A PROCEDURE FOR ISOLATION AND CHARACTERIZATION OF OUTER MEMBRANE COMPONENTS FROM *NEISSERIA GONORRHOEAE*

by

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A THESIS

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INTRODUCTION

Gonorrhea, a disease that has been known since ancient times, is one of the most prevalent bacterial diseases in modern society. According to the Center for Disease Control, the incidence of gonorrhea is the highest of any reportable disease in the United States (98). This high incidence has, in part, been responsible for rejuvenating studies on the causative agent, Neisseria gonorrhoeae.

The discovery of penicillin and its subsequent use in the treatment of gonorrhea led to a marked reduction in research involving the gonococcus. However, the high incidence of gonorrhea coupled with the development of increased resistance to antibiotics, has led to a resurgence in studies on the biology of the gonococcus. This is especially true in light of the recent appearance of penicillinase-producing strains of N. gonorrhoeae (99).

Kellogg et al. (48) reported the existence of four colonial variants of N. gonorrhoeae which have been designated T1, T2, T3, and T4. This finding was confirmed by Reyn et al., who reported the existence of still another colonial variant, designated T5 (81). Based on studies with human volunteers, cells from colony types 1 and 2 are considered virulent, while cells from colony types 3 and 4 are considered avirulent (48, 49). Maintenance of virulent colony types requires selective in vitro passage to prevent the conversion of T1 and T2 colonies to T3 and T4 colonies (48).

In 1975, Chan et al. reported a new colonial variant, designated as $T1^1$, which exhibited characteristics of T1, T2 and T5 colonial variants (19). The morphology and properties of the gonococcal colonial variants are shown in Table 1.

Considerable attention has been given to cell surface components thought to be related to the virulence of the gonococcus. Jephcott et al. (43) demonstrated by electron microscopy that T1 and T2 gonococci possess pili on their surfaces, while T3 and T4 cells lack these appendages. This was also confirmed by Swanson et al. (94). Therefore, the presence of pili like colony type 1 and 2 morphology was associated with virulence.

Pili are approximately 80 Å in diameter and extend up to 2 microns in length from the cell surface (43). Many studies have investigated the attachment properties of piliated gonococci. Piliated gonococci attached more readily than non-piliated organisms to erythrocytes (16, 52, 80, 100), tissue culture cells (93, 95), human vaginal epithelium (61), cultured human fallopian tube (102), and human sperm cells (41). Piliated gonococci were also shown to be more virulent for chimpanzees (10, 58), chick embryos (15, 17), and more resistant to phagocytosis by human polymorphonuclear leukocytes (71, 97) than non-piliated cells.

These findings, which associated piliated gonococci with virulence, stimulated further investigation into methods for isolating and purifying pili. Gonococcal pili are composed of protein and have an aggregate molecular weight of 1 to 20 million daltons (12, 14).

Table 1. Morphology and properties of gonococcal colonial variants. Data taken from references 19, 43, 48.

Character	Colonial type 1	1[1]	2	3	4	5
Average diameter (mm)	0.5-0.7	0.7	0.5-0.7	1.0-1.4	1.0-1.4	1.4
Elevation (convexity)	++	++	++	+	+	+
Reflection of light	+	++	++	-	-	+
Surface granularity	-	++	+	+	-	++
Edge	Entire	Slightly crenated	Entire	Entire	Entire	Slightly crenated
Opacity	Translucent	Opaque	Translucent	Translucent	Translucent	Opaque
Color	Grey-gold	Very dark gold	Dark gold	Light brown	Colorless	Brown
Structure	Amorphous	?	Amorphous	Granular	Amorphous	Granular
Consistency	Viscid	?	Friable	Viscid	Viscid	?
Auto-agglutinability in saline	+	++	+	-	-	++
Hemadsorption with rabbit erythrocytes	+	+	+	-	-	-
Hemagglutination	+	+	+	-	-	+/-
Presence of pili	+	+	+	-	-	-
Competent for transformation	+	?	+	-	-	?
Approximate LD_{50} for chick embryos inoculated intravenously[b]	$<10^4$	$<10^3$	$<10^2$	$<10^7$	$<10^7$	$<10^7$

a. As observed under light conditions described by Kellogg et al. (48). Cells were grown on GC base agar containing glucose and defined supplement (48) for 20-24 hrs in 5% CO_2 in air, at 37 C.

b. Approximate dose in colony-forming units at 72 hrs.

The weight of the monomeric pilus protein is approximately 19,000 ± 2500 daltons and varies slightly for different strains of gonococci (78). Much remains to be elucidated about the nature of pili: such as its biosynthesis and assembly, antigenic regions, and the nature of pili attachment (13).

Novotny and Turner (70) have shown by immunoelectron microscopy that although cells of colony types 3 and 4 do not possess pili, they possess pili antigens in their cell envelopes. This suggests that pili may arise from the cell envelope of N. gonorrhoeae, but are not expressed phenotypically in T3 and T4 cells.

Because of their location on the exterior of the gonococcus and their accessibility to the hosts's immune mechanisms, pili and other antigenic components of the cell envelope are of interest as potential immunoprophylactic agents. Therefore, methods for preparing relatively pure membrane fractions would facilitate this approach. Several investigators have developed techniques for this purpose, which will be discussed later in this section.

Cell envelopes of Gram-Negative Bacteria. There are distinct differences between cell walls of gram-positive and gram-negative bacteria. A typical gram-positive cell wall consists primarily of a thick peptidoglycan layer (200-800 Å), which surrounds the cytoplasmic membrane. The remainder of the wall is generally composed of polysaccharides and polyphosphate polymers (teichoic acids). While teichoic acids are not necessarily important in determining the shape of the cell, they have

been implicated as bacteriophage receptors (20, 34, 108). Considerable evidence has accumulated demonstrating a covalent linkage between peptidoglycan and cell wall polysaccharides and teichoic acids (32, 40, 51). Membrane teichoic acids extend from membrane glycolipid through the peptidoglycan layer to the cell surface and may be involved in the binding of divalent cations (25, 39). The peptidoglycan layer helps provide rigidity and support to the cell and aids in maintaining its integrity in spite of an internal osmotic pressure of 5-20 atmospheres resulting from active transport processes (42).

In contrast to the gram-positive cell wall, the cell envelope of gram-negative bacteria is a more complex structure, consisting of a cytoplasmic membrane, peptidoglycan layer, and a lipopolysaccharide-containing outer membrane. The greater structural complexity of the gram-negative envelope has made the task of isolating and defining the chemical structure of individual components more difficult. The integrity of the gram-negative cell envelope is dependent upon weak interactions including hydrophobic bonding of various molecular species as well as covalent bonding between some of the macromolecular species (29). The separation and purification of gram-negative cell envelope components presents certain difficulties.

Cytoplasmic (Inner) Membrane. Both gram-negative and gram-positive bacteria have cytoplasmic membranes surrounding their intracellular contents. This structure is responsible for maintaining the osmotic stability of the cell. The cytoplasmic membrane is comprised of 53-75%

protein and 20-30% lipid (22, 37) and constitutes between 10 and 25% of the dry weight of the cell (87).

Freeze-etching studies revealed that the frozen cytoplasmic membrane cleaves along a median hydrophobic zone traversed by protein "studs" (2, 68). These protein "studs" are thought to be permease molecules (26). The cytoplasmic membrane plays a vital role in the life of the bacterial cell. The biosynthesis of peptidoglycan precursors (83), lipopolysaccharide (38, 77) and phospholipid (3, 38, 76, 105) occur in the cytoplasmic membrane. Other enzymes have also been located within the cytoplasmic membrane including reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase, succinic dehydrogenase, malic oxidase, lactic dehydrogenase, cytochromes and ATPase (46, 63, 85).

Peptidoglycan. The peptidoglycan layer, although much thinner in gram-negative organisms (15-20 Å) than in gram-positive organisms (33), can be considered as a simple macromolecule of high tensile strength surrounding the cytoplasmic membrane. Peptidoglycan is comprised of glycan strands that are interconnected by peptide chains. Despite many variations there is a considerable consistency of peptidoglycan structure among bacteria (33).

The glycan moiety consists of alternating β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid residues. The only variations encountered involve phosphorylation or acetylation of the muramyl 6-hydroxyl groups (31).

Short peptide substituents consisting of a limited group of amino acids interconnect the glycan strands. The peptide subunit is bound through its N-terminus to the carboxyl group of muramic acid, and usually consists of four alternating L- and D-amino acids (33). Most frequently, L-alanine is bound to muramic acid by means of an amide linkage, followed by D-glutamic acid which is linked to a diamino acid (such as lysine in gram-positive cells or meso-diaminopimelic acid in gram-negative cells), which in turn is linked to a terminating D-alanine. Interpeptide bridges sometimes cross-link the primary peptide subunits. Ghuysen (31) enumerates different categories of peptidoglycan, designated chemotypes I-V. These chemotypes are based on the amino acids and the types of cross-bridging present.

All gram-negative peptidoglycans so far tested appear to be of chemotype I (Figure 1). In this chemotype, the peptide subunits are composed of L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanine units, some of which are, in turn, cross-linked through C-terminal D-alanyl-meso-diaminopimelic acid linkages (31, 62). Peptidoglycans from Moraxella glucidolytica, Pseudomonas alcaligenes, Neisseria perflava and Proteus vulgaris differ from that of E. coli in that the former peptidoglycans are O-acetylated at the C6 position, while the latter is not. Cross-linking varied greatly among the peptidoglycans studied.

Outer membrane. The outer membrane differs from the cytoplasmic (inner) membrane in both structure and function. Both membranes are

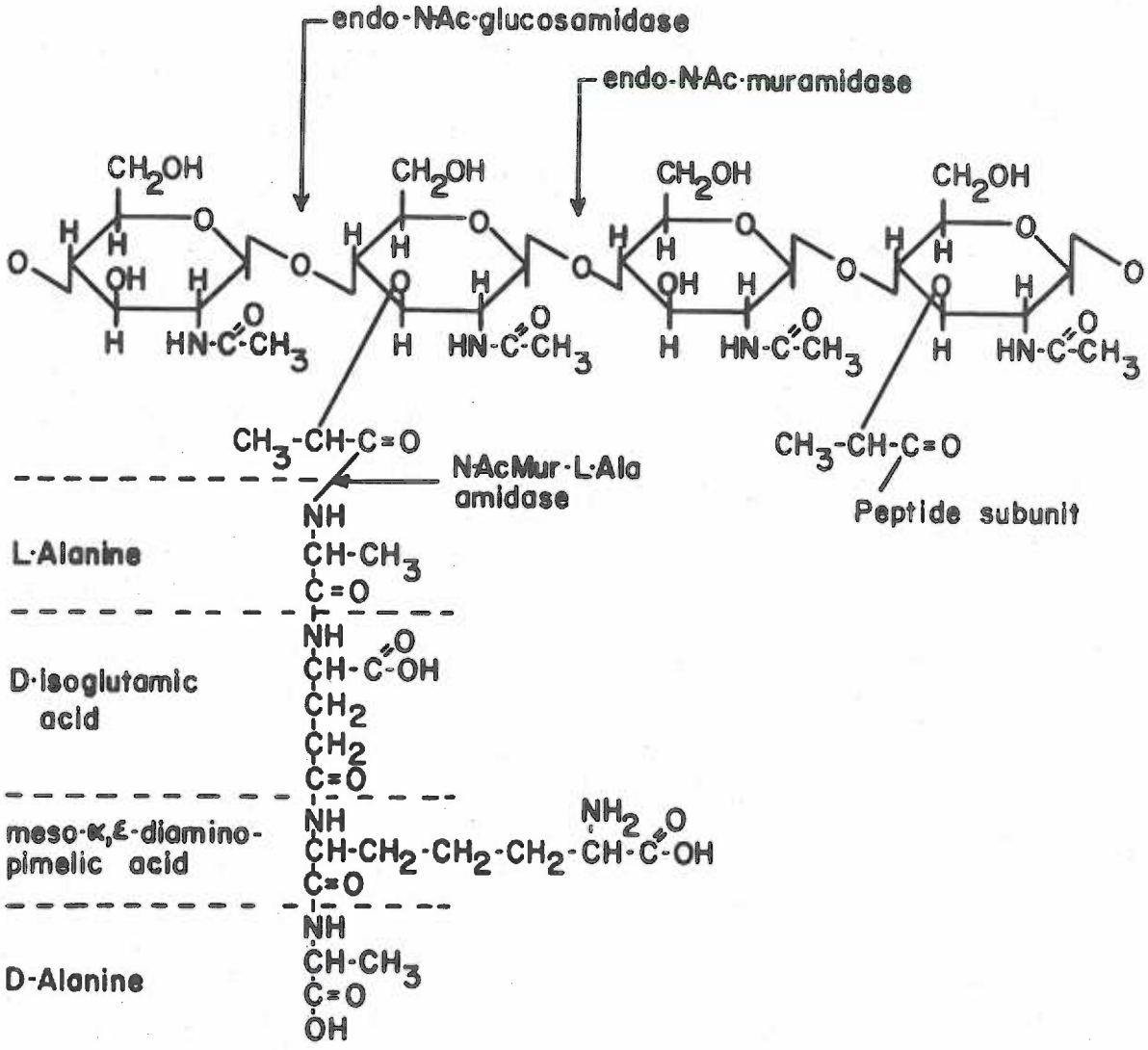


Figure 1. Structure of gram-negative peptidoglycan.

Drawing courtesy of Dr. Bruce Hebeler.

similar in their protein-phospholipid bilayer appearance, but the outer membrane is more dense when sedimented by sucrose density gradient centrifugation, owing to its lipopolysaccharide (LPS) content (22).

Variation exists among gram-negative organisms as to the means of attachment of their outer membrane to the rest of the cell envelope. Braun and Rehn (8) described a lipoprotein in the cell envelope of E. coli that is covalently attached to the peptidoglycan and extends into the outer membrane, forming membrane-peptidoglycan bridges. Further investigation has revealed lipoprotein linkages in Salmonella typhimurium and Serratia marcescens (9), while none could be demonstrated in Proteus vulgaris, P. mirabilis, Pseudomonas fluorescens, or Neisseria gonorrhoeae (9, 107). Those organisms lacking a covalently-linked lipoprotein evidently retain their outer membrane by a weaker, unknown mechanism, because under some conditions of chemical fixation the outer membrane is seen under the electron microscope as a loose, wrinkled layer (22).

The outer membrane appears to function as a non-selective permeability barrier (86) to moderate- and large-sized molecules. DeVoe et al. demonstrated that cells from which the outer membrane had been removed were able to transport a specific amino acid analogue suggesting that the outer membrane is not involved in active transport (23).

In many gram-negative bacteria, lysozyme cannot enter and attack the peptidoglycan unless the outer membrane is first treated with ethylenediaminetetraacetic acid (EDTA) (5, 22, 106). Gram-negative bacteria are also impermeable to actinomycin D unless treated with EDTA (53, 55).

When unsaturated fatty acids are substituted for saturated ones in the outer membrane, permeability of the cell to lysozyme increases, as does the ease of periplasmic enzyme release following osmotic shock (84). All of this evidence points to the role of the outer membrane as a permeability barrier.

Lipopolysaccharide (LPS) is a component of the outer membrane which has an important role in its function. This was shown by a comparison of isogenic strains of S. typhimurium which differ only in that the rough strain has a specific and well-understood defect in its LPS (56). This single defect, which shortens the chain of sugars distal to lipid A, produces a marked increase in the inward penetration of antibiotics and lysozyme as well as an increase in the release of periplasmic enzymes during growth (21, 56).

LPS molecules consist of long-chain phosphate-containing heteropolymers which compose a three-part structure. Region I is represented by the O-specific sidechains, consisting of repeating oligosaccharide units containing specific linkages. In this manner, these O-specific sidechains carry the immunological specificities of the O-antigen, and enable the differentiation of the lipopolysaccharides of the various serotypes (60).

The O-specific chains are linked to Region II, or the basal core. The basal core can be divided into two sections: (a) an innermost backbone region which is linked to lipid A and contains L-glycero-D-mannoheptose, 2-keto-3-deoxyoctonate (KDO), phosphate, and ethanolamine; and (b) an outer core which is composed of sugar residues which

vary among bacterial groups (69, 75). Although the most common sugars in bacterial LPS include glucosamine, KDO, galactose, glucose, and L-glycero-D-mannoheptose, many more sugars are found less commonly. For this reason, lipopolysaccharides have been classified into over 50 chemotypes according to their sugar composition (60, 72).

The structure of the core oligosaccharide seems to vary little among Salmonella (60); however, deviations are apparent as more bacterial groups are compared. The overall trend appears to be that the outer core consists of a pentasaccharide that varies in composition between groups of bacteria, while the inner core is usually composed of two heptose residues hooked to three KDO molecules (22). Attached to heptose and KDO are phosphate residues that are linked to ethanolamine molecules (69).

The core oligosaccharide is connected to region III, lipid A, by a ketosidic linkage through KDO. The lipid A of Salmonella is a glycophospholipid consisting of β-1,6-D-glucosamine disaccharide units, connected by 1-4-pyrophosphate bridges. To the hydroxy and amino groups of glucosamine residues are linked fatty acids such as lauric (12:0), myristic (14:0), palmitic (16:0) and D-β-hydroxymyristic acid (22, 60). Lipid A is a common constituent of both S and R strains of bacteria, R mutants being deficient in their ability to synthesize a complete lipopolysaccharide moiety. It has been suggested that the hydrophobic lipid A portion of the LPS molecule associates with the hydrophobic zone of the outer membrane while the polysaccharide portion of the molecule projects from the inner and outer surfaces (21).

If portions of the LPS molecules do project from the surface of the cell, then any deletion (i.e., those observed in R-mutant strains) would shorten these projections as well as the thickness of the barrier imposed by them. Reynolds and Pruul (82) propose that an intact barrier of O-antigen bearing polysaccharide chains could protect smooth strains of gram-negative bacteria by reacting with antibodies and complement at a defined distance from the outer membrane. In this way, the outer membrane would be protected from injury caused by the action of specific antibody and complement. If, however, the polysaccharides are shortened or absent as a result of mutation, these cells would be more susceptible to complement-mediated damage. It has also been shown (82) that tris(hydroxymethyl)-aminomethane or EDTA treatment renders smooth strains more susceptible to immune sera, suggesting the protective value of an intact LPS molecular network.

Another function attributed to LPS is as bacteriophage or bacteriocin receptors. The specificity is thought to reside in the terminal sugar portions of the LPS molecule (21).

Several studies on the LPS of Neisseria species have yielded conflicting results. Stead et al. (92) analyzed and compared LPS extracted from several strains of N. gonorrhoeae, and found that the LPS from each strain was similar in composition. The values obtained by these investigators showed that lipid A accounted for 37 to 44% (w/w) of LPS and contained 44 to 73% fatty acid, 1.0 to 1.3% phosphorus and 14.2 to 15.5% glucosamine. All the lipid A preparations contained the same fatty acids: β-hydroxy-decanoic acid, β-hydroxylauric acid,

β-hydroxy-myristic acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid and oleic acid (92). The LPS from these strains consisted of lipid A covalently linked to a core region composed of 3 molecules of KDO, 2 of heptose, 1 of glucose, 2 of galactose and 4 of glucosamine. This structure is analogous to the basal region of the LPS of the Enterobacteriaceae (74). Stead et al. (92) concluded that under their experimental conditions, no significant difference in the composition of LPS existed among those strains of N. gonorrhoeae tested. Two of the strains examined were of colony type 1 morphology, three of colony type 4 morphology, and one was resistant to 0.5 μg penicillin/ml. Even though two different procedures were used to isolate LPS from the organisms, no evidence was obtained for the presence of any O-antigen sidechains (92).

At the same time, Perry et al. (79) published a study suggesting that T1 and T2 gonococci produced an "S" type LPS complete with high molecular weight O polysaccharides, while T3 and T4 gonococci produced an "R" type incomplete LPS in which only lipid A and a core oligosaccharide were present. In this study (79), T4 organisms all had similar LPS composition, that included 50-56% lipid A and a core oligosaccharide comprised of glucosamine, glucose, galactose, heptose and KDO. LPS from the T1 organisms examined contained significantly lower amounts of insoluble lipid A (5-15%) which the authors said was consistent with results obtained by partial hydrolysis of "S" type LPS preparations from other gram-negative organisms (59, 79). The major difference between T1 and T4 LPS was the presence of a high molecular

weight polysaccharide in the T1 LPS which eluted in the void volume of a Sephadex G-50 column. The authors believe this molecular entity to be O-polysaccharide, and though its composition showed great interstrain variation, it did contain what is probably N-acetyl-D-galactose and N-acetyl-D-glucosamine. The intrastrain variation was thought to be responsible for different gonococcal serotypes (79).

Studies on LPS isolated from nonpathogenic Neisseria (44, 45) confirm the wide variation in composition which occurs throughout the genus Neisseria. Differences in core oligosaccharide type were found between species, with two oligosaccharide types being found within one species, N. cinerea. Unlike the LPS obtained from all the other nonpathogenic Neisseria, those of N. canis and N. subflava possess O-specific sidechains, and may be unique among the nonpathogenic members of the genus (45). The importance of these findings remains to be elucidated.

Many studies have centered on structural proteins found in the gram-negative envelope as well as those found in the outer membrane (4, 76, 88, 89). Schnaitman disrupted cells of E. coli with a French pressure cell and resolved the membrane fraction into 20 to 30 protein bands by SDS-polyacrylamide gel electrophoresis (89). In subsequent studies (91) differences were shown to occur in outer membrane protein profiles when organisms were subjected to catabolite repression or when they were harvested at different phases of growth. These variations in proteins were mainly quantitative in nature.

Birdsell and Cota-Robles (5), developed a method for spheroplasting which employs plasmolysis of cells in hypotonic sucrose to permit the penetration of lysozyme to the peptidoglycan layer. The resulting osmotically sensitive cell suspension is diluted 1:1 to produce spheroplasts, after which EDTA is added to weaken the outer membrane. Upon further dilution, the cell ruptures and the outer membranes assume a highly coiled configuration.

Osborn et al. (76) used this method to obtain spheroplasts of S. typhimurium which were subsequently lysed by sonication or osmotic shock. After centrifugation to equilibrium on a sucrose gradient, the material banding at a density of 1.22 g/cm^3 was shown to represent the outer membrane. Other material appearing as a band of lower buoyant density was identified as the cytoplasmic membrane fraction.

Identification of the isolated bands was based on chemical composition and distribution of specific enzyme activities. Components of the electron transport system and of sugar transport were specifically localized in the cytoplasmic membrane, with usually less than 3% contamination of the outer membrane fraction. The only enzymatic activity found in the outer membrane was a combination of phospholipase A and lysophospholipase activity (76). Not only did the outer membrane fraction contain large amounts of lipopolysaccharide, but it had a lower ratio of phospholipid to protein than in the cytoplasmic membrane. In addition, the ratios of phosphatidylglycerol and cardiolipin to phosphatidylethanolamine were reduced 50 and 25%, respectively, over those found in cytoplasmic membrane. These results confirmed the

earlier findings of Miura and Mizushima (64), Schnaitman (89) and Fox et al. (27) on the chemical and enzymatic composition of the two membranes.

The protein composition of the membranes of S. typhimurium (76) were shown to be very similar to those of E. coli (89) by SDS polyacrylamide gel electrophoresis. The total membrane fraction of S. typhimurium was resolved into approximately 20 protein bands, including two major components having apparent molecular weights of 32,000 and 40,000 daltons. These bands as well as several others appeared to be located in the outer membrane (76).

The techniques used in the previously mentioned studies have been employed to characterize the outer membrane of Neisseria gonorrhoeae. In addition, several techniques have been developed in order to obtain envelope fractions of relatively high purity. It is clear that techniques such as these would facilitate studies regarding gonococcal surface antigens, as well as any structural proteins that may have a role in the permeability on the outer membrane.

Isolation of the outer membrane often involves the use of lysozyme, EDTA, or both. Zollinger et al. (109) incubated N. meningitidis in buffer with EDTA for 30 minutes at 60 C before passing the cell suspension twice through a 1-inch, 23-gauge hypodermic needle using manual pressure. The final product obtained following ultracentrifugation was reported to be native cell wall complex, consisting primarily of outer membrane fragments. This conclusion was based on several lines of evidence: (a) electron microscopy revealed that the product was

similar in appearance to the outer membrane; (b) intact extracted organisms lacked outer membrane; (c) the end product was homogeneous when sedimented in a sucrose density gradient; (d) the complex was completely soluble in 2% SDS, which would not occur if peptidoglycan were present. SDS-polyacrylamide gel electrophoresis revealed simple (one to eight major bands), reproducible and strain-dependent protein band patterns (109).

Johnston and Gotschlich (46) isolated the outer membrane of N. gonorrhoeae utilizing isopycnic centrifugation of osmotically ruptured spheroplasts formed by the action of EDTA and lysozyme. The authors concluded that the chemical and enzymatic composition of the isolated membrane was similar to those of other gram-negative bacteria (64, 76, 89). The buoyant densities for the gonococcal cytoplasmic and outer membranes, 1.141 and 1.219 g/cm^3, respectively, were similar to values found for E. coli and S. typhimurium (64, 76, 89). The outer membrane preparation was practically devoid of cytoplasmic membrane contamination, as the specific activities of succinic dehydrogenase, D-lactate dehydrogenase and NADH oxidase were only 1.0, 1.3, and 2.5%, respectively, of the activities found in the cytoplasmic membrane (46). EDTA can chelate metal ions required for the structural integrity of the cell envelope (54). The presence of EDTA was necessary; otherwise maximum separation of the membranes could not be achieved during sucrose gradient centrifugation (46).

The majority of outer membrane proteins could be accounted for by three proteins having apparent molecular weights of 34,500, 22,000, and

11,500 daltons. The 34,500 dalton protein accounted for over 60% of the outer membrane protein. Johnston and Gotschlich (46) were unable to resolve this large protein into subunits, but felt that this protein had immunological significance due to its quantity, location on the outer membrane, and apparent electrophoretic homogeneity. Later studies showed that the molecular weight of the major outer membrane protein varies with the strain studied (47).

Wolf-Watz et al. (107) adapted an isolation procedure originally developed for use with E. coli (106) for comparison of the outer membrane composition of penicillin-sensitive and -resistant strains of N. gonorrhoeae. Lysozyme and EDTA were used to treat plasmolyzed cells, after which the cell suspension was passed three times through a 22-gauge needle. Following centrifugation, the pH of the supernatant was lowered to 5.0 and aggregation of the outer membrane occurred. Following isopycnic sucrose density gradient centrifugation the outer membrane appeared as a homogeneous band with an apparent density of 1.25 g/cm^3 (107). Specific activities of phospholipase A and lysophospholipase, which are the only known enzymatic activities of E. coli and Salmonella outer membranes (23), increased sixfold in the outer membrane fraction. The strains used in this study differed in their sensitivity to penicillin and were not isogenic. SDS-polyacrylamide gel electrophoresis of their extracted outer membranes revealed no differences in their protein patterns (107). There were at least six major proteins with a predominant protein having an apparent molecular weight of 35,000 daltons.

Lipopolysaccharide from both penicillin-sensitive and -resistant strains was compared, with similar findings (107). The core oligosaccharide in both sets of strains contained glucose, galactose, glucosamine, and small amounts of heptose in similar ratios. Gonococcal LPS differed in its heptose content from that of E. coli and Salmonella.

The outer membrane of heptose-deficient mutants of E. coli (6) is highly permeable. If the low heptose content of this species is consistent (107), it may correlate with the high permeability of the gonococcus to antibiotics.

Use of Chaotropic Agents to Extract Outer Membrane Proteins. To facilitate the study of particulate proteins as well as other hydrophobic membrane components, Hatefi and Hanstein (35) investigated the use of chaotropic agents. Chaotropic agents promote the solubilization of many hydrophobic compounds in aqueous solvents. Because of the predominance of hydrophobic interactions in membrane systems, the stability of these systems in aqueous media has been a major obstacle in resolving them and elucidating their molecular organization.

Hatefi and Hanstein (35) suggested that hydrophobic interactions are the most significant forces contributing to the stability of membranes. This apparently results because van der Waals attractions between apolar groups are weak and hydrogen bonds of the type C=O···H-N and C=O···H-O are unstable if exposed to water (50). Thus, transferring an apolar molecule from a lipophilic environment to water is accompanied by an entropy decrease (35). If the structure of water

could be changed in the direction of greater disorder, the negative entropy difference could theoretically be eliminated, resulting in greater ease of solubilization for hydrophobic entities. Such a situation can occur in the presence of certain inorganic anions (35). These anions tend to make water more disordered and lipophilic and in this way weaken hydrophobic bonds within membranes, thereby increasing the water solubility of particulate proteins. In their words, Hatefi and Hanstein describe chaotropic agents as "those inorganic anions which favor the transfer of apolar groups of water (35)". According to their findings, the order of effectiveness of those agents tested was $SCN^{-}>ClO_4^{-}>$guanidine>urea>$Cl^{-}>F^{-}$.

Moldow et al. (65) isolated E. coli membranes and extracted them with varying concentrations of chaotropic agents and detergents. Guanidinium thiocyanate (6 M) was considered the most effective agent tested, solubilizing 84% of the membrane proteins. Among the other agents tested, 2% SDS solubilized 82% and 6 M sodium thiocyanate (NaSCN) solubilized 42% of the protein, as determined by either isotopic or direct methods. The degree of solubilization was proportional to the concentration of the chaotropic agent used. Varying the temperature between 4 and 37 C did not affect the amount of membrane protein solubilized. Extracted membrane proteins were fractionated by column chromatography. Because the thiocyanate ion is known to react with free amino groups forming thiocarbamyl derivatives, this could have produced a heterogeneous population of polypeptides differing only in their derivatization and resulting in artificial fractionation

(65). However, studies with ^{14}C-thiocyanate showed that less than one in 5,000 amino acid residues in the fractions was thiocarbamylated. Unfortunately, this could still be a disadvantage if renaturation of the proteins is to be attempted, and could be remedied by employing a less chemically reactive, but equally effective, chaotropic ion.

The major E. coli outer membrane protein described by Schnaitman (89) was examined in the above study (65). Each of the chromatographically-separated fractions contained polypeptides that migrated in the position of the major outer membrane protein band after SDS-polyacrylamide gel electrophoresis. However, urea gel patterns and the chromatographic elution patterns indicated that the polypeptides of these fractions differed in charge, suggesting that the major band contained more than a single structural protein (65).

A gentle technique for isolating the serotype antigen from N. meningitidis employed treatment of whole cells for 2 hours at 45 C in 0.2 M lithium chloride (28). Frasch and Gotschlich (28) demonstrated by gel diffusion that the isolated proteins obtained by this method retained the immunological characteristics of the native cellular proteins. In the case of type 2 meningococci, the major outer membrane protein contained the serotype antigenic determinant (28). The protein banding pattern of the extracted serotype antigen was compared by SDS-polyacrylamide gel electrophoresis to that of isolated spheroplast membranes and isolated outer membranes. The serotype antigen appeared to be derived exclusively from the outer membrane, with little or no cytoplasmic membrane contamination. The lithium

chloride-extracted antigen consisted primarily of five major protein species, of which a protein with an apparent molecular weight of 41,000 daltons represented about 50% of the total outer membrane protein (28). This serotype antigen was also an effective immunogen producing high titered type-specific bactericidal antisera in rabbits.

Recently, Johnston et al. (47) developed a method to isolate type-specific antigens from N. gonorrhoeae and to use these extraction products as the basis of a serotyping system for the gonococcus. Treatment with 0.2 M lithium acetate for 2 hours at 45 C extracted outer membrane antigens from log-phase cells. Intact cells rather than isolated outer membranes were extracted because during the spheroplasting procedure surface proteins are released into the spheroplasting medium, lowering the final yield (47). Gel electrophoresis demonstrated that serotype antigen was located in the outer membrane and accounted for over 60% of the total protein. Other investigators have been able to resolve the major outer membrane protein of other bacteria into more than one group of polypeptides (7, 90). However, using these methods (7, 90), Johnston et al. (47) were unable to do so with N. gonorrhoeae as also was the case with Frasch and Gotschlich (28) with N. meningitidis.

Electron microscopy revealed that the extracted cells were not extensively lysed by treatment with lithium acetate; rather, the outer membrane was peeled away in small vesicles (47). The outer membrane was the site of the serotype antigen complex, as shown by the almost identical SDS-polyacrylamide gel patterns of outer membrane and extracted antigen.

A variation was observed in the electrophoretic mobility of serotype antigens of different strains of N. gonorrhoeae thus enabling a classification scheme to be devised. Antisera raised in rabbits to the different serotype antigens showed that strains having antigens with the same electrophoretic mobility had immunological identity. Conversely, strains differing in the mobility of their major protein showed immunological nonidentity. When all four colony types of one strain (T1, T2, T3, T4) were examined for serotype designation, no difference was detected between colonial types. Repeated analysis showed that serotype specificity is a stable property of each strain of N. gonorrhoeae (47). The antigenic heterogeneity among 81 strains of N. gonorrhoeae examined and the differences observed offer a possible explanation for the lack of immunity to gonorrhea.

STATEMENT OF PROBLEM

The study of the interaction of outer membrane components of pathogenic gram-negative bacteria with the defense mechanisms of its host is necessary for a complete understanding of its pathogenesis, the antigenic nature of outer membrane components, and the development of vaccines.

In order that outer membrane components can be studied in more detail, methods for the isolation of these components with little or no contamination from other cellular components must be developed. The antigenic nature of N. gonorrhoeae is only now beginning to be elucidated. It remains unclear as to why some colonial variants of N. gonorrhoeae are pathogenic while others are not. Methods for isolation of outer membrane components from gonococci have been developed (28, 46, 47, 107), but problems such as low yields, undesirable levels of contaminating materials, and lengthy procedures have not been completely solved.

The objectives addressed in this thesis will be:

1. The development and characterization of a technique designed to extract outer membrane components from Neisseria gonorrhoeae.
2. A comparison with other extraction techniques to evaluate the new method's usefulness.
3. An examination of the outer membrane composition of colonial variants of different strains of Neisseria gonorrhoeae.

MATERIALS AND METHODS

Organisms. Clinical strains of N. gonorrhoeae were used in this study. The specific properties of strains CS-7 (type 4) and JW-31 (type 4) have been previously described (66, 67). N. gonorrhoeae F62 was provided by K. Holmes, United States Public Health Service Hospital, Seattle, Washington. Additional clinical strains from non-disseminated gonococcal infections were obtained from the Multnomah County Health Department, Portland, Oregon. The identity of all organisms was confirmed by cell morphology in gram-stained smears, oxidase reaction, and the production of acid from glucose, but not from maltose, fructose or sucrose. N. gonorrhoeae strains CS-7 and JW-31 were maintained as previously described (66, 67). Colony types T1, T2, T3 and T4 (11, 48, 49, 81) from fresh clinical and laboratory strains were selected, subcultured and maintained by selective passage on GC agar plates.

Throughout this study, "strain" will be used to indicate a clinical isolate from which colonial variants have been selected. There is no uniform definition for what constitutes a gonococcal "strain". Therefore, in order to avoid confusion between "strain" and "isolate", "strain" will be used throughout.

Salmonella typhimurium DB-21, a prototrophic strain of LT-2, was obtained from D. Botstein, Massachusetts Institute of Technology, Cambridge, Massachusetts. Pseudomonas aeruginosa PS-7 was obtained through the courtesy of E. Fisher, Portland State University, Portland,

Oregon. E. coli K-12 was a departmental strain. S. typhimurium DB-21 and E. coli K-12 were maintained on trypticase soy agar (Difco) slants at 4 C. P. aeruginosa PS-7 was stored at -70 C in trypticase soy broth containing 1.25% bovine serum albumin and 6% sucrose.

Media and growth conditions. The basal medium contained the following per liter: proteose peptone no. 3 (Difco), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; and soluble starch (Difco), 1 g. The final pH of the medium was 7.2. When used for the growth of N. gonorrhoeae, glucose (5 g/liter), $NaHCO_3$ (42 mg/liter) and a growth factor supplement, identical in composition to IsoVitalex enrichment (BBL) but lacking glucose, were added after autoclaving. GC agar (Difco) plates containing glucose (5 g/liter) and growth factor supplement (1% vol/vol) were used for daily maintenance of gonococcal colony types. Glucose (5 g/liter) was added to the basal medium for growth of E. coli and S. typhimurium; glycerol (1% vol/vol) and monosodium glutamate (8.46 g/liter) were added to the basal medium for growth of P. aeruginosa.

Cells from a 16 h overnight culture were washed and resuspended to one-half their original volume in a solution containing 0.85% NaCl and 0.1% cysteine hydrochloride (pH 6.5). Cultures were inoculated (1% vol/vol) and incubated on a gyrotory shaker at 37 C. Agar-grown cultures (18 h) of single colony types were microscopically checked for characteristic colonial morphology and removed from GC agar plates by resuspension in the NaCl-cysteine hydrochloride solution. Liquid cultures were inoculated to an initial turbidity of 10-20 Klett units (1.5×10^7 CFU/ml; 25-50 µg [dry weight]/ml) and grown to log phase at

37 C on a gyrotory shaker. At time of harvest, cultures were streaked onto GC agar plates and examined for colonial morphology after 18 h growth at 37 C (5% CO_2). T1 and T2 cultures typically contained >80% of the inoculated colony type, while T3 and T4 cultures generally contained >95% of the inoculated colony type.

Extraction of outer membrane components. Cultures were harvested at a turbidity of 100-150 Klett units (Klett-Summerson colorimeter, 540 nm) by centrifugation at 7,000 x g for 15 min. Cells were resuspended and washed once with 0.15 M NaCl in pre-weighed centrifuge tubes. The cell pellets were resuspended in a 1 M solution of the chaotropic agent (20 ml/g wet weight cells) and extracted by shaking in the presence of glass beads (5 mm diam.) on a gyrotory shaker (70 rpm; Model G-25 Gyrotory Shaker, New Brunswick Scientific Company, New Brunswick, N.J.) for 2 h at room temperature. The suspension was then decanted into centrifuge tubes and the glass beads washed with a small volume of a 1 M solution of the chaotropic agent to remove any adherent material. The solutions were pooled and centrifuged at 12,000 x g for 20 minutes at 4 C. The resulting supernatant was decanted and recentrifuged at 30,000 x g for 45 min at 4 C to remove any remaining large aggregates of cellular material. The 30,000 x g supernatant was then subjected to ultracentrifugation at 100,000 x g for 2 h in a Beckman L5-65 preparative ultracentrifuge using a type 30 fixed angle rotor (Beckman Instruments, Inc., Palo Alto, Ca.). The gel-like pellet was washed twice with 0.15 M NaCl. The final pellet

was resuspended as completely as possible in a small volume (0.5-1.0 ml) of 0.15 M NaCl and centrifuged at 2,000 x g for 15 minutes. The supernatant was retained as the final product.

Extraction of gonococcal lipopolysaccharide. Lipopolysaccharide was extracted from N. gonorrhoeae strains according to the method of Galanos et al. (30).

Analytical procedures. Protein was assayed by the method of Lowry et al. (57) with bovine serum albumin as standard. Total lipid phosphorus was measured by the procedure of Ames and Dubin (1). Phospholipid concentrations were calculated assuming an average molecular weight of 700 (76). Samples (0.01-0.1 ml) were added to 0.075 ml of 10% $Mg(NO_3)_2 \cdot 6H_2O$ and ashed over a hot flame. After cooling, 0.6 ml of 0.5 N HCl was added and the samples incubated in a 100 C water bath for 15 min. Inorganic phosphate was determined after cooling by adding 1.4 ml of a mixture of 1 part 10% ascorbic acid and 6 parts 0.42% $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ in 1 N H_2SO_4. Samples were incubated at 37 C for 60 min and the absorbancy at 800 nm measured in a Beckman Acta CIII spectrophotometer. Unknown phosphorous concentrations were calculated from a standard curve prepared with KH_2PO_4.

Heptose was measured by the cysteine-H_2SO_4 reaction (24) as modified by Osborn (73). H_2SO_4 (2.25 ml of 6 parts concentrated H_2SO_4:1 part H_2O) was slowly added to duplicate samples (0.5 ml) in an ice water bath, and mixed by shaking in the cold. After 3 min, the tubes

were transferred to a 20 C water bath for 3 min and then heated in a boiling water bath for 10 min. After cooling, 0.05 ml of 3% cysteine HCl was added to one sample, the other serving as a blank. The tubes were stored in the dark at room temperature. Absorbancy at 505 and 545 nm was determined 2 h after addition of cysteine and corrected for nonspecific absorbance in the blank without cysteine [A_{505} sample-A_{545} sample]-[A_{505} blank-A_{545} blank]). Unknown values were determined from a standard curve obtained using D-mannoheptulose.

2-Keto-3-deoxyoctonate (KDO) was determined by a modification (73) of the thiobarbituric acid method of Weissbach and Hurwitz (104). A 0.2 ml volume of 0.4 N H_2SO_4 was added to 0.2 ml of the solution to be tested, and the mixture incubated for 20 min in a boiling water bath. A 0.2 ml aliquot of this solution was removed and to it was added 0.25 ml of H_5IO_6 (containing 10 μmoles), and allowed to stand for 20 min at room temperature. Next, 0.5 ml of 2% $NaAsO_2$ in 0.5 N HCl was added with shaking, and the mixture allowed to stand for 2 min. At this time, 2.0 ml of 0.3% thiobarbituric acid in H_2O (adjusted to pH 2) was added, the solution was mixed and then incubated in a boiling water bath for 10 min. After cooling, absorbancy at 548 nm was determined. Unknown values were determined from a standard curve prepared with 2-keto-3-deoxyoctonate.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out essentially as described by Weber and Osborn (103) with minor

modifications. A stock acrylamide solution consisted of 4.17 g acrylamide and 0.113 g methylenebisacrylamide was made to 25 ml total volume with water. The solution was filtered through Whatman No. 1 filter paper and stored in an amber bottle at 4 C. The gel buffer (pH 7.2), contained per liter: $NaH_2PO_4 \cdot H_2O$, 7.8 g; Na_2HPO_4, 20.4 g; and sodium dodecyl sulfate, 2.0 g. Gel tubes used for 7.5% gels were 7.5 cm in length and had a 5 mm inside diameter. Before use they were soaked in a dichromate cleaning solution, rinsed thoroughly, coated with a 1:200 dilution of Photo-Flo (Eastman Kodak Co., Rochester, N.Y.) and dried in an oven. In order to prepare 18 gels, 15 ml of gel buffer were deaerated under vacuum for at least 1 h and then mixed with 13.5 ml of the acrylamide solution in a foil-covered flask. After further deaeration for 4-5 min, 1.5 ml of freshly prepared ammonium persulfate (3 mg/ml H_2O) and 0.045 ml of N,N,N^1,N^1,-tetramethylethylenediamine were added. After a brief, gentle mixing, gel tubes were filled to within 1 cm from the top, and distilled water was carefully overlaid using a syringe equipped with a 22-gauge needle. The gels were allowed to polymerize for a minimum of 1 h under a fluorescent light. For storage, the water was drawn off the top of the gel and the tubes submerged in test tubes filled with gel buffer, diluted 1:1 with water.

Samples for SDS-PAGE were adjusted to equal phospholipid content. In this way, quantitative and qualitative intrastrain variations in outer membrane proteins could be determined. To equalize phospholipid concentrations between samples, appropriate dilutions were made with sodium phosphate buffer (pH 7.2). An aliquot (120 µl) of sample was mixed with an equal volume of 0.01 M sodium phosphate buffer (pH 7.2)

containing 1% SDS and 1% β-mercaptoethanol and incubated at 100 C for 5 min. After cooling, 200 µl of this mixture was combined with 20 µl β-mercaptoethanol, 10 µl tracking dye (0.05% bromphenol blue in water) and 3 drops glycerol. After mixing, 100 µl of the sample solution was layered on gels preelectrophoresed for 20 min at 8 mA/gel. Samples layered on the gels usually contained 0.3-0.6 µmole phospholipid and 10-25 µg protein. Gel buffer, diluted 1:1 with water, was carefully overlaid to fill the tubes. The upper and lower chambers of the electrophoresis apparatus were filled with a 1:1 dilution of gel buffer, the anode connected to the top chamber, and electrophoresis carried out at a constant current of 8 mA/gel. Under these conditions, the tracking dye moved to within 5 mm of the bottom of the gel in 3.5-4 h. Gels were removed from the tubes and the length of the gel and the dye front measured. Gels were stained with Coomassie blue (1.25 g Coomassie blue dissolved in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid, filtered through Whatman No. 1 filter paper) for 2 h at room temperature. Gels were then rinsed in distilled water and destained overnight at 60 C in a solution of glacial acetic acid, 75 ml; methanol, 50 ml; and distilled water, 875 ml containing 20 g Bio-Rex RG 501-X8 (BioRad Laboratories, Richmond, Ca.) per 500 ml of destaining solution. After destaining, the length of the gels and the positions of the bands were recorded. Stained gels were stored in 7.5% acetic acid. Apparent molecular weights of proteins were obtained by calculating relative mobilities according to Weber and Osborn (103). Values thus obtained were used to obtain apparent molecular weights using known molecular weight markers as a comparison.

Sucrose density gradients. Linear sucrose density gradients (10% to 60% wt/wt) were prepared in 0.05 M Tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.4). Aliquots (0.5 ml) of NaSCN-extracted material were layered on top and the gradients centrifuged to equilibrium at 120,000 x g at 4 C for 14 h (SW 41 rotor, L5-65 ultracentrifuge, Beckman Instruments, Inc., Palo Alto, Ca.). Gradients were fractionated (Densi-Flow density gradient remover, Polystaltic pump, Buchler Instruments, Fort Lee, N.J.) and 0.25-0.30 ml fractions collected. The density of the sucrose was obtained using a refractometer (Bausch and Lomb, Inc., Rochester, N.Y.).

Electron microscopy. Acetate-barbital buffer consisted of sodium acetate, 19.43 g; sodium barbital, 29.43 g; and sodium chloride, 34.0 g per liter of distilled water. Fixative consisted of acetate-barbital buffer, 6.25 ml; 10 N HCl, 8.75 ml; 1.25 M $CaCl_2$, 0.25 ml; and H_2O, 10.0 ml. The fixative was prepared by combining 3 parts of this solution with 1 part 4% OsO_4. Uranyl acetate solution was prepared by dissolving 0.5 g in 100 ml of acetate barbital buffer. Cells of N. gonorrhoeae CS-7 were extracted with 1 M NaSCN as previously described. After the 12,000 x g centrifugation, the pellet was rapidly resuspended in the buffered osmium tetroxide fixative and centrifuged immediately (2,000 x g for 5 min). The resulting pellet was resuspended in 1.0 ml of the fixative and allowed to stand 16-18 h at room temperature. After the addition of 8 ml of the stock acetate-barbital buffer the suspension was centrifuged (2,000 x g for

5 min). The pellet was then mixed with 1 ml of a 2% melted Noble agar (Difco) solution in acetate-barbital buffer and drops placed on appropriately marked microscopic slides. When the agar had solidified the drops were cut into cubes (2-3 mm) and placed in 0.5% uranyl acetate buffer, dehydrated in successive changes of ethanol, imbedded and sectioned. The sections were examined in a Philips EM-200 electron microscope at 60 kV. Non-extracted cells of strain CS-7 were used as a control.

For negatively-stained whole cell preparations, colonial variants of N. gonorrhoeae F62 were subcultured and cloned on GC agar plates. One or two drops of 0.1 M ammonium acetate buffer (pH 7.0) were dropped onto the surface of a colony of N. gonorrhoeae which had been grown for 18 h at 37 C in 5% CO_2. After allowing the buffer to remain in contact with the colony for 2 min, the liquid which contained some cells was drawn off with a Pasteur pipette and dropped onto a paraffin block. Carboned, Formvar-coated copper grids were placed onto a drop of the sample for 1.5-2 min and dried by touching the edge of the grid to bibulous paper. These grids were placed onto a drop of 1.5% ammonium molybdate (pH 7.0) for 30 s. Excess fluid was removed with bibulous paper. Samples were examined in an RCA EMU-3G electron microscope at 50 kV.

Chemicals and radioisotopes. 2-Keto-3-deoxyoctonate, D-mannoheptulose, phenazine methosulfate (PMS), 2,5-diphenyl-3-(3,4-dimethyl-2-thiazolyl) monotetrazolium bromide (thiazolyl blue),

Tris(hydroxymethyl)aminomethane (Tris), ribonuclease A and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Ovalbumin and chymotrypsinogen A were from Schwarz/Mann (Orangeburg, N.Y.). Electrophoresis grade acrylamide, methylenebisacrylamide, sodium dodecyl sulfate, ammonium persulfate, N,N,N^1,N^1-tetramethylethylenediamine, β-mercaptoethanol, and Bio-Rex RG 501-X8 mixed bed resin were products of Bio-Rad Laboratories (Richmond, Ca.). D-[^{14}C(U)]-glucose (specific activity 5 mCi/mmole), sodium [1,2-^{14}C] acetate (specific activity 52 mCi/mmole), [2-^{3}H]-adenine (specific activity 31.7 mCi/mmole), and L-[^{3}H]-amino acid mixture (1.0 mCi/ml) were obtained from New England Nuclear Corp. (Boston, Ma.). All other reagents were of analytical grade and obtained from standard commercial sources.

Enzyme assays. Succinate dehydrogenase (EC 1.3.99.1) and D-lactate dehydrogenase (EC 1.1.1.28) were measured by coupling the enzymes via PMS to the reduction of thiazolyl blue. Incubation mixtures contained 60 mM phosphate buffer, pH 7.5; 10 mM KCN; 15 µg thiazolyl blue; 15 µg PMS; 25 mM succinate or 2.5 mM D-lactate; and membrane-rich sample (10-135 µg protein) in a volume of 0.5 ml. Increase in the absorbance in 550 nm was followed in a Beckman Model 25 recording spectrophotemeter at 25 C.

Comparison of different extraction techniques. Duplicate log-phase cultures of *N. gonorrhoeae* CS-7 were grown in basal medium containing either [^{3}H]-amino acids or [2-^{3}H]-adenine. Each culture was

divided into four equal aliquots that were extracted by one of four methods: 1) 1 M NaSCN, as previously described; 2) 0.2 M LiCl, a technique similar to that used with NaSCN, except that the extraction was performed with 0.2 M LiCl for 2 h at 45 C and the extracted material washed with distilled water (28); 3) the "O" preparation of Zollinger et al. (109) in which cells are resuspended in 0.05 M sodium phosphate buffer (pH 7.4), containing 0.15 M NaCl and 0.01 M ethylenediaminetetraacetic acid (EDTA), incubated for 30 min at 60 C and subjected to mild shear by passing twice through a 23-gauge hypodermic needle. After centrifugation at 12,000 x g for 20 min, the supernatant was centrifuged at 80,000 x g for 2 h; the pellet is the final product; and 4) the OM isolation procedure of Wolf-Watz et al. (107), which involves passing osmotically protected lysozyme-EDTA spheroplasts three times through a 23-gauge needle. After centrifugation, the pH of the supernatant is lowered to 5.0 to aggregate the membrane. The aggregated membrane is obtained by centrifugation (10,000 x g for 10 min).

Dry weight determinations. Dry weights were determined by centrifuging samples at 5,000 x g for 10 min to concentrate the cells. The cells were washed with distilled water and dried to constant weight in tared aluminum dishes at 80 C in a vacuum oven.

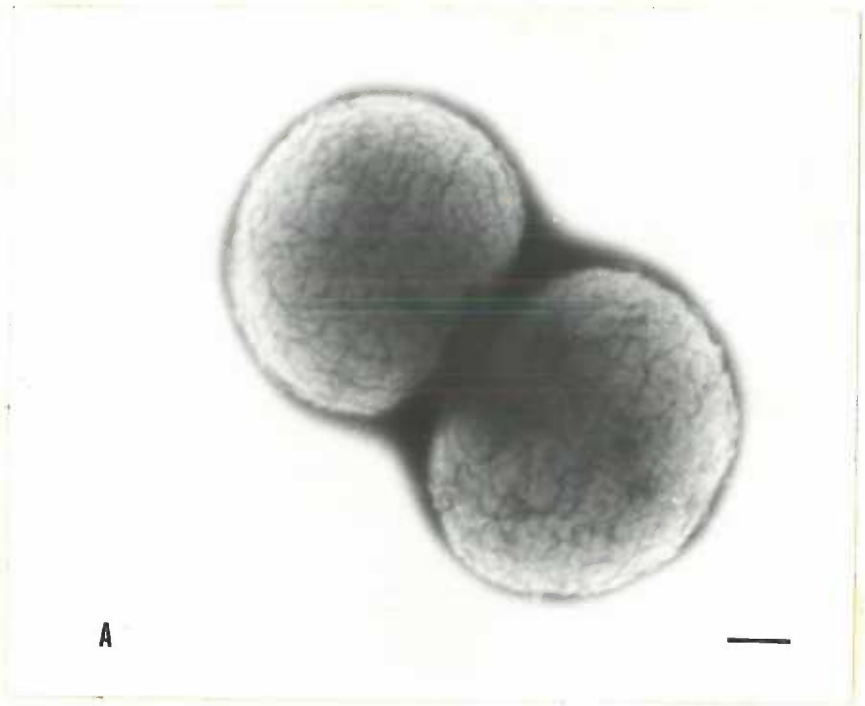
RESULTS

Surface morphology of N. gonorrhoeae. The appearance of negatively-stained preparations of N. gonorrhoeae F62 is shown in Fig. 2. Fig. 2(a) shows an electron micrograph of a diplococcus with a rugose outer membrane surface. In Fig. 2(b), Tl cells of N. gonorrhoeae F62 are shown. Pili, which are characteristic of pathogenic N. gonorrhoeae when passaged in vivo, are shown in abundance. The gentle technique by which the cells in this micrograph were prepared (see Materials and Methods) enabled numerous long pili to be visualized.

Effect of NaSCN extraction on cell morphology. Control (non-extracted) and NaSCN-extracted organisms were fixed and thin sections were prepared in order to evaluate structural changes which occurred during the extraction procedure. Fig. 3 compares the structure of log-phase control organisms with similar organisms after a 2 h extraction with NaSCN. Control cells possess the characteristic cell envelope of gram-negative bacteria, which includes a peptidoglycan layer sandwiched between the cytoplasmic (inner) membrane and the outer membrane. In contrast, the extracted organisms characteristically display disrupted outer membrane that is pulled away from the cell or nearly absent. However, completely disrupted cells (ghosts) are rare, indicating that lysis during extraction is not a common occurrence and therefore suggests that the material extracted from these organisms

Figure 2. a) Electron micrograph demonstrating the surface morphology of N. gonorrhoeae F62 T4. The outer membrane of the gonococcus is visible in this preparation. The cells have been negatively stained with ammonium molybdate according to the procedure described in Materials and Methods, and were examined with the RCA EMU-3G electron microscope. Bar = 0.25 μm.

b) Electron micrograph demonstrating the presence of pili on N. gonorrhoeae F62 T1. Pili are visualized in this negatively stained specimen which was prepared as above. Bar = 0.25 μm.


A

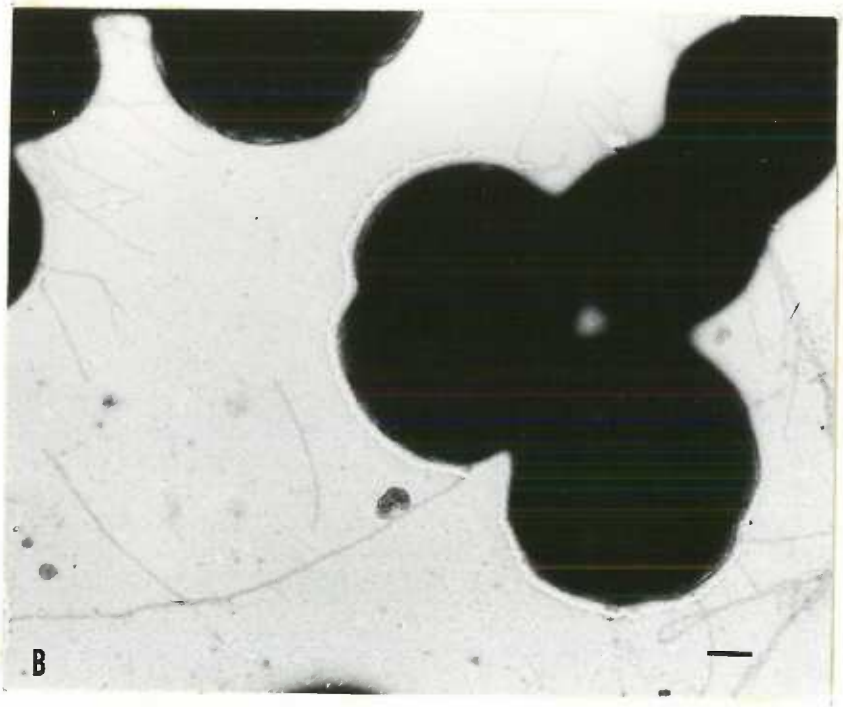
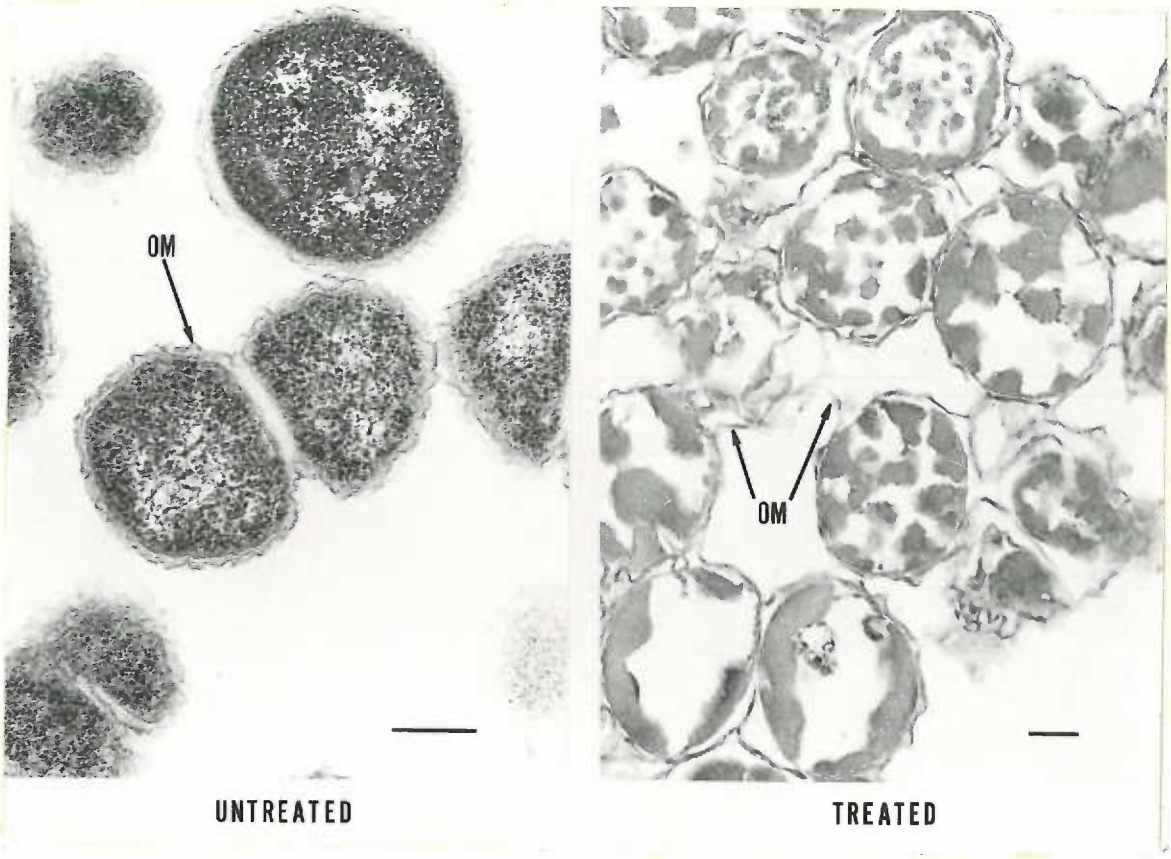

B

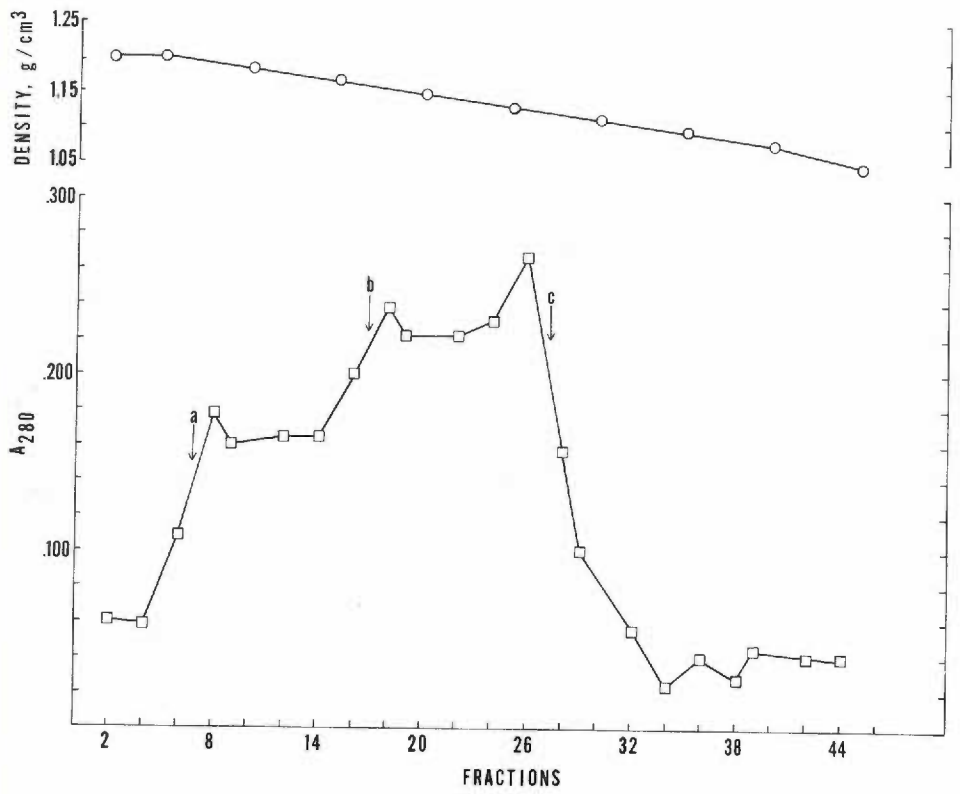
Figure 3. Electron micrographs comparing N. gonorrhoeae CS-7 before (untreated) and after (treated) 2 h extraction with 1 M NaSCN. OM = outer membrane. Treated cells were extracted according to the procedure in Materials and Methods, collected by centrifugation at 12,000 x g for 10 min, and stained, imbedded and sectioned as described. Specimens examined with a Philips EM-200 electron microscope. Bars = 0.25 μm.


OM
OM
UNTREATED
TREATED

should contain relatively low amounts of cytoplasmic membrane contamination. There was also noted what appears to be aggregation of cytoplasmic material in the NaSCN-extracted organisms. No explanation for this has been obtained. However, extracted organisms were not viable when plated onto GC agar. The electron micrographs in Fig. 2 are very similar to those of N. meningitidis whose outer membranes were extracted by a different technique (109).

Sucrose gradient centrifugation. The homogeneity of the NaSCN-extracted material from N. gonorrhoeae CS-7 was investigated by isopycnic centrifugation on 10-60% (wt/wt) sucrose density gradients (Fig. 4). Centrifugation resulted in a broad peak with two apparent shoulders. The homogeneity of the peak was further investigated by SDS-PAGE. Samples designated by arrows (a, b, c) were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie blue. The gels depicted in Fig. 4 indicate the relative similarity of the protein species in each peak. The samples applied to the gels were undiluted and the intensity of the bands is a direct reflection of the protein concentration of each of the fractions tested. In earlier experiments, shorter centrifugation times of 4 h, or sucrose gradients from 15-65% (wt/wt) without Tris were used. Under these conditions, incomplete centrifugation to equilibrium was achieved, as noted by the majority of the protein remaining at the top of the gradient. A sample volume of 0.5 ml was used throughout all sucrose gradient runs. The technique for sucrose gradient ultracentrifugation described in Materials and Methods yielded the best results of any technique used.

Figure 4. Sucrose density gradient (10%-60% sucrose, wt/wt) centrifugation of product obtained by 1 M NaSCN extraction of log phase cells of N. gonorrhoeae CS-7. The bottom of the gradient is at the left. Arrows (a, b, c) indicate points at which samples were taken for SDS-PAGE (gels a, b, c). Gels are stained with Coomassie blue.


DENSITY, g / cm3
1.25
1.15
1.05
.300
.200
.100
A280
a
b
c
2
8
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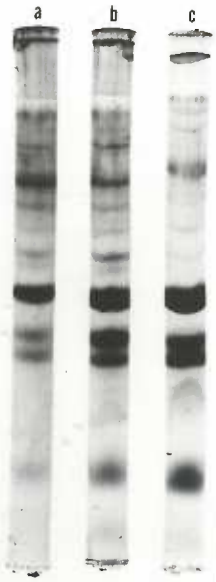
38
44
FRACTIONS


a
b
c

Comparison of different extraction techniques. The NaSCN-extraction technique was compared with three other procedures. Ideally, techniques which extract outer membrane components without extensive cell lysis should give preparations containing minimal amounts of intracellular components. Duplicate log-phase cultures of N. gonorrhoeae CS-7 were grown in basal medium containing either [^{3}H]-amino acids, or [2-^{3}H]-adenine. Each culture was divided into four equal aliquots, which were extracted by one of four methods listed in Materials and Methods (28, 107, 109). The results are summarized in Table 2. LiCl-treatment was shown to be the most gentle method, followed by NaSCN, as judged by the percent of whole cell [^{3}H]-adenine found in the final product. It has been previously reported that much of the DNA released from lysed bacteria will adhere to cells and cell envelopes (18). Therefore, the final product was examined to obtain an indication of the degree of nucleic acid contamination. As shown in Table 2, the procedure using 1.0 M NaSCN results in less nucleic acid contamination in the final product than two other methods tested, while at the same time providing a comparable yield of protein, as indicated by the percent of whole cell [^{3}H]-amino acids extracted. Even though the procedure using LiCl resulted in low amounts of nucleic acid contamination, the protein yield was also low when compared to the other procedures tested. Samples obtained with each of the protocols were subjected to SDS-PAGE (data not shown). The SDS-PAGE protein pattern exhibited by samples obtained from the NaSCN technique or the "O" preparation of Zollinger et al. (109) were essentially identical. The gels of the

Table 2. Comparison of methods for extracting outer membrane components of *N. gonorrhoeae* CS-7.

Method	Percent of whole cell [^{3}H]-amino acids extracted	Percent of whole cell [^{3}H]-adenine extracted
1.0 M NaSCN	.56 (.54 - .59)	.05 (.036 - .056)
0.2 M LiCl (28)	.14 (.014 - .27)	.013 (.009 - .019)
"O" Preparation (109)	.50 (.042 - .96)	.068 (.058 - .078)
"OM" Preparation (107)	.69 (.55 - .84)	.199 (.165 - .236)

Values shown are averages from three determinations. Ranges of values are shown in parentheses.

LiCl-extracted product obtained by the method of Frasch and Gotschlich (28) also appeared to be similar, and contained the major outer membrane protein. Due to the low protein concentration of the sample, however, it was not known if some of the minor bands evident on gels of samples obtained by other techniques were absent on the LiCl gel because the proteins were not extracted, or because the concentration of those proteins was so low that they escaped detection. The protein bands obtained in the "OM" preparation of Wolf-Watz et al. (107) provided a much more complex pattern than those patterns obtained with the other three extraction techniques. Some of those bands may have originated in the cytoplasmic membrane, as suggested by the increased nucleic acid contamination with this method.

Enzyme assays. Enzymatic analyses were conducted to determine the extent of cytoplasmic membrane contamination in the final product. Two cytoplasmic enzymes, succinate dehydrogenase (SDH) and D-lactate dehydrogenase (LDH), were used as markers to provide a basis for comparison with the data from the experiment shown previously (Table 2). To first investigate the effects of chaotropic agents on intact cell envelopes, aliquots of cell envelopes obtained after sonication were incubated with 1 M solutions of either NaSCN, NaCl, or LiCl for 2 h at room temperature. Control preparations were resuspended in either water or the 0.15 M NaCl solution used to wash the cells prior to sonication. After treatment, cell envelopes were centrifuged at 100,000 x g for 2 h and washed twice with 0.15 M NaCl. Enzyme assays were conducted on this

final pellet. The same approximate enzymatic activity was detected in all preparations, with the exception of the 1 M NaSCN preparation, which contained no detectable activity.

The chaotropic agent LiCl was previously shown to be similar to NaSCN in its usefulness in our extraction procedure. Therefore, LDH and SDH activities were assayed in an outer membrane preparation obtained by extraction with 1 M LiCl. Assays were performed after each wash to ascertain the value of two washes of the final product. A cytoplasmic membrane preparation was obtained by the "OM" technique of Wolf-Watz et al. (107). The lysozyme-EDTA spheroplasts were centrifuged from the outer-membrane containing supernatant (osmotically stabilized by sucrose) and lysed by resuspension in distilled water. This method appeared to be superior to sonication, as less membrane disruption is thought to occur, and enzyme activities are higher. After low speed centrifugation (4,000 x g) to remove cellular debris, the cytoplasmic membrane-rich pellet was obtained by centrifugation of the 4,000 x g supernatant for 2 h at 100,000 x g. The enzymatic activities from the various preparations are shown in Table 3. The activities are expressed per mg dry weight of the total cell volume extracted to avoid any biasing which may occur when preparations of different protein concentrations are compared. The enzymatic activities in the cytoplasmic membrane preparation were taken as 100% and the activities in the LiCl preparations are compared as a percentage of the activity found in the cytoplasmic membrane. It is worth noting the value of the second wash in removing LDH and SDH activity. The preparations were always washed twice before anything was done with them.

Table 3. Lactate dehydrogenase and succinate dehydrogenase activities of various membrane preparations

	Activity[a]			
Preparation	Lactate dehydrogenase	Percent of inner membrane activity	Succinate dehydrogenase	Percent of inner membrane activity
Cytoplasmic membrane[b]	1090.6	100	217.3	100
Outer membrane 1 M LiCl (1 wash)[c]	98.6	9.0	5.1	2.3
Outer membrane 1 M LiCl (2 washes)	3.3	0.3	3.3	1.5

[a]All enzyme activities expressed as nanomoles of thiazolyl blue reduced per minute per milliliter per milligram dry weight of total cell volume extracted.

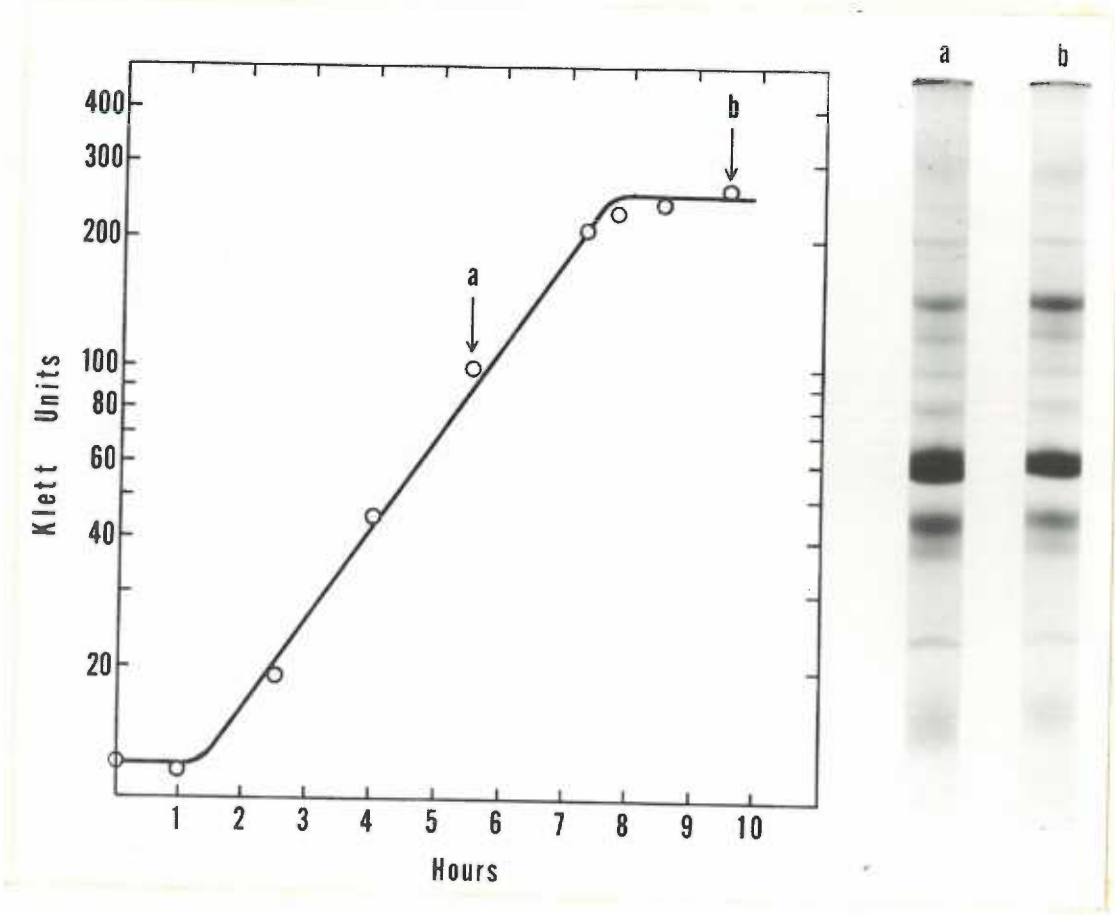
[b]Obtained by lysis of outer membrane depleted spheroplasts according to the method of Wolf-Watz et al. (107).

[c]Obtained by the method outlined in Materials and Methods.

It is felt that the data presented in Table 3 suggest that the NaSCN-extracted outer membrane product is as free of cytoplasmic membrane contamination as the LiCl-extracted product. This would seem reasonable when the percent of enzymatic contamination (Table 3) is compared to the percent of whole cell [^{3}H]-adenine appearing in the final product (Table 2).

Influence of growth phase on membrane components extracted from N. gonorrhoeae CS-7. To determine whether changes occurred in outer membrane proteins during growth, cultures of N. gonorrhoeae CS-7 were harvested during the logarithmic (100 Klett units) and stationary phases (260 Klett units) of growth (Fig. 5). Cells were extracted with 1 M NaSCN and the products assayed for protein and phospholipid. The extraction product from logarithmic phase cells yielded 1.85 µg protein/mg dry weight and 0.144 µmole phospholipid/mg dry weight while the extraction product from stationary phase cells yielded 1.61 µg protein/mg dry weight and 0.157 µmole phospholipid/mg dry weight. Samples were adjusted to identical phospholipid concentrations and subjected to SDS-PAGE. No qualitative differences in protein species in the outer membranes of cells harvested at either point in the growth curve (Fig. 5) were observed. The differences in the intensities of similar bands is probably due to a higher protein/phospholipid ratio in log-phase cells. These differences were not significant and so all cells extracted in subsequent experiments were harvested during logarithmic growth.

Figure 5. Comparison of outer membrane proteins extracted from cells of N. gonorrhoeae CS-7. Duplicate cultures were grown and harvested at either logarithmic (point a) or stationary phase (point b). After extraction, products were adjusted to equal amounts of phospholipid and subjected to SDS-PAGE (gels a and b).


Klett Units
400
300
200
100
80
60
40
20
a
b
1
2
3
4
5
6
7
8
9
10
Hours
a
b

Effect of NaSCN concentration. Batch cultures of N. gonorrhoeae CS-7 were grown, harvested, pooled, and equal aliquots extracted with various concentrations of NaSCN (0.25, 0.50, 0.75, 1.0 and 2.0 M) according to the extraction procedure outlined in Materials and Methods. In one experiment, cells were grown in a small quantity of basal medium containing 0.1 μCi/ml of sodium [1,2-^{14}C] acetate. Cells labeled in this manner were combined with a large quantity of unlabeled cells, mixed and distributed into four equal aliquots. These aliquots of cells were then extracted with either 0.25, 0.50, 1.0, or 2.0 M NaSCN. Aliquots of unextracted cells were counted and compared with counts obtained from the extracted material. These values were used to calculate the product yield. The results are summarized in Table 4. Recovery increases to a maximum obtained by extraction with either 1.0 or 2.0 M NaSCN. Based on these data, which indicated no significant increase in product yield when the concentration of NaSCN was increased beyond 1.0 M, concentrations greater than 2.0 M NaSCN were not tested.

The thiocyanate-extracted material was assayed for protein, phosphorus (phospholipid), KDO, and heptose. Results of these assays are shown in Table 5. The yields of the various membrane components increase as the NaSCN concentration is increased to 1.0 M. When the thiocyanate concentration is raised to 2.0 M, however, the yield of the membrane components appears to level off or actually decrease. This may be partly due to increased solubilization of extracted materials which cannot then be sedimented in the ultracentrifuge.

Table 4. Effect of thiocyanate concentration on extraction of [1,2-^{14}C] acetate-labeled N. gonorrhoeae.

NaSCN concentration (M)	Initial activity of whole cells (cpm)	Total cpm in product	Recovery[a] (percent)
0.25	4.69×10^5 ($4.52 - 4.85 \times 10^5$)	3624 (3521-3727)	0.75 (.72 - .78)
0.50	4.13×10^5 ($3.85 - 4.62 \times 10^5$)	4989 (4505-5473)	1.18 (.97 - 1.4)
1.00	4.20×10^5 ($3.83 - 4.78 \times 10^5$)	5568 (5118-6019)	1.35 (1.1 - 1.6)
2.00	4.39×10^5 ($3.76 - 5.23 \times 10^5$)	5745 (5242-6248)	1.31 (1.0 - 1.6)

Values shown are averages of three determinations. Ranges of values are shown in parentheses.

[a]Recovery = $\frac{\text{Total cpm in final product}}{\text{Total cpm in whole cells prior to extraction}} \times 100$

Table 5. Partial characterization of product extracted from N. gonorrhoeae CS-7 with NaSCN.

NaSCN concentration (M)	Protein (mg/g dry weight)[a]	Phospholipid (μmole/g dry weight)	KDO[b] (μmole/g dry weight)	Heptose (μmole/g dry weight)
0.25	1.56	3.28	0.055	0.225
	(0.76 - 2.13)	(2.90 - 3.62)	(0.052 - 0.062)	(0.080 - 0.361)
0.50	2.39	4.28	0.074	0.243
	(2.08 - 2.75)	(4.12 - 4.32)	(0.071 - 0.082)	(0.117 - 0.372)
0.75	2.44	4.37	0.065	0.239
	(2.08 - 2.79)	(4.19 - 4.42)	(0.059 - 0.071)	(0.085 - 0.375)
1.00	2.62	4.59	0.106	0.329
	(2.08 - 3.03)	(4.52 - 4.61)	(0.074 - 0.126)	(0.163 - 0.510)
2.00	1.142	4.14	0.089	0.307
	(0.49 - 1.52)	(3.94 - 4.36)	(0.061 - 0.111)	(0.099 - 0.553)

Values shown are averages of three determinations. Ranges of values are shown in parentheses.

[a]Dry weight is of cells prior to extraction.

[b]KDO = 2-Keto-3-deoxyoctonate.

To examine the phenomenon of selective extraction of membrane components at higher NaSCN concentrations, the ratios of heptose, KDO and protein to phospholipid were calculated. The ratios of heptose/phospholipid and KDO/phospholipid (Fig. 6) are relatively constant as the concentration of NaSCN is varied from 0.25 to 2.0 M. However, the protein/phospholipid ratio reaches a maximum with a NaSCN concentration of 0.75 to 1.0 M and then decreases rapidly at a concentration of 2.0 M (Fig. 7). This suggests that at NaSCN concentrations from 0.75 M to 1.0 M, more protein is associated with the extracted phospholipid-containing membrane. At a concentration of 2.0 M, the protein appears to be extracted from the phospholipid matrix and probably remains in the 100,000 x g supernatant. The constant ratios of heptose/phospholipid and KDO/phospholipid indicate that only proteins are primarily involved, as the ratios of KDO and heptose (lipopolysaccharide markers) to phospholipid would decrease if they were also extracted. Based on these findings, 1.0 M NaSCN was used throughout the remaining experiments.

Effect of extraction time. Equal aliquots of logarithmic phase cells of N. gonorrhoeae were extracted for various times. The results (Fig. 8) show that the protein/phospholipid ratio remains relatively constant throughout the range of extraction times. The actual amounts of protein and phospholipid remain steady until approximately 150 min, at which time a gradual increase in both is noted. SDS-PAGE indicated that all samples contained similar protein bands.

Figure 6. Effect of NaSCN concentration on the ratios of KDO/phospholipid and heptose/phospholipid in outer membrane components extracted from N. gonorrhoeae CS-7. Symbols: ○, μmoles heptose/μmoles phospholipid; △, μmoles KDO/μmole phospholipid.

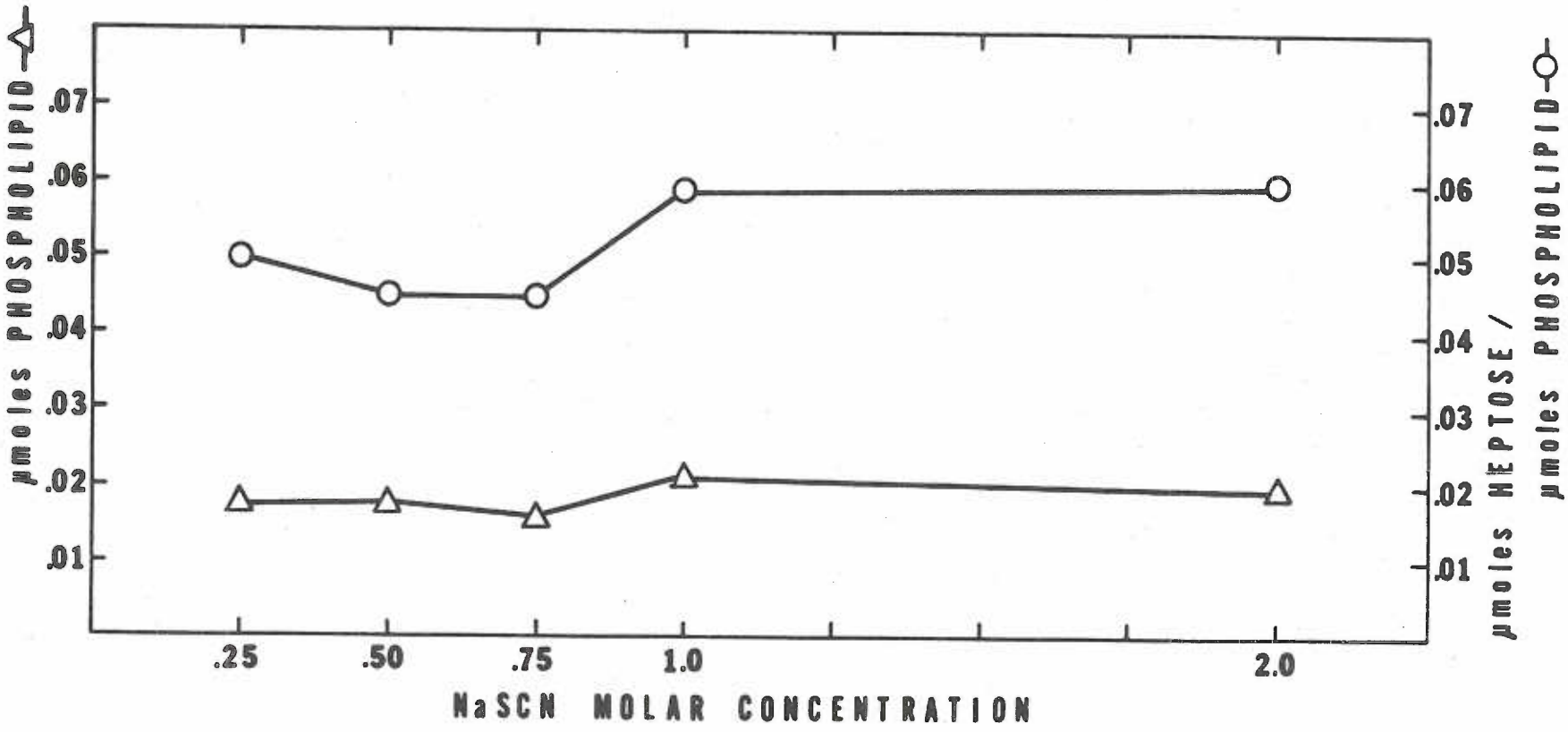

μmoles PHOSPHOLIPID
.07
.06
.05
.04
.03
.02
.01
μmoles HEPTOSE / μmoles PHOSPHOLIPID
.25
.50
.75
1.0
2.0
NaSCN MOLAR CONCENTRATION

Figure 7. Effect of NaSCN concentration on relative protein concentration of outer membrane components extracted from N. gonorrhoeae CS-7.

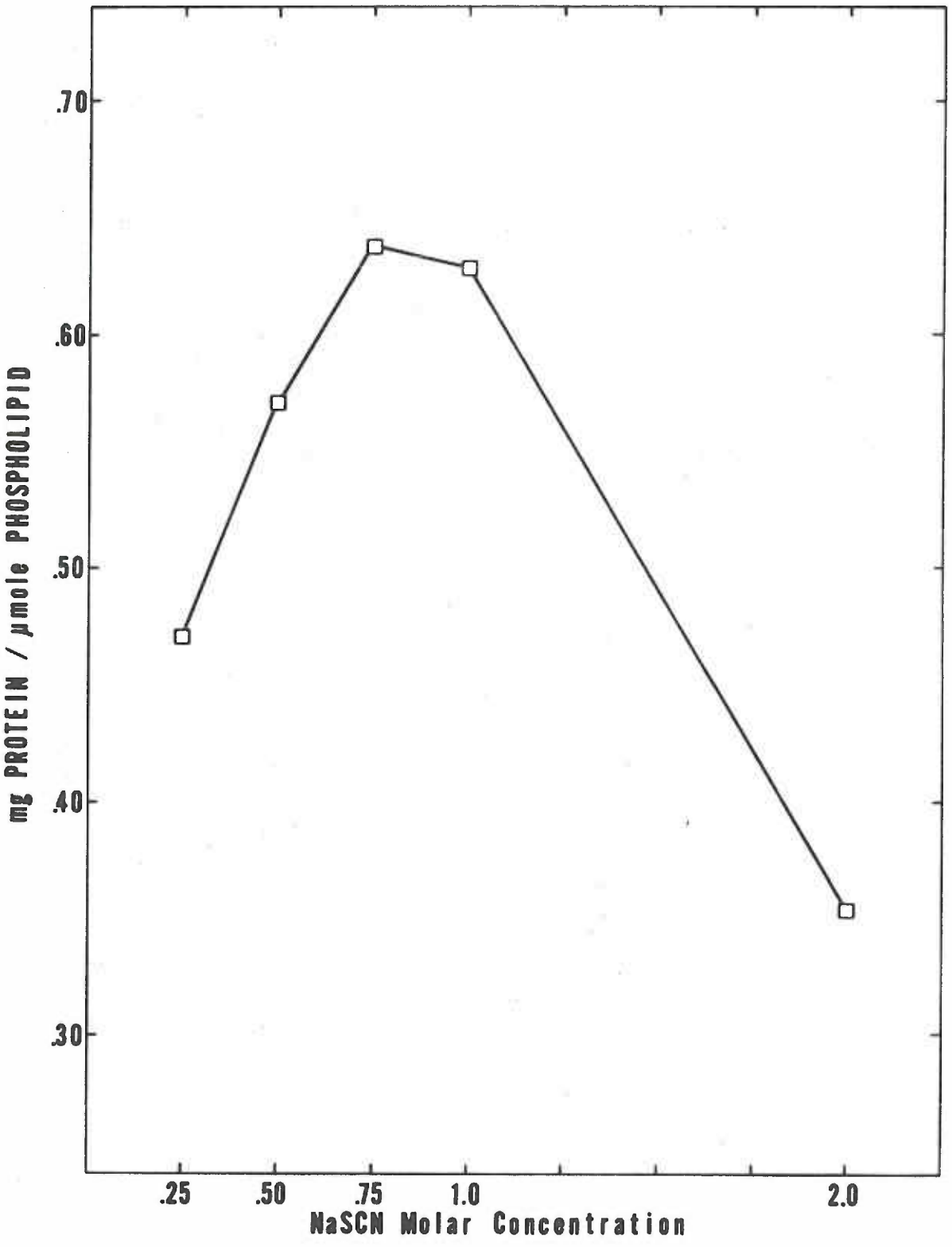
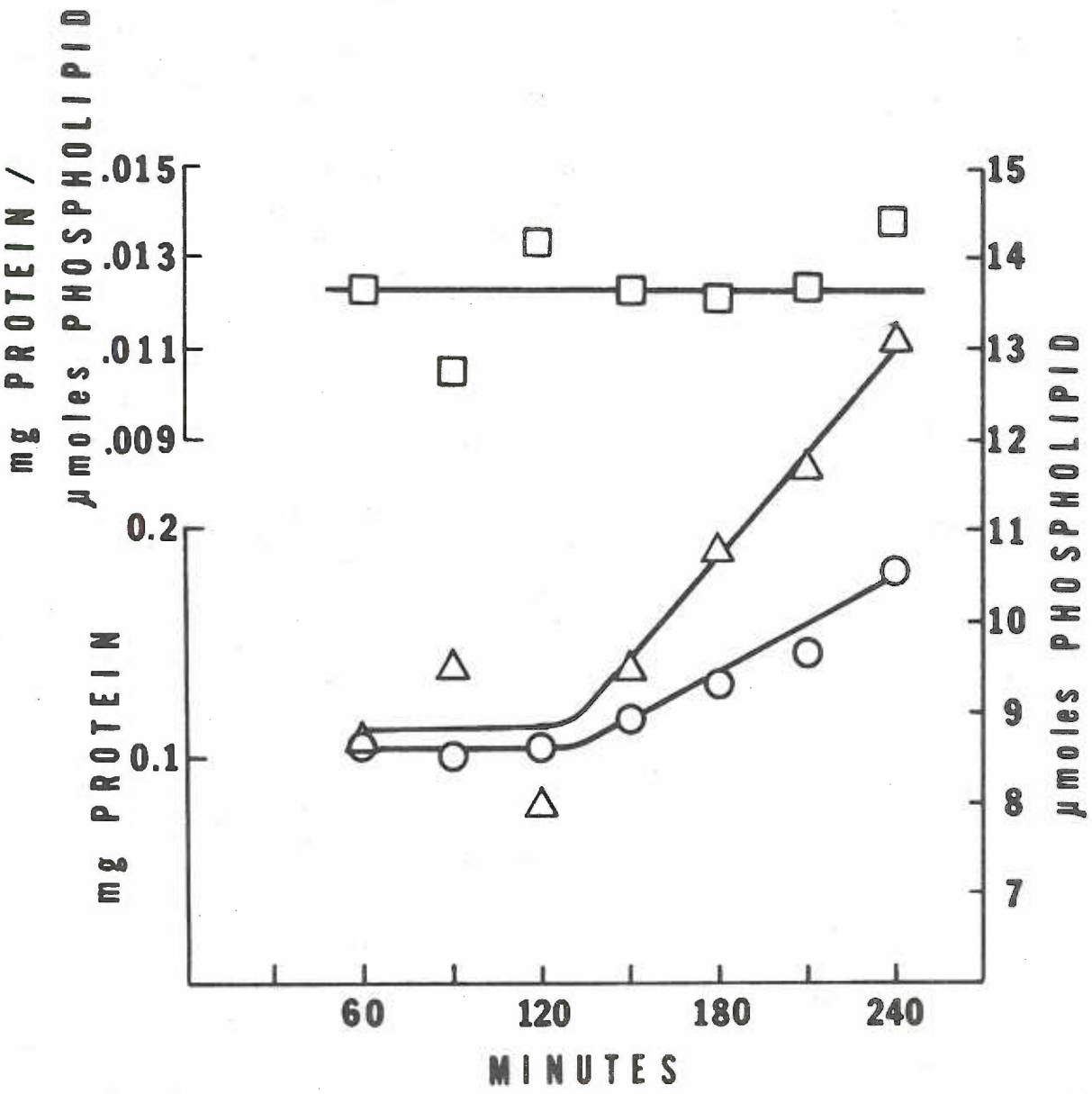

.70
.60
.50
.40
.30
mg PROTEIN / µmole PHOSPHOLIPID
.25
.50
.75
1.0
2.0
NaSCN Molar Concentration

Figure 8. Effect of time of extraction by 1 M NaSCN on the composition of the final product extracted from N. gonorrhoeae CS-7. Symbols: □, mg protein/μmole phospholipid; △, μmoles phospholipid; ○, mg protein.


mg PROTEIN / µmoles PHOSPHOLIPID
.015
.013
.011
.009
0.2
0.1
mg PROTEIN
15
14
13
12
11
10
9
8
7
µmoles PHOSPHOLIPID
60
120
180
240
MINUTES

Extraction of other gram-negative organisms. The presence of lipoprotein molecules in the cell envelope of gram-negative organisms is thought to be at least partly responsible for anchoring the outer membrane to the rest of the multilayered complex (21). However, the distribution of lipoprotein is not ubiquitous among gram-negative bacteria. To determine whether the presence of lipoprotein in the cell envelope would affect the ease of extraction of membrane components, organisms known to have lipoprotein (E. coli and S. typhimurium) as well as organisms having little or no lipoprotein (P. aeruginosa, N. gonorrhoeae) were extracted with 1 M NaSCN. The extraction products were assayed chemically for protein and phospholipid and examined by SDS-PAGE.

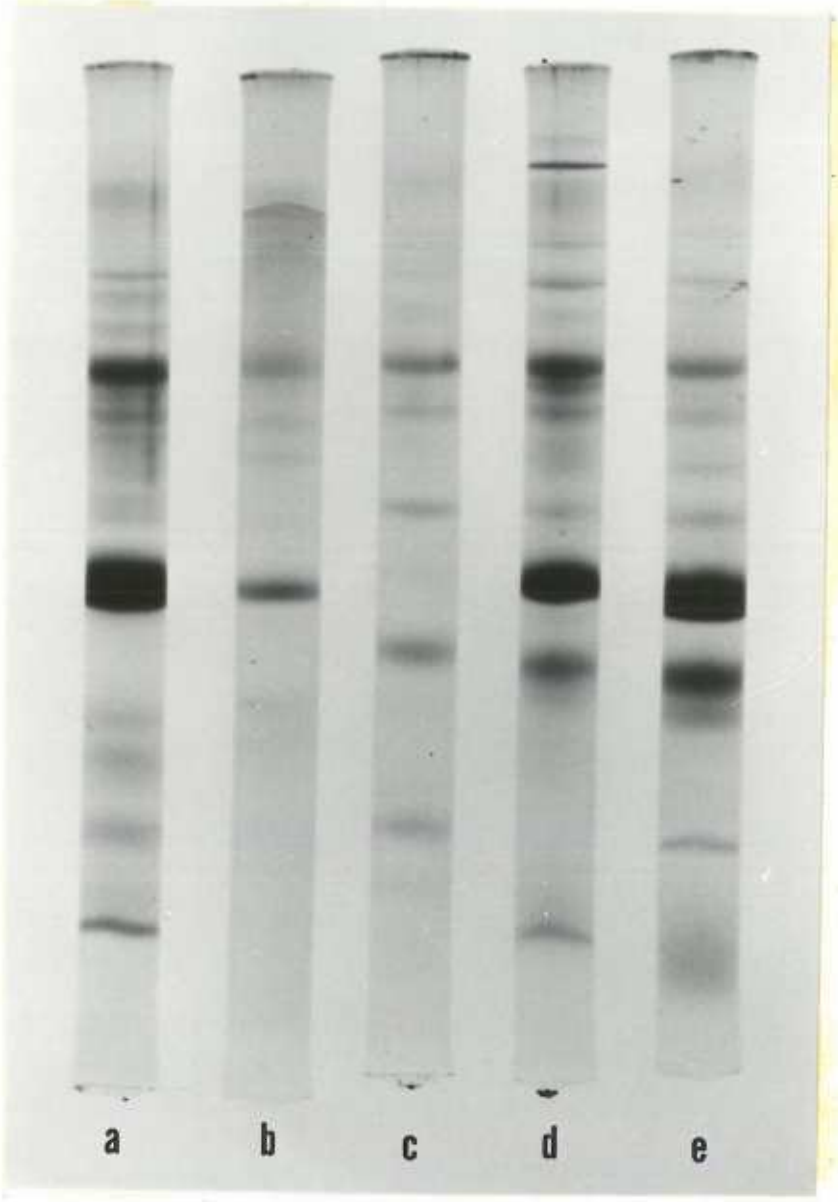
The results in Table 6 show that with the organisms examined, the presence or absence of lipoprotein has no direct bearing on the extractability of outer membrane components. However, only small amounts of extractable material were obtained from S. typhimurium DB-21. The reasons for these differences will be discussed later. Fig. 9 shows polyacrylamide gels of the thiocyanate-extracted material from organisms listed in Table 6. With the exception of P. aeruginosa, all organisms have a major outer membrane protein with an apparent molecular weight of 39,000 to 45,000 daltons. In addition to this major protein, a limited number of minor bands are also visible. These results compare favorably to those observed with other outer membrane extraction procedures (28, 46, 107, 109). The variation in the ratio of protein/phospholipid between bacterial species is apparent in the stained gels which were prepared using equal amounts of phospholipid.

Table 6. Partial characterization of thiocyanate-extracted product from various gram-negative bacteria.

Organism	µg protein/ mg dry weight	µmole phospholipid/ mg dry weight
N. gonorrhoeae CS-7	1.85 (1.40 - 2.25)	0.14 (0.11 - 0.17)
P. aeruginosa PS-7	1.74 (1.26 - 2.22)	0.46 (0.35 - 0.52)
E. coli K-12	2.43 (2.10 - 2.76)	0.15 (0.10 - 0.18)
S. typhimurium DB-21	0.08 (0.068 - 0.092)	0.04 (0.03 - 0.05)

Values shown are averages of three determinations. Ranges of values are shown in parentheses.

Figure 9. SDS-polyacrylamide gels of thiocyanate-extracted product from various gram-negative bacteria. Gels: a) E. coli K-12; b) S. typhimurium DB-21; c) P. aeruginosa PS-7; d) N. gonorrhoeae CS-7; e) N. gonorrhoeae JW-31. Samples were adjusted to equal amounts of phospholipid. After SDS-PAGE, gels were stained with Coomassie blue.


a
b
c
d
e

Comparison of chaotropic agents. To compare the usefulness of 1 M NaSCN to 1 M solutions of other chaotropic agents, 4 liters of a log phase culture of N. gonorrhoeae CS-7 was grown and pooled together with 50 ml of cells labeled during growth in the presence of 0.2 μCi/ml of D-[U-^{14}C]-glucose. After mixing, equivalent aliquots were extracted as per the protocol listed in Materials and Methods. The molecular weights of the chaotropic agents used and the pH of a 1 M solution in water are shown in Table 7. The extraction product was assayed for radioactivity and protein and phospholipid determinations were also performed. The results are summarized in Table 8 and Fig. 10. NaSCN (1 M) compares favorably with the other chaotropic agents tested. The protein/phospholipid ratio (Table 8) of the extraction product shows some variation with the agents used and this probably reflects the differential ability of the various agents to extract proteins from their phospholipid matrix. A comparison of Table 7 and Fig. 10 shows that there is no correlation between the pH of a 1 M solution of the chaotropic agent and its relative effectiveness in extracting outer membrane components.

A comparison of the data in Table 8 shows that some agents, such as guanidine SCN and KBr appear to extract low amounts of protein (reflected in the protein/phospholipid ratios). This may be a reflection of the differential ability of these agents to extract proteins from their phospholipid matrix, or it may indicate a solubilization of certain proteins which are not sedimented by ultracentrifugation. Samples of outer membrane components extracted by NaSCN, guanidine SCN, LiCl,

Table 7. Properties of chaotropic agents.

Formula	Molecular weight	pH of 1 M solution[a]
NaSCN	81.07	6.7
NaCl	58.4	6.15
LiCl	52.39	6.1
$NH_2C(NH)NH_2 \cdot HSCN$	118.16	5.18
KCl	74.56	5.19
NaBr	102.89	5.65
KBr	119.01	5.9
KI	166.01	8.57

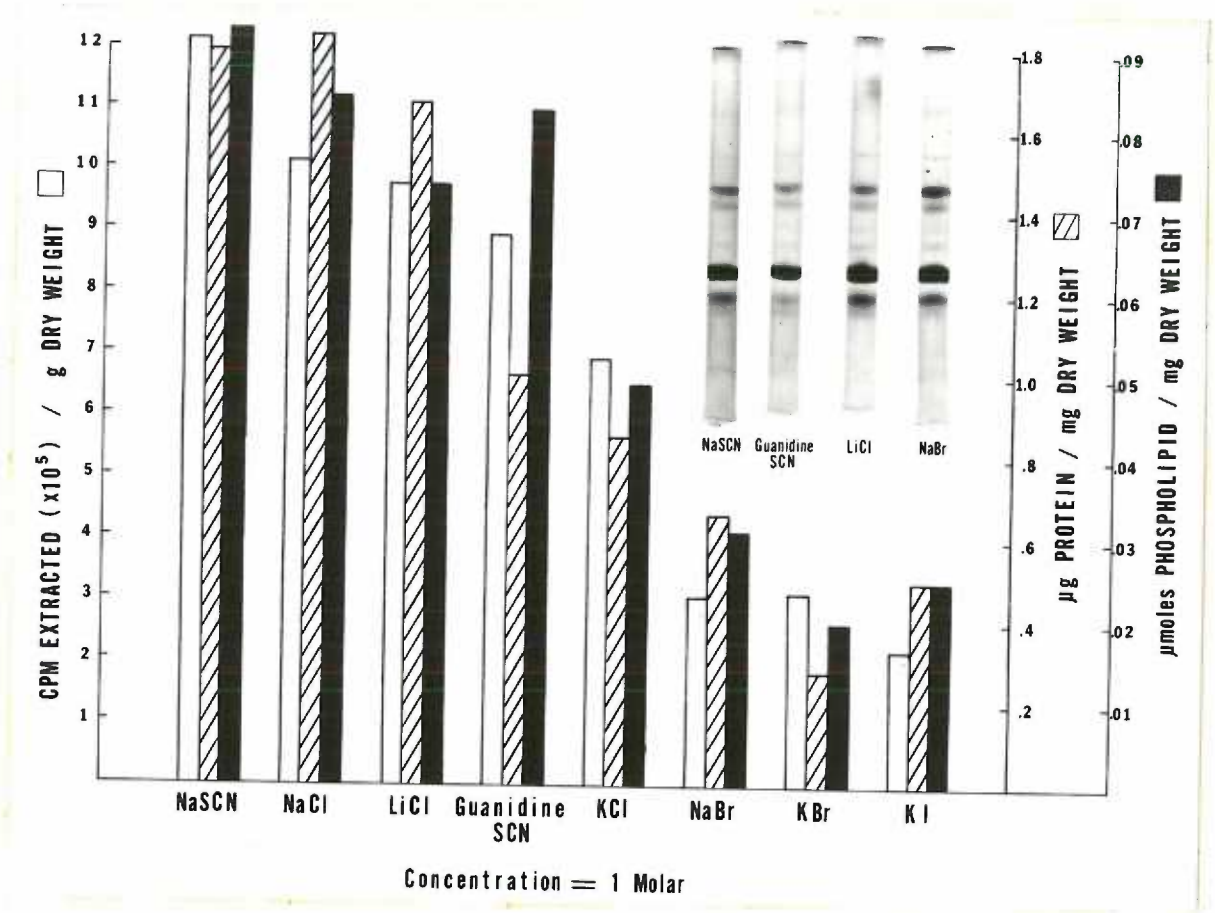
[a]Distilled water used for preparing the solutions was pH 6.4.

Table 8. Partial characterization of products extracted from [U-^{14}C]-glucose labeled N. gonorrhoeae CS-7 by various chaotropic agents (1 M).

Chaotropic agent	CPM extracted/ g dry weight	µg protein/ mg dry weight	µmole phospholipid/ mg dry weight	µg protein/ µmole phospholipid
NaSCN	12040 (11597-13494)	1.79 (1.68 - 1.90)	0.093 (0.083 - 0.097)	19.8 (19.5 - 20.2)
NaCl	10158 (9876-12791)	1.85 (1.73 - 1.97)	0.084 (0.079 - 0.089)	22.0 (21.8 - 22.1)
LiCl	9732 (9264-1159)	1.66 (1.40 - 1.71)	0.073 (0.065 - 0.075)	22.2 (21.5 - 22.8)
Guanidine SCN	8967 (7549-9488)	1.00 (0.95 - 1.12)	0.083 (0.081 - 0.090)	12.0 (11.7 - 12.6)
KCl	6923 (5421 - 7561)	0.85 (0.76 - 0.93)	0.049 (0.032 - 0.055)	17.3 (16.9 - 22.3)
NaBr	3085 (2739-3472)	0.66 (0.62 - 0.69)	0.031 (0.024 - 0.035)	21.3 (19.7 - 25.8)
KBr	3135 (2505-3716)	0.28 (0.19 - 0.35)	0.020 (0.018 - 0.022)	14.0 (10.5 - 15.9)
KI	2257 (1905-2617)	0.50 (0.41 - 0.62)	0.025 (0.018 - 0.031)	20.0 (19.3 - 22.7)

Values shown are averages from three determinations. Ranges of values are shown in parentheses.

Figure 10. Histogram representing characteristics of [U-^{14}C]-glucose labeled outer membrane components extracted. Equal aliquots of cells were extracted by one of the indicated chaotropic agents (1 M). Symbols: ☐, counts per minute; ▨, µg protein; and ■, µmole phospholipid are all expressed relative to dry weight of cells extracted. Insert: SDS-polyacrylamide gels of outer membrane proteins extracted by NaSCN, guanidine SCN, LiCl, and NaBr.


CPM EXTRACTED (x10⁵) / g DRY WEIGHT
μg PROTEIN / mg DRY WEIGHT
μmoles PHOSPHOLIPID / mg DRY WEIGHT
NaSCN
NaCl
LiCl
Guanidine SCN
KCl
NaBr
KBr
KI
Concentration = 1 Molar

and NaBr were examined by SDS-PAGE at equivalent phospholipid concentrations. The results are shown in Fig. 10 (inset). The stained protein-containing bands in the guanidine SCN gel are the faintest of the four gels, a direct reflection of the low protein/phospholipid ratio.

Comparison of outer membrane protein profiles from colonial variants of N. gonorrhoeae. Colonial variants of strains 1362, 1365, 1407 and F62 were selected and maintained by passage on GC agar. Broth-grown cultures were prepared as previously described in Materials and Methods. After extraction with 1 M NaSCN, the outer membrane complex was analyzed for protein, phospholipid, KDO and heptose. Differences in the ratio of protein/phospholipid are shown in Table 9. An interesting observation is that within the individual strains examined, T3 colonial variants appear to yield lower amounts of protein and phospholipid than other colonial variants.

The ratios of KDO/heptose were usually between 0.5 and 1.2; most often less than 1.0. The ratio of KDO/heptose in purified lipopolysaccharide from N. gonorrhoeae strains CS-7 and JW-31 was 0.83 and 0.79 respectively. Previously published values for the KDO/heptose ratio in gonococcal lipopolysaccharide are between 0.69 and 1.85 (79, 92). With the exception of N. gonorrhoeae 1407, all other strains and colony types examined gave values that were similar to published findings.

Interstrain as well as intrastrain variations as shown in SDS-PAGE of outer membrane proteins extracted from N. gonorrhoeae strains 1362,

Table 9. Comparison of outer membrane components extracted from four strains of *N. gonorrhoeae*.

µg Protein/µmole phospholipid

Colony Type	Strain 1362	1365	1407	F62
T1	30.22 (26.92-33.52)	17.87 (15.39-20.35)	27.87 (25.92-29.82)	18.67 (17.22-20.12)
T2	17.22 (16.65-17.79)	29.30 (27.90-30.70)	22.17 (18.54-25.80)	22.12 (19.31-24.93)
T3	0.78 (0.41-1.15)	6.67 (4.61-8.73)	10.00 (8.30-11.70)	- -
T4	65.40 (59.77-71.03)	35.17 (33.65-36.69)	34.60 (31.17-38.03)	12.55 (10.72-14.38)

Values shown are averages from duplicate determinations. Ranges of values are shown in parentheses.

1365, 1407, and F62 are shown in Figures 11, 13, 15, and 17. Qualitative as well as quantitative differences are evident, especially in strains 1362, 1365 and F62. In strain 1407 (Fig. 17), the differences are more quantitative in nature as can be seen in bands B, C, and D. Densitometer scans of the gels substantiate differences in protein band patterns within and between strains (Figs. 12, 14, 16, 18). Positions of the positive and negative electrodes relative to the scanned gels are also noted. It is apparent that there is one predominant outer membrane protein which accounts for approximately 40-60% of the total outer membrane proteins extracted. The apparent molecular weight of this protein is strain-specific, and remains constant in all colonial variants from that strain. However, the apparent molecular weight of the major outer membrane protein varies between strains (Figs. 11, 13, 15, 17).

Higher molecular weight proteins (45,000-120,000 daltons) are also present as minor components (Figs. 11-18). These proteins appear relatively constant within strains, and due to their low concentration, will not be discussed further.

The only adequate outer membrane protein pattern from a T3 colonial variant was obtained from strain 1362 (Figs. 15, 16). Since T3 colonial variants rapidly convert to T4 variants, it was difficult to grow them in pure culture. The identity of the large peak appearing at the left of peak A in the scan of 1362 T2 (Fig. 16) is unknown, and was not observed in subsequent preparations of 1362 T2.

Figure 11. SDS-PAGE of outer membrane proteins of N. gonorrhoeae F62 T1, T2 and T4. Positions of standards and their relative molecular weights are shown on the left. On the right, bands A, B, C, D, and E are indicated along with their apparent molecular weights. Gels are stained with Coomassie blue.

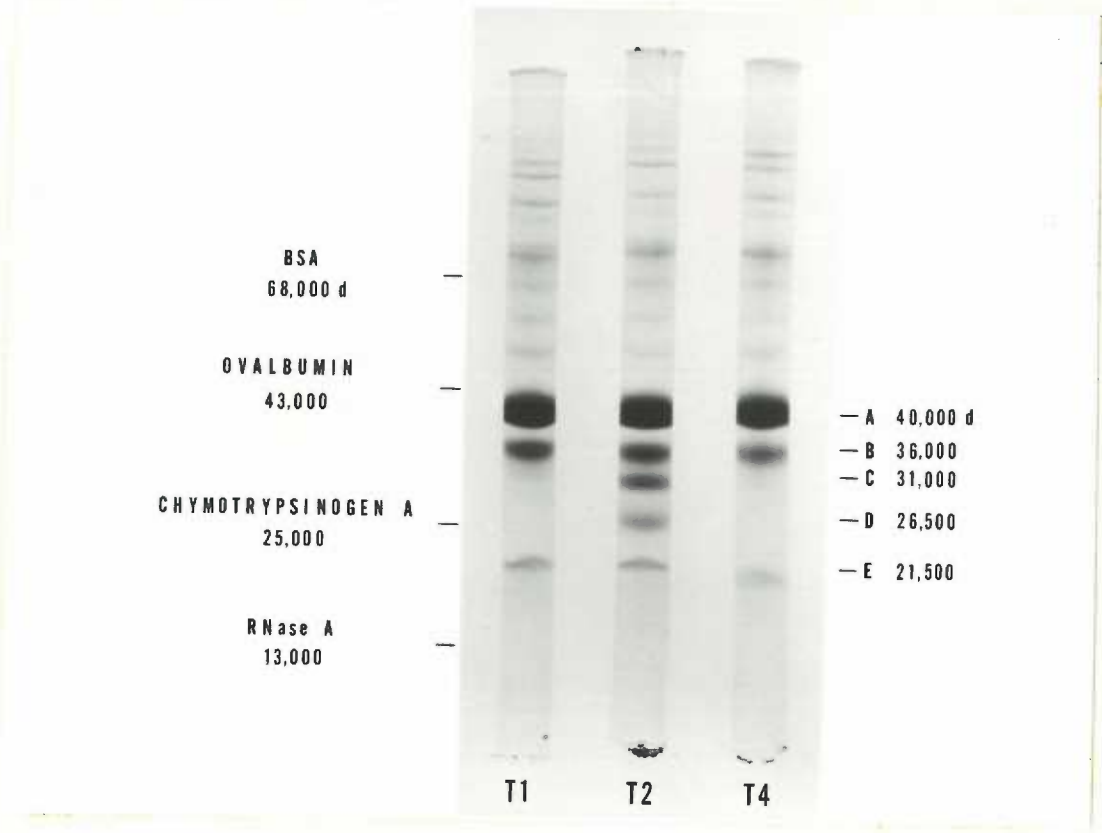

BSA
68,000 d
OVALBUMIN
43,000
CHYMOTRYPSINOGEN A
25,000
RNase A
13,000
A 40,000 d
B 36,000
C 31,000
D 26,500
E 21,500
T1
T2
T4

Figure 12. Densitometer profiles of SDS-polyacrylamide gels of outer membrane proteins of N. gonorrhoeae F62 colony types T1, T2, and T4. Labeled peaks correspond to labeled bands shown in Fig. 11.

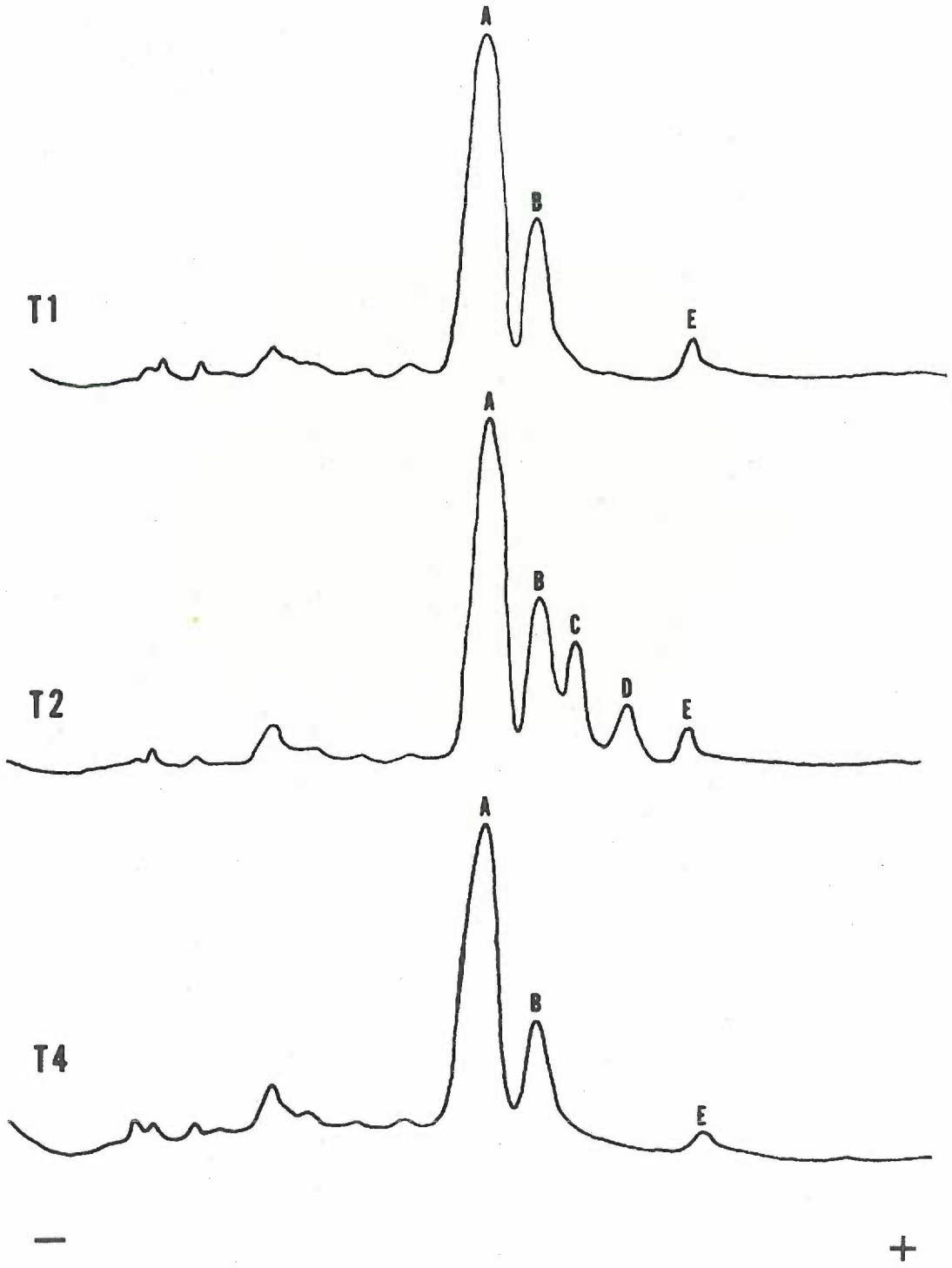

T1
A
B
E
T2
A
B
C
D
E
T4
A
B
E
—
+

Figure 13. SDS-PAGE of outer membrane proteins of N. gonorrhoeae 1365 T1, T2 and T4. Positions of standards and their relative molecular weights are shown on the left. On the right, bands A, B, C, D, and E are indicated, along with their apparent molecular weights. Gels are stained with Coomassie blue.

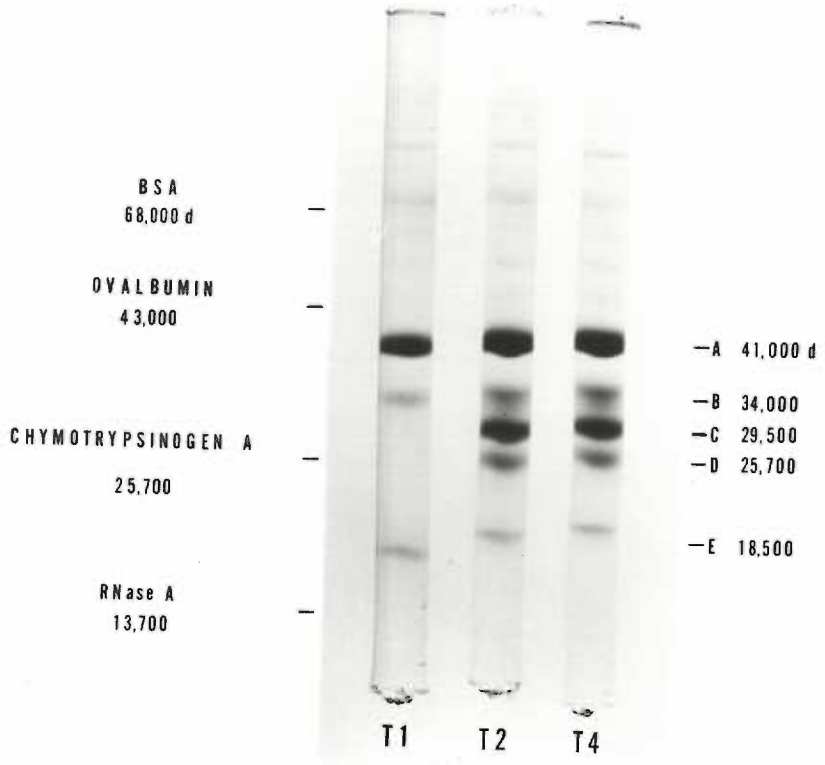

BSA
68,000 d
OVALBUMIN
43,000
CHYMOTRYPSINOGEN A
25,700
RNase A
13,700
A 41,000 d
B 34,000
C 29,500
D 25,700
E 18,500
T1
T2
T4

Figure 14. Densitometer profiles of SDS-polyacrylamide gels of outer membrane proteins of N. gonorrhoeae 1365 colony types T1, T2, and T4. Labeled peaks correspond to labeled bands shown in Fig. 13.

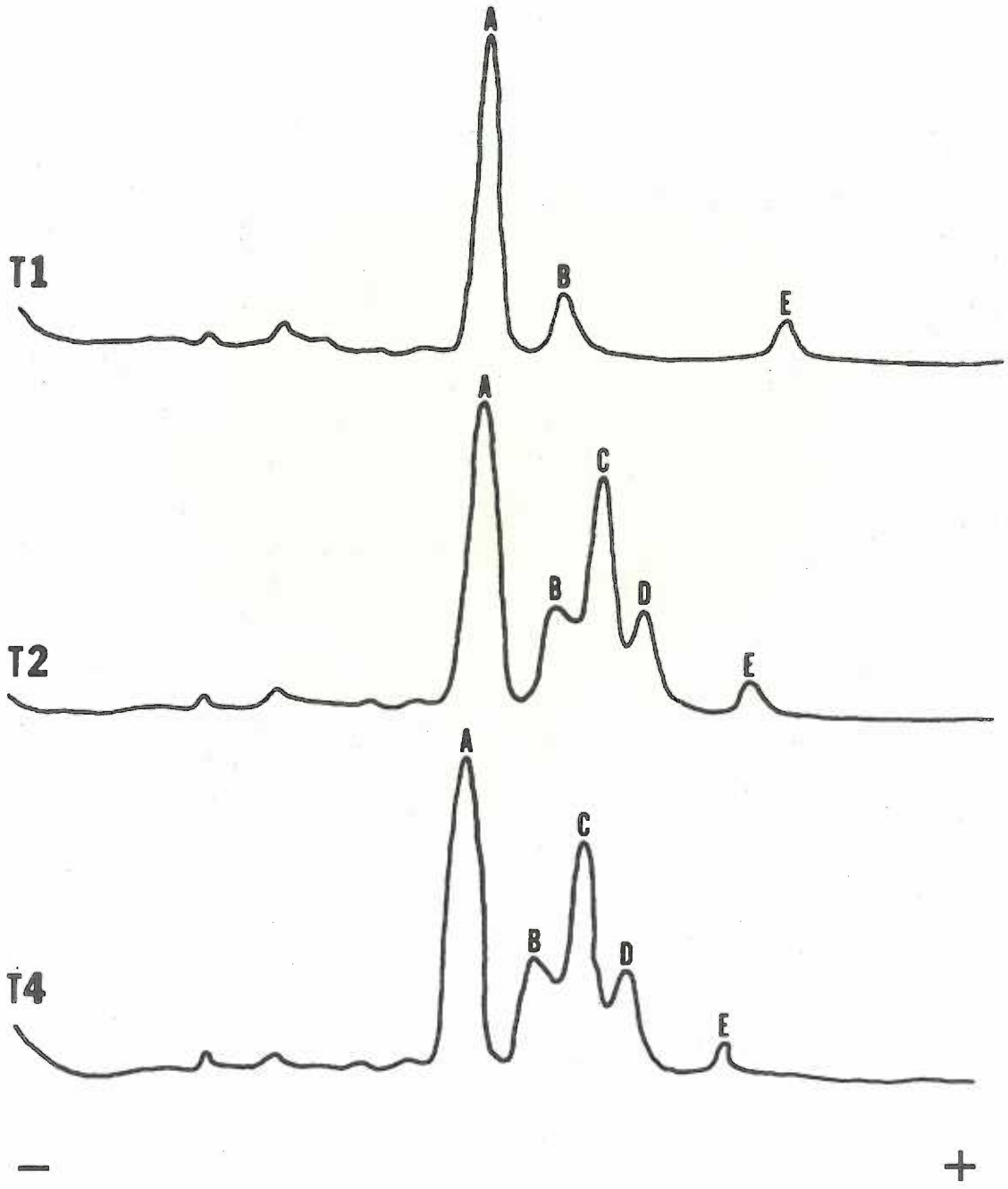

T1
A
B
E
T2
A
B
C
D
E
T4
A
B
C
D
E
−
+

Figure 15. SDS-PAGE of outer membrane proteins of N. gonorrhoeae 1362 colony types T1, T2, T3, and T4. Positions of standards and their relative molecular weights are shown on the left. On the right, bands A, B, C, D, and E are indicated along with their relative molecular weights. Gels are stained with Coomassie blue.

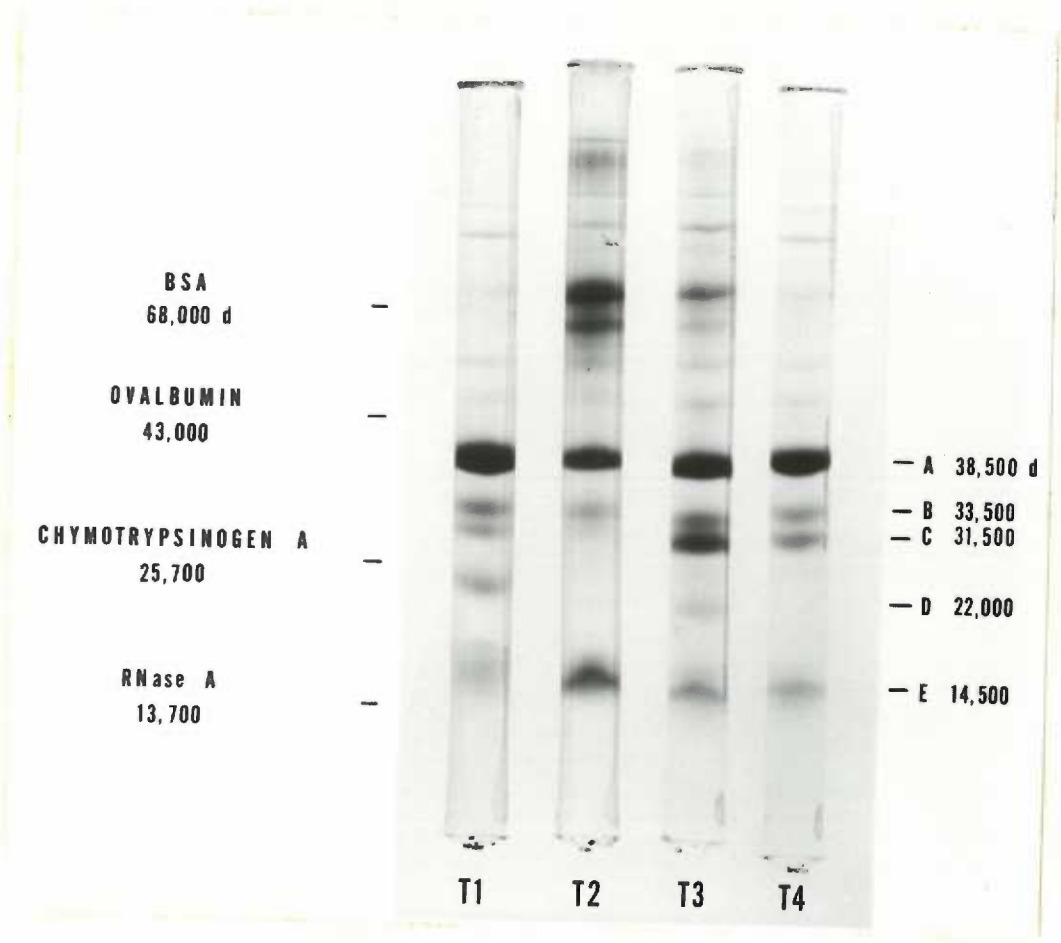

BSA
68,000 d
OVALBUMIN
43,000
CHYMOTRYPSINOGEN A
25,700
RNase A
13,700
A 38,500 d
B 33,500
C 31,500
D 22,000
E 14,500
T1
T2
T3
T4

Figure 16. Densitometer profiles of SDS-polyacrylamide gels of outer membrane proteins of N. gonorrhoeae 1362 colony types T1, T2, T3, and T4. Labeled peaks correspond to labeled bands shown in Fig. 15.

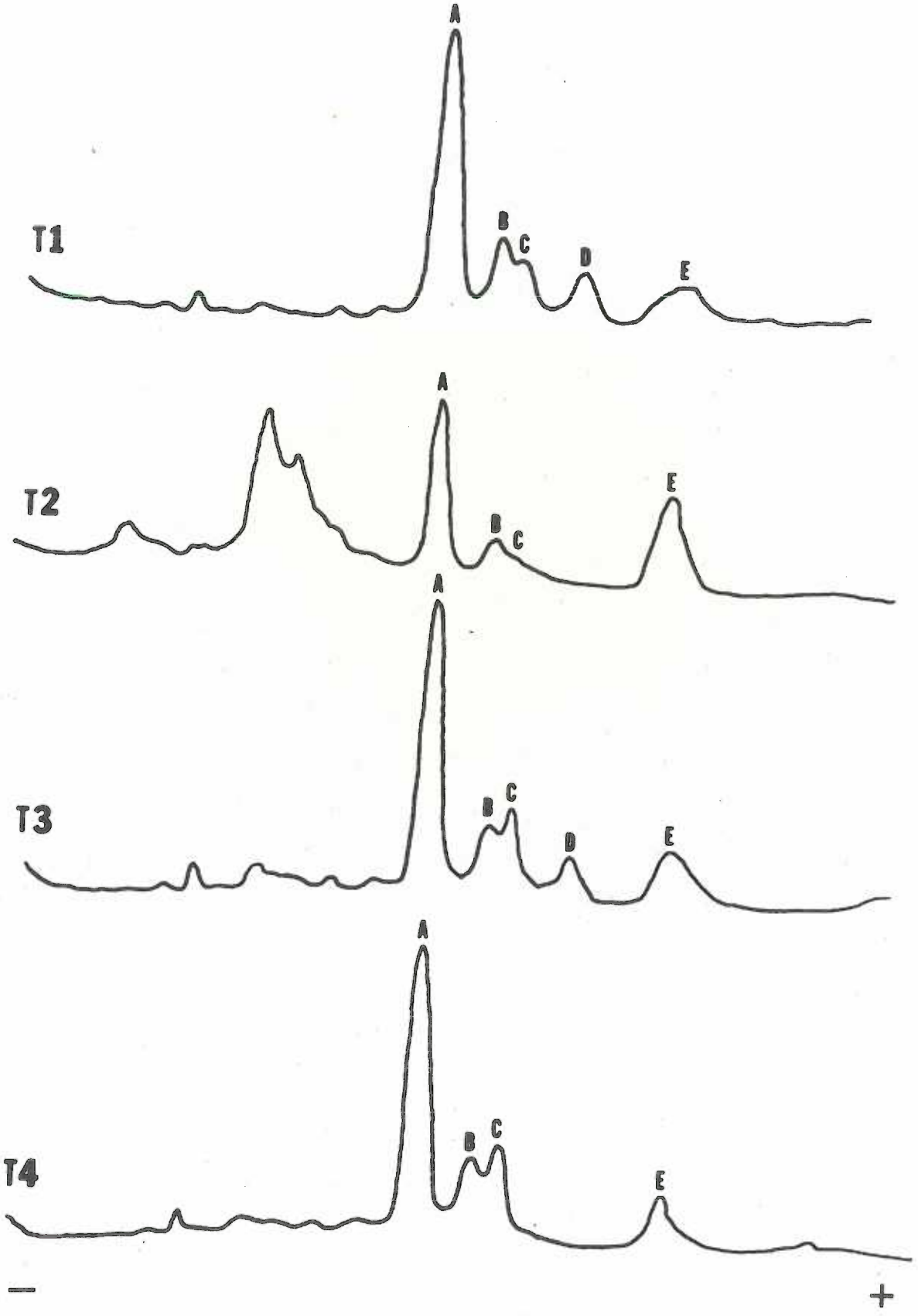

T1
A
B
C
D
E
T2
A
B
C
E
T3
A
B
C
D
E
T4
A
B
C
E
−
+

Figure 17. SDS-PAGE of outer membrane proteins of N. gonorrhoeae 1407 colony types T1, T2, and T4. Positions of standards and their relative molecular weights are shown on the left. On the right, bands A, B, C, D, and E are indicated along with their relative molecular weights. Gels are stained with Coomassie blue.

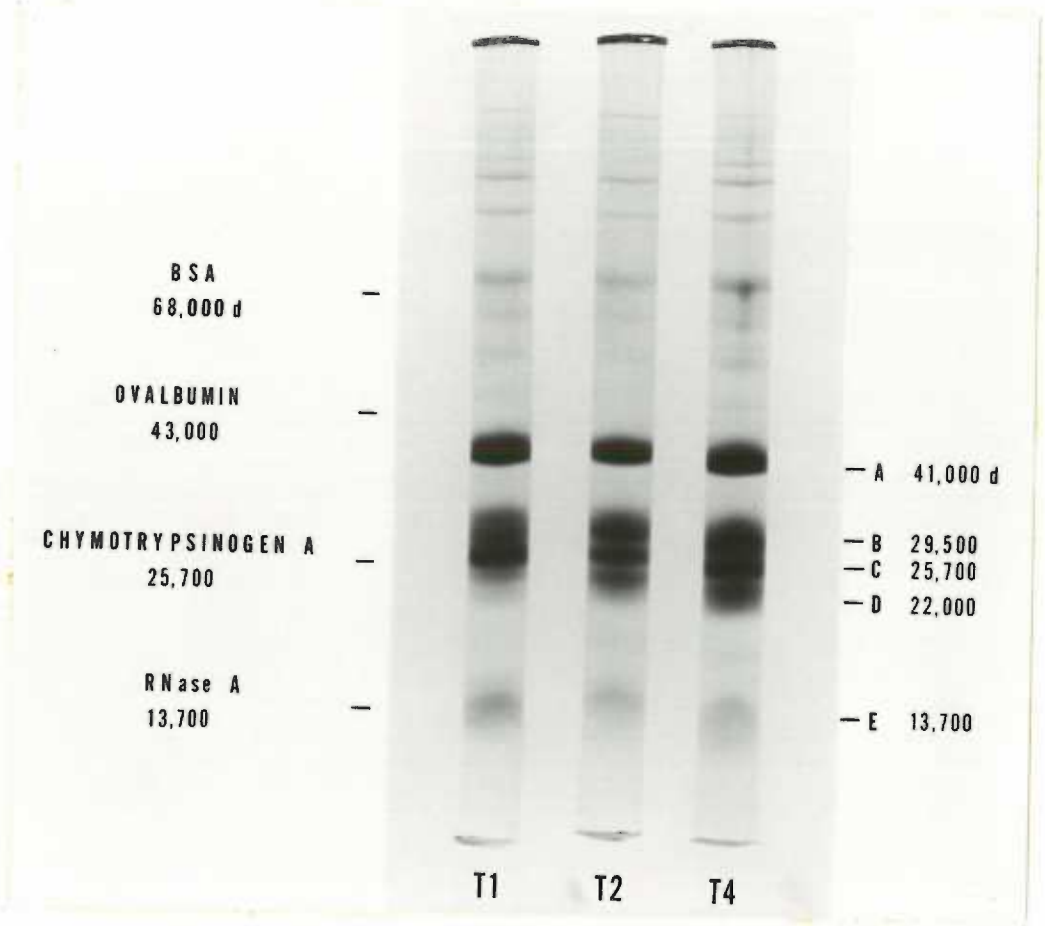
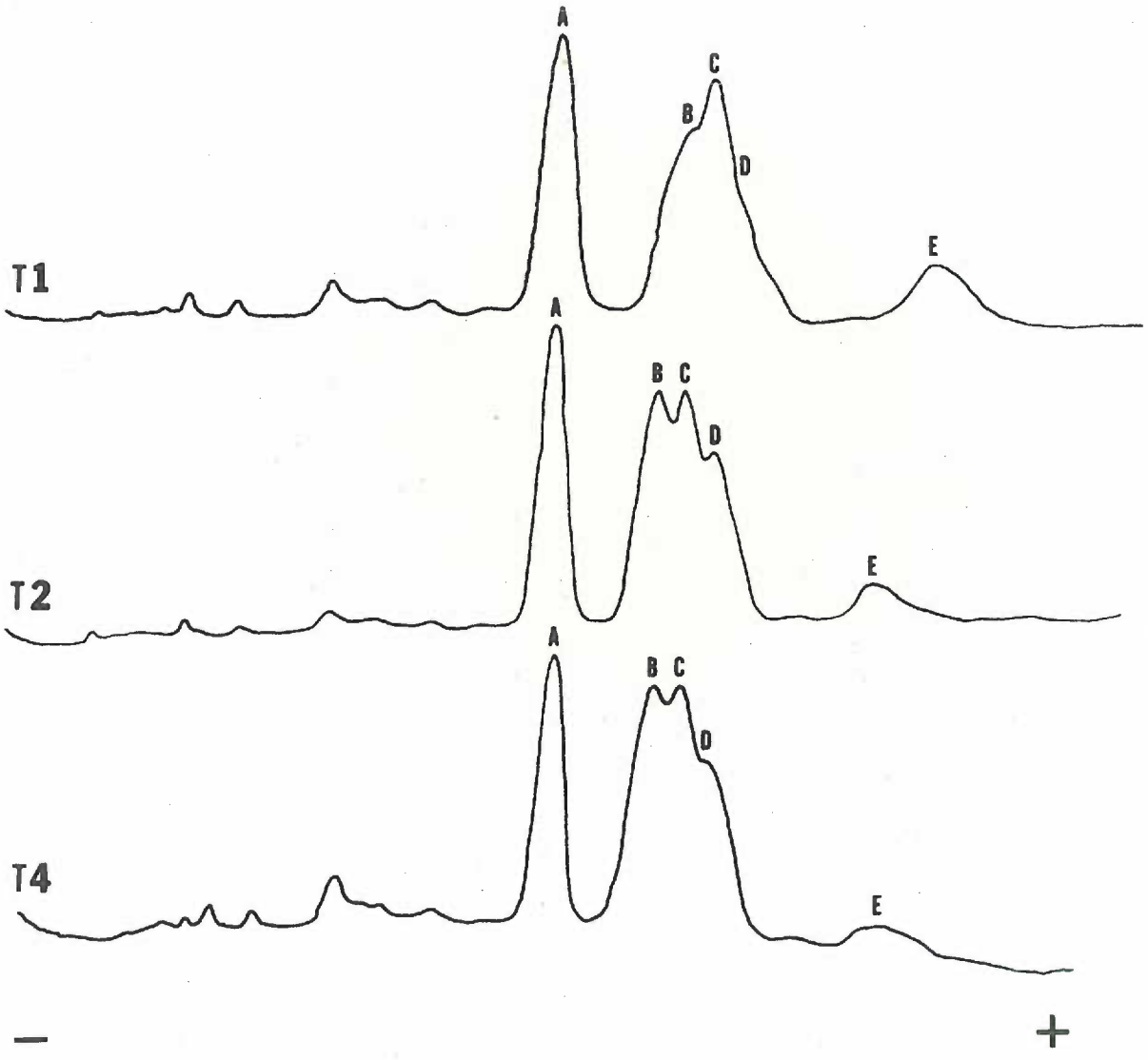

BSA
68,000 d
OVALBUMIN
43,000
CHYMOTRYPSINOGEN A
25,700
RNase A
13,700
A 41,000 d
B 29,500
C 25,700
D 22,000
E 13,700
T1
T2
T4

Figure 18. Densitometer profiles of SDS-polyacrylamide gels of outer membrane proteins of N. gonorrhoeae 1407 colony types T1, T2, and T4. Labeled peaks correspond to labeled bands shown in Fig. 17.


A
B
C
D
E
T1
A
B C
D
E
T2
A
B C
D
E
T4
–
+

The major outer membrane protein remains constant within each strain and is a stable strain characteristic. The position of this protein on SDS-PAGE remains constant within each strain, independent of other more variable proteins. Among the strains examined, the major outer membrane protein has a range in molecular weight of 38,500 to 43,000 daltons. A variation in the molecular weight of the major outer membrane protein of N. gonorrhoeae has also been reported by Johnston et al. (47).

To determine whether intrastrain protein variations are uniquely associated with colonial morphology, a T1 colonial variant of strain 1362 was isolated from a T2 variant. The variant was grown as previously described and the outer membrane extracted. The product from the T1 revertant (from T2) was subjected to SDS-PAGE in parallel with extracts from T1 and T2 variants of strain 1362. The results (Fig. 19) indicate that a change occurs in the outer membrane protein pattern when a colony reverts from one type to another.

The differences observed between outer membrane proteins of isogenic colonial variants may have resulted from dilution of minor protein species. In order to test this hypothesis, outer membrane preparations from strains F62 T1, 1365 T1, and 1365 T2 were subjected to SDS-PAGE at a two-fold increase in sample concentration. These samples were run simultaneously with samples at the normal concentration. The results (Fig. 20) show that although the stained protein bands are darker in the gels run with double strength samples, no new bands appeared.

Figure 19. SDS-PAGE of outer membrane proteins extracted from colonial variants of N. gonorrhoeae 1362. Gels: a) T1; b) T1, a spontaneous revertant from T2; c) T2.

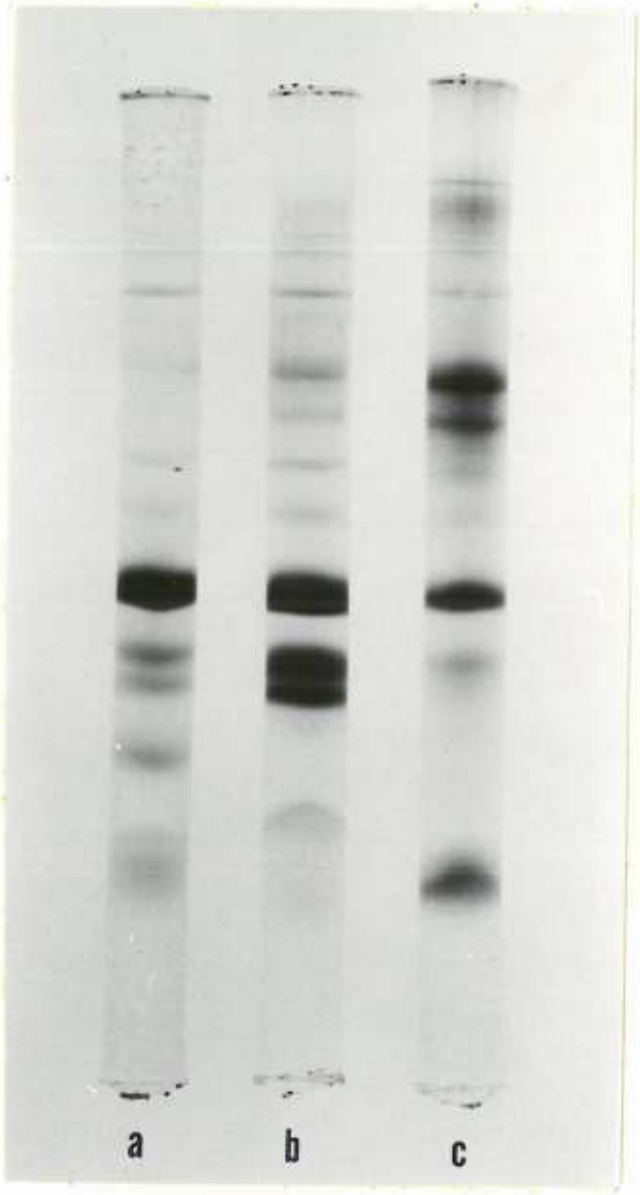
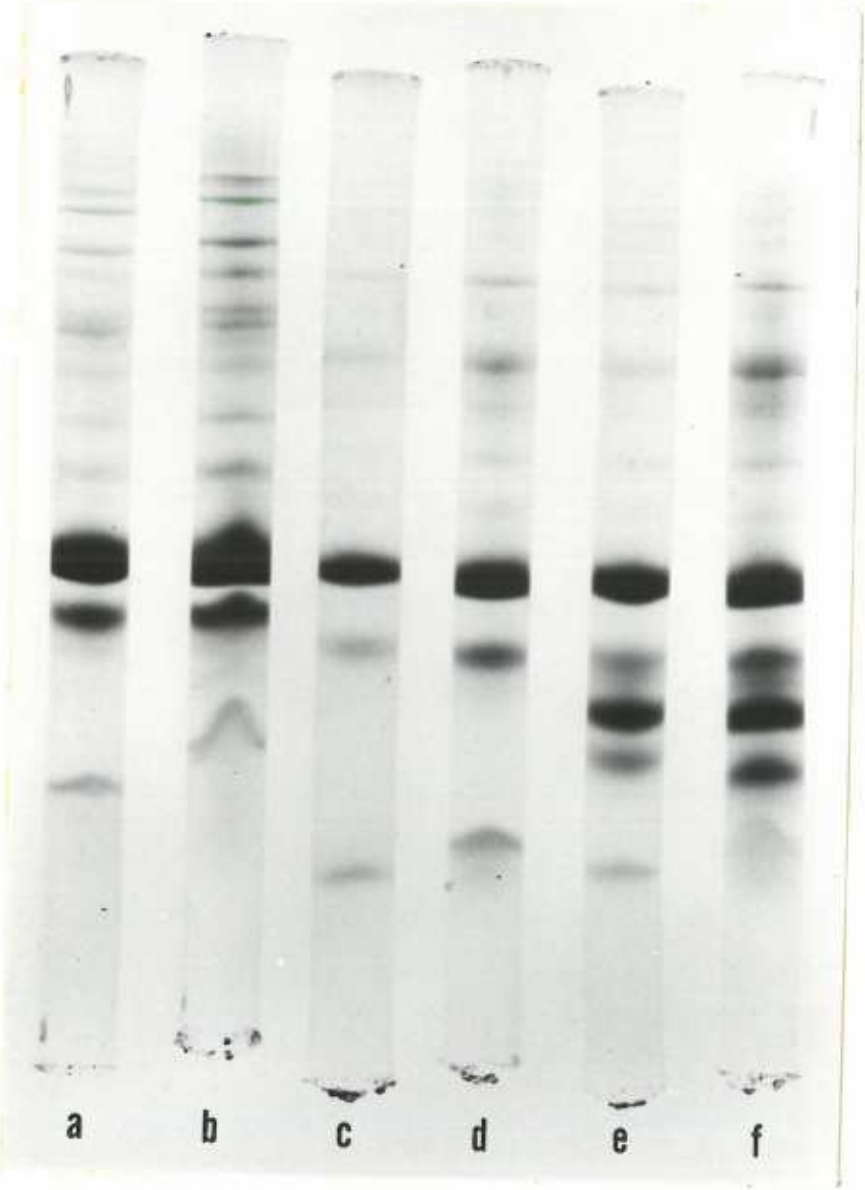

a
b
c

Figure 20. SDS-polyacrylamide gels of outer membrane proteins of different strains of N. gonorrhoeae. Samples were run in duplicate at twice the original concentration to demonstrate the absence of additional protein-containing bands. Gels: a) F62 T1; b) F62 T1 (2x); c) 1365 T1; d) 1365 T1 (2x); e) 1365 T2; f) 1365 T2 (2x).


a
b
c
d
e
f

DISCUSSION

Outer membrane components of gram-negative cells have been the subject of many studies. Some of these studies have sought to gain insight into the interaction of these outer membrane components with the immune defense mechanisms of the host. Only when a better understanding is reached of the antigenic nature of outer membrane components and their relationship to the immune system of the human host, can serological classification schemes, development of vaccines, and elucidation of structure-function relationships take place.

Research with organisms from several genera has been successful in elucidating the answers to some of these questions. Vaccines to several diseases have been developed, as well as methods of serological identification that are useful in diagnosis. However, until a few years ago, there was a paucity of information regarding the biology of Neisseria gonorrhoeae.

To date, a successful vaccine to provide protection against gonorrhea has not been developed. Patients can contract the disease numerous times with no apparent acquired immunity (47). Recently, however, a more fully developed picture of the gonococcus has begun to emerge. Current research includes efforts to identify virulence factors responsible for disease, so that the pathogenesis of gonorrhea may be fully understood and a vaccine developed.

Pili have been associated with virulent colony types (43, 80), but aside from a possible means of attachment, their contribution to disease (16) is not known. Lipopolysaccharide, which may act as a "picket fence" barrier to keep antibody and complement at a distance from the outer membrane (21) is only now beginning to be characterized in N. gonorrhoeae (79, 92).

Proteins are potent antigens and may offer the key to developing a vaccine as well as a serological classification scheme (47). Therefore, several techniques have been developed for isolating outer membrane proteins and native outer membrane complexes from N. gonorrhoeae and N. meningitidis. Some of these techniques involve the isolation of outer membranes from spheroplasts or whole organisms (46, 107, 109). However, cell lysis is often extensive during these procedures. Utilization of chaotropic agents (47, 65) permits solubilization of outer membrane components while decreasing the amount of cell lysis. Techniques such as these should facilitate studies of gonococcal surface antigens and their possible association with virulence. This study has attempted to show the development of a technique which can be used quite easily to isolate and examine differences in outer membrane composition within and between strains of N. gonorrhoeae.

It was initially important to characterize the components removed from the cell and to show that what was being extracted from gonococci was in fact outer membrane components. Several different experiments were conducted to discover the degree of contamination by inner or cytoplasmic membrane components. The results of these experiments

show that this technique is gentle and capable of isolating gonococcal outer membrane components with negligible amounts of inner membrane contaminants.

The low degree of cellular lysis was confirmed by electron microscopic examination of thin sections of cells after extraction with a chaotropic agent. The electron micrographs demonstrated that after 2 h of extraction with 1 M NaSCN, the cells were largely intact and the trilaminar gram-negative envelope could still be discerned on some cells. However, in comparison to the control cells, the extracted cells showed blebbing and peeling away of the outer membrane. Electron micrographs published of meningococci subjected to another extraction procedure (109) bear a striking resemblance to electron micrographs presented herein. In the former study, Zollinger et al. (109) reported that they were able to remove the outer membrane of N. meningitidis in its native state with a low degree of cell lysis.

One of the traditional methods of demonstrating the homogeneity of a preparation is by centrifugation on a sucrose gradient. This technique has been used to achieve a separation of inner and outer cell membranes from various gram-negative bacteria (46, 76, 106, 107). Outer and inner membranes of N. gonorrhoeae have been shown to have densities of 1.21-1.25 g/cm^3 (46, 107) and 1.14 g/cm^3 (46), respectively. These values are similar to data obtained for E. coli and S. typhimurium (76, 106). Sucrose gradients of the extracted membrane components in this study yielded one broad peak, rather than two, indicating that cytoplasmic membrane contamination was not present. SDS-PAGE

electrophoresis of samples taken from the top, middle and bottom of the peak demonstrated the homogeneity of the preparation. The fact that only part of the band had a density of that documented for outer membrane (1.2 g/cm^3) may be explained by the large sample (0.5 ml) that had been placed on the gradient (12 ml) and which had perhaps not completely sedimented to equilibrium. An alternative explanation might be that only part of the extracted material was associated with lipopolysaccharide and therefore banded lower in the gradient than other outer membrane material which could have been dissociated from the LPS during the extraction (22). The SDS-polyacrylamide gels from this experiment, as well as from subsequent experiments demonstrated a protein banding pattern that closely resembled the profile described by Johnston et al. (47) for isolated outer membranes of N. gonorrhoeae.

In order to characterize the effectiveness and selectivity of NaSCN extraction, comparative studies involving three other extraction procedures (28, 107, 109) were conducted. To determine whether cytoplasmic components were present in the extraction product, cells were grown in the presence of [^{3}H]-adenine and then extracted. DNA resulting from cell lysis will adhere to the outside surfaces of other cells in the culture (18). The product from NaSCN-extracted cells was monitored for [^{3}H]-adenine activity to ascertain whether there were nucleic acids adhering to the extracted outer envelopes. There was no evidence indicating a significant nucleic acid contamination in the extracted product.

Of the four extraction procedures which were compared, two employed chaotropic agents to extract outer membrane components, while the other two methods subjected spheroplasts to shearing forces in order to remove the outer membrane. Each mode of extraction has advantages as well as disadvantages. Methods employing LiCl (28) or NaSCN were easier to perform, and yielded (as measured by ^{3}H-amino acid) nearly as much material as the other two techniques. NaSCN extraction was superior to the LiCl extraction (28) because of the greater yield (Table 2). Analysis by SDS-PAGE revealed that the protein banding patterns were similar.

The "O" (109) and "OM" (107) extraction procedures gave greater yields of material than the first two procedures, but were more time-consuming and difficult to perform. The "OM" preparation of Wolf-Watz et al. (107) had the greatest amount of [^{3}H]-adenine contamination and showed numerous bands on SDS-PAGE revealing a more complex pattern than that obtained with the other extraction methods.

Succinic dehydrogenase (SDH) and flavoprotein-dependent lactate dehydrogenase (LDH) are cytoplasmic membrane markers which are easy to assay and are found in many gram-negative bacteria (21). It is possible to assess the degree of cytoplasmic membrane contamination in outer membrane preparations if SDH and LDH activity is present. The inability to assay directly for SDH and LDH activity in NaSCN-extracted outer membrane was due to an apparent inactivation of these enzymes by the SCN^{-} ion. This inactivation apparently results from the reaction of the thiocyanate anion with free amino groups and subsequent formation of thiocarbamyl derivatives (65). It is possible that such modification

of amino acid residues either at the active site or at some other location in the molecule could result in inactivation of the enzyme. Other studies using guanidinium thiocyanate (65) indicated that less than one in 5,000 amino acid residues in proteins extracted from bacterial membranes was thiocarbamylated. However, the same authors were quick to point out that even a minor degree of thiocarbamylation is a disadvantage if renaturation of the proteins is to occur. Since the modification of only one amino acid residue in an enzyme could conceivably alter its activity, thiocarbamylation of essential amino groups may have been responsible for the absence of enzyme activity. Data from enzyme assays of LiCl-extracted outer membranes provides additional evidence that extraction by chaotropic agents does not result in extensive cytoplasmic membrane contamination arising through cell lysis. In other studies, outer membrane extraction techniques were evaluated by the amount of cytoplasmic membrane enzymatic activity present in the final preparation. Johnston and Gotschlich (46) separated gonococcal inner and outer membranes on a sucrose gradient, experiencing contamination of their outer membrane fraction with 1-2% of the enzymatic activity of the inner membrane. Wolf-Watz et al. (107) were not able to achieve this low level of inner membrane contamination with their procedure, experiencing a six- to eight-fold decrease in cytoplasmic membrane enzymatic markers in their outer membrane preparation as compared to the total membrane fraction. The results of the present study, in comparison to the above studies, appear quite favorable, as cytoplasmic membrane contamination of the extraction product was between 0.3-1.5%.

Therefore, it seems reasonable to extrapolate from the purity of the LiCl-extracted material to the purity of the preparations extracted with NaSCN. The data obtained from electron microscopic examination of NaSCN-treated cells, as well as the low levels of [^{3}H]-adenine in the final preparation appear to support this assumption.

The operational effectiveness of NaSCN was tested by varying its concentration as well as the time of extraction. The overall effectiveness of NaSCN extraction was determined by the presence of membrane components, some of which were uniquely associated with the outer membrane. Heptose and 2-keto-3-deoxyoctonate (KDO) are useful outer membrane markers as they are present in almost all wild-type gram-negative bacteria. Mutant strains that are heptose-deficient are known as Re or "extreme rough" mutants because all lipopolysaccharide components distal to the KDO residues are absent (22). However, these mutants are rare and were not among the strains of N. gonorrhoeae examined in this study.

Values for protein, KDO and heptose, (Figs. 6, 7) were reported relative to the concentration of phospholipid. In this way, a total picture of the outer membrane during and after extraction can be obtained.

The ratios of KDO and heptose to phospholipid does not change appreciably throughout the range of NaSCN concentrations tested. These constant ratios show that no preferential extraction of either KDO or heptose occurs at high NaSCN concentrations. However, the ratio of protein to phospholipid (Fig. 7) is affected, with the ratio rising to

a maximum at 0.75 to 1 M NaSCN, and then declining at an NaSCN concentration of 2 M. A possible explanation can be afforded if it is remembered that the mode of action of chaotropic agents is to facilitate the solubilization of hydrophobic molecules (i.e., membrane-associated proteins) and to make water more lipophilic (35). Hence at lower concentrations of NaSCN, fragments of outer membrane appear to be extracted from the gonococci. An optimum concentration exists at 0.75 or 1 M, at which point proteins are still imbedded in fragments of the phospholipid matrix. At higher concentrations of NaSCN, membrane proteins appear to be removed from their phospholipid matrix and are not recovered in the 100,000 x g pellet following extraction.

Varying the time of extraction also yielded interesting results. Even when extraction was continued for as long as 4 h, the protein/phospholipid ratio remained essentially constant. Surprisingly, the absolute amounts of protein and phospholipid obtained from cells extracted for up to 2.5 h were very similar. When cells were extracted for longer periods of time, the amounts of both protein and phospholipid began to increase. The effects of prolonged extraction on the integrity of the cells were not investigated, and so to minimize the danger of cellular lysis, the extraction time was standardized at 2 h.

Conflicting results were obtained when lipoprotein-containing cells as well as cells having no covalently bound lipoprotein were extracted. S. typhimurium and E. coli appear to have similar lipoprotein molecules which occur with approximately the same frequency in the cell envelope (9). Both types of lipoprotein appear to be covalently linked

by lysine residues to the peptide chains of the peptidoglycan and occur with a frequency of one molecule of lipoprotein for every ten repeat units of peptidoglycan (9). P. aeruginosa PS-7 and N. gonorrhoeae CS-7 contain no detectable covalently bound lipoprotein, but considerable differences were observed when the ratios of protein to phospholipid in extracted outer membranes were compared. In light of this information, the presence of lipoprotein appears to have no bearing on the ease with which outer membrane components are extracted from the cells used in this study. There are apparently other differences in the cell envelopes of these organisms which contribute to the relative ease of outer membrane extraction. It is uncertain at the present time what effects such bacterial components as capsules, flagella, or pili may have on the ease of extraction of outer membrane components.

The various chaotropic agents investigated demonstrated varying effectiveness in extracting outer membrane components when employed in the protocol described. Hatefi and Hanstein (35) suggested that there is an inverse relationship between chaotropic potencies and the charge densities of related chaotropes. This appears to correlate well with the results shown in Table 6 and Fig. 8 which demonstrate that both NaSCN and guanidine SCN are quite effective in extracting outer membrane components. When predicting the chaotropic potencies of the other anions investigated, one might expect that, based on charge densities, the potencies would be $I^- > Br^- > Cl^-$. However, as Hatefi and Hanstein (35) point out, the order of effectiveness of these chaotropes will often be reversed when both hydrophobic and electrostatic forces in a

membrane need to be overcome. Therefore, it was of interest to note that this is exactly what was observed in this study. The chaotropic potency of I^-, Br^- and Cl^-, as judged by the relative amounts of membrane components extracted, was $Cl^->Br^->I^-$ (Table 8, Fig. 10) and not the opposite order, which would have been according to charge density.

Striking examples of interstrain as well as intrastrain variation in outer membrane proteins were noted in the isolates of N. gonorrhoeae studied. Not only did the protein/phospholipid ratio shift within strains when colonial variants were compared, but variation between outer membrane proteins was also evident. Worth noting was the observation that when a colonial variant reverted to another colonial type, it resulted in a phenotypic change that could be detected in the outer membrane proteins (Fig. 19).

These findings are similar to a recent study in which a serotyping system for gonococci based upon differences in the major outer membrane protein was developed (47), and which reinforces the concept that the antigenic heterogeneity of the gonococcus may be a major reason why no acquired immunity has been described for human gonococcal infections.

A report has been published recently corroborating the intrastrain variation which exists in strains of N. gonorrhoeae (101). The authors (101) describe a heat-modifiable outer membrane protein which had different apparent molecular weights depending on the conditions of sample preparation. I had earlier observed that this phenomenon was not present in all strains. A similar finding was reported by Heckels (36). It was for this reason that all SDS-PAGE was carried out under identical

conditions so that comparisons could be made within and between strains. The role of the variable outer membrane proteins has not been established, as their presence or absence cannot be correlated solely with either pathogenic or nonpathogenic colonial variants.

Previous investigators have developed techniques for removal of the outer membrane of N. gonorrhoeae; however, no parallel comparisons have been made. This study has compared the extraction procedure described herein with three other techniques (28, 107, 109), and determined that the former technique compared quite favorably, according to the parameters investigated. A major advantage that this method has is that both large and small volumes can be easily extracted, the technique is not comparatively time consuming, and it is easy to perform. This technique will hopefully aid investigators as studies on the outer membrane of N. gonorrhoeae continue.

SUMMARY AND CONCLUSIONS

A technique has been developed for the extraction of outer membrane components from N. gonorrhoeae. The purity of the preparations thus obtained was ascertained by a) electron microscopy, which revealed minimal levels of cellular lysis; b) isotopic studies, which indicated extremely low levels of labeled nucleic acid contamination; and c) enzyme assays, which revealed cytoplasmic membrane enzyme activities comparable to, or lower than that obtained by other extraction procedures (28, 107).

Partial characterization of the outer membrane components obtained by this technique include protein, phospholipid, heptose and 2-keto-3-deoxyoctonate. Examination of colonial variants of different strains of N. gonorrhoeae demonstrated interstrain and intrastrain variations by protein banding patterns on SDS-PAGE and protein/phospholipid ratios of the extraction products obtained.

Various parameters of the extraction technique were investigated and discussed. This extraction technique, which is not technically difficult or time-consuming, may prove useful in current research involving the gonococcus. The antigenic mosaic nature of the outer membrane of N. gonorrhoeae is now being explored, and this method will provide a useful means for the extraction and examination of certain components.

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