tailed t-test).

Simonson et al., (1982) demonstrated that cells grown under iron limited conditions expressed a specific uptake mechanism for transferrin iron which was enhanced during growth at low pH. It was suggested that the enhanced virulence was due to a more efficient iron uptake mechanism. In order to circumvent this possibility, the virulence of cells grown under nitrogen starvation conditions was examined. Interestingly, the enhanced virulence exhibited by these cells was similar to that of low pH/iron starved cells. It is important to note that both types of limitations resulted in much higher levels of CP, suggesting a firm relationship of polysaccharide production to virulence. However, iron uptake kinetics were not examined on the nitrogen starved cells and the possibility of enhanced iron uptake as an additional virulence component could not be ruled out.

iv. Summary.

The loss of CP biosynthetic capability in serogroup B N. meningitidis strain M986 resulted in the loss of virulence of strains PRM101 and PRM102 for mice which was regained upon reversion to the wildtype phenotype (strain PRM101r). Possession of cell surface-associated CP conferred resistance to the bactericidal effects of normal serum as shown by M986 but not PRM102. A decrease in the growth rate as evidenced by growth under nitrogen or iron limitation as well as low pH or low pH/iron limiting conditions resulted in enhanced cell-associated and cell-free CP production. Proportionately decreased cell surface hydrophobicity and membrane associated CMP-NANA hydrolase levels correlated with the higher levels of cell-associated CP. Strain M986, grown under these conditions, demonstrated an increased virulence for mice which also correlated with the increased

production of CP levels.

DISCUSSION

DISCUSSION

i. Introduction.

It remains an enigma as to why the meningococcus can be carried asymptotically in the nasopharynx by 5-30% of the normal population, but on the other hand, is capable of causing either sporadic or large scale epidemics of invasive disease (DeVoe, 1982). Our current understanding of the factors determining whether or not meningococcal infection will lead to overt disease is scanty at best. The literature appears divided into two distinct schools of thought which hold that meningococcal disease is due either to the inherent susceptibility of certain individuals or the extraordinary virulence of certain meningococcal strains.

Most infections acquired by the respiratory route are asymptomatic and localized to the nasopharynx. Infrequent dissemination from this site probably occurs only in the small percentage of infected individuals who have low antibody titers to the serogroup and/or the serotype antigens of the infecting strain. However, various lines of evidence indicate that invasive meningococcal disease is much more complex than susceptibility per se. Acute respiratory disease has been suggested as a predisposing factor to meningococcal disease (Edwards et al., 1977; Peltola, 1983). However, until more conclusive evidence is acquired, this hypothesis remains purely speculative.

The wide spectrum of clinical manifestations of meningococcal disease observed during epidemics, and the ability of this organism to grow in a variety of anatomical sites (DeVoe, 1982), lend support to differential

virulence of meningococcal strains. In fact, it has been demonstrated that carrier strains of meningococci were less virulent for mice than disease isolates (Holbein et al., 1979; Holbein, 1981). Lack of antibiotic treatment during meningococcal disease results in an approximately 90% fatality rate (DeVoe, 1982) indicating that this powerful pathogen possesses virulence properties capable of evading or subverting host defence mechanisms.

It is reasonable to assume that alterations in the physicochemical and antigenic surface properties of an invasive pathogen could be advantageous to the invading organism since it is the bacterial surface which interacts with and determines the host immune response. Evidence supporting phenotypic alterations of the organism have been emerging in the literature. Griffiss et al., (1974) described a case of recurring infection with an antigenically identical meningococcal strain in a patient possessing bactericidal antibodies. These authors suggested that alterations in the organism's antigenic configuration may have occurred during in vivo growth. A variety of investigators have shown that nutritional and environmental alterations during in vitro growth have a profound effect on the metabolism, phenotypic expression, and virulence of both gonococci and meningococci. Tyeryar et al., (1974) have demonstrated the reversion of avirulent gonococci to a virulent phase after tissue culture passage. Recent studies with the meningococcus revealed that in vitro growth under low pH/iron limiting conditions greatly enhanced the virulence of this organism for mice. These conditions are thought to mimic the in vivo conditions of heavy nasopharyngeal carriage of meningococci or the infected abdominal cavity of mice (Brener et al., 1981; DeVoe, 1982). However, the enhanced virulence of this organism was attributed, only in

part, to an enhanced iron uptake expressed by the bacterium under these conditions. Since bacteria appear quite adept at altering their surface components in relation to their surroundings (Smith, 1977; Magnusson et al., 1979a, 1979b; Brener et al., 1981; Stephens and McGee, 1983), examination of meningococcal physicochemical surface properties under conditions of nutrient limitation and conditions of low pH would be relevant to a better understanding of its pathogenicity.

Bacterial capsular polysaccharides have long been suspected to play a critical role in the ability of meningitis-causing bacterial strains to invade and proliferate in the central nervous system (Tofte et al., 1979; Peterson and Quie, 1981; Stephens and McGee, 1983; Klegerman et al., 1984). The mechanisms of how CP exerts its effects during pathogenesis are only recently becoming understood and are described primarily in terms of resistance to the serum bactericidal action (Gemski et al., 1980) as well as opsonin dependent (Horowitz and Silverstein, 1980; Bortolussi et al., 1983) and independent phagocytosis (Van Oss, 1978; Ohman and Stendahl, 1982; Peterson et al., 1984). In the case of serogroup B meningococci, the role that CP plays in virulence is obscure. It is generally accepted that the incidence of meningococcal disease is correlated to the absence of serum bactericidal antibodies directed towards surface antigens and the lack of the complete terminal attack sequence of complement. However, failure to elicit bactericidal antibodies directed towards the sialic acid CP of serogroup B meningococci is not uncommon (Kasper et al., 1973b). Although immunological susceptibility most certainly plays a crucial role in the pathogenesis of meningococcal disease, the studies on which this concept is based have been performed primarily with serogroup A and C meningococci (Goldschneider et al., 1969a).

Few studies have addressed the important question of how the production of this important virulence factor might be regulated in vivo. This question assumes even greater importance in light of a variety of observations correlating enhanced CP synthesis with virulence (Nicholson and Glynn, 1975; Craven et al., 1980; Klegerman et al., 1984).

The natural habitat of the meningococcus, the human nasopharynx, is where this organism encounters a variety of environmental and nutritional conditions many of which would exert a pronounced effect on determining the expression of various virulence factors important to the invasiveness of this organism. A complete understanding of how the sialic acid CP of serogroup B N. meningitidis functions as a virulence factor necessitates an understanding of not only the structure of the polymer, but also how its precursors are synthesized, the regulatory mechanisms involved in its production, and its mechanism of assembly.

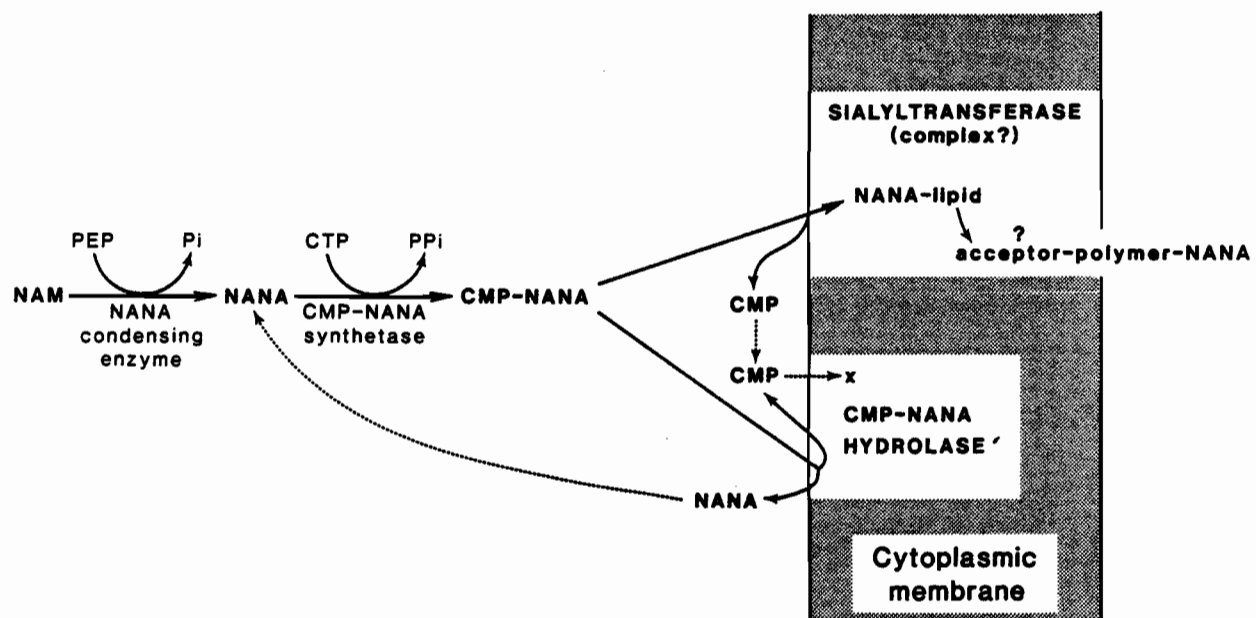
ii. Production and assembly of meningococcal capsular polysaccharide.

This dissertation has illuminated some important aspects concerning the pathway for sialic acid CP biosynthesis in serogroup B N. meningitidis. The mutant strain PRM102 has a greatly reduced ability to elaborate detectable levels of CP as evidenced by growth on antibody-agar detection plates, rocket immunoelectrophoresis and immunoelectron microscopy. Surprisingly, this strain possesses all the enzymes known to be necessary for polymer production. The major difference between the encapsulated wild type strain M986 and strain PRM102 was determined at the molecular level and concerned the levels of cytoplasmic membrane-associated sialyltransferase.

PRM102 demonstrated only 25% of the wild type activity. K1 E. coli, which elaborates a sialic acid CP identical to serogroup B meningococci, was found to possess even less sialyltransferase activity but was still capable of synthesizing surface polysaccharide. The discovery of a CMP-NANA hydrolase in the cytoplasmic membrane of both strains of N. meningitidis but not in K1 E. coli, revealed an important difference in polysaccharide synthesis between these two bacterial pathogens. It is also interesting to note that despite the similarity of the CP produced by serogroup B meningococci and K1 E. coli, there appears to be no homology in the DNA sequences encoding for their respective sialyltransferase genes (Echarti et al., 1983). The discovery of the CMP-NANA hydrolase in serogroup A meningococci but its absence in nonpathogenic neisseriae was intriguing. It is possible that serogroup A organisms may have evolved from either serogroup B or C organisms or on the other hand, this enzyme may be plasmid encoded. Bhatti et al., (1981) have detected the presence of plasmid DNA in serogroup B meningococci with unknown functions.

These results and those of other studies can be incorporated into an overall working hypothesis for sialic acid polysaccharide synthesis shown in figure 22. CMP-NANA is produced in the cytoplasm through the sequential action of the NANA condensing enzyme and the CMP-NANA synthetase on N-acetylmannosamine (NAM) and is available to both the sialyltransferase and the CMP-NANA hydrolase within the cytoplasmic membrane. The sialyltransferase can incorporate NANA into high molecular weight polymer, which emerges on the external surface of the cytoplasmic membrane. In this model, CMP-NANA delivered to the cytoplasmic membrane is also accessible to the CMP-NANA hydrolase, which returns both CMP and NANA to the cytoplasm. The composition of the acceptor fixing the CP to the meningococcal outer membrane is

Figure 22. Working hypothesis for the biosynthetic pathway for sialic acid polysaccharide synthesis in serogroup B N. meningitidis and its regulation. PEP = phosphoenolpyruvate.



uncertain. Gotschlich et al., (1981) have identified various 1,2-diacylglycerols covalently linked to the reducing termini of purified high molecular weight polysaccharides from serogroup A, B, and C meningococci and K29 E. coli.

The biosynthesis of complex microbial cell envelope polymers is multifactorial in nature. High molecular weight carbohydrate polymers such as peptidoglycan, capsular polysaccharides, lipopolysaccharides and teichoic acids are synthesized from precursors initially synthesized in the bacterial cytoplasm. They are assembled, in many cases, through the use of lipid carriers at the cytoplasmic membrane by membrane-associated glycosyltransferases and, through a mechanism as yet unknown, appear in a completed form on the outer surface of the cytoplasmic membrane (Sutherland, 1982). This temporal mode of synthesis demands a high degree of organization of the appropriate enzymes in the membrane environment to permit coordination between the appropriate glycosyltransferases, lipid carriers, and acceptor sites. Troy et al., (1975) have demonstrated the involvement of a C₅₅-polyisoprenyl lipid carrier, undecaprenyl phosphate (UP), mediating the transfer of NANA from CMP-NANA to an acceptor in the case of E. coli. If the transfer of the initial NANA residue to a terminal acceptor was mediated by a lipid carrier, then it would be likely that more than one enzyme possessing sialyltransferase activity would be involved in polysaccharide synthesis; one for priming the lipid carrier and one for polymer chain elongation (Troy and McCloskey, 1979). Thus, the sialyltransferase shown in figure 22 might represent a complex composed of more than one enzymatic activity. Previous attempts by Vann et al., (1978) to determine the involvement of such a lipid carrier in the production of serogroup C meningococcal CP were unsuccessful based on their inability to

detect organic solvent extractable radioactivity in particulate membranes.

Evidence has been provided demonstrating the involvement of glycosyl lipid-linked intermediates in serogroup B N. meningitidis. These intermediates were extractable in organic solvents but this alone is insufficient to prove the actual participation of lipid intermediates especially in light of the results from Gotschlich et al., (1981) demonstrating the presence of lipid covalently attached to meningococcal CP. Although the extracted glycosyl-linked lipid was not isolated and its composition determined directly, the results strongly suggest that it is undecaprenyl phosphate. The isomer ficaprenyl phosphate, which differs from UP by the cis-trans configuration at one internal olefinic bond (Willoughby et al., 1972), could substitute for UP.

The first demonstration of lipid carrier participation in the biosynthesis of a bacterial CP was in Aerobacter aerogenes (Troy et al., 1971). The responsible lipid carrier isolated in this organism was identical to that involved in the biosynthesis of PG (Anderson et al., 1967), LPS (Wright et al., 1967) and membrane mannan (Lahav et al., 1969). In the case of serogroup B N. meningitidis, addition of purified UP to meningococcal particulate membranes or to delipidated membrane proteins revealed a specific requirement for UP. Additional evidence for the identification of the lipid carrier is shown in table 7. Cross-competition experiments between CP and PG synthesis demonstrated that CP precursors were able to inhibit PG synthesis by competing for available endogenous UP. The same appeared to hold true for the opposite case as well. This serves to further illustrate the central role for undecaprenyl phosphate in the biosynthesis of complex microbial polysaccharides.

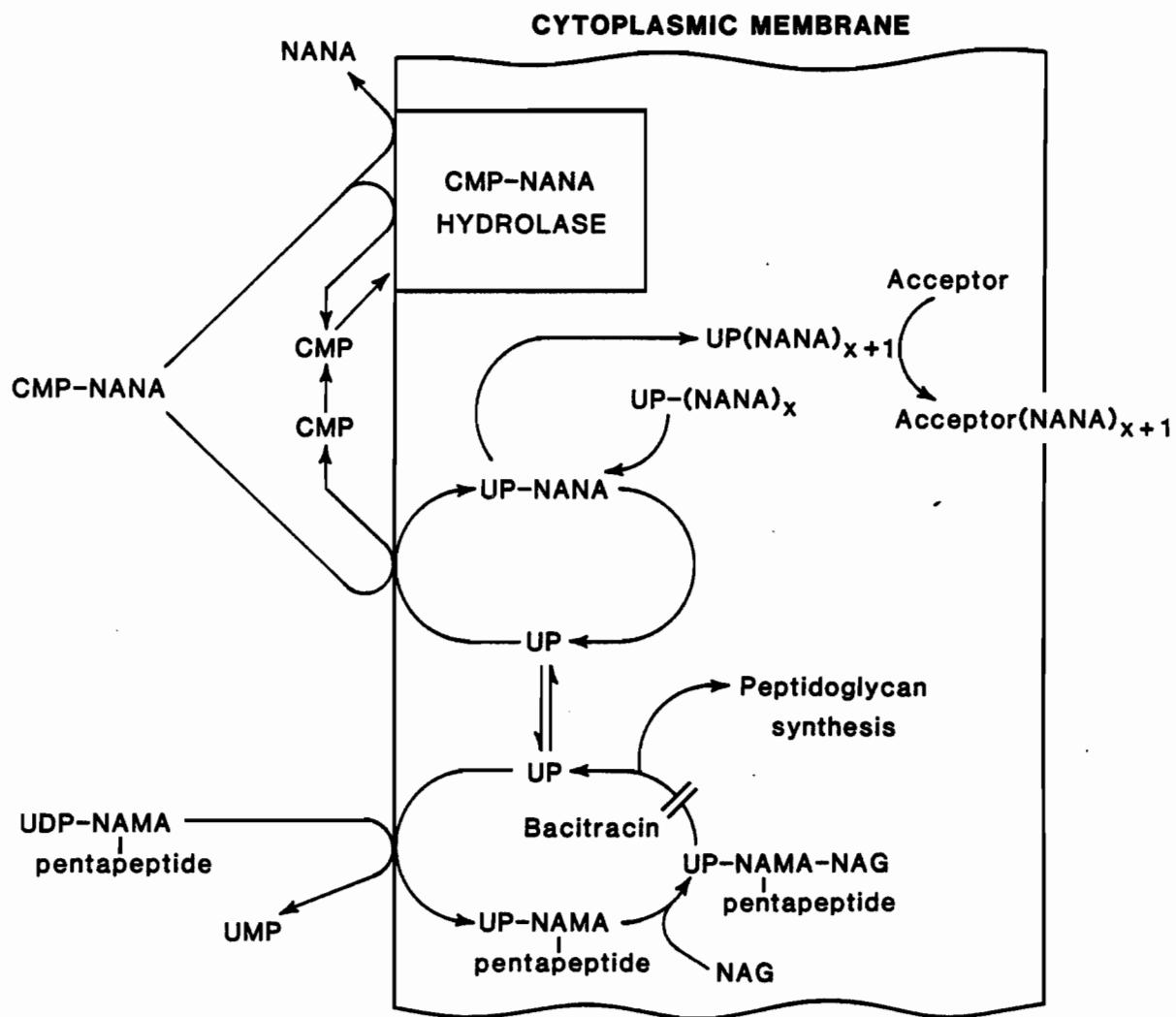
The actual mechanism of CP elongation via UP is unclear. In view of other biosynthetic processes that employ UP, there exists 2 major possibilities. First, polymerization of NANA residues, transferred from UP-NANA, may occur directly onto an acceptor in a manner similar to that of peptidoglycan (Anderson et al., 1967). Alternatively, polymerization may occur directly on UP itself, which in turn, transfers the completed polysaccharide (or oligosaccharide) to a membrane acceptor in a manner similar to O-antigen (Wright et al., 1967) or teichoic acid linkage unit (Ward, 1981) synthesis. In either case, the sialyltransferase would appear to be the penultimate enzyme in this pathway since the involvement of at least one other enzyme must occur. The evidence presented in this dissertation tends to support the latter hypothesis since in the former, the only lipid-linked intermediate one could isolate would be UP-NANA, an amphipathic molecule able to migrate on thin-layer silica gel chromatographic plates in C:M:Water. The reason for not detecting significant levels of UP-NANA is unclear but was probably due to a rapid turnover of this material i.e. a rapid buildup of UP with more than one residue. This was especially evident in the pulse-chase experiments since addition of unlabeled CMP-NANA caused an immediate decrease in the extractable radioactivity suggesting a rapid turnover of labeled lipid intermediates. This would also provide a possible explanation for the inability of Vann et al., (1978) to detect lipid soluble radioactivity. The bulk of the radioactivity in the C:M extracts remained at the origin after developing thin-layer silica gel plates in C:M:Water, indicating an oligomeric form of NANA. Direct examination of the C:M extracts revealed that NANA was present in a polymeric form with an average degree of polymerization of approximately 4 to 5 residues per chain. This provided direct support for the elongation of NANA on UP. This

oligosaccharide-lipid is consistent with similar experiments reported for E. coli (Troy et al., 1975), as well as with O-antigen synthesis of LPS (Wright et al., 1967), and the linkage unit of teichoic acid in Staphylococcus aureus (McArthur et al., 1978).

A model for CP synthesis and its interaction with peptidoglycan synthesis, at the cytoplasmic membrane level, is presented in figure 23. This model illustrates the central role of UP in the synthesis of meningococcal capsular and peptidoglycan polysaccharides. Moreover, the proposed model displays UP as a shared component between PG and CP synthesis rather than being a unique, integral component of the enzyme complexes of either pathway. How this lipid carrier physically transports, or assists in transportation, charged hydrophilic molecules from one side of a hydrophobic membrane to the opposite side is unknown. Unfortunately, various aspects of polyisoprenol biochemistry remain obscure including information concerning the microenvironment of UP, the nature of the organization of UP in the membrane, intramembranal translocation of glycan chains, and the motional properties of UP. However, studies by Weppner and Neuhaus, (1978) have demonstrated that the microenvironment of the initial lipid-linked intermediate of PG synthesis is in a hydrophobic environment within 4 to 6 angstroms of the lipid matrix.

Anderson et al., (1972) proposed that enzymes involved in the synthesis of peptidoglycan and teichoic acid are organized in the membrane within multienzyme complexes, ensuring the orderly synthesis of glycan chains. Existing theories propose that the synthesis of wall polymers occurs at the inner surface of the membrane, and that the polymers are extruded through the membrane (Fiedler and Glaser, 1973). The data in table 6 demonstrating polymerization of NANA residues on UP would support this mode

Figure 23. Working hypothesis for the involvement of undecaprenyl phosphate in the synthesis of the capsular polysaccharide of N. meningitidis strain M986. NAG = UDP-N-acetylglucosamine



of translocation. Bertram et al., (1981) demonstrated that protoplasts of Bacillus subtilis were able to synthesize poly(ribitol phosphate) from nucleotide precursors supplied in the surrounding medium. These authors proposed a model whereby the enzyme complex spans the membrane. They hypothesized that a component reorientates in such a way that normally substrates gain access when the appropriate active sites are exposed at the inner surface of the membrane. They further suggest that the polymer dissociates at the outer surface of the membrane in such a way that the sites of nucleotide interaction are temporarily exposed to the outer surface of the membrane.

iii. Regulation of capsular polysaccharide synthesis.

The regulation of bacterial polysaccharide synthesis is only partially understood. Most of the available information is concerned primarily with the effects of environmental and nutritional conditions on total polysaccharide production. In this study, regulation of the serogroup B sialic acid CP was examined at the molecular level.

Production of sialyl polymer in vitro is influenced by the presence of a membrane-associated CMP-NANA hydrolase. Both the CMP-NANA hydrolase and the sialyltransferase compete for the same substrate but the hydrolase displays a higher rate of CMP-NANA consumption than the sialyltransferase. Kinetic examination of the two activities confirmed the greater affinity and reaction rates of the hydrolase for CMP-NANA than the sialyltransferase. A product of both enzymes, CMP, selectively inhibits the CMP-NANA hydrolase strongly suggesting that both CMP and the CMP-NANA

hydrolase are involved in the regulation of the sialic acid CP. Thus, due to the higher affinity for substrate, the CMP-NANA hydrolase can limit the amount of NANA which can be incorporated into high molecular weight polymer. However, with high levels of both CMP-NANA hydrolase and sialyltransferase, CMP would accumulate and negatively modulate the hydrolase. The latter would favor polymer formation.

The hypothesis of enzyme competition can explain the inability of N. meningitidis strain PRM102 to elaborate polysaccharide i.e. in terms of enzyme competition for a common substrate. Substantial levels of sialyltransferase would be required due to the high activity of the CMP-NANA hydrolase. Evidence supporting this hypothesis is shown in table 1 whereby K1 E. coli, which lacks a CMP-NANA hydrolase, possesses sialyltransferase levels similar to strain PRM102 yet still elaborates CP. Since the intracellular levels of CMP in the serogroup B meningococcus were not determined, the effects CMP may have on modulating CMP-NANA hydrolase activity and consequently CP production in vivo are uncertain. Significant quantities of cell-associated dialyzable sialic acid were found in strain PRM102. Since CMP concentrations between 10 to 40 μ M readily depress CMP-NANA hydrolase activity, it is not unreasonable to conclude that intracellular CMP levels can approach regulatory levels and modulate the hydrolytic activity.

The CMP-NANA hydrolase of serogroup B meningococci may also serve another important conservatory role. N-acetylglucosamine is a precursor to both peptidoglycan and NANA synthesis. Thus, activity of the CMP-NANA hydrolase which releases NANA may indirectly conserve N-acetylglucosamine for PG synthesis. This is especially important in view of the fact that polysaccharide synthesis is a dispensible function, at least in

vitro, whereas PG synthesis is indispensable. Thus, rapid growth in vitro may favor necessary PG synthesis and restrict dispensible CP synthesis. A completely different situation could occur in vivo where generation times might be somewhat slower and where polysaccharide synthesis is essential for survival in the host.

It has been well documented that changes in environmental conditions profoundly effect cellular metabolism, and phenotypic expression (Sutherland, 1982; Ombaka et al., 1984). Furthermore, it has been demonstrated that the rate of exopolysaccharide synthesis in Pseudomonas NCIB decreased as the growth rate of the organism increased (Williams and Wimpenny, 1980). Limiting concentrations of nitrogen, iron, and phosphate for P. aeruginosa resulted in an increase of extracellular polysaccharide and secondary metabolites (Ombaka et al., 1984). Interestingly, it has been shown that the gonococcus may produce a capsule especially when in the presence of a lactic acid producing bacteria which lowers culture pH (Richardson and Sadoff, 1977). Morse and Hebeler, (1978) had shown that gonococci grown in low pH media elaborate more total hexose and suggested that it may be capsular in nature.

A decrease in the growth rate of N. meningitidis strain M986 caused by the limitation of an essential nutrient or conditions of low pH results in an increase in the level of cell-surface associated serogroup B CP (Table 10). Enhancement of CP synthesis appears to be a nonspecific phenomenon triggered by unfavorable growth conditions. The cross competition data presented in table 7 provides a possible explanation at the molecular level. The inverse relationship between growth rate and CP production may be explained in terms of availability of endogenous C₅₅-lipid carrier (undecaprenyl phosphate) for the synthesis of PG and CP synthesis in

serogroup B N. meningitidis. Precursors of one biosynthetic pathway will inhibit synthesis of the other since both processes compete for undecaprenyl phosphate.

The sharing of UP from a common pool during PG and CP synthesis raises a number of important questions concerning the regulation of these polymers in N. meningitidis. It should be noted that meningococci do not appear to possess O-antigenic side chains in their lipopolysaccharides (Jennings et al., 1980). Consequently, UP would be involved primarily in two rather than three major cell envelope biosynthetic pathways suggesting a more direct control over the regulation of CP and PG biosynthesis. Since the common pool of UP, in vitro, appears to be a rate-limiting factor for polymer production, the factors controlling both the production and the availability of UP would be crucial in determining the actual amounts of these polymers to be synthesized. It has been suggested (Anderson et al., 1972; Willoughby, 1972; Sutherland, 1982) that UP availability may be determined by the balance between phosphorylation and dephosphorylation of UP so that the rate of wall synthesis could be controlled by a single common factor (ie. lipid intermediates). Since the rate of peptidoglycan synthesis under conditions of low pH or nutrient limitation has presumably decreased, this could result in increased availability of UP for CP synthesis. Furthermore, N-acetylglucosamine could be shunted for synthesis of NANA for CP production rather than for PG synthesis. Thus, it would appear that the environment plays a large role in determining the rate of synthesis of essential peptidoglycan in relation to the relatively nonessential CP polymer. This regulation could occur through controlling the availability of UP, therefore, when conditions are suitable for rapid growth, synthesis of the dispensible capsule decreases while synthesis of essential peptidoglycan

is favored. The evidence concerning the availability of UP as a regulatory mechanism appears to parallel the regulation of protein glycosylation in eucaryotic cells. Availability of the eucaryotic counterpart of UP, a C₈₀- to C₁₀₀-polyisoprenyl phosphate collectively known as dolichol phosphate, appears to be the rate limiting factor of N-linked glycoprotein production (Hubbard and Robbins, 1980).

The stimulation of sialyltransferase activity by addition of exogenous UP (see Figure 14) implies that UP is present in meningococcal membranes at less than saturating levels for competing CP and PG synthesis. A competition for UP has also been demonstrated in Bacillus licheniformis and Staphylococcus lactis I3 between PG and teichoic acid syntheses. Therefore, UP availability may well prove to be an extremely flexible, central bacterial control mechanism coordinating which cell envelope biopolymers are to be produced and their relative amounts.

The data suggest that another mechanism may be operating in concert with the mechanism discussed above to enhance CP synthesis during growth under conditions of low pH or nutrient limitation. Data presented in table 11 show a reduction in membrane-associated CMP-NANA hydrolase activity while the sialyltransferase levels remained unchanged in cells limited in their growth. Such a reduction would help to explain, in part, the increase in CP production since repression of hydrolytic activity would enable available CMP-NANA to be incorporated directly into CP production. However, it is possible that the NANA condensing enzyme and/or the CMP-NANA synthetase activities were also altered by these growth conditions and can not be ruled out. How the regulatory mechanisms described in this dissertation interact with each other and how much each contributes to CP regulation in serogroup B meningococci is uncertain but would be determined, in part, by the

surrounding environmental conditions.

iv. Capsular polysaccharide and virulence.

The pathogenesis of meningococcal disease remains incompletely understood, primarily at the level concerning the factors which determine whether the interaction of the meningococcus and the human host will result in overt disease or asymptomatic carriage. This problem is a consequence of our limited knowledge about the relationships between meningococcal physiology and the pathogenesis of the disease. In the absence of obvious secreted virulence factors, like exotoxins, the physicochemical cell surface properties may determine the relative virulence of the meningococcus.

A vast amount of epidemiological data and experimental studies have implicated capsular polysaccharides in the pathogenesis of infectious diseases (Pluscke et al., 1983; Agüero and Cabello, 1983; Cross et al., 1984; Klegerman et al., 1984). However, only in recent years have investigators provided direct evidence for the role of CP in virulence through the use of isogenic mutants deficient in their abilities to produce CP. Most of the earlier studies had employed various strains differing in many characteristics (Gemske et al., 1980; Yeung and Mattingly, 1983; Verweij-Van Vught et al., 1983). The capsular polysaccharides of Gram-negative bacteria are usually high molecular weight, hydrophilic, polyanionic polymers and therefore possession of CP imparts a charged, hydrophilic character to the outer surface of these organisms (Costerton et al., 1981). Recently, investigations have been concentrating on the mechanisms governing how these polymers confer virulence

properties to the bacterium. Possession of capsular polysaccharides as a virulence factor has been usually described in terms of conferring resistance to the serum bactericidal reaction (Sutton et al., 1982; Cross et al., 1984), in addition to antibody dependent (Verweij-Van Vught et al., 1983) and independent (Horwitz and Silverstein, 1980; Pluscke et al., 1983) phagocytosis.

The results presented in this study clearly demonstrates that the virulence of the meningococcus for mice is linked with the ability to produce the serogroup B CP. Loss of the CP renders the organism essentially avirulent which is regained upon reversion to the wild type phenotype. The data presented in table 9 illustrates one function that the sialic acid CP imparts to the organism. Possession of the CP confers resistance to the serum bactericidal reaction. Unlike P. aeruginosa, the encapsulated organisms are sensitive to killing by complement in the presence of immune antibodies (Ombaka et al., 1984). The capsule appears to act by impeding the deposition of functional membrane attack complexes of complement into the membrane presumably by virtue of their physicochemical properties (Gemski et al., 1980; Taylor, 1983). This finding is significant in light of emerging evidence demonstrating the requirement for complement to effectively phagocytose and kill encapsulated bacteria thus imposing a requirement for specific immune antibodies for complement fixation (Horwitz and Silverstein, 1980; Bortolussi et al., 1983).

Since host defence mechanisms recognize the bacterial surface as foreign and consequently direct their actions to the surface components, it would be of advantage to invading pathogens to be able to modify their surface compositions allowing them to resist such mechanisms. The in vivo environment and the adaptability of a pathogen to adverse conditions may

mirror a pathogen's virulence and ability to establish disease. Various investigators have been directing their efforts at determining what metabolic and phenotypic changes occur with pathogenic bacteria in response to such adverse environmental conditions. Of particular importance is the effect of low pH and limiting iron conditions since these may mimic conditions found in vivo, especially during inflammation (DeVoe, 1982). Brener et al., (1981) demonstrated a dramatic increase in meningococcal virulence during growth under low pH/iron limiting conditions. The enhanced virulence correlated with the appearance of new outer membrane proteins. Simonson et al., (1982) demonstrated a highly efficient iron acquisition mechanism in iron starved meningococci that specifically recognized the serum glycoprotein transferrin. Apart from inflammation, the host further responds to bacterial invasion by sequestering serum iron rendering it unavailable for bacterial use (Weinberg, 1978). Thus the ability to remove iron from transferrin can be considered a virulence factor, confirmed in part by the inability of nonpathogenic neisseriae to utilize transferrin-bound iron (Simonson et al., 1982). Magnusson et al. (1979a, 1979b) concluded that N. gonorrhoeae, grown under low pH/iron limiting conditions, appeared to abolish the negative surface charge. However, if the gonococcus was grown in low pH/iron sufficient media, the cells possessed a higher negative surface charge and displayed an increased hydrophilicity. The lowering of medium pH enhances total hexose production in the gonococcus and activates the tricarboxylic acid cycle (Morse and Hebel, 1978). These authors suggested that the increased total hexose may be capsular in nature. Craven et al., (1980) observed that blood or CSF isolates produce CP in greater amounts than closely related strains therefore suggesting that an increase in CP synthesis may occur in vivo. The data appears

consistent with this observation.

When meningococci are grown in low pH/iron limiting medium, an increase in the amount of cell-associated serogroup B sialic acid CP occurs. The data also indicates a direct correlation between enhanced virulence and enhanced CP production. Of particular importance is the finding that nitrogen starved meningococci exhibited a high level of CP synthesis and virulence for mice similar to meningococci grown in low pH/iron limited medium. This data suggests that increased levels of CP appear to be a general virulence mechanism in response to unfavorable environmental conditions rather than a specific response to iron as an essential growth requirement. The data further revealed a correlation between an increase in cell-surface hydrophilicity and an increase in virulence. Since phagocytosis may occur by opsonin-independent mechanisms and is mediated by specific bacterial surface components primarily through their effect on surface hydrophobicity and charge (Van Oss, 1978; Ohman et al., 1982; Peterson et al., 1984), the increase in the polyanionic surface polysaccharide could enhance resistance to phagocytosis by an increase in cell-surface hydrophilicity. The increase in cell-surface hydrophilicity is proportional to the level of CP suggesting that the observed hydrophilicity is probably due to the enhanced synthesis of the polyanionic sialic acid CP. This hypothesis is supported, in part, by the observation by Magnusson et al., (1979b) that iron-induced proteins had little effect on the surface charge of the gonococcus.

This study serves to demonstrate that meningococcal virulence may not be a fixed property of the organism as previously thought but rather that the meningococcus responds to adverse conditions in a manner which increases its virulence for a susceptible host. Interestingly, what originally appeared to

be a beneficial host defense mechanism (i.e. inflammation) may well prove to be a detrimental response under certain circumstances. The latter underlines the adaptability of this rather remarkable pathogen. During pathogenesis, the enhancement of capsular polysaccharide levels by low pH and nutrient limiting conditions may be temporal in nature. Pathogenic bacteria must colonize tissue in order to establish a disease and avoid being removed by various host clearance mechanisms. Indeed, Goldschneider et al., (1969a) demonstrated that colonization of the nasopharyngeal mucosa by the meningococcus precedes invasive disease. However, it has been shown that encapsulation by this organism reduces adherence to human epithelial cells by the demonstration that carrier isolates tend to adhere more avidly than invasive isolates (Salit and Morton, 1981). Similar results have been found with H. influenzae type b whereby two strains isolated from the nasopharynx adhered better to buccal epithelial cells than the same two strains isolated from the cerebrospinal fluid of the same patients (Kaplan et al., 1983). It would appear that a decrease in piliation may be necessary before invasive disease may occur, but whether low pH or nutrient limiting conditions can suppress piliation is only speculative at this time. However, the low virulence of carrier strains may be due to decreased levels of CP since most carrier strains are ungroupable and the lack of CP would render these organisms avirulent consistent with the findings of Holbein, (1981). One may then speculate that an invasive organism, producing lower levels of CP in order to adhere firmly to the nasopharyngeal mucosa but yet able to resist host defense mechanisms, may then multiply causing heavy nasopharyngeal carriage and subsequently an inflammatory response on behalf of the host. Due to lowered pH and limited iron, an invasive meningococcal strain may consequently undergo various phenotypic changes such as increasing

CP synthesis and the production of an efficient iron uptake mechanism. These phenotypic alterations would cause a dramatic increase in its virulence and consequently its capacity to cause invasive disease. Although it is speculative at this time, it is not unreasonable to assume that carrier strains may not possess the ability to adapt rapidly to unfavorable alterations in the host environment and are consequently susceptible to the host defences.

One must exercise caution when interpreting data generated by environmental studies since under varying environmental conditions not examined or not yet identified virulence determinants may also be important. The finding that increased CP levels from nutrient limitation and low pH conditions results in increased virulence is most interesting. However, further work is needed to identify other changes in surface components (e.g. LPS, piliation, outer membrane proteins) and the consequences of these changes in relation to the overall pathogenesis of meningococcal disease. It would appear that other virulence factors may be operating during invasive disease since some carrier strains are groupable but possess low virulence (Holbein, 1981).

When considering the CP as a virulence factor, it is also necessary to take into consideration the actual composition of the polymer. The three major meningococcal serogroups, A, B, C, associated with invasive disease, are acidic linear polymers composed of a single monosaccharide in contrast to the serogroups associated with lesser virulence, Y, W135, and 29E. The latter elaborate polymers of repeating subunits of two different monosaccharides. A similar relationship exists with H. influenzae. Of the six CP types identified for this organism, the two most virulent and invasive types share a similar structure in their respective CP, composed of a neutral sugar,

ribitol, and a phosphodiester unlike the less virulent types (Sutton et al., 1982). These authors demonstrated a relationship among CP composition in this organism, the ability to resist the bactericidal effects of serum and virulence. Unfortunately, little work has been done in this field and thus the relationship between CP composition and structure to virulence is poorly understood. Most convalescent immune sera after meningococcemia demonstrate antibodies directed primarily to outer membrane protein antigens, and the lack of killing by antipolysaccharide antibodies is unique to serogroup B meningococci (Kasper et al., 1973b). This is probably due to the composition of the sialic acid serogroup B CP which is not immunogenic in humans. This polysaccharide could effectively camouflage the outer surface of the meningococcus rendering it less foreign to the host immune system. It is interesting to note the results of Yeung and Mattingly, (1983) demonstrating that the sialic acid moiety greatly enhances the virulence of the type III antigen in group B streptococci suggesting an important biological role for this sugar.

v. Summary.

This study has provided evidence which clearly links the production of the serogroup B meningococcal sialic acid capsular polysaccharide to the virulence of this organism for mice. The results further demonstrated that virulence may not be a "fixed" property of this organism but rather that it may undergo various phenotypic alterations resulting in enhanced virulence and invasive potential in response to unfavorable environmental conditions. One of the most important alterations is enhanced synthesis of the serogroup

B CP which confers resistance to the bactericidal effects of serum and an increase in cell-surface hydrophilicity. Mechanisms providing an explanation for the regulation of meningococcal CP synthesis were demonstrated at the molecular level.

Although this study illuminated various aspects concerning the pathogenesis of meningococcal disease, many questions remain unanswered. Of paramount importance is the physiological differences between carrier and disease isolates which could reveal better clues as to the ability of disease isolates in causing invasive disease, and the role of host defense in its prevention.

A vaccine is urgently required for the prevention of meningococcal disease caused by serogroup B organisms. A clearer understanding of the phenotypic alterations during in vivo growth in the human host may determine which surface antigens would be the best vaccine candidates. Finally, a better understanding of polyisoprenol biochemistry would shed light on what may prove to be a central regulatory mechanism directing the synthesis of outer membrane antigens and virulence determinants for not only the meningococcus but for other invasive pathogens.

LITERATURE CITED

LITERATURE CITED

- Aguero, M. E., and F. C. Cabello. 1983. Relative contribution of Col V plasmid and K1 antigen to the pathogenicity of Escherichia coli. Infect. Immun., 40:359-368.
- Anderson, R. G., H. Hussey, and J. Baddiley. 1972. The mechanism of wall synthesis in bacteria. Biochem. J. 127:11-25.
- Anderson, J. S., M. Matsushashi, M. A. Haskin, and J. L. Strominger. 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. II. Phospholipid carriers in the reaction sequence. J. Biol. Chem., 242:3180-3190.
- Archibald, A. R. 1974. The structure, biosynthesis and function of teichoic acids. Adv. Microbiol. Physiol., 11:53-95.
- Archibald, F. S., and I. W. DeVoe. 1978. Iron in Neisseria meningitidis: minimum requirements, effects of limitation, and characteristics of uptake. J. Bacteriol., 136:35-48.
- Arko, R. J. 1974. An immunological model in laboratory animals for the study of Neisseria gonorrhoeae. J. Inf. Dis., 129:415-455.
- Artenstein, M. S. 1967. Studies on meningococcal meningitis. p. 260-292. In Department of Bacteriology, Annual report, vol. 2. Walter Reed Army Institute of Research, Washington, D. C.

- Artenstein, M. S. 1975. Prophylaxis for meningococcal disease. J. Am. Med. Assoc., 231:1035-1037.
- Artenstein, M. S., H. Schneider, and M. D. Tingley. 1971. Meningococcal infections. I. Prevalence of serogroups causing disease in U. S. Army personnel in 1964-1970. Bull. W. H. O., 45:275-278.
- Artenstein, M. S., P. E. Winter, R. Gold, and C. D. Smith. 1974. Immunoprophylaxis of meningococcal infection. Milit. Med., 139:91-95.
- Ashton, F. E., A. Ryan, B. B. Diena, and C. E. Frasch. 1979. Evaluation of the antiserum agar method for the serogroup identification of Neisseria meningitidis. Can. J. Microbiol., 25:784-787.
- Bae, H. C., E. H. Cota-Robles, L. E. Casida Jr. 1972. Microflora of soil as visualized by transmission electron microscopy. Appl. Microbiol., 23:637-648.
- Banks, H. S. 1948. Meningococcosis. A protean disease. Lancet, ii:635-640.
- Barry, G. T., and W. F. Goebel. 1957. Colominic acid, a substance of bacterial origin related to sialic acid. Nature, 179:206.
- Beachey, E. H. 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Inf. Dis., 143:325-344.

- Bertram, K. C., I. C. Hancock, and J. Baddiley. 1981. Synthesis of teichoic acid by Bacillus subtilis protoplasts. J. Bacteriol., 148:406-412.
- Beuvery, E. C., F. Miedema, R. W. van Delft, J. Haverkamp, A. B. Leussink, B. J. te Pas, K. S. Teppema, and R. J. Tiesjema. 1983. Preparation and physicochemical and immunological characterization of polysaccharide-outer membrane protein complexes of Neisseria meningitidis. Infect. Immun., 40:369-380.
- Bhatti, A. R., K. O'Hara, L. A. White, and L. E. Bryan. 1981. Presence of plasmid deoxyribonucleic acid in Neisseria meningitidis. FEMS Microbiol. Lett., 10:175-177.
- Blacklow, R. S., and L. Warren. 1962. Biosynthesis of sialic acids by Neisseria meningitidis. J. Biol. Chem., 237:3520-3526.
- Bortolussi, R., P. Ferrieri, and P. G. Quie. 1983. Influence of growth temperature of Escherichia coli on K1 capsular antigen production and resistance to opsonization. Infect. Immun., 39:1136-1141.
- Bovre, K., E. Holten, H. Vik-Mo, A. Brondbo, D. Bratlid, P. Bjark, and P. J. Moe. 1977. Neisseria meningitidis infections in northern Norway: an epidemic in 1974-1975 due mainly to group B organisms. J. Infect. Dis., 135:669-672.
- Braatz, J. A., and E. C. Heath. 1974. Role of polysaccharide in the secretion of protein by Micrococcus sodonensis. J. Biol. Chem., 249:2536-2547.

- Brandtzaeg, P., and K. Tolo. 1977. Mucosal penetrability enhanced by serum-derived antibodies. *Nature*, 266:262-263.
- Braun, V. 1973. Molecular organization of the rigid layer and the cell wall of Escherichia coli. *J. Inf. Dis.*, 128:S9-S16.
- Bray, D., and P. W. Robbins. 1967. The direction of chain growth in Salmonella anatum O-antigen biosynthesis. *Biochem. Biophys. Res. Commun.*, 28:334-339.
- Breeze Jr., S. S., and K. C. Hsu. 1971. Techniques of ferritin-tagged antibodies. *Meth. Virol.*, 5:399-422.
- Brener, D., I. W. DeVoe, and B. E. Holbein. 1981. Increased virulence of Neisseria meningitidis after in vitro iron-limited growth at low pH. *Infect. Immun.* 33:59-66.
- Brener, D., J. E. Gilchrist, and I. W. DeVoe. 1977. Relationship between colonial variation and pili morphology in a strain of Neisseria meningitidis. *FEMS Microbiol. Lett.*, 2:157-161.
- Brinton Jr., C. C. 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram-negative bacteria. *Trans. N. Y. Acad. Sci.*, 27:1003-1054.
- Brooks, D., and J. Baddiley. 1969. A lipid intermediate in the synthesis of a poly-(N-acetylglucosamine 1-phosphate) from the wall of Staphylococcus

lactis N. C. T. C. 2102. Biochem. J. 115:307-314.

Brunetti, P., A. Swanson, and S. Roseman. 1963. Enzymatic determination of sialic acid. Meth. Enzymol., 6:465-473.

Buddingh, G. J., and A. D. Polk. 1939. Experimental meningococcus infection of the chicken embryo. J. Exp. Med., 70:485-490.

Bullen, J. J., H. J. Rogers, and E. Griffiths. 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol., 80:1-35.

Bundle, D. R., I. C. P. Smith, and H. J. Jennings. 1974. Determination of the structure and conformation of bacterial polysaccharides by carbon 13 nuclear magnetic resonance. Studies on the group-specific antigens of Neisseria meningitidis serogroups A and X. J. Biol. Chem., 249:2275-2281.

Calver, G. A., C. P. Kenny, and G. Laverne. 1976. Iron as a replacement for mucin in the establishment of meningococcal infection in mice. Can. J. Microbiol., 22:832-838.

Cassidy, J. T., G. W. Jourdan, and S. Roseman. 1965. The sialic acids. VI. Purification and properties of sialidase from Clostridium perfringens. J. Biol. Chem., 240:3501-3506.

Catlin, B. W. 1973. Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing. J.

Inf. Dis., 128:178-194.

Cesarini, J. P., M. Vandekerkov, R. Faucon, and J. Nicoli. 1967. Ultrastructure de la paroi de Neisseria meningitidis. Ann. Inst. Pasteur. (Paris), 113:833-841.

Chen Jr., P. S., T. J. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem., 28:1756-1759.

Colacicco, G., A. R. Buckelew Jr., and E. M. Scarpelli. 1973. Protein and lipid-protein fractions of lung washings: chemical characterization. J. Appl. Physiol., 34:743-749.

Comb, D. G., D. R. Watson, and S. Roseman. 1966. The sialic acids. IX. Isolation of cytidine 5'-monophospho-N-acetylneuraminic acid from Escherichia coli K-235. J. Biol. Chem., 241:5637-5642.

Conte, J. E., and S. L. Barriere. 1981. In Manual of antibiotics and infectious diseases. p. 54-55. Lea and Febiger, Philadelphia, Pa.

Corpe, W. A. 1980. In Adsorption of microorganisms to surfaces. p. 105-144. G. Bitton, K. C. Marshall (eds). Wiley, New York.

Costerton, J. W., and R. T. Irvin. 1981. The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol., 35:299-324.

Costerton, J. W., R. T. Irvin, and K. -J. Cheng. 1981. The role of bacterial cell

surface components in pathogenesis. Crit. Rev. Microbiol., 8:303-338.

Councilman, W. T., F. B. Mallory, and J. H. Wright. 1898. Epidemic cerebro-spinal meningitis and its relation to other forms of meningitis. p. 1-178. Report of the State Board of Health of Massachusetts. Boston.

Craven, D. E., and C. E. Frasch. 1979a. Protection against group B meningococcal disease: Evaluation of serotype 2 protein vaccines in a mouse bacteremia model. Infect. Immun., 26:110-117.

Craven, D. E., and C. E. Frasch. 1979b. Serogroup identification of meningococci by a modified antiserum agar method. J. Clin. Microbiol., 9:547-548.

Craven, D. E., C. E. Frasch, J. B. Robbins, and H. A. Feldman. 1978. Serogroup identification of Neisseria meningitidis: comparison of an antiserum agar method with bacterial slide agglutination. J. Clin. Microbiol., 7:410-414.

Craven, D. E., M. S. Peppler, C. E. Frasch, L. F. Mocca, and P. P. McGrath. 1980. The role of Neisseria meningitidis in the adherence of case and carrier isolates. p. 189-192. In S. Normark and D. Danielsson (ed.). Genetics and immunobiology of pathogenic Neisseria. Norrlands-tryck, Umea.

Cross, A. S., P. Gemski, J. C. Sadoff, F. Orskov, and I. Orskov. 1984. The importance of the K1 capsule in invasive infections caused by Escherichia coli. J. Inf. Dis., 149:184-193.

Danielson, L., and E. Mann. 1806. The first american account of cerebrospinal meningitis. The Medical and Agricultural Register, volume 1.

Devine, L. F., and C. R. Hagerman. 1970. Relationship of serogroups of Neisseria meningitidis. I. Microagglutination, gel diffusion, and slide agglutination studies of meningococcal antisera before and after absorption with RAS-10 strain of meningococci. Infect. Immun. 1:226-231.

Devine, L. F., S. L. Rhode III, and C. R. Hagerman. 1972. Relationship of serogroups of Neisseria meningitidis. II. R variants of Neisseria meningitidis. Infect. Immun., 5:48-54.

DeVoe, I. W., and J. E. Gilchrist. 1973. Release of endotoxin in the form of cell wall blebs during in vitro growth of Neisseria meningitidis. J. Exp. Med. 138:1156-1167.

DeVoe, I. W., and J. E. Gilchrist. 1974. Ultrastructure of pili and annular structures on the cell wall surface of Neisseria meningitidis. Infect. Immun. 10:872-876.

DeVoe, I. W., and J. E. Gilchrist. 1975. Pili on meningococci from primary cultures of nasopharyngeal carriers and cerebrospinal fluid of patients with acute disease. J. Exp. Med., 141:297-305.

DeVoe, I. W., and J. E. Gilchrist. 1976. Localization of tetramethylphenylenediamine-oxidase in the outer cell wall layer in

Neisseria meningitidis. J. Bacteriol., 128:144-148.

DeVoe, I. W., and J. E. Gilchrist. 1978. Piliation and colonial morphology among laboratory strains of meningococci. J. Clin. Microbiol., 7:397-384.

DiRienzo, J. M., K. Nakamura, and M. Inouye. 1978. The outer membrane proteins of gram-negative bacteria: biosynthesis, assembly, and functions. Ann. Rev. Biochem., 47:481-532.

Dopter, C. 1909. Etude de quelques germes isolés du rhinopharynx, voisins du meningocoque (parameningocoques). Compt. Rend. Soc. Biol., 67:74-76.

Duguid, J. P., E. S. Anderson, and I. Campbell. 1966. Fimbriae and adhesive properties in Salmonellae. J. Pathol. Bacteriol., 92:107-138.

Duguid, J. P., and J. F. Wilkinson. 1953. The influence of cultural conditions on polysaccharide production by Aerobacter aerogenes. J. Gen. Microbiol., 9:174-189.

Echarti, C., B. Hirschel, G. J. Boulnois, J. M. Varley, F. Waldvogel, and K. N. Timmis. 1983. Cloning and analysis of the K1 capsule biosynthesis genes of Escherichia coli: lack of homology with Neisseria meningitidis group B DNA sequences. Infect. Immun., 41:54-60.

Edwards, E. A., L. F. Devine, C. H. Sengbusch, and H. W. Ward. 1977. Immunological investigations of meningococcal disease. III. Brevity of group C acquisition prior to disease occurrence. Scand. J. Inf. Dis. 9:105-110.

- Ehrenworth, L., and H. Baer. 1956. The pathogenicity of Klebsiella pneumoniae for mice: the relationship to the quantity and rate of production of type-specific capsular polysaccharide. J. Bacteriol., 72:713-717.
- Ellwood, D. C., and D. W. Tempest. 1972. Effect of environment on bacterial wall content and composition. Adv. Microbiol. Physiol., 7:83-117.
- Farries, J. S., W. Dickson, E. Greenwood, T. R. Malhotra, J. D. Abbot, and D. M. Jones. 1975. Meningococcal infections in Boulton, 1971-1974. Lancet ii:118-121.
- Fearon, D. T. 1978. Regulation by membrane sialic acid of β 1H-dependent decay-dissociation of amplification C3 convertase of the alternate complement pathway. Proc. Nat. Acad. Sci. U. S. A. 78:1971-1975.
- Feingold, D. S. 1969. The serum bactericidal reaction. IV. Phenotypic conversion of Escherichia coli from serum-resistance to serum-sensitivity by diphenylamine. J. Inf. Dis., 120:437-444.
- Feldman, H. A. 1971. Meningococcus and gonococcus: never the twain...well, hardly ever. N. Engl. J. Med., 285:518-520.
- Ferguson, J. H., and O. D. Chapman. 1948. Fulminating meningococcic infections and the so-called Waterhous-Friderichsen syndrome. Am. J. Pathol., 24:763-795.

Fiedler, F., and L. Glaser. 1973. Assembly of bacterial cell walls. *Biochim. Biophys. Acta*, 300:467-485.

Fiedler, F., and L. Glaser. 1974. Purification of polyribitol phosphate polymerase and lipotechoic acid carrier. *J. Biol. Chem.* 249:2690-2695.

Finne, J., M. Leinonen, and P. H. Makela. 1983. Antigenic similarities between brain components and bacteria causing meningitis. *Lancet*, ii:355-357.

Fletcher, M., and G. D. Floodgate. 1973. An electron microscope demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. *J. Gen. Microbiol.*, 74:325-344.

Folch, J., M. Lees, and G. H. Sloanes-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497-509.

Frasch, C. E., and S. S. Chapman. 1972. Classification of Neisseria meningitidis serogroup B into distinct serotypes II. Extraction of type specific antigens for serotyping by precipitin techniques. *Infect. Immun.*, 6:127-133.

Frasch, C. E., and S. S. Chapman. 1973. Classification of Neisseria meningitidis group B into distinct serotypes. III. Application of a new bactericidal inhibition technique to distribution of serotypes among cases and carriers. *J. Inf. Dis.*, 127:149-154.

- Frasch, C. E., and E. C. Gotschlich. 1974. An outer membrane protein of Neisseria meningitidis serogroup B responsible for serotype specificity. J. Exp. Med., 140:87-104.
- Frasch, C. E., R. M. McNelis, and E. C. Gotschlich. 1976. Strain-specific variation in the protein and lipopolysaccharide composition of the group B meningococcal outer membrane. J. Bacteriol., 127:973-981.
- Frasch, C. E., and J. B. Robbins. 1978. Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guinea pig model. J. Exp. Med., 147:629-644.
- Gayda, R. C., L. T. Yamamoto, and A. Markovitz. 1976. Second site mutations in CapR (lon) strains of Escherichia coli K-12 that prevent radiation sensitivity and allow bacteriophage lambda to lysogenize. J. Bacteriol., 127:1208-1216.
- Gayda, R. C., H. Avni, P. E. Berg, and A. Markovitz. 1979. Outer membrane protein a and other polypeptides regulate capsular polysaccharide synthesis in Escherichia coli K-12. Mol. Gen. Genet., 175:325-331.
- Gemski, P., A. S. Cross, and J. C. Sadoff. 1980. K1 antigen-associated resistance to the bactericidal activity to serum. FEMS Letts., 9:193-197.
- Ginsburg, V. 1964. Sugar nucleotides and the synthesis of carbohydrates. Adv. Enzymol., 26:35-88.

- Glaser, L. 1965. The synthesis of teichoic acid. IV. On the regulation of cytidine 5'-diphosphateglycerol concentration. *Biochem. Biophys. Acta.*, 101:6-15.
- Glaser, L., and A. Loewy. 1979. Regulation of teichoic acid synthesis during phosphate limitation. *J. Biol. Chem.*, 254:2184-2187.
- Glaser, L., A. Melo, R. Paul. 1967. Uridine diphosphate sugar hydrolase. Purification of enzyme and protein inhibitor. *J. Biol. Chem.*, 242:1944-1954.
- Glover, J. A. 1920. Cerebrospinal fever: studies in the bacteriology, preventive control and specific treatment of cerebrospinal fever among the military forces 1915-1919, In Med. Res. Council Spec. Series no. 50, Section 20. His Majesty's Stationary Office, London.
- Glynn, A. A., and C. J. Howard. 1970. The sensitivity to complement of strains of Escherichia coli related to their K antigens. *Immunol.*, 18:331-346.
- Gold, R., and F. A. Wyle. 1970. New classification of Neisseria meningitidis by means of bactericidal reactions. *Infect. Immun.*, 1:479-484.
- Gold, R., M. L. Lepow, I. Goldschneider, T. F. Draper, and E. C. Gotschlich. 1978a. Antibody responses of human infants to three doses of group A Neisseria meningitidis polysaccharide vaccine administered at two, four and six months of age. *J. Inf. Dis.*, 138:731-735.

Gold, R., I. Goldschneider, M. S. Artenstein, T. F. Draper, and M. Randolph. 1978b. Carriage of Neisseria meningitidis and Neisseria lactamica in infants and children. J. Inf. Dis., 137:112-121.

Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969a. Human immunity to the meningococcus. I. The role of humoral antibodies. J. Exp. Med., 129:1307-1326.

Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969b. Human immunity to the meningococcus. II. Development of natural immunity. J. Exp. Med., 129:1327-1348.

Goldschneider, I., T. F. Draper, and E. C. Gotschlich. 1978. Antibody responses of human infants to three doses of group A Neisseria meningitidis polysaccharide vaccine administered at two, four and six months of age. J. Inf. Dis., 138:731-735.

Gordon, M. H., and E. G. D. Murray. 1915. Identification of the meningococcus. J. Roy. Army. Med. Corps., 25:411-423.

Gotschlich, E. C. 1975. Development of polysaccharide vaccines for the prevention of meningococcal diseases. Monogr. Allergy, 9:245-258.

Gotschlich, E. C. 1980. The Neisseria. p. 636-643. In B. Davis, R. Dulbecco, H. Eisen, and H. S. Ginsburg. (eds) : Microbiology, Harper and Row Publishers, 3rd edition, Hagerstown, Maryland.

- Gotschlich, E. C., B. A. Fraser, O. Nishimura, J. B. Robbins, and T. -Y. Liu. 1981. Lipid on capsular polysaccharides of gram-negative bacteria. J. Biol. Chem. 256:8915-8921.
- Gotschlich, E. C., I. Goldschneider, and M. S. Artenstein. 1969a. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers. J. Exp. Med., 129:1367-1384.
- Gotschlich, E. C., T.-Y. Liu., and M. S. Artenstein. 1969b. Human immunity to the meningococcus. III. Preparation and immunochemical properties of the group A, group B, and group C meningococcal polysaccharides. J. Exp. Med. 129:1349-1365.
- Govan, J. R. W. 1975. Muroid strains of Pseudomonas aeruginosa: the influence of culture medium on the stability of mucus production. J. Med. Microbiol., 8:513-522.
- Grados, O., and W. H. Ewing. 1970. Antigenic relationship between Escherichia coli and Neisseria meningitidis. J. Inf. Dis., 122:100-103.
- Griffiss, J. M., and M. S. Artenstein. 1976. The ecology of the genus Neisseria. Mount Sinai J. Med., 43:746-761.
- Griffiss, J. M., R. M. Bannatyne, M. S. Artensein, and C. S. Anglin. 1974. Recurrent meningococcal infection with an antigenically identical strain. J. Am. Med. Assoc., 229:68-70.

- Hebeler, B. H., and F. E. Young. 1976. Chemical composition and turnover rate of peptidoglycan of Neisseria gonorrhoeae. J. Bacteriol. 126:1180-1185.
- Heckels, J. E. 1981. Structural comparison of Neisseria gonorrhoeae outer membrane proteins. J. Bacteriol., 145:736-742.
- Heckels, J. E., B. Blackett, J. S. Everson, and M. E. Ward. 1976. The influence of surface charge on the attachment of Neisseria gonorrhoeae to human cells. J. Gen. Microbiol., 96:359-364.
- Higashi, Y., G. Siewert, and J. L. Strominger. 1970. Biosynthesis of the peptidoglycan of bacterial cell walls. XIX. Isoprenoid alcohol phosphokinase. J. Biol. Chem., 245:3683-3690.
- Hildebrandt, J. F., L. W. Mayer, S. P. Wang, and T. M. Buchanan. 1978. Neisseria gonorrhoeae acquire a new principal outer membrane protein when transformed to resistance to serum bactericidal activity. Infect. Immun., 20:267-273.
- Hill, W. R., and T. D. Kinney. 1947. The cutaneous lesions in acute meningococcemia. J. Am. Med. Assoc., 134:513-518.
- Holbein, B. E. 1980. Iron-controlled infection with Neisseria meningitidis in mice. Infect. Immun., 29:886-891.

- Holbein, B. E. 1981. Differences in virulence for mice between disease and carrier strains of Neisseria meningitidis. Can. J. Microbiol., 27:738-741.
- Holbein, B. E., K. W. F. Jericho, and G. C. Likes. 1979. Neisseria meningitidis infection in mice: influence of iron, variations in virulence among strains and pathology. Infect. Immun. 24:545-551.
- Horowitz, M. A., and S. C. Silverstein. 1980. Influence of the Escherichia coli capsule on complement fixation and on phagocytosis and killing by human phagocytes. J. Clin. Invest., 65:82-94.
- Howard, C. J., and A. A. Glynn. 1971. The virulence for mice of strains of Escherichia coli related to the effects of K antigens on their resistance to phagocytosis and killing by complement. Immunol., 20:767-777.
- Hubbard, S. C., and P.W. Robbins. 1980. Synthesis of the N-linked oligosaccharides of glycoproteins. J. Biol. Chem., 255:11782-11793.
- Hughes, R. C., P. D. Palmer, and B. H. Sanford. 1972. Factors involved in the cytotoxicity of normal guinea pig serum for cells of murine tumor TA3 sublines treated with neuraminidase. J. Immunol., 111:1071-1080.
- Ison, C., A. A. Glynn, and S. Bascomb. 1982. Acquisition of new genes by oral Neisseria. J. Clin. Pathol., 35:1153-1157.

Jacobs, S. A., and C. W. Norden. 1974. Pneumonia caused by Neisseria meningitidis. J. Am. Med. Assoc., 227:67-68.

Janssen, W. A., W. D. Lawton, G. M. Fikui, and M. J. Surgalla. 1963. The pathogenesis of plague. I. A study of the correlation between virulence and relative phagocytosis resistance of some strains of Pasteurella pestis. J. Inf. Dis., 113:139-143.

Jennings, H. J., A. K. Bhattacharjee, D. R. Bundle, C. P. Kenny, A. Martin, and I. C. P. Smith. 1977. Structures of the capsular polysaccharides of Neisseria meningitidis as determined by ^{13}C -nuclear magnetic resonance spectroscopy. J. Inf. Dis., 136:S78-S83.

Jennings, H. J., A. K. Bhattacharjee, L. Kenne, C. P. Kenny, and G. Calver. 1980. The R-type lipopolysaccharides of Neisseria meningitidis. Can. J. Biochem., 58:128-136.

Jennings, H. J., G. B. Hawes, G. A. Adams, and C. P. Kenny. 1973. The chemical composition and serological reactions of lipopolysaccharides from serogroups A, B, X, and Y Neisseria meningitidis. Can. J. Biochem., 51:1347-1354.

Jennings, H. J., and C. Lugowski. 1981. Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J. Immunol., 127:1011-1018.

Jephcott, A. E., A. Reyn, and A. Birch-Andersen. 1971. Brief report: Neisseria

gonorrhoeae. III. Demonstration of presumed appendages of cells from different colony types. Acta. Pathol. Microbiol. Scand. Sect. B., 79:437-439.

Johnston, K. H., and E. C. Gotschlich. 1974. Isolation and characterization of the outer membrane of Neisseria meningitidis. J. Bacteriol., 119:250-257.

Kalmanson, G. M., H. J. Harwick, M. Turck, and L. B. Guze. 1975. Urinary tract infection: localization and virulence of Escherichia coli. Lancet, 1:134-136.

Kaplan, S. L., E. O. Mason Jr., and B. L. Weidemann. 1983. Role of adherence in the pathogenesis of Haemophilus influenzae type b infection in infant rats. Infect. Immun., 42:612-617.

Kasahara, M., and Y. Anraku. 1974. Succinate dehydrogenase of Escherichia coli membrane vesicles. J. Biochem., 76:959-966.

Kasper, D. L., J. L. Winkelhake, W. D. Zollinger, B. L. Brandt, and M. S. Artenstein. 1973a. Immunochemical similarity between polysaccharide antigens of Escherichia coli 07:K1(L):NM and group B Neisseria meningitidis. J. Immunol., 110:262-268.

Kasper, D. L., J. L. Winkelhake, B. L. Brandt, and M. S. Artenstein. 1973b. Antigenic specificity of bactericidal antibodies to Neisseria meningitidis. J. Inf. Dis., 127:378-386.

- Kazatchkine, M. D., D. T. Fearon, and K. F. Austen. 1979. Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and β 1H for cell bound C3b. *J. Immunol.*, 122:75-81.
- Kean, E. L., and K. J. Bighouse. 1974. Cytidine 5'-monophosphosialic acid hydrolase. Subcellular location and properties. *J. Biol. Chem.*, 249:7813-7823.
- Kellogg, D. S., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reissig. 1968. Neisseria gonorrhoeae. II. Colonial variation and pathogenicity during 35 months in vitro. *J. Bacteriol.*, 96:596-605.
- Kellogg, D. S., W. L. Peacock, W. E. Deacon, and C. I. Pirkle. 1963. Neisseria gonorrhoeae. I. Virulence genetically linked to clonal variation. *J. Bacteriol.*, 50:1274-1279.
- Kingsbury, D. T. 1967. Deoxyribonucleic acid and homologies among species of the genus Neisseria. *J. Bacteriol.*, 94:870-876.
- Klegerman, M. E., K. M. Boyer, C. K. Papierniak, L. Levine, and S. P. Gotoff. 1984. Type-specific capsular antigen is associated with virulence in late-onset group B streptococcal type III disease. *Infect. Immun.*, 44:124-129.
- Kopmann, H. J., and K. Jann. 1975. Biosynthesis of the O9 antigen of Escherichia coli. The polysaccharide component of E. coli O9:K29-. *Eur. J. Biochem.*, 60:587-601.

- Kornfeld, S., R. Kornfeld, E. F. Neufeld, and P. J. O'Brien. 1964. The feedback control of sugar nucleotide biosynthesis in liver. *Proc. Nat. Acad. Sci. U. S. A.*, 52:371-379.
- Kornfeld, S. J., and A. G. Plaut. 1981. Secretory immunity and the bacterial IgA proteases. *Rev. Inf. Dis.*, 3:521-534.
- Kundig, F. D., D. Aminoff, and S. Roseman. 1971. The sialic acids. XII. Synthesis of colominic acid by a sialyltransferase from Escherichia coli K-235. *J. Biol. Chem.* 246:2543-2550.
- Kuwahara, S. S. 1980. Carbohydrate interference in assays based on the periodate-coupled thiobarbituric acid reagent. *Anal. Biochem.*, 101:54-60.
- Kuwahara, S. S., and M. C. Snetting. 1979. Sialic acid interference with the thiobarbituric acid method for measuring 3-deoxyoctulosonic acid. *Anal. Biochem.*, 100:118-121.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, 227:680-685.
- Lahav, M., T. H. Chiu, and W. J. Lennarz. 1969. Studies on the biosynthesis of mannan in Micrococcus lysodeikticus. II. The enzymatic synthesis of mannosyl-1-phosphoryl-undecaprenol. *J. Biol. Chem.*, 233:5890-5898.
- Lambden, P. R., J. E. Heckels, L. T. James, and P. J. Watt. 1979. Variations in surface protein composition associated with virulence properties in opacity

types of Neisseria gonorrhoeae. J. Gen. Microbiol., 114:305-312.

Lee, T. J., P. D. Utsinger, R. Snyderman, W. J. Yount, and P. F. Sparling. 1978. Familial deficiency of the seventh component of complement associated with recurrent bacteremic infections due to Neisseria. J. Inf. Dis., 138:359-368.

Lifely, M. R., A. S. Gilbert, and C. Moreno. 1981. Sialic acid polysaccharide antigens of Neisseria meningitidis and Escherichia coli: esterification between adjacent residues. Carbohydrate Res., 94:193-203.

Lin, J. J. C., and H. C. Wu. 1980. Assembly of membrane proteins in Escherichia coli. J. Biol. Chem., 255:802-806.

Liu, T.-Y., E. C. Gotschlich, E. K. Jonssen, and J. R. Wysocki. 1971a. Studies on the meningococcal polysaccharides. I. Composition and chemical properties of the group A polysaccharides. J. Biol. Chem., 246:2849-2858.

Liu, T.-Y., E. C. Gotschlich, F. T. Dunne, and E. K. Jonssen. 1971b. Studies on the meningococcal polysaccharides. II. Composition and chemical properties of the group B and Group C polysaccharide. J. Biol. Chem., 246:4703-4712.

Liu, T.-Y., E. C. Gotschlich, W. Egan, and J. B. Robbins. 1977. Sialic acid-containing polysaccharides of Neisseria meningitidis and Escherichia coli strain BOS-12: structure and immunology. J. Inf. Dis., 136:S71-S77.

- Loeb, M. R., A. L. Zachary, and D. H. Smith. 1981. Isolation and partial characterization of outer and inner membranes from encapsulated Haemophilus influenzae type b. J. Bacteriol., 145:596-604.
- Lowell, G. H., L. F. Smith, M. S. Artenstein, G. S. Nash, and R. P. MacDermott. 1979. Antibody-dependent cell-mediated antibacterial activity of human mononuclear cells. I. K lymphocytes and monocytes are effective against meningococci in cooperation with human sera. J. Exp. Med., 150:127-137.
- Lowell, G. H., L. F. Smith, J. M. Griffiss, and B. L. Brandt. 1980. IgA-dependent, monocyte mediated, antibacterial activity. J. Exp. Med., 152:452-457.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
- MacLeod, M. N., and I. W. DeVoe. 1981. Location of carbonic anhydrase in the cytoplasmic membrane of Neisseria sicca (strain 19). Can. J. Microbiol., 27:87-92.
- Magnusson, K.-E., E. Kihlstrom, L. Norlander, A. Norqvist, J. Davies, and S. Normark. 1979a. Effect of colony type and pH on surface charge and hydrophobicity of Neisseria gonorrhoeae. Infect. Immun. 26:397-401.
- Magnusson, K.-E., E. Kihlstrom, A. Norqvist, J. Davies, and S. Normark. 1979b. Effect of iron on surface charge and hydrophobicity of Neisseria gonorrhoeae. Infect. Immun. 26:402-407.

- Makela, P. H., H. Kavhty, P. Weckstrom, A. Sivonen, O. V. Rekonen. 1975. Effect of group A meningococcal vaccine in army recruits in Finland. *Lancet*, ii:883-886.
- Mandrell, R. E., and W. D. Zollinger. 1982. Measurement of antibodies to meningococcal group B polysaccharide: low avidity binding and equilibrium binding constants. *J. Immunol.*, 129:2172-2177.
- Masson, L., and B. E. Holbein. 1983. Physiology of sialic acid capsular polysaccharide synthesis in serogroup B *Neisseria meningitidis*. *J. Bacteriol.*, 154:728-736.
- Masson, L., B. E. Holbein, and F. E. Ashton. 1982. Virulence linked to polysaccharide production in serogroup B *Neisseria meningitidis*. *FEMS Lett.*, 13:187-190.
- McArthur, H. A. I., F. M. Roberts, I. C. Hancock, and J. Baddiley. 1978. Lipid intermediates in the biosynthesis of a linkage unit between teichoic acids and peptidoglycan. *FEBS Lett.*, 86:193-200.
- McLeod, C. M., and M. R. Krauss. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharides formed in vitro. *J. Exp. Med.*, 92:1-9.
- McSharry, J. J., R. J. Pickering, and L. A. Caliguiri. 1981. Activation of the alternate complement pathway by enveloped viruses containing limited amounts

of sialic acid. Virology, 114:507-515.

Melancon, J., R. A. Murgita, and I. W. DeVoe. 1983. Activation of murine B lymphocytes by Neisseria meningitidis and isolated meningococcal surface antigens. Infect. Immun., 42:471-479.

Miller, C. P. 1933. Experimental meningococcal infection in mice. Science, 78:340-341.

Miller, M. A., P. Millikin, P. S. Griffin, R. A. Sexton, and M. Yousaf. 1979. Neisseria meningitidis urethritis - a case report. J. Am. Med. Assoc., 242:1651-1656.

Mooney, J. K., J. J. Mooney, and F. Hinman. 1976. The antibacterial effects of the bladder: an electron microscopic study. J. Urol., 115:381-386.

Morell, A. G., G. Bregoriadis, I. H. Scheinberg, J. Hickman, and G. Ashwell. 1971. The role of sialic acid in determining the survival of glycoproteins in the circulation. J. Biol. Chem., 246:1461-1467.

Moreno, C., J. Hewitt, K. Hastings, and D. Brown. 1983. Immunological properties of monoclonal antibodies specific for meningococcal polysaccharides: the protective capacity of IgM antibodies specific for polysaccharide group B. J. Gen. Micro., 129:2451-2456.

Morse, S. A., and B. H. Hebel. 1978. Effect of pH on the growth and glucose metabolism of Neisseria gonorrhoeae. Infect. Immun., 21:87-95.

- Morse, S. A., C. S. Mintz, S. K. Sarafian, L. Bartenstein, M. Bertram, and M. A. Apicella. 1983. Effect of dilution rate on lipopolysaccharide and serum resistance of Neisseria gonorrhoeae grown in continuous culture. *Infect. Immun.*, 41:74-82.
- Muhlradt, P., J. Menzl, J. R. Golecki, and V. Speth. 1973. Outer membrane of *Salmonella*. Sites of export of newly synthesized lipopolysaccharide on the bacterial surface. *Eur. J. Biochem.*, 35:471-481.
- Mulks, M. H., A. G. Plaut, H. A. Feldman, and B. Frangione. 1980. IgA proteases of two distinct specificities are released by Neisseria meningitidis. *J. Exp. Med.*, 152:1442-1447.
- Munford, R. S., C. M. Patton, and G. W. Gorman. 1975. Epidemiological studies of serotype antigens common to group B and C Neisseria meningitidis. *J. Inf. Dis.*, 131:286-290.
- Munford, R. S., A. Taunay, J. S. de Moraes, D. W. Fraser, and R. A. Feldman. 1973. Spread of meningococcal infection within households. *Lancet*, i:1275-1278.
- Nicholson, A., and I. H. Lepow. 1979. Host defense against Neisseria meningitidis requires a complement-dependent bactericidal activity. *Science*, 205:298-299.
- Nicholson, A. M., and A. A. Glynn. 1975. Investigation of the effect of K antigen in Escherichia coli urinary tract infections by use of a mouse model.

Br. J. Exp. Path. 56:549-553.

Ohman, L., J. Hed., and O. Stendahl. 1982. Interaction between human polymorphonuclear leukocytes and two different strains of fimbriae-bearing Escherichia coli. J. Inf. Dis., 146:751-757.

Ombaka, E. A., R. M. Cozens, and M. R. W. Brown. 1984. Influence of nutrient limitation of growth on stability and production of virulence factors of mucoid and nonmucoid strains of Pseudomonas aeruginosa. Rev. Inf. Dis., 5:S880-S888.

Onderdonk, A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett. 1977. The capsular polysaccharide of Bacteroides fragilis as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. J. Inf. Dis., 136:82-89.

Orskov. I., F. Orskov, B. Jann, and K. Jann. 1977. Serology, chemistry, and genetics of Escherichia coli. Bacteriol. Rev., 41:667-710.

Osborn, M. J.. 1969. Structure and biosynthesis of the bacterial cell wall. Ann. Rev. Biochem. 38:501-538.

Osborn, M. J. 1972. Synthesis of bacterial O-antigens. Methods Enzymol. 28:583-601.

Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of Salmonella typhimurium: isolation and

characterization of cytoplasmic and outer membrane. J. Biol. Chem.,
247:3962-3972.

Ottow, J. C. G. 1975. Ecology, physiology and genetics of fimbriae and pili. Annu.
 Rev. Microbiol., 29:79-108.

Parodi, A. J., and L. F. Leloir. 1979. The role of lipid intermediates in the
 glycosylation of proteins in the eucaryotic cell. Biochim. Biophys. Acta.
659:1-37.

Peetz, R. H., and G. E. Kenny. 1978. Prevention of autolysis in suspensions of
Neisseria gonorrhoeae by mercuric ions. J. Bacteriol.,
135:283-285.

Peltola, H. 1983. Meningococcal disease: still with us. Rev. Inf. Dis., 5:71-91.

Petersen, B. H., T. J. Terrence, R. Snyderman, and G. F. Brooks. 1979. Neisseria
meningitidis and Neisseria gonorrhoeae bacteremia associated with
 C6, C7, or C8 deficiency. Ann. Int. Med., 90:917-920.

Peterson, P. K., G. Gekker, R. Shapiro, M. Freiberg, and W. F. Keane. 1984.
 Polyamino acid enhancement of bacterial phagocytosis by human
 polymorphonuclear leukocytes and peritoneal macrophages. Infect. Immun.,
43:561-566.

Peterson, P. K., and P. G. Quie. 1981. Bacterial surface components and the
 pathogenesis of infectious diseases. Ann. Rev. Med., 32:29-43.

- Plaut, A. G., J. V. Gilbert, M. S. Artenstein, and J. D. Capra. 1975. Neisseria gonorrhoeae and Neisseria meningitidis: extracellular enzyme cleaves human immunoglobulin A. *Science*, 190:1103-1105.
- Pluschke, G., J. Mayden, M. Achtman, and R. P. Levine. 1983. Role of the capsule and the O antigen in resistance of O18:K1 Escherichia coli to complement-mediated killing. *Infect. Immun.*, 42:907-913.
- Pricer, W. E., and G. Ashwell. 1971. The binding of desialylated glycoproteins by plasma membranes of rat liver. *J. Biol. Chem.*, 246:4825-4833.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493-497.
- Reller, L. B., R. R. MacGregor, and H. N. Beaty. 1973. Bactericidal antibody after colonization with Neisseria meningitidis. *J. Inf. Dis.*, 127:56-62.
- Reyn, A. 1974. Gram-negative cocci and coccobacilli; Family Neisseriaceae. p. 427-432. In R. E. Buchanan and N. E. Gibbons (eds) : *Bergey's manual of determinative bacteriology*. Williams and Wilkins. Baltimore.
- Richardson, W. P., and J. C. Sadoff. 1977. Production of a capsule by Neisseria gonorrhoeae. *Infect. Immun.*, 15:663-664.
- Robbins, J. B., G. H. McCracken, E. C. Gotschlich, F. Orskov, I. Orskov, and L. A.

Hanson. 1974. Escherichia coli K1 capsular polysaccharide associated with neonatal meningitis. N. Engl. J. Med., 290:1216-1220.

Robbins, J. B., R. L. Myerowitz, J. K. Whisnant, M. Argaman, R. Schneerson, Z. T. Handzel, and E. C. Gotschlich. 1972. Enteric bacteria cross-reactive with Neisseria meningitidis groups A and C and Diplococcus pneumoniae types I and III. Infect. Immun., 6:651-656.

Rohr, T. E., and F. A. Troy. 1980. Structure and biosynthesis of surface polymers containing polysialic acid in Escherichia coli. J. Biol. Chem., 255:2332-2342.

Roseman, S. 1962. Enzymatic synthesis of cytidine 5'-monophospho-sialic acids. Proc. Nat. Acad. Sci. U. S. A., 48:437-441.

Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Lett. 9:29-33.

Salit, I. E., and G. Morton. 1981. Adherence of Neisseria meningitidis to human epithelial cells. Infect. Immun., 31:430-435.

Schiffer, M. S., E. Oliveira, M. P. Glode, H. G. McCracken Jr., L. M. Sarff., and J. B. Robbins. 1976. Relationship between invasiveness and the K1 capsular polysaccharide of Escherichia coli. Pediatr. Res., 10:82-87.

Schnaitman, C. A. 1970. Comparison of the envelope protein compositions of several

gram-negative bacteria. J. Bacteriol., 104:1404-1405.

Scott, W. A., and E. L. Tatum. 1971. Purification and partial characterization of glucose-6-phosphate dehydrogenase from Neurospora crassa. J. Biol. Chem., 246:6347-6352.

Siewert, G., and J. L. Strominger. 1967. Bacitracin: An inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in biosynthesis of the peptidoglycan of bacterial cell walls. Proc. Nat. Acad. Sci. U. S. A., 57:767-773.

Simonson, C., D. Brener, and I. W. DeVoe. 1982. Expression of a high-affinity mechanism for aquisition of transferrin iron by Neisseria meningitidis. Infect. Immun., 36:107-113.

Sjostedt, S. 1946. Pathogenicity of certain serological types of B. coli. Acta Pathol. Micribiol. Scand., 63:1-148.

Skevakis, L., C. E. Frasch, J. M. Zahradnik, and R. Dolin. 1984. Class-specific human bactericidal antibodies to capsular and noncapsular surface antigens of Neisseria meningitidis. J. Inf. Dis., 149:387-396.

Smit, J., and H. Nikaido. 1978. Outer membrane of gram-negative bacteria. XVIII. Electron microscopic studies on porin insertion sites and growth of cell surface of Salmonella typhimurium. J. Bacteriol., 135:687-702.

Smith, H. 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Rev.,

41:475-500.

Sparkes, B. G. 1983. Immunomodulating activity of meningococcal antigens. Can. J. Microbiol., 29:1611-1618.

Sparling, P. F., and A. R. Yobs. 1967. Colonial morphology of Neisseria gonorrhoeae isolated from males and females. J. Bacteriol., 93:513-518.

Stephens, D. S., and Z. A. McGee. 1981. Attachment of Neisseria meningitidis to human mucosal surfaces: influence of pili and type of receptor cell. J. Inf. Dis., 143:525-532.

Stephens, D. S., and Z. A. McGee. 1983. Association of virulence of Neisseria meningitidis with transparent colony type and low-molecular-weight outer membrane proteins. J. Inf. Dis., 147:282-292.

Stevens, P., C. L. Liu, and L. S. Young. 1980. K-1 antigen content and the presence of an additional sialic acid-containing antigen among bacteremic K-1 Escherichia coli: correlation with susceptibility to opsonophagocytosis. Infect. Immun., 29:1055-1061.

Sturgess, J. M. 1977. The mucous lining of major bronchi in the rabbit lung. Am. Rev. Respr. Dis., 115:819-827.

Smith, H. 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Rev., 41:475-500.

- Sugasawara, R. J., J. G. Cannon, W. J. Black., I. Nachamkin, R. L. Sweet, and G. F. Brooks. 1983. Inhibition of Neisseria gonorrhoeae attachment to HeLa cells with monoclonal antibody directed against a protein II. Infect. Immun., 42:980-985.
- Sutherland, I. W. 1977. In Surface carbohydrates of the procaryotic cell. I. W. Sutherland (ed), New York: Academic.
- Sutherland, I. W. 1982. Biosynthesis of microbial exopolysaccharides. Adv. Microbiol. Physiol., 23:79-150.
- Sutton, A., R. Schneerson, S. Kendall-Morris, and J. B. Robbins. 1982. Differential complement resistance mediates virulence of Haemophilus influenzae type b. Infect. Immun., 35:95-104.
- Svanborg-Eden, C., and L. A. Hansson. 1978. Escherichia coli pili as possible mediators of attachment to human urinary tract epithelial cells. Infect. Immun., 21:229-237.
- Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta, 24:604-611.
- Swanson, L. 1977. Surface components affecting interactions between Neisseria gonorrhoeae and eucaryotic cells. J. Inf. Dis., 136:S138-S143.
- Swanson, J. 1978. Studies on gonococcus infection. XII. Colony color and opacity

variants of gonococci. Infect. Immun., 19:320-331.

Swanson, J. 1978. Studies on gonococcal infection. XIV. Cell wall protein differences among color/opacity colony variants of Neisseria gonorrhoeae. Infect. Immun., 21:292-302.

Swanson, J., S. J. Kraus, and E. C. Gotschlich. 1971. Studies on gonococcal infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med., 134:886-905.

Taubin, H. L., and L. Landsberg. 1971. Gonococcal meningitis. N. Engl. J. Med., 285:504-505.

Taylor, P. W. 1983. Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. Microbiol. Rev., 47:46-83.

Taylor, P. W., and M. K. Robinson. 1980. Determinants that increase the serum resistance of Escherichia coli. Infect Immun., 29:278-280.

Tofte, R. W., P. K. Peterson, Y. Kim, and P. G. Quie. 1979. Opsonic activity of normal human cerebrospinal fluid for selected bacterial species. Infect. Immun., 26:1093-1098.

Tomasi Jr., T. B. 1972. Secretory immunoglobulins. N. Engl. J. Med., 287:500-506.

Tonn, S. J., and J. E. Gander. 1979. Biosynthesis of polysaccharides by procaryotes.

Ann. Rev. Microbiol., 33:169-199.

Troy, F. A. 1979. The chemistry and biosynthesis of selected bacterial capsular polymers. Ann. Rev. Microbiol., 33:519-560.

Troy, F. A., F. E. Frerman, and E. C. Heath. 1971. The biosynthesis of capsular polysaccharide in Aerobacter aerogenes. J. Biol. Chem., 246:118-133.

Troy, F. A., and M. A. McCloskey. 1979. Role of a membranous sialyltransferase complex in the synthesis of surface polymers containing polysialic acid in Escherichia coli. J. Biol. Chem., 254:7377-7387.

Troy, F. A., I. K. Vijay, and N. Tesche. 1975. Role of undecaprenyl phosphate in synthesis of polymers containing sialic acid in Escherichia coli. J. Biol. Chem., 250:156-163.

Tyeryar Jr., F. J., A. L. Quan, A. A. Rene, and E. Weiss. 1974. Phase transition of gonococci in mammalian cell cultures. Infect. Immun., 10:1401-1406.

Vahlne, G. 1945. Serological typing of the colon bacteria with special reference to the occurrence of B. coli in man under normal and pathological conditions, particularly in appendicitis. Acta. Pathol. Microbiol. Scand., 62:1-127.

Van Dijk, W. C., H. A. Verbrugh, M. E. Van der tol, R. Peters, and J. Verhoef. 1979. Role of Escherichia coli K1 capsular antigens during complement

activation, C3 fixation and opsonization. *Infect. Immun.*, 25:603-609.

Van Lenten, L., and G. Ashwell. 1971. Studies on the chemical and enzymatic modification of glycoproteins. A general method for the titration of sialic acid containing glycoproteins. *J. Biol. Chem.*, 246:1889-1894.

Vann, W. F., T.-Y. Liu, and J. B. Robbins. 1978. Cell-free biosynthesis of the O-acetylated N-acetyl neuraminic acid capsular polysaccharide of group C meningococci. *J. Bacteriol.*, 133:1300-1306.

Van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. *Annu. Rev. Microbiol.*, 32:19-39.

Van Snick, J. L., P. L. Masson, and J. F. Heremans. 1978. The involvement of lactoferrin in the hyposideremia of acute inflammation. *J. Exp. Med.*, 140:1068-1084.

Vedros, N. Neisseria Repository Bullitin. U. S. Naval Research Unit no. 1, School of Public Health, University of California, Berkeley, California.

Verbrugh, H. A., W. C. van Dijk, M. E. van Erne, R. Peters, P. K. Peterson, and J. Verhoef. 1979. Quantitation of the third component of human complement attached to the surface of opsonized bacteria: opsonin-deficient sera and phagocytosis-resistant strains. *Infect. Immun.*, 26:808-812.

Verweij-Van Vught, A. M. J. J., J. F. Van den Bosch, F. Namavar, M. Sparrius, and D. M. MacLaren. 1983. K antigens of Escherichia coli and virulence in

urinary-tract infection: studies in a mouse model. J. Med. Microbiol.,
16:147-155.

Vijay, I. K., and F. A. Troy. 1975. Properties of membrane-associated
 sialyltransferase of Escherichia coli. J. Biol. Chem.,
250:164-170.

Vieusseux, G. 1805. Memoire sur la maladie qui a regne a Geneve au printemps de
 1805. J. Med. Chi. Parm., 11:163-168.

Ward, J. B. 1981. Teichoic and teichuronic acids: biosynthesis, assembly, and
 location. Microbiol. Rev., 45:211-243.

Warren, L., and R. S. Blacklow. 1962. Biosynthesis of N-acetylneuraminic acid and
 cytidine 5'-monophospho-N-acetylneuraminic acid in Neisseria
meningitidis. Biochem. Biophys. Res. Commun., 7:433-438.

Warren, L., and H. Felsenfeld. 1962. The biosynthesis of sialic acids. J. Biol.
 Chem., 237:1421-1431.

Watkinson, R. J., H. Hussey, and J. Baddiley. 1971. Shared lipid phosphate carrier
 in the biosynthesis of teichoic acid and peptidoglycan. Nature New Biol.
 (London)., 229:57-59.

Weeke, B. 1973. Rocket immunoelectrophoresis. Scand. J. Immunol., 2(Suppl.
 1):37-46.

- Wegener, W. S., B. H. Hebeler, and S. A. Morse. 1977. Cell envelope of Neisseria gonorrhoeae: relationship between autolysis in buffer and the hydrolysis of peptidoglycan. *Infect. Immun.* 18:210-219.
- Weichselbaum, A. 1887. Ueber die aetiologie der akuten meningitis cerebrospinalis. *Fortschr. Med.*, 5:573-583.
- Weinberg, E. D. 1978. Iron and infection. *Microbiol. Rev.*, 42:45-66.
- Weiner, I. M., T. Higuishi, L. Rothfield, M. Saltmarsh-Andrew, M. J. Osborn, and B. L. Horecker. 1965. Biosynthesis of bacterial lipopolysaccharide. V. Lipid-linked intermediates in the biosynthesis of the O-antigen groups of Salmonella typhimurium. *Proc. Nat. Acad. Sci. U. S. A.*, 54:228-235.
- Weppner, W. A., and F. C. Neuhaus. 1978. Biosynthesis of peptidoglycan. *J. Biol. Chem.*, 253:472-478.
- Whitfield, C., E. R. Vimr, J. W. Costerton., and F. A. Troy. 1984. Protein synthesis is required for in vivo activation of polysialic acid capsule synthesis in Escherichia coli K1. *J. Bacteriol.*, 159:321-328.
- Wiggins, G. L., D. G. Hollis, and R. Weaver. 1973. Prevalence of serogroups and sulfonamide resistance from the civilian population in the United States, 1964-1970. *Am. J. Pub. Health.*, 63:59-65.
- Williams, A. G., and J. W. T. Wimpenny. 1980. Extracellular polysaccharide

biosynthesis by pseudomonas 11264. Studies on precursor-forming enzymes and factors affecting exopolysaccharide production by washed suspensions. J. Gen. Microbiol., 116:133-141.

Williams, R. C., and R. J. Gibbons. 1972. Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. Science, 177:697-699.

Willoughby, E., Y. Higashi, and J. L. Strominger. 1972. Enzymatic dephosphorylation of C₅₅-isoprenylphosphate. J. Biol. Chem., 247:5113-5115.

Wright, A., M. Dankert, P. Fennessey, and P. W. Robbins. 1967. Characterization of a polyisoprenoid compound functional in O-antigen biosynthesis. Proc. Nat. Acad. Sci. U. S. A., 57:1798-1803.

Wyle, F. A., M. S. Artenstein, B. L. Brandt, E. C. Tramont, D. L. Kasper, P. L. Altieri, S. L. Berman, and J. P. Lowenthal. 1972. Immunologic response of man to group B meningococcal polysaccharide vaccines. J. Inf. Dis., 126:514-522.

Yanagawa, R., and K. Otsuki. 1970. Some properties of the pili of Corynebacterium renale. J. Bacteriol., 101:1063-1069.

Yeung, M. K., and S. J. Mattingly. 1983. Isolation and characterization of type III group B streptococcal mutants defective in biosynthesis of the type specific antigen. Infect. Immun., 42:141-151.

- Yeung, M. K., and S. J. Mattingly. 1984. Isolation and characterization of type III group B streptococcal mutants defective in biosynthesis of the type-specific antigen. *Infect. Immun.*, 42:141-151.
- Yurewicz, E. C., M. A. Ghalambor, D. H. Duckworth, and E. C. Heath. 1971. Catalytic and molecular properties of a phage-induced capsular polysaccharide depolymerase. *J. Biol. Chem.*, 246:5607-5616.
- Zollinger, W. D., and R. E. Mandrell. 1977. Outer membrane protein and lipopolysaccharide serotyping of Neisseria meningitidis by inhibition of a solid-phase radioimmunoassay. *Infect. Immun.*, 18:424-433.
- Zollinger, W. D., R. E. Mandrell, and J. M. Griffiss. 1982. Enhancement of immunologic activity by noncovalent complexing of meningococcal group B polysaccharide and outer membrane proteins. p. 254-262. In: L. Weinstein and B. L. Fields (eds). *Seminars in infectious disease*. Vol. IV: Bacterial vaccines. New York: Thieme-Stratton Inc..
- Zollinger, W. D., R. E. Mandrell, J. M. Griffiss, P. Altieri, and S. Berman. 1979. Complex of meningococcal group B polysaccharide and type 2 outer-membrane protein immunogenic in man. *J. Clin. Invest.*, 63:836-848.
- Zollinger, W. D., C. L. Pennington, and M. S. Artenstein. 1974. Human antibody response to three meningococcal outer membrane antigens: comparison by specific hemagglutination assays. *Infect. Immun.*, 10:975-984.

APPENDIX

Figure 24. Standard curve for the determination of protein concentration by the method of Lowry et al., (1951). Bovine serum albumin was used as a standard at the concentrations indicated.

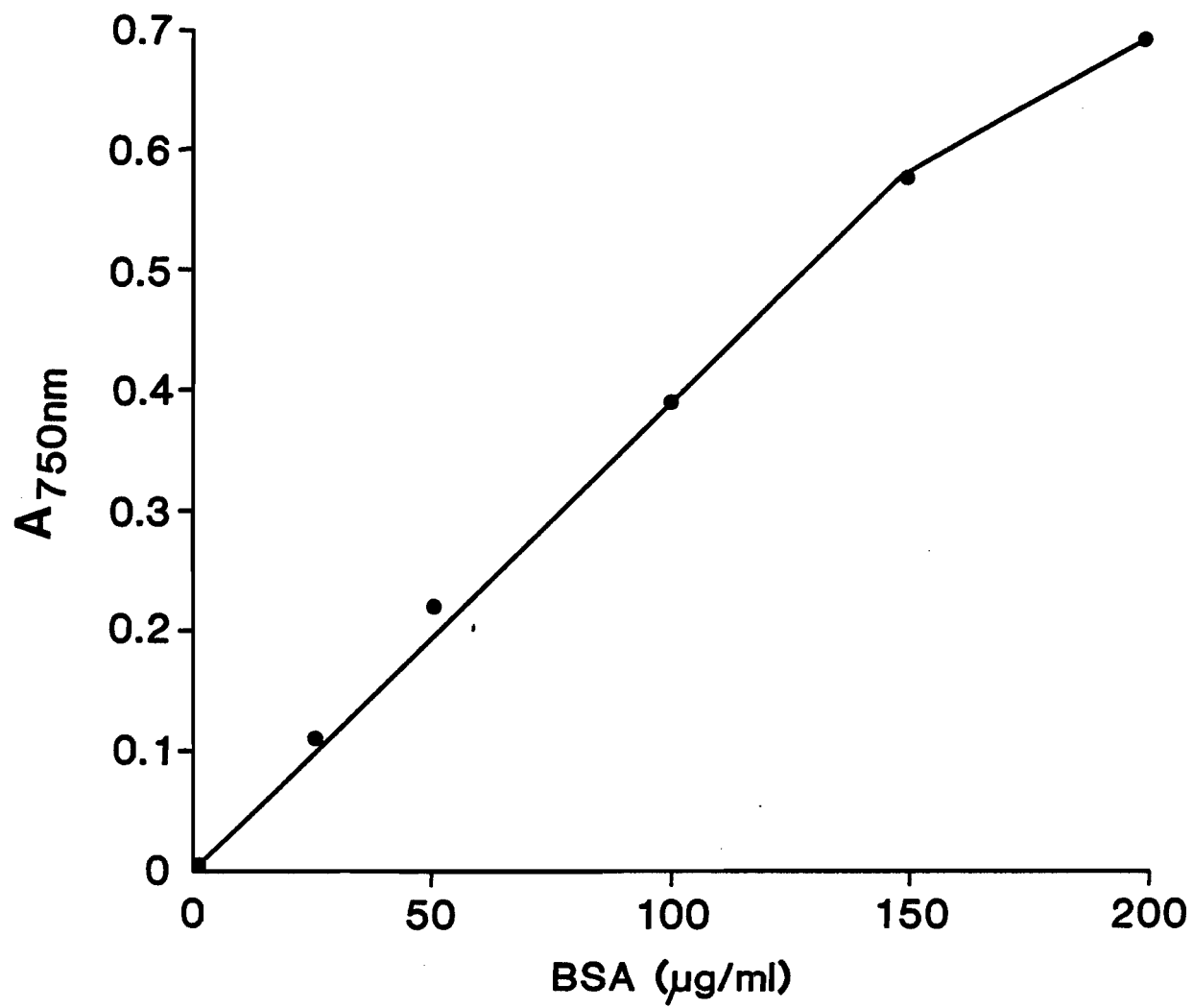


Figure 25. Radioactive decay curve for ^{125}I . Theoretical half-life = 60 days. The calculated half-life of 61 days is in close agreement with the theoretical half-life.

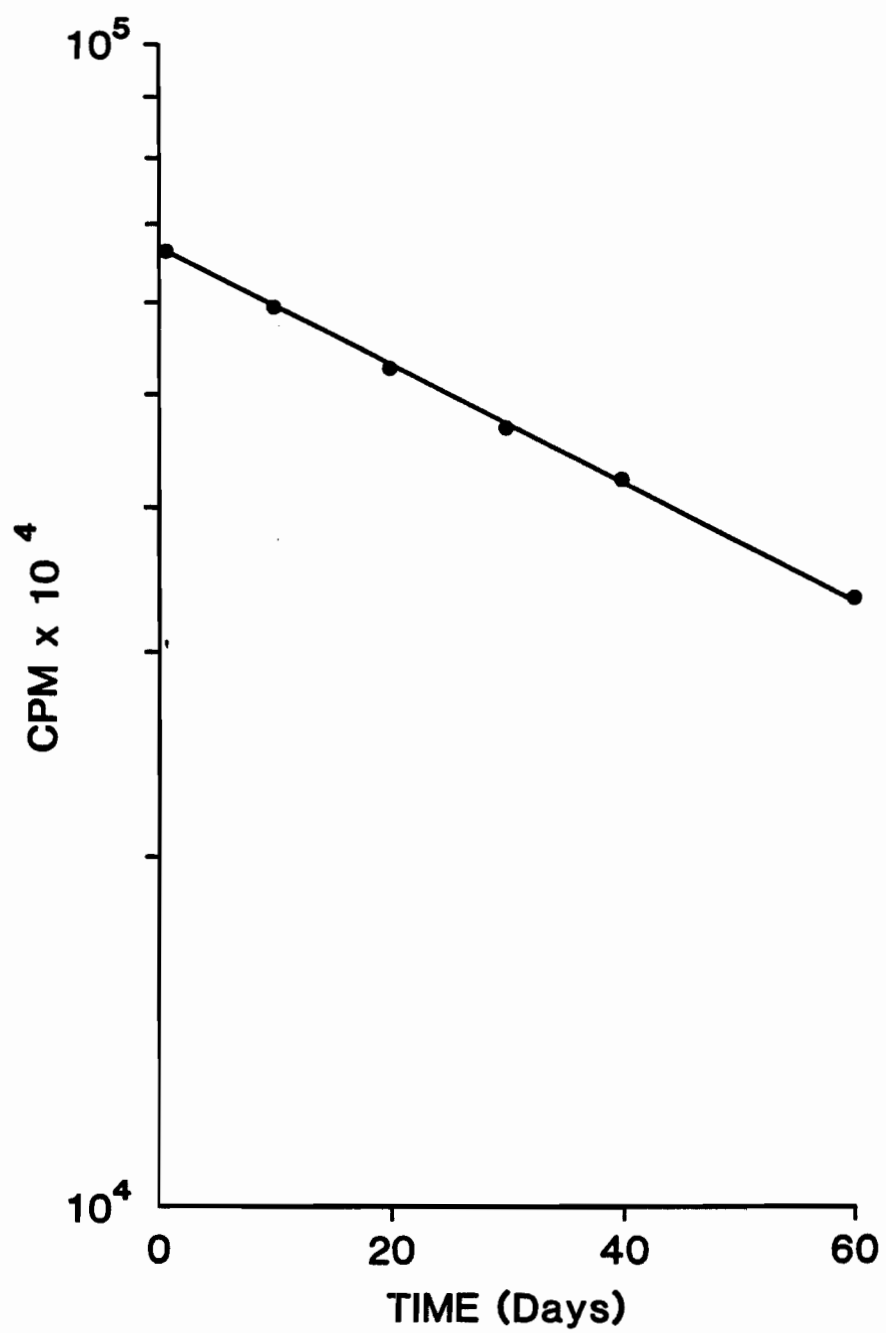


Figure 26. Standard curve for the determination of molecular weights of proteins in Sodium Dodecyl Sulfate-polyacrylamide gels. The protein standards used were: phosphorylase b, 94K; bovine serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 20.1K; lactalbumin, 14.4K.

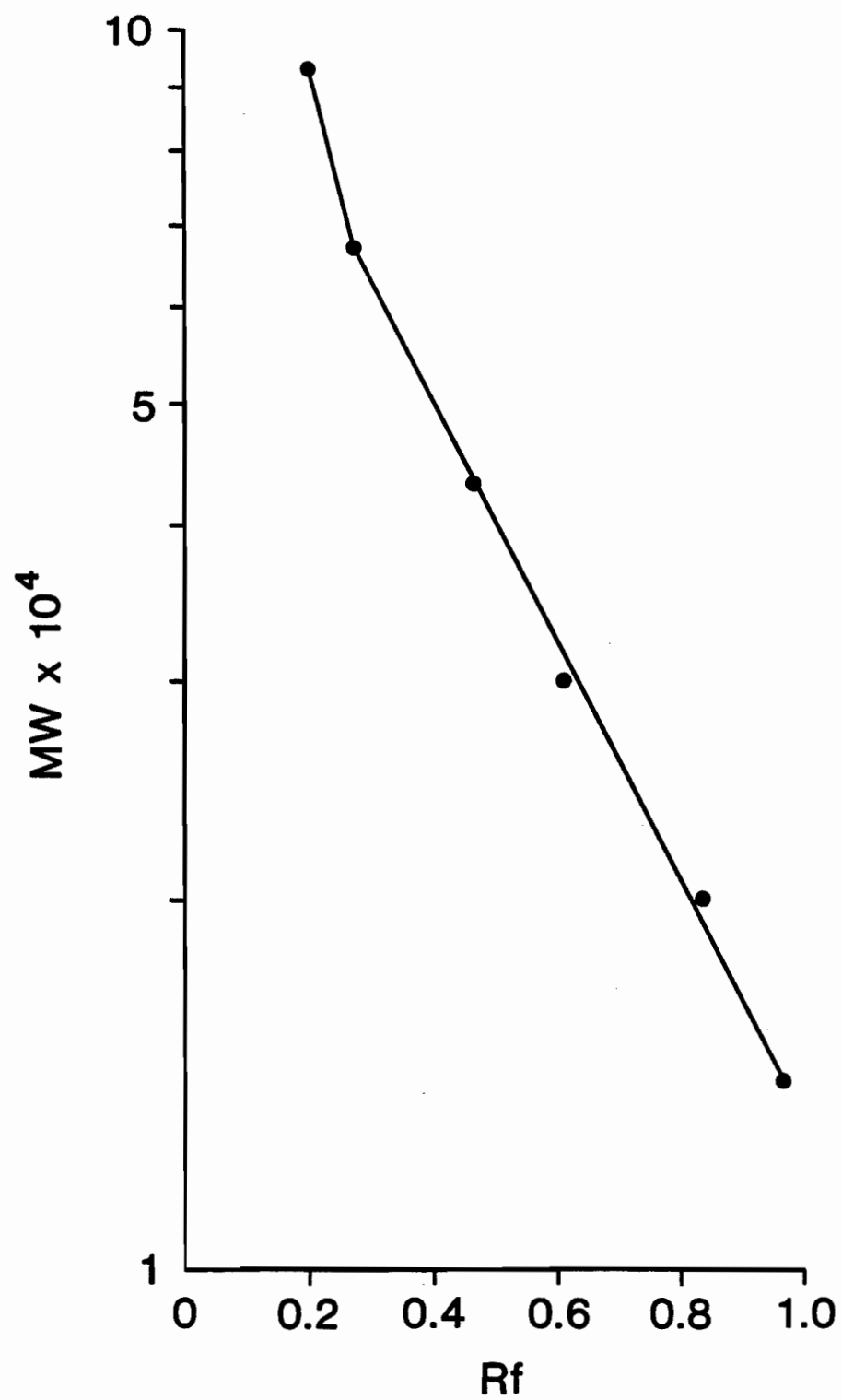


Figure 27. Standard curve for the determination of phosphorus concentration by the method of Chen et al., (1956). KH_2PO_4 was used as a standard at the concentrations of phosphorus indicated.

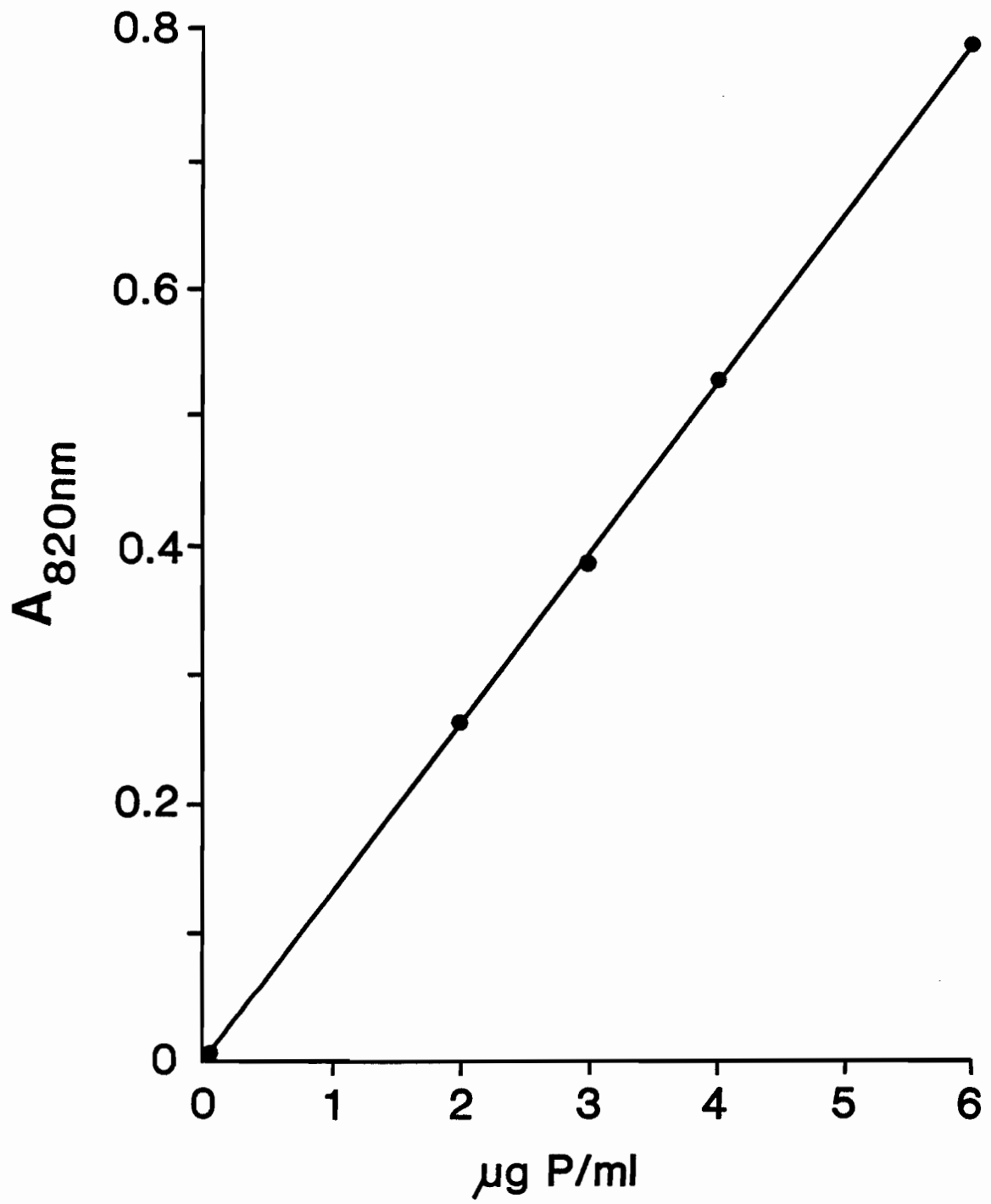


Figure 28. Standard curve for the determination of sialic acid capsular polysaccharide concentration of serogroup B N. meningitidis by the method of Weeke, (1973). Purified sialic acid capsular polysaccharide from N. meningitidis serogroup B was used as a standard at the levels indicated.

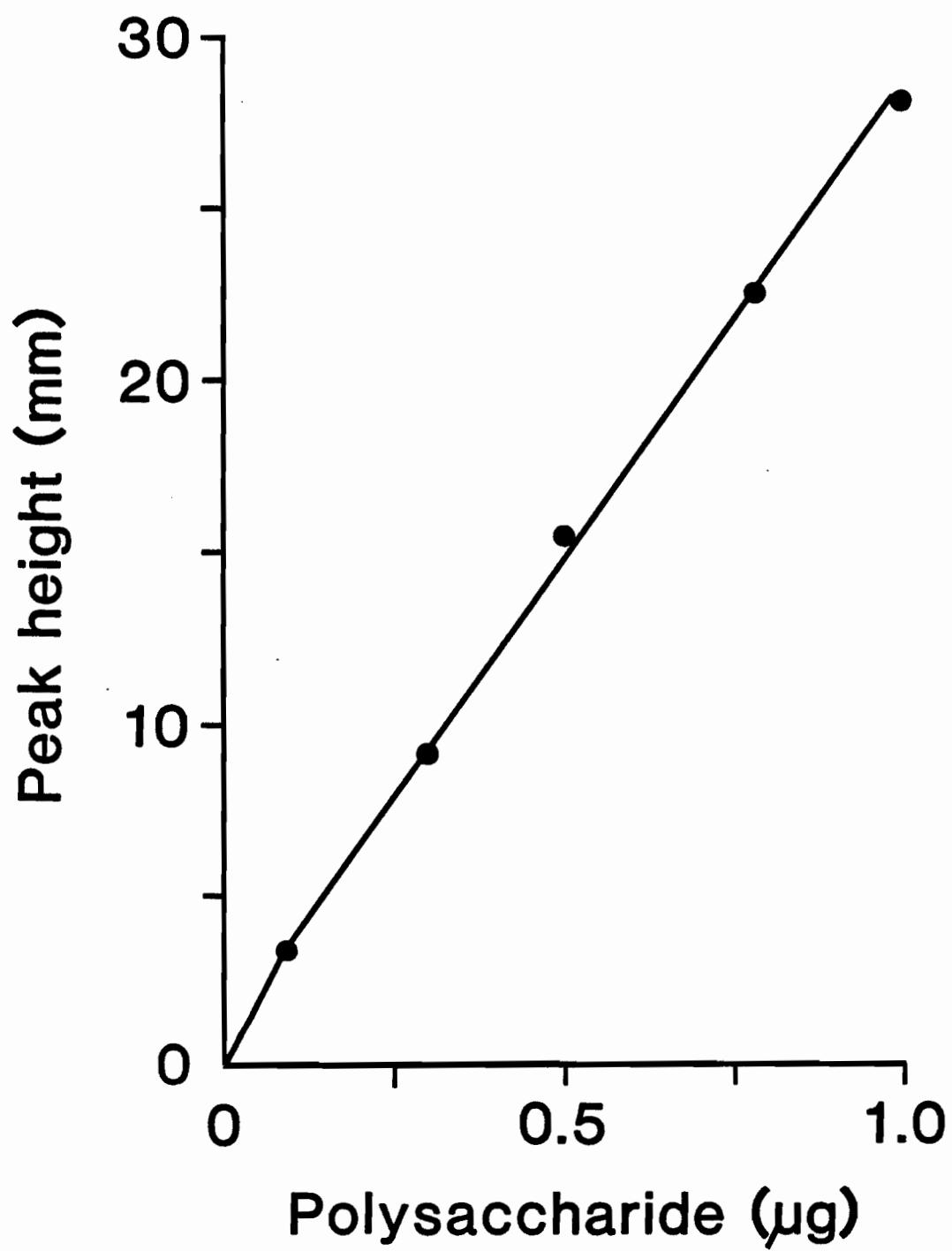


Figure 29. Standard curve for the determination of sialic acid by the method of Svennerholm, (1957). Various concentrations of N-acetylneuraminic acid (●) were measured as indicated. Various concentrations of 2-keto-3-deoxyoctonate (○) were also examined for their reactivity with the HCl-resorcinol reagent.

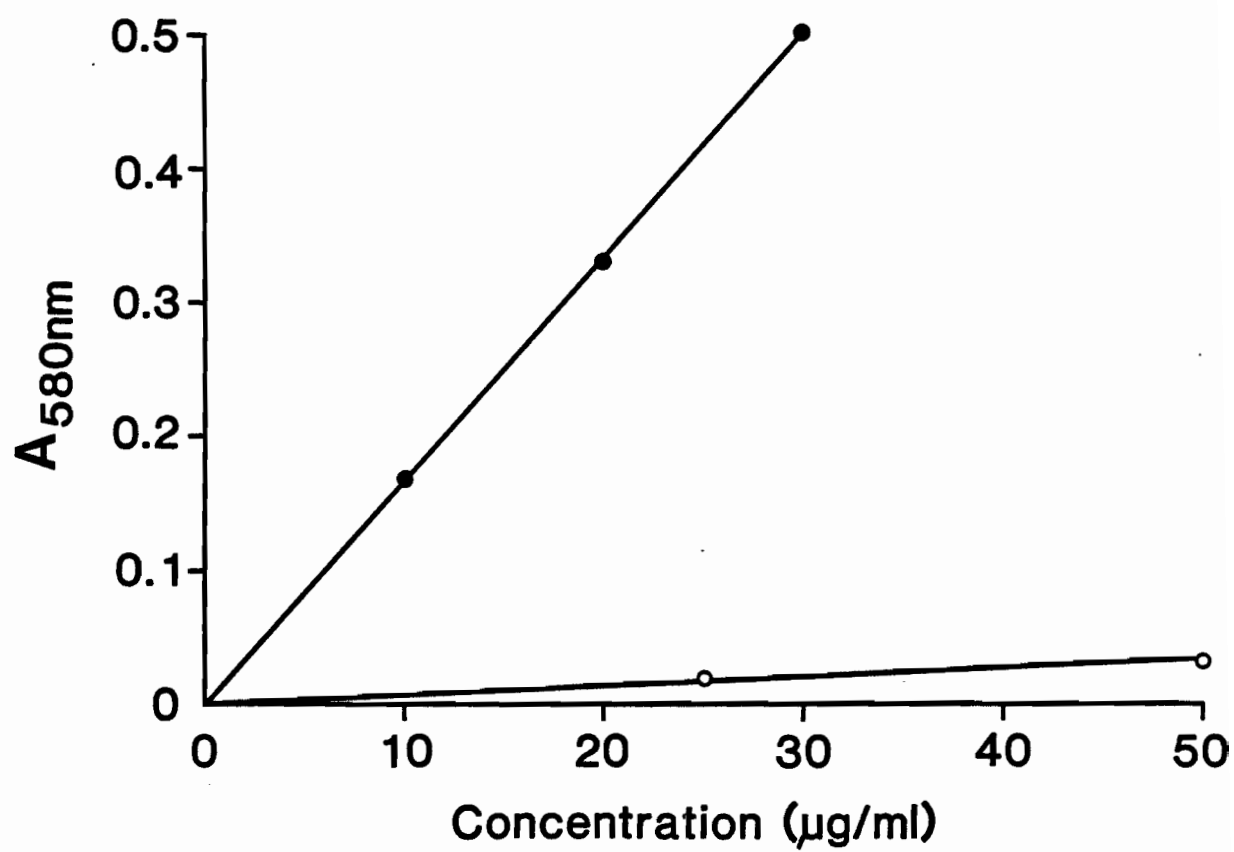


TABLE 13. Purity examination of various strains

Organism	Strain	Serogroup ^a	Gram stain	Morphology
<u>Neisseria meningitidis</u>	M986	B	Gram-negative	diplococcus
<u>Neisseria meningitidis</u>	PRM101	B ^c	Gram-negative	diplococcus
<u>Neisseria meningitidis</u>	PRM102	B ^c	Gram-negative	diplococcus
<u>Neisseria meningitidis</u>	PRM101r	B	Gram-negative	diplococcus
<u>Neisseria meningitidis</u>	M990	B	Gram-negative	diplococcus
<u>Neisseria meningitidis</u>	Dres 03	B ^c	Gram-negative	diplococcus
<u>Neisseria meningitidis</u>	604-A	A	Gram-negative	diplococcus
<u>Neisseria meningitidis</u>	2241-C	C	Gram-negative	diplococcus
<u>Neisseria flava</u>	M953	NG ^d	Gram-negative	diplococcus
<u>Neisseria perflava</u>	ATCC #14799	NG	Gram-negative	diplococcus
<u>Escherichia coli</u> (K1)	O1:K1:HNM	B	Gram-negative	rod
<u>Escherichia coli</u> (K12)	ATCC #10798	NG	Gram-negative	rod

^a Tested by slide agglutination using serogroup A, B or C rabbit antisera.

^b All meningococcal strains examined were able to ferment glucose and maltose but not sucrose or lactose.

^c Light agglutination.

^d NG = nongroupable.

TABLE 14. Composition of Neisseria Defined Medium (NDM-C)^a

Component	Final concentration
L-Glutamic acid	10 mM
D-Glucose	10 mM
L-Cysteine	1 mM
Uracil	1 mM
L-Arginine	1 mM
Trizma base	40 mM
NaCl	140 mM
NaH ₂ PO ₄ ·H ₂ O	1 mM
Na ₂ SO ₄	2.3 mM
CaCl ₂ ·2H ₂ O	500 uM
MgSO ₄ ·7H ₂ O	200 uM
KCl	2 mM
NH ₄ Cl	10 mM
Zn ²⁺ , Cu ²⁺ , Mn ²⁺ (as chlorides)	0.02 uM
Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O	as appropriate
Casein hydrolysate	0.1%

^a Taken from Archibald and DeVoe (1978) and Masson et al. (1982).

TABLE 15. Chromatographic identification of substrates and products of various enzymes involved in sialic acid synthesis

Compound	Method of detection	R_f
Sialic acid polymer	^{14}C	0.00
CMP	UV quenching	0.10
CMP-NANA	^{14}C	0.22
NANA	^{14}C	0.54
Cytidine	UV quenching	0.63
NAM	^3H	0.78

cells grown under nutrient limitation and low pH conditions could be an increase in the synthesis of enzymes involved in CP synthesis. To this end, the two principle enzymes, sialyltransferase and CMP-NANA hydrolase, which are involved in the both synthesis and regulation of the serogroup B polysaccharide, were examined. The data in table 11 suggest that sialyltransferase activity appears not to be inducible by the conditions employed, although cells grown under low pH/iron limiting conditions demonstrated an actual reduction in sialyltransferase activity to a level comparable with the noncapsular mutant strain PRM102. This apparently anomalous result could be partially explained by a decrease in CMP-NANA hydrolase activity. In fact, all cells producing increasingly higher levels of CP than cells grown in Normal NDM-C were found to possess a proportional decrease in CMP-NANA hydrolase activity. Although cells grown in low pH/iron limited NDM-C medium possessed a similar level of sialyltransferase as strain PRM102, the CMP-NANA hydrolase activity was much reduced. This reduction would help to explain, in part, why there was a significant increase in CP content in cells grown under conditions of nutrient limitation and low pH. The possibility that other enzyme activities involved in CP synthesis (i.e. NANA condensing enzyme, CMP-NANA synthetase) had been altered, was not investigated and cannot not be ruled out.

To ascertain whether enhanced polysaccharide production correlated with enhanced virulence of N. meningitidis strain M986 for mice, cultures were grown under conditions of nitrogen limitation, low pH and low pH/iron limitation. The data presented in table 12 revealed that cells enhanced in their ability to produce CP were more virulent for mice. Therefore our findings were consistent with those of Brener et al., (1981), in that cells grown in low pH/iron limited medium were the most virulent for mice; the decrease in LD₅₀ being almost two orders of magnitude.

the growth of strains M986 and PRM102 in batch cultures of NDM-C medium. Although there have been several reports in the literature concerning interference of 2-keto-3-deoxyoctonate (KDO) with assays developed for the determination of sialic acid (Kuwahara and Snetting, 1979; Kuwahara, 1980), KDO did not interfere with the determination of NANA by the HCl-resorcinol method (see appendix, Figure 29). A comparative analysis of the synthesis and release kinetics of sialic acid during growth revealed that sialic acid was produced during the exponential phase of growth of strain M986 and significant release of the polysaccharide to the surrounding medium occurred only when the culture has entered the stationary phase of growth (Figure 2). There was no net synthesis of sialic acid after cessation of growth and the appearance of sialic acid in the culture medium corresponded with a loss of cell-associated sialic acid. Released polysaccharide was non-dialyzable thus having a relative molecular weight (M_r) greater than 12000. Glucose-6-phosphate dehydrogenase was also released during the stationary phase suggesting that the release of polysaccharide may have been related to cellular autolysis (data not shown). A similar pattern of growth, sialic acid synthesis and sialic acid release was obtained with strain PRM101r. All the sialic acid which was cell-associated after seven hours of growth of strain M986 was released by sonication of the bacterial cells and this material was soluble at $220000 \times g$ and was non-dialyzable. This evidence indicated that the cell-associated sialic acid was easily sheared from the cells as free high M_r polysaccharide. Strains PRM102 and PRM101 exhibited similar patterns of growth and polysaccharide synthesis and release, therefore only the results obtained with PRM102 are shown in figure 2. It is important to note that CP production appears unessential for growth as evidenced by the similar rates of growth between strains PRM102 and M986 in NDM-C. There was evidence for synthesis of cell-associated sialic acid by PRM102 during the



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