

ISOLATION & PARTIAL CHARACTERIZATION OF THE  
CAPSULE PRODUCED BY Neisseria gonorrhoeae

Submitted in Partial Fulfillment of the Requirements for  
Graduation with Honors to the Department of Biology  
Carroll College, Helena, Montana

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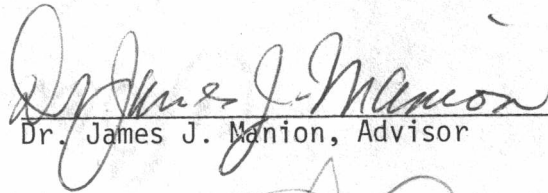
Research performed at NIH, Rocky Mountain Laboratory, Hamilton  
Montana, under the supervision of Penny Hitchcock, D.V.M.

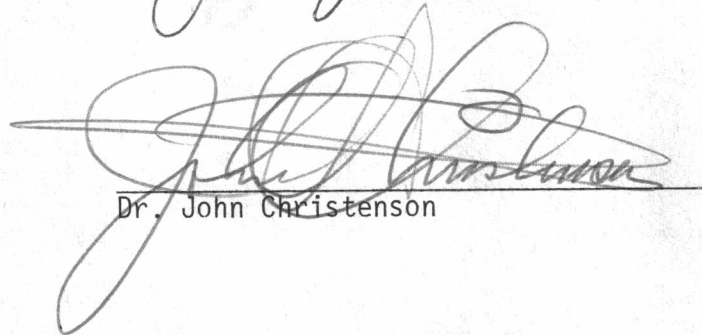


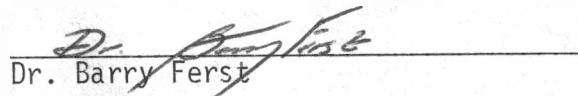
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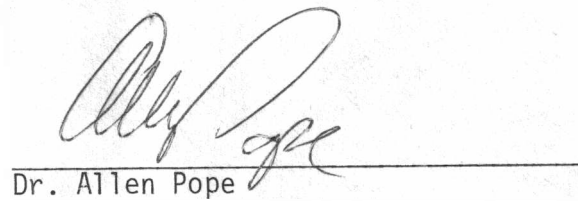
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## TABLE OF CONTENTS

Acknowledgements . . . . .	ii
Abstract . . . . .	iii
List of Illustrations . . . . .	iv
Introduction and Literature Review . . . . .	1
Materials and Methods	
Bacteria and Culture Conditions . . . . .	6
Capsule Extraction I . . . . .	6
Phenol Extraction . . . . .	9
Capsule Extraction II . . . . .	10
Spectrophotometric Properties . . . . .	13
Lowry Protein Assay . . . . .	13
KDO Assay . . . . .	14
Neutral Sugar Assay . . . . .	14
Orcinol Assay . . . . .	15
Phosphorus Assay . . . . .	15
Limulus Amebocyte Lysate Assay . . . . .	16
Chick Embryo Lethality Test . . . . .	16
SDS-PAGE Electrophoresis . . . . .	17
Scanning Electron Microscopy . . . . .	18
Results	
Spectrophotometric Properties . . . . .	19
Biochemical Properties . . . . .	19
Biological Properties . . . . .	25
Electrophoretic Properties . . . . .	26
Scanning Electron Microscopy . . . . .	29
Discussion and Conclusions . . . . .	36
Literature Cited . . . . .	40

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## ABSTRACT

The capsule of Neisseria gonorrhoeae strains JS1 and JS3 was extracted according to the procedure of Wolfgang Casper (1937). The extracted products were biologically and biochemically characterized. The extracts were comprised primarily of carbohydrate and other constituents detected are ribonucleic acids, protein, lipopolysaccharide, phosphorus, and 2-keto-3-deoxyoctonate. The presence of these impurities has greatly interfered with the identification of the specific polysaccharide components.

Examination of Neisseria gonorrhoeae by scanning electron microscopy indicated that extracellular material was stabilized by low temperatures. In order to maximize the amount of extracted polysaccharide, the bacterial suspensions were refrigerated whenever possible during the purification procedure.

## LIST OF ILLUSTRATIONS

1. Capsule Extraction Procedures . . . . .	.8,12
2. Spectrophotometric Scan	
Casper's Antigen . . . . .	.20
Phenol Extracted CA . . . . .	.21
Dialyzed CA . . . . .	.22
DOC CA . . . . .	.23
PSS CA (Sol & insol) . . . . .	.24
3. Electrophoretic gels	
Stains-A11 . . . . .	Fig.A,27
Stains-A11 . . . . .	Fig.B,26
Coomassie Brilliant Blue . . . . .	Fig.C,27
Silver Stain . . . . .	Fig.D,27
6. Scanning Electromicrographs	
JS1 (refrigerated 24h) . . . . .	Fig.01,31
JS1 (no refrigeration) . . . . .	Fig.02,31
JS3 (refrigerated 24h) . . . . .	Fig.03,32
JS3 (no refrigeration) . . . . .	Fig.04,32
JS1 O (group 1) . . . . .	Fig.05,33
JS3 N (group 2) . . . . .	Fig.06,33
JS1 O (group 3) . . . . .	Fig.07,33
JS3 N (group 3) . . . . .	Fig.08,33
JS1 O (group 4) . . . . .	Fig.09,34
JS3 N (group 4) . . . . .	Fig.10,34
JS3 N (group 5) . . . . .	Fig.11,34
JS1 N (group 5) . . . . .	Fig.12,34
JS3 O (group 5) . . . . .	Fig.13,35
JS1 O (group 5) . . . . .	Fig.14,35

## INTRODUCTION AND LITERATURE REVIEW

Symptoms of gonococcal infections were recognized in ancient China, Egypt, and Rome. The urethral exudate was believed to be semen. In 1879, Neisser first reported observing the bacterial pathogen after successfully staining smears of urethral, vaginal and conjunctival exudates. In the United States there were 1,004,129 reported cases in 1980 (equivalent to 443 cases per 100,000) and it is estimated that an additional 1 to 1.5 million diagnosed cases are not reported (17). Most men and women who acquire symptomatic gonorrhoea cease sexual activity and are treated. Approximately 40% of infected individuals are asymptomatic or have mild symptoms and are the primary transmitters of disease (17). Effective antimicrobial therapy was first applied in the 1930's using sulfonamides. By the mid 1940's nearly all of the sulfa-sensitive strains were wiped out and sulfa-resistant strains prevailed (17). Large quantities of penicillin became readily available by 1943 and proved to be extremely effective against bacterial infections. In the last 25 years the effective dose of penicillin, and several other related antibiotics, has increased steadily in proportion to the identification of antibiotic resistant gonococci. Understanding the biological characteristics of the pathogen is the first step toward eliminating this international epidemic.

Neisseria gonorrhoeae is a gram negative diplococcus. In vitro gonococci are able to alter their surface components. Several degrees of opacity variations within the colonies of a given strain and from different strains has been reported (37). Colonial opacity has been

correlated with cellular modifications of the outer membrane protein II constituents. Opacity variants are designated as O- (transparent), O+, O++, and O+++ . Virtually all Neisseria gonorrhoeae isolates can shift their opacity phenotypes and apparently do so in vivo (36). The transparent colony phenotype appears to be the invasive form of N. gonorrhoeae, as it has been recovered from blood and joint fluid, urethral mucosa of asymptomatic males, uterine cervical specimens taken at menstruation, and fallopian tube or peritoneal specimens. Specimens from males with symptomatic urethritis and cervical specimens obtained at mid-cycle usually form opaque colonies (17,36).

Changability of colony morphology is an important key to understanding host-pathogen interactions. The gonococcus adjusts pilin production such that the presence of pili is highly variable. Colonies that are piliated (P+, P++) appear as small spherical masses with distinct edges when grown on solid medium. Non-piliated colonies are larger, flatter and exhibit rough edges. Pili appear to be important for the attachment of virulent gonorrhea to mucosal and epithelial cells of the urethra, which is the primary site of infection (24,33).

Neisseria gonorrhoeae produces a capsule that may increase its virulence by interfering with phagocytosis by lymphocytes. Studies suggest that encapsulation plays a role in resistance to serum killing (5,6,12) by blocking antibody and outer membrane antigen interactions. Demarco de Hormaeche et al. have shown that only encapsulated variants are found in infected subcutaneous chambers implanted in mice (11). They have also shown that specific rabbit serum raised against encapsulated variants killed both capsulated and noncapsulated gonococci,

but serum raised against noncapsulated killed only that variant. Difficulty in maintaining virulence in laboratory cultures, and complications in determining the specific capsular components has hampered research into the immunogenic characteristics of gonorrhoea.

In 1937, Dr. Wolfgang A. Casper, developed a procedure to extract a protein-free capsular polysaccharide from fresh isolates of acute gonococcal infections (4). Casper identified the carbohydrate to be a complete antigen that was type specific. Three years later he tested the capsule vaccine in one woman and ten men (7). He was certain the vaccine would not cause the venereal disease because it was not made of whole organisms, but secondary effects and possible allergic reactions could not be determined prior to administering the antigen. The woman was a gonorrhoeic prostitute that was being treated in Casper's hospital ward and the men were volunteers who sought food and lodging during the harsh years of economic depression. Five of the ten men were inoculated with Casper's vaccine. All of the men had intercourse with the prostitute. The five who had been vaccinated did not become infected and four of the five not inoculated contracted the disease (8). Experimentation on such a small population did not prove that the capsular extract was the long sought after cure for gonorrhoea, but it did set precedence for further clinical investigation. Unfortunately Casper's work was discontinued because, being of Jewish faith, he was forced to flee from Nazi Germany. He came to the United States and was commissioned as a surgeon in the Public Health Service. The use of antibacterial sulfonamide drugs became widespread, and lack of funding brought Casper's research on N. gonorrhoeae to an end.

Researchers have tried to determine the specific immunogenic importance of capsule production. It has been shown that in vitro cultivation methods are unsuccessful in maintaining virulence in all N. gonorrhoeae strains (4,11,24,32,40). Martin Walsh and coworkers suggested that the chick embryo is an economical laboratory animal that can be used to maintain and reconstitute virulence in gonococcal strains (40). They postulated that the chick embryo allantoic cavity was a selective medium for the rapid growth of virulent gonococci. Kellogg, and others, (25) reported that gonococci maintained for 35 months on selective media retain virulence and that the virulent characteristics are related to colony morphology.

Visualization of capsule on Neisseria gonorrhoeae has been demonstrated by Richardson and Sadoff (32) using light microscopy and India Ink stain. James and Swanson (21) have also identified capsule using a variety of staining methods. Demarco de Hormaeche and Thornley (11) have shown the presence of capsule on gonococcal stains using light and electron microscopy. They cultured two strains in subcutaneous chambers in guinea pigs and prepared them with Alcian Blue. Transmission electron micrographs revealed disperse masses of capsule on the bacterial surfaces and strands connecting adjacent cells (11). Hendley, et al. (18) have reported that  $\frac{1}{2}$ GPH agar seems to support capsule production that surrounds a majority of the gonococci. They concluded that the capsule is composed of acidic polysaccharides because it stains with Ruthinium Red prior to observation by electron microscopy.

Understanding the importance of capsule production in Neisseria gonorrhoeae leads to improvements in diagnostic techniques and advance-

ment in eradicating gonorrhoea.

## MATERIALS AND METHODS

### Bacteria and Culture Conditions

Neisseria gonorrhoeae strains JS1 and JS3 (provided by Penny Hitchcock, NIH, Rocky Mountain Laboratory, Hamilton, Montana) were used. They were grown on the modified, clear typing medium previously described by James and Swanson (28,34). Organisms were cultured at 37°C, for 18 - 24h under a moist atmosphere containing 5% CO<sub>2</sub>. Single colonies were selected and passaged daily.

### Capsule Extraction I

Gonococcal strain JS1 (P-,0+) was grown on clear typing medium (34). The agar was scraped and the bacteria were lyophilized in distilled water and stored at room temperature. (Refer to p.8)

Two and one-half grams of the lyophilized gonococci were suspended in 100 ml of a 0.1% sodium desoxycholate and physiological saline solution. The pH was adjusted to 8 with 0.1 N NaOH. The suspension was incubated for 24h at 37°C.

After the 24h period, the suspension was centrifuged at 6,000 rpm for 20 min. The supernatant fluid was decanted and 500% (vol/vol) of 90% ethyl alcohol (EtOH) was added. The alcohol of this first alcohol extraction, will cause the capsular polysaccharides and some proteins to precipitate out of solution.

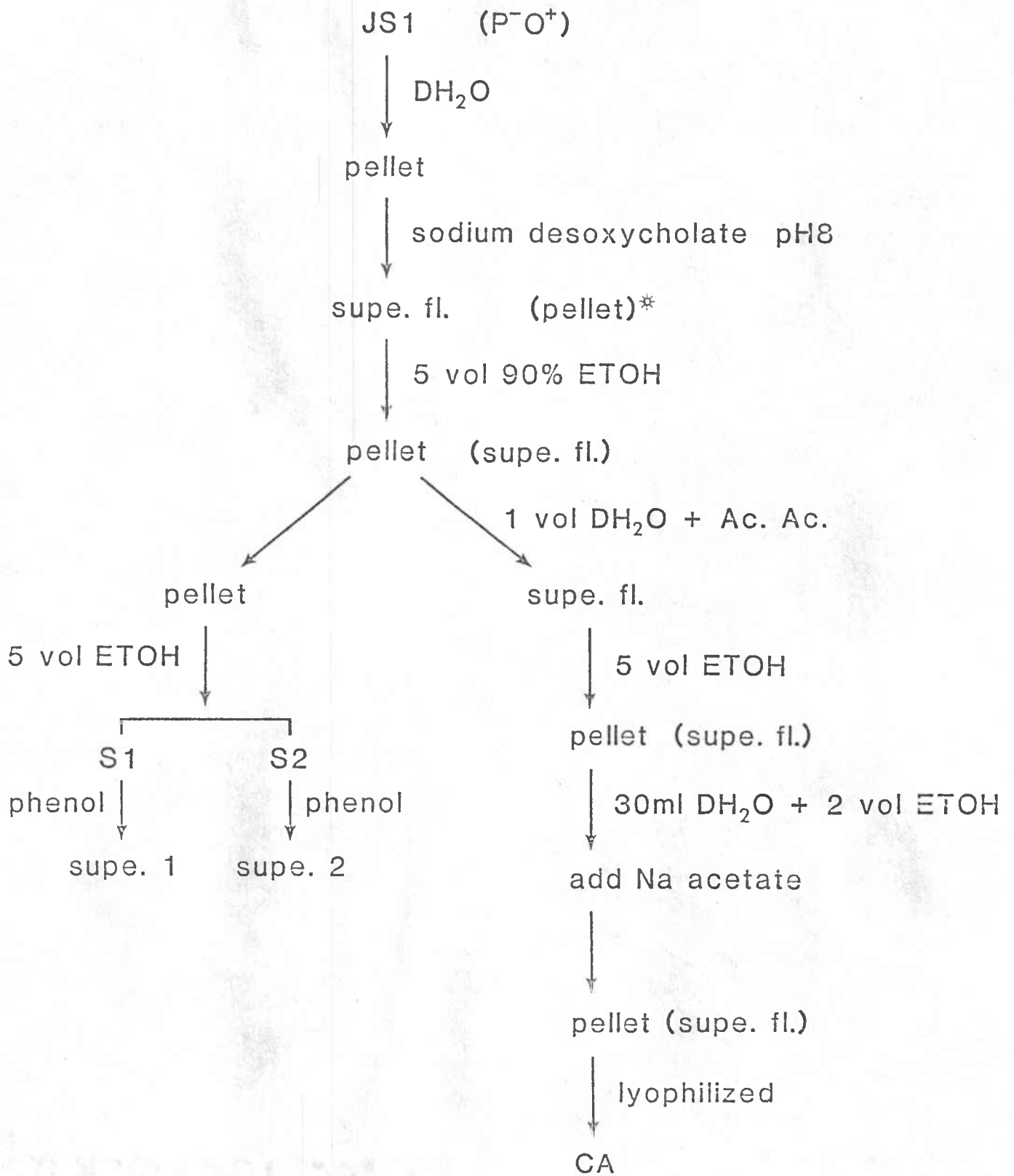
The solution was centrifuged at 8,000 rpm for 20 min. The supernatant fluid was decanted and 100% (vol/vol) of distilled water was mixed

with the pellet. In order to remove protein 10 ml of glacial acetic acid was added drop by drop to the pellet-water suspension. The solution sat for 20 min at room temperature, then it was centrifuged at 5,000 rpm for 10 min.

A second alcohol extraction was performed by adding 500% (vol/vol) of 90% EtOH to the supernatant fluid. The alcohol solution was mixed for 24h at 4°C. The solution was centrifuged at 8,000 rpm for 20 min. The pellet was suspended in 30 ml of distilled water. 200% (vol/vol) of 90% EtOH was mixed with the pellet suspension, and to facilitate polysaccharide precipitation approximately 1 g sodium acetate was added. The polysaccharide precipitated for 3h at room temperature, then the suspension was centrifuged (8,000 rpm, 20 min). The pellet was dissolved in 45 ml of distilled water and lyophilized. The lyophilized product is referred to as Casper's Antigen (CA).

The pellet that resulted from centrifuging the acetic acid solution was dissolved in 20 ml of 1 N NaOH. The pH was adjusted to 7 with glacial acetic acid, and 200% (vol/vol) of 90% EtOH was added. The solution sat for 41 days at 4°C.

During the 41 days of refrigeration the suspension separated into two parts. The lighter, coagulated portion was carefully removed and centrifuged (9,000 rpm, 20 min). The pellet was collected and labeled S-1. The dense, heavier portion was centrifuged (5,000 rpm, 5 min) and the resulting pellet was referred to as S-2. All of the alcohol was evaporated from both pellets.



\* fractions in parenthesis not utilized further

### Phenol Extraction

A hot phenol extraction (44) was performed on S-1 and S-2. The procedure for S-1 extraction was carried out as follows, with the volumes used for S-2 in parenthesis.

1. Dissolve in 5 ml (15 ml) of 70°C glass distilled water and 5 ml (15 ml) of 70°C 90% distilled phenol.
2. Vortex for 15 min (maintain 70°C temperature)
3. Cool on ice for 15 min.
4. Centrifuge @ 5,000 rpm for 40 min.
5. Carefully extract the aqueous phase and repeat steps 1 - 4.

The final aqueous phases were separately dialyzed against distilled water (4°C, 24h). Following dialysis, the solutions were lyophilized. S-1 yielded a flocculent, opalescent substance that was called Supernatant 1 (Supe 1). S-2 produced a similar substance that was less dense, this was referred to as Supernatant 2 (Supe 2).

A hot phenol extraction was also performed on the CA to remove proteins. In a microfuge tube, 2 mg CA was dissolved in 0.5 ml (70°C) glass distilled water and 0.5 ml (70°C) 90% distilled phenol. The solution was mixed thoroughly at 2-min intervals for 15 min, while maintaining a constant temperature of 70°C. It was cooled on ice for 15 min and centrifuged at 7,000 rpm for 20 min. The aqueous phase was removed and the process was repeated using the aqueous phase. The concentrated aqueous phase was dialyzed against distilled water (as previously described). The dialyzed solution was lyophilized and the product was labeled dia CA.

## Capsule Extraction II

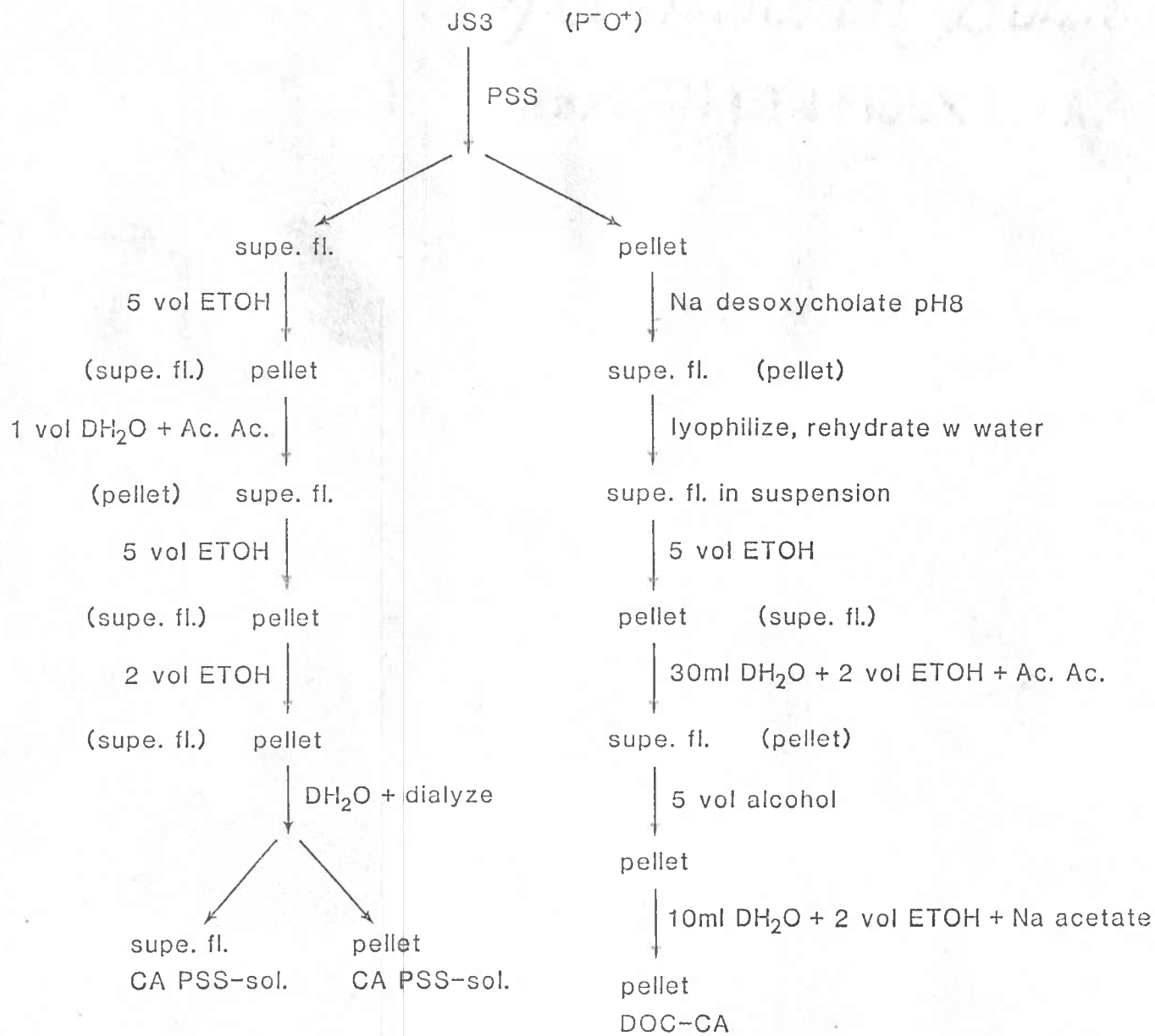
Gonococcal strain JS3 (P-,0+) had been frozen on May 22, 1982 and maintained at -70°C until thawed on July 25, 1985 and cultured on clear typing medium. The bacteria were incubated for 18h at 37°C and 5% CO<sub>2</sub>. The colonies were harvested with sterile Dacron swabs and suspended in trypticase soy broth (TSB). The bacterial suspension was used to inoculate 70 large petri plates that contained clear typing medium, and the cultures incubated for 18h-20h. The colonies were harvested in physiological saline solution (PSS) by gentle scraping with a glass L-rod. The suspension was kept on ice for 5h, and then centrifuged (10,000 rpm, 20 min). The supernatant fluid and the pellet from the PSS suspension were processed separately. (Refer to p.12)

The supernatant fluid was mixed with 500% (vol/vol) of 90% EtOH for 24h at 4°C. It was centrifuged for 20 min at 8,000 rpm, and the water soluble pellet was lyophilized and later redissolved in 150 ml of glass distilled water. Glacial acetic acid (1N) was added to the solution until a large amount of precipitate formed. The suspension was centrifuged at 5,000 rpm for 10 min. The supernatant fluid was decanted and mixed with 500% (vol/vol) of 90% EtOH (24h, 4°C). The alcohol suspension was centrifuged (8,000 rpm, 20 min) and the resulting pellet was dissolved in 25 ml distilled water. 200% (vol/vol) of 90% EtOH was added to the dissolved pellet with approximately 1 g of sodium acetate to facilitate precipitation. A precipitate formed for one hour at room temperature before the suspension was centrifuged (8,000 rpm, 20 min). The supernatant fluid was decanted and 10 ml of distilled water was mixed with

the pellet. The dissolved pellet was dialyzed against distilled water for 5h at 4°C. The dialyzed solution was centrifuged for 5 min at 10,000 rpm. The supernatant was lyophilized and the product was labeled, PSS CA. The pellet was mixed with 20 ml of distilled water and lyophilized. The lyophilized pellet was called PSS CA insol.

The pellet of the PSS suspension was suspended in 600 ml of a 0.1% sodium desoxycholate and physiological saline solution. The pH was adjusted to 8 with 1N NaOH. The solution mixed for 24h, at 37°C. The solution was centrifuged (6,000 rpm, 20 min) and the supernatant fluid was lyophilized and later rehydrated with 200 ml of glass distilled water. An alcohol extraction was performed by mixing 500% (vol/vol) of 90% EtOH with the solution and letting it stir for 24h at 4°C. Centrifuging (8,000 rpm, 20 min) the alcohol solution yielded a pellet that was re-suspended in 100 ml of distilled water and a small amount of glacial acetic acid dropwise until a precipitate formed. The suspension was centrifuged at 8,000 rpm for 20 min. The pellet was resuspended in 50 ml glass distilled water and the pH was adjusted to 7 with 1N NaOH.

A second alcohol extraction was performed by adding 500% (vol/vol) of 90% EtOH to the pellet suspension and mixing for 24h at 4°C. The alcohol suspension was centrifuged at 8,000 rpm for 20 min. The pellet was dissolved in 40 ml distilled water and 200% (vol/vol) of 90% EtOH. Approximately 1 g sodium acetate was added to the alcohol suspension and it stirred for 24h at room temperature. The suspension was centrifuged for 20 min at 8,000 rpm. The resulting pellet was suspended in 20 ml distilled water and lyophilized. The product was named DOC CA.



### Spectrophotometric Properties

The lyophilized Casper's Antigen (CA) was assayed on a Beckman DU-8 spectrophotometer at a concentration of 250 ug/ml. The wavelength ranged from 300nm to 220nm, and the resulting absorption data was recorded graphically (Fig.1,p.20). The hot phenol extracted antigen (dia CA) was assayed at a concentration of 1.5 mg/ml (Fig.2,p.21).

One ml of CA was dissolved in 4 ml distilled water and dialyzed against a large quantity of distilled water for 24h at 4°C. The dialyzed solution was lyophilized, and a spectrophotometric scan was performed on a 100 ug/ml sample (Fig.3,p.22). All other conditions were the same as previously mentioned.

A spectrophotometric assay of a 100 ug/ml sample of DOC CA and distilled water. A graph of the absorbance values over 300-220nm (Fig.4, p.23) shows no significant peaks. Samples of PSS CA (100 ug/ml distilled water) and PSS CA insol (100 ug/ml distilled water) were also assayed (Fig.5,p.24).

### Lowry Protein Assay

The Lowry protein assay is a colorimetric indicator of the percentage of protein in a sample. The Lowry assay was published by Oliver Lowry (27). Bovine serum albumin was used as a standard from which to determine the linear relationship between known amounts of protein and absorbance at 660nm.

A Gifford spectrophotometer measured the absorbance of a 100 ug/ml sample of CA at a wavelength of 660nm. One hundred ug samples of Supe 1,

Supe 2 and DOC CA were assayed at 660nm by a Beckman DU-8 spectrophotometer.

#### KDO Assay

The KDO (2-keto-3-deoxyoctonate) assay determines the percentage of KDO in a sample by measuring the absorbance differences of colorimetric indicator added to standard concentration solutions. The presence of KDO indicates the concentration of lipopolysaccharide in the sample. The procedure was designed by Weisbach and Hurwitz (43). Absorbance data (550nm) was compared to the absorbances of standard concentrations.

#### Neutral Sugar Assay

The neutral sugar assay was performed as described by Dubois (15). It is a colorimetric assay that indicates the relative amount of simple sugars, oligosaccharides, and polysaccharides and their derivatives with respect to a glucose standard. The absorbance was determined at wavelengths 480nm and 490nm. The protocol used is as follows.

1. Dissolve 100 ug samples in 200 ml glass distilled water.
2. Make sugar standard (glucose) dilutions 60, 50, 40, 30, 20, 10 ug/2 ml. Blank with 2 ml glass distilled water.
3. Add 50 ul of 80% phenol to each sample.
4. Add 5 ml conc. sulfuric acid to each sample, and mix well. Let stand for 10 min.
5. Place sample vials in waterbath at 25-30'C for 20 min.
6. Read absorbances at 480nm and 490nm.

### Orcinol Assay

The orcinol assay described by Grinswold, et al. qualitatively and quantitatively determines the concentration of ribonucleic acids in a preparation (16). Dialyzed CA, Supe 1 and Supe 2 were dissolved in glass distilled water and processed as follows.

1. RNA standards as follows:  
500 ug RNA in 2.5 ml of 0.25 N perchloric acid, heat at 70°C for 30 min, add 2.5 ml of 0.1 N hydrochloric acid, dilute to 100, 50, 25, 12.5, 6.25 ug/ml concentrations.
2. Dissolve 100 ug of each sample in 1 ml 0.1 N HCl.
3. Add 2 ml Orcinol reagent to each tube. Orcinol reagent is as follows:  
11 ml ferric chloride reagent (100 mg FeCl<sub>3</sub> · 6 H<sub>2</sub>O/100 ml conc. HCl)  
11 ml ethanolic Orcinol reagent (6.0 g Orcinol/100 ml EtOH)
4. Cover tubes with marbles and heat to 90°C for 30 min. Cool to room temperature. Read at 665nm.

### Phosphorus Assay

The phosphorus assay determines the percentage of phosphorus present in a sample. The procedure used is a modification of the method of Dryer, Tammes and Routh (14). The protocol used in determining the percentage of phosphorus in CA, Supe 1 and Supe 2 is as follows:

1. Add 0.5 ml of distilled water and 0.1 ml of 5 N sulfuric acid to 500 ug samples.
2. Heat tubes in a heating block, starting cold to 160 - 180°C. Remove the tubes, allow to cool and add 1 drop of 30% H<sub>2</sub>O<sub>2</sub> to each.
3. Return the tubes to the heating block for at least 30 min. Digest should be clear and colorless at this point.

4. Remove the tubes, cool and add 2 drops of 5% urea solution.
5. Return the tubes to heat for 30 min.
6. Remove the tubes, cool and add 2 ml of water. Place in boiling waterbath for 5 min.
7. Cool to room temperature and add 0.2 ml of dilute (0.008 M) molybdate reagent. Mix by shaking.
8. Add 2 ml of semidine reagent, dilute to 5 ml with water and mix by covering the tube with parafilm and inverting twice.
9. Let stand 10 min and read the absorbance at 345nm.

For reagent composition refer to Dryer, Tammes and Routh (14).

In order to calculate the percentage of phosphorus in the sample, the following equation was used:

$$\%P = \frac{A \text{ (sample)}}{A \text{ (standard)}} \times \frac{\text{Weight (sample)}}{\text{Weight (standard)}} \times 100$$

#### Limulus Amebocyte Lysate Assay

A Limulus amebocyte lysate assay was performed on CA, DOC CA, Supe 1, and Supe 2. The test is a highly sensitive indicator of minute quantities of bacterial lipopolysaccharides. The samples were serially diluted to 1 ug, 10 ng, 1 ng, 0.1 ng, 0.01 ng, and 0.001 ng concentrations. The Limulus amebocyte lysate will solidify in the presence of lipopolysaccharide.

#### Chick Embryo Lethality Test

Eleven day old chick embryos were injected with dilutions of DOC CA & PSS CA in order to test the bacterial endotoxicity level of the sub-

stances. Mortality is assessed at 24h and the estimated mean lethal dosage ( $LD_{50}$ ) was calculated by the method of Reed and Muench (28).

The embryos were inoculated with 0.1 ml dilutions of 1,000 ng, 500 ng, 100 ng, 50 ng, and 10 ng concentrations of DOC CA and PSS CA. Purified JS3 lipopolysaccharide (50 ng/dose) was used as a control. The inoculated eggs were incubated for 24h at 37°C.

#### SDS-PAGE Electrophoresis

Samples prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were solubilized in a solution containing Tris (6.8), glycerol, B-mercaptoethanol and sodium dodecyl sulfate as described by Laemmli (26). The 5% stacking gel and the 12.5% separating gel did not contain SDS (19). Electrophoresis was done at 40 mA constant current with the Tris-glycine Laemmli buffer system (26). Molecular weight markers (MW) (Bio-Rad Laboratories, Richmond, California) were used in several of the gels.

The gels were fixed overnight in 25% (vol/vol) isopropanol- 7% (vol/vol) acetic acid before being stained. Coomassie brilliant blue and Stains -All (BioRad, Richmond, CA) were used to dye the gels. The silver stain described by Hitchcock & Brown (19) for LPS-protein identification was also used.

Whole cell lysates (WC) were processed by harvesting the gonococci from solid agar with a sterile Dacron swab and suspending them in cold Dulbecco phosphate buffer saline (10 ml, pH 7.2) (10). The turbidity was adjusted to 200 Klett units (blue filter, Klett-Summerson colori-

meter), and 1.5 ml of this suspension was centrifuged for 1.5 min. The pellet was solubilized in 50  $\mu$ l of lysing buffer containing 2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris (pH 6.8) and bromophenol blue. The lysates were heated for 10 min at 100°C.

Protein digestion was performed using 25  $\mu$ g of proteinase K (PK) (Boehring Mannheim GmbH, West Germany) solubilize in 10  $\mu$ l of lysing buffer. The solution was added to each boiled lysate and incubated at 60°C for 60 min (19).

#### Scanning Electromicroscopy

The procedure for scanning electromicroscopy (SEM) has been described (20). Agar plates streaked with colonies were inverted over osmium tetroxide crystals for 3 to 5 min. Small squares (5 mm) of agar were removed from the petri dishes and fixed for variable periods of time by placement in glass dishes containing 3% glutaraldehyde in 0.1 M sodium cocodylate buffer (pH 7.5). Care was taken not to allow the liquid to contact the colonies. The fixative was removed by aspiration, and the specimens were rinsed twice (5 min each) with buffer. The rinsed colonies were saturated with 2% osmium tetroxide in cocodylate buffer for 45 min at room temperature. The specimens were rinsed four times (5 min each) with distilled water. They were dehydrated at room temperature in a graded series of acetone and critical-point dried in CO<sub>2</sub> with a Balzer critical-point dryer (Balzers Union, Hudson, N.H.). Specimens were mounted with metallic silver paint and then coated with 10 nm of gold-palladium with a Technics Hummer X (Anatech, Ltd., Alexandria, Va.) and examined.

## RESULTS

Casper's Antigen was white and flocculent with no apparent odor. It could be easily compressed but undisturbed it remained frothy. The second extracts of the antigen were similar in texture. The PSS CA was opalescent and light. The DO CA was white, flocculent and was more densely compact than PSS CA. Supe 1 was white and clumpy. Supe 2 had a light, compressible texture and was opalescent.

### Spectrophotometric Properties

The spectrophotometric scans were taken over wavelengths 220nm to 300nm. Peaks which respond to protein and nucleic acid absorb at 280nm and 260nm respectively.

The lyophilized Casper's Antigen was assayed at a concentration of 250 ug/ml. The resulting absorption data was recorded graphically (fig.1, p.20). The graph shows significant amounts of impurities. The spectrophotometric scan of the phenol extracted Casper's Antigen peaked in the 280nm - 260nm range (Fig.2,p.21). The dialyzed Casper's Antigen had decreased absorbance at 280nm and 260nm (Fig.3,p.22).

The Beckman DU-8 measured the absorbance of a 100 ug/ml sample of DOC CA within the ultraviolet spectrum and showed no substantial peaks (Fig.4,p.23). Solutions of PSS CA and PSS CA insol (100 ug/ml) were also assayed (Fig.5,p.24).

### Biochemical Properties

A 100 ug sample of Casper's Antigen was assayed by the Lowry

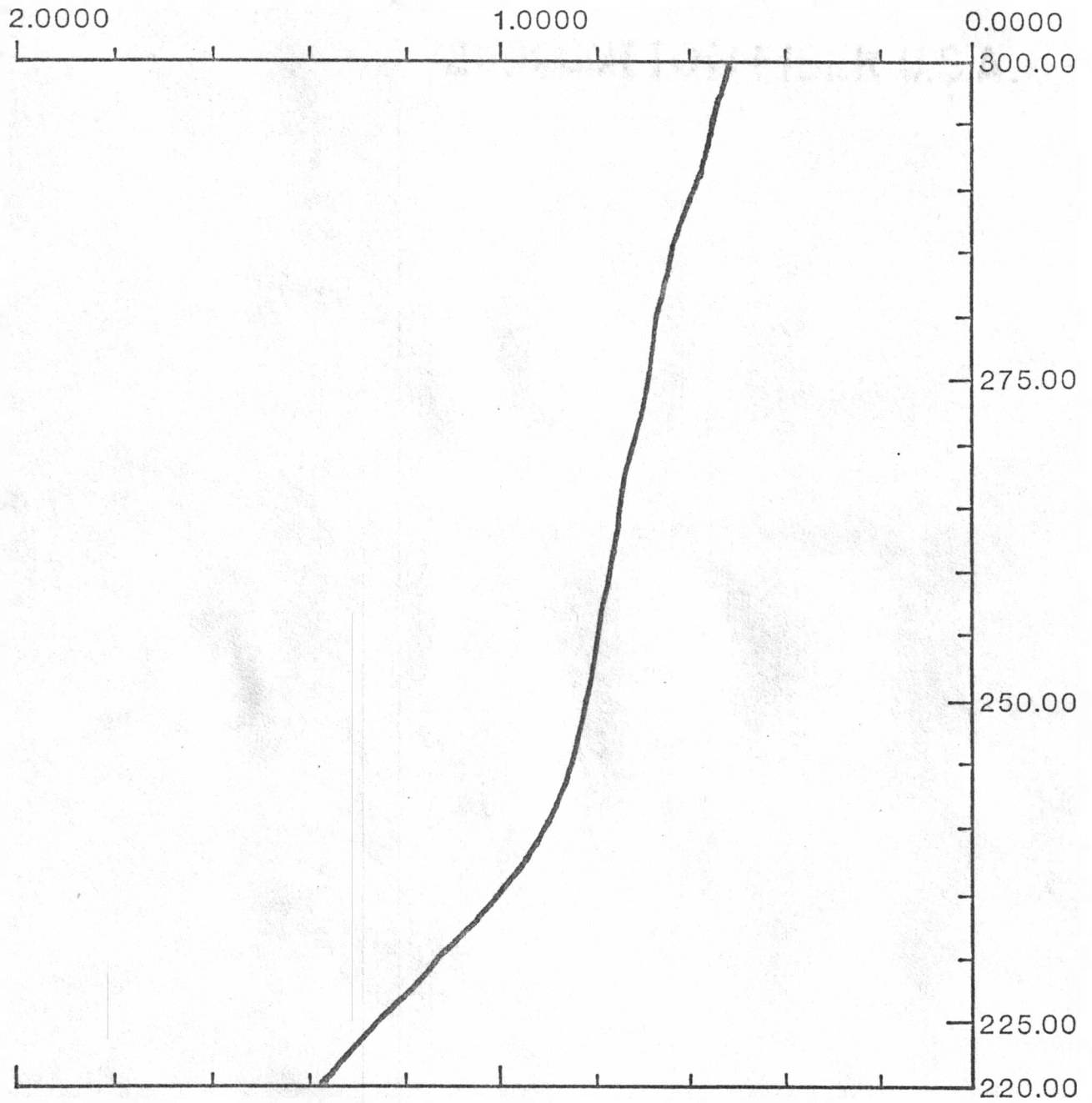


Figure 1. Spectrophotometric scan of CA at 250 ug/ml concentration.

(note: 2.5X more concentrated than preps shown in Fig. 3,4,5)

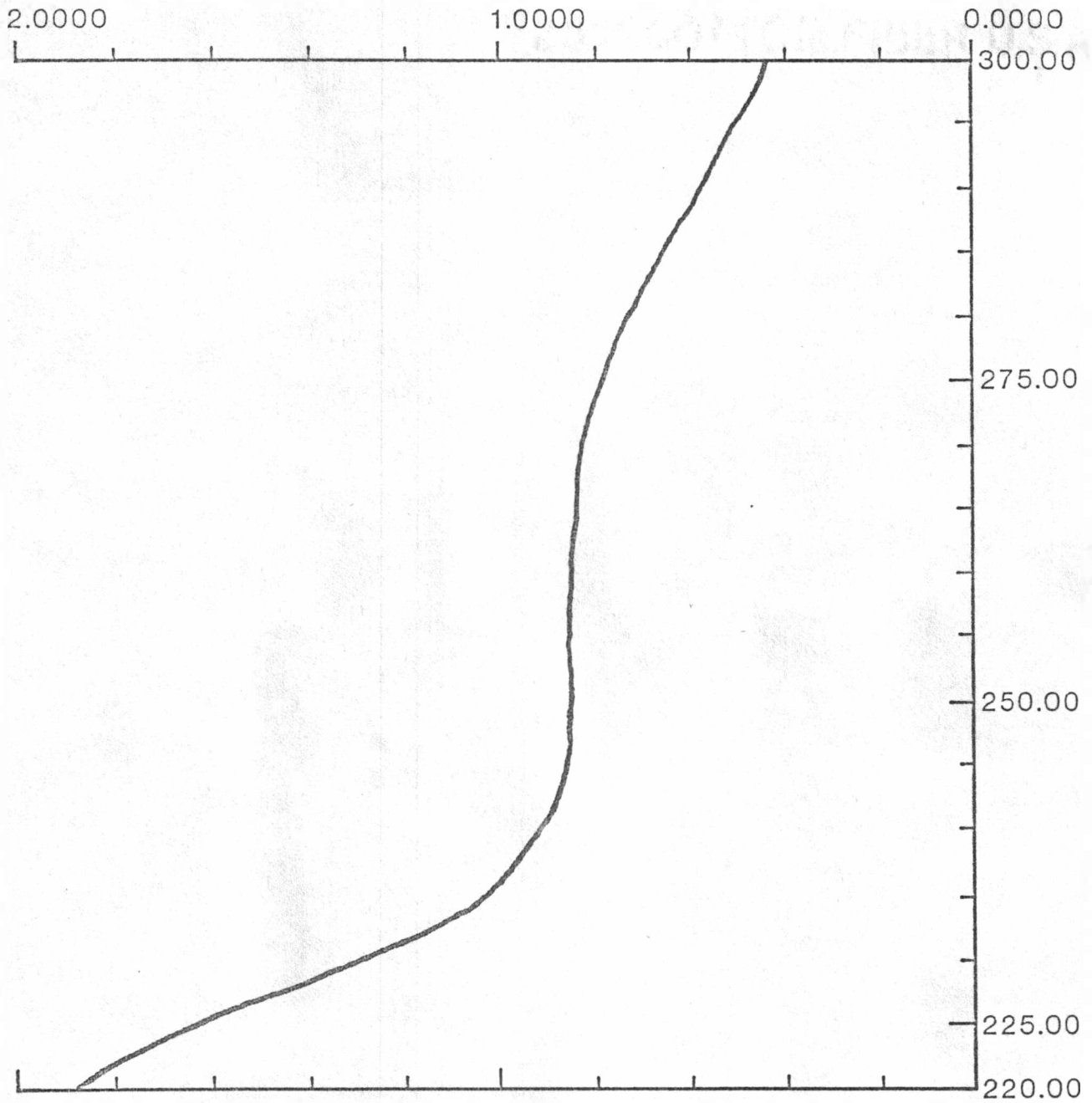


Figure 2. Spectrometric scan of Phenol extracted CA(1.5 mg/ml).

(note: 15X more concentrated than preps shown in Fig. 3,4,5)

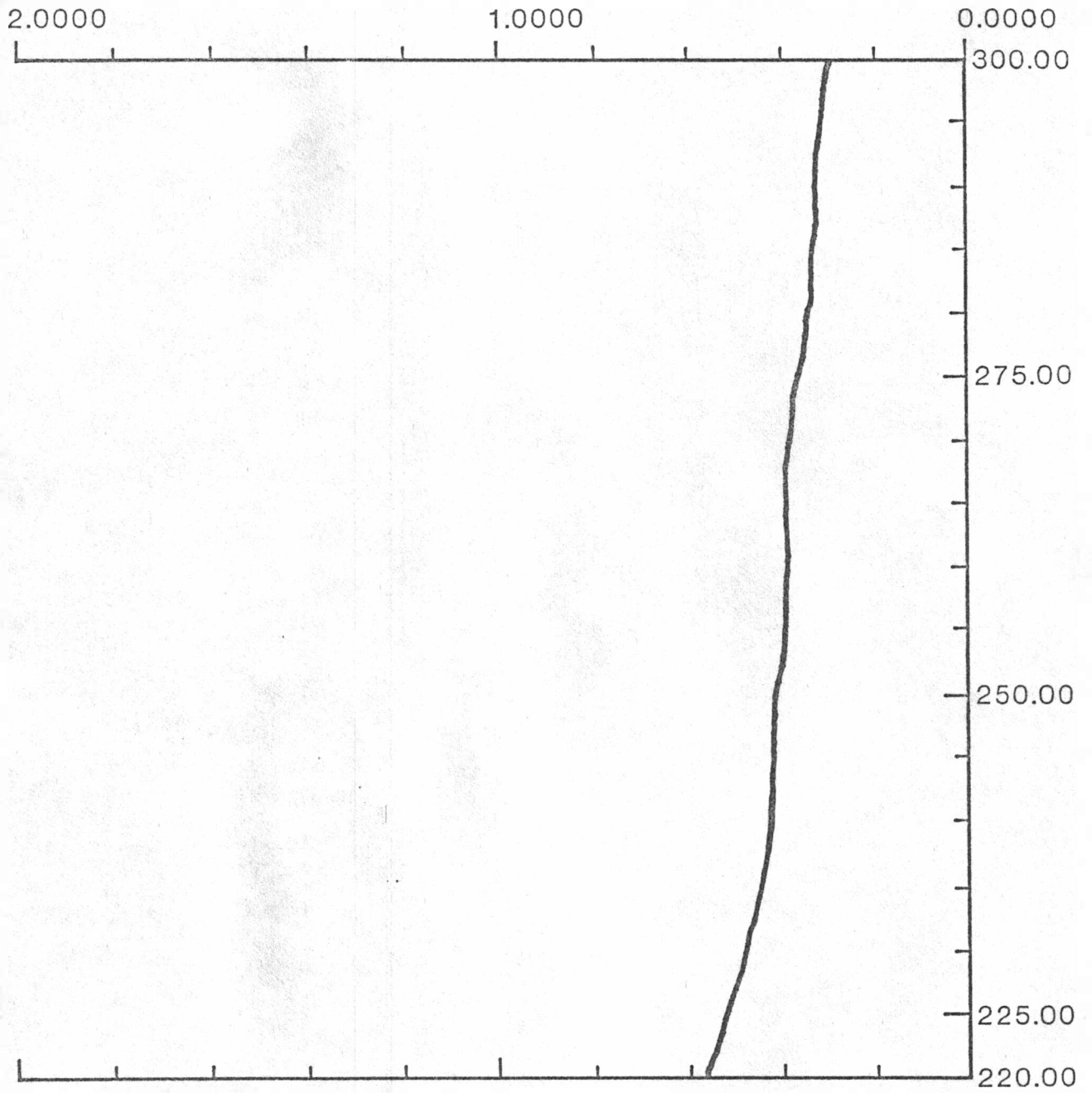


Figure 3. Spectrophotometric scan of dialyzed CA at 100 ug/ml Concentration.

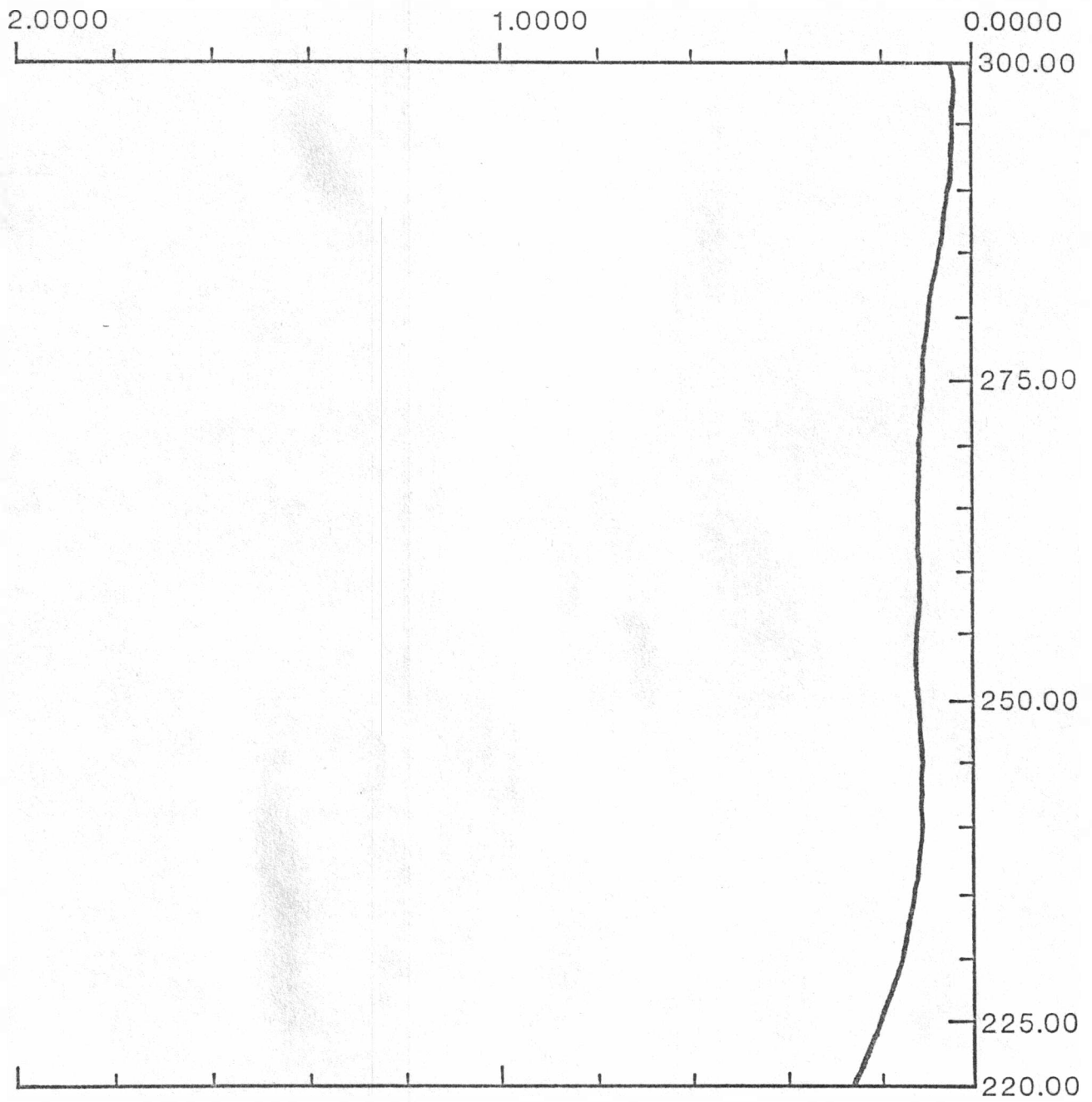


Figure 4. Spectrophotometric scan of DOC CA at 100 ug/ml concentration.

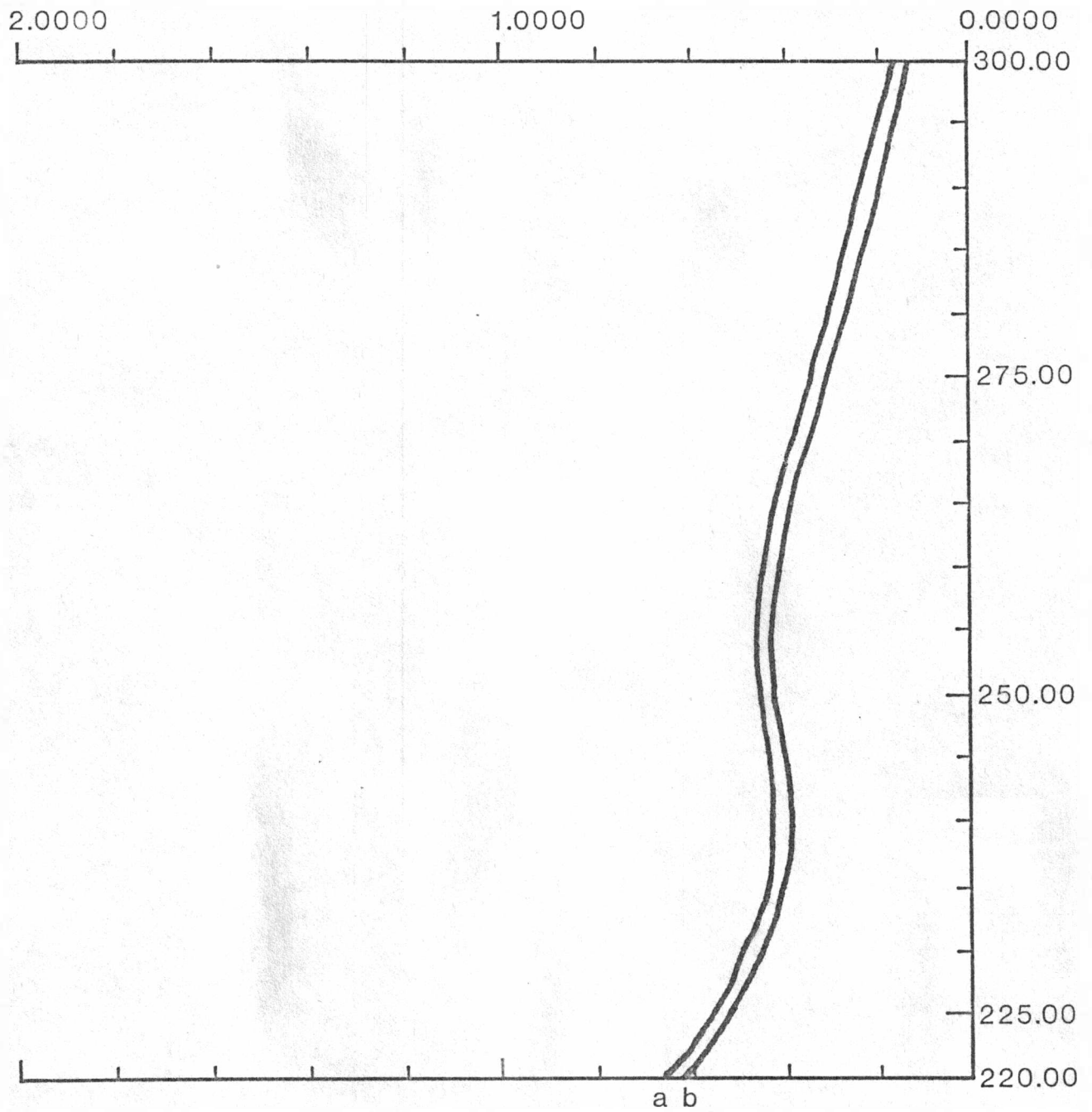


Figure 5. Spectrophotometric scan of PSS CA (a) and PSS CA insol (b) at 100 ug/ml concentrations.

method and yielded approximately 36 ug/ml protein which corresponds to 36% contamination. The results of protein assays suggested that there was a dramatic reduction of contaminants in DOC CA, Supe 1, and Supe 2 extracts. One hundred ug samples of Supe 1, Supe 2 and DOC CA indicate there is 4.8 mg/ml protein contamination in Supe 1 and 2.5 mg/ml in Supe 2. Absorbance data suggests there is 7.6 mg/ml protein in the DOC CA sample. This corresponds to 4.8% protein in Supe 1, 2.5% in Supe 2 and 7.6% in DOC CA.

The neutral sugar assay confirmed there were significant amounts of polysaccharides in the extracts. The 50 ug sample of dia CA was composed of 12.5 ug/ml carbohydrate and equivalent amounts of Supe 1 and Supe 2 contain 20.5 ug/ml and 22.5 ug/ml, respectively. This corresponds to 25% polysaccharide in dia CA, 41% in Supe 1 and 45% in Supe 2.

It was speculated that there was ribonucleic acid contamination in the capsule extracts. The orcinol assay determined there was 12 ug/ml RNA in dia CA, 26 ug/ml in Supe 1, and 29 ug/ml in Supe 2. This corresponds to 12% RNA in dia CA, 26% in Supe 1 and 29% in Supe 2.

### Biological Properties

The Limulus Amebocyte Lysate and the Chick Embryo Lethality assays confirmed that the level of bacterial endotoxins found in the extracts were considerably low. The DOC CA and Supe 1 showed no lipopolysaccharide contamination. The CA preparation exhibited unsafe endotoxin levels above 10 ng, and Supe 2 could be potentially hazardous

at concentrations above 1 ng.

Results of the Chick Embryo Lethality assay were inconclusive. Seven of the ten eggs injected with 50 ng/0.1ml JS3 LPS were killed (this coincides with the expected 50%). One embryo of the eight given 1,000 ug/0.1 ml dosage of PSS CA was killed. Two embryos of the eight that received 50 ng/0.1 ml dosage of DOC CA died. Hemorrhaging due to technique is attributed to be the cause of all three deaths, rather than fatal endotoxicity levels in the capsule extracts.

#### Electrophoretic Properties

The electrophoretic mobility of the Casper's Antigen extracts was suggestive of a high molecular weight polymer because it remained in the upper portion of the 12.5% SDS-Page separating gel. Several of the gels exhibited a light staining area that masked the proteins expected to be seen in the upper  $\frac{1}{4}$  of the gel (Fig. A & C, p.27 & 28). The silver stained (Fig. D, p.28) gel shows a more densely stained region near the top of the gel, possibly due to capsular material.

Stains - All was used to dye two of the gels (Fig. A & B, p.27). This method stains polysaccharide a distinct red. The upper portion of the gels was stained red.

Casper's Antigen extracts were electrophoresed at 20 ug/u1 concentrations.

Gonococcal strains JS3 and JS1 were used for most of the gel preparations. The bacteria were harvested from June-August 1985 laboratory stock and from May 1981 stock (frozen at -70'C) which was known to be producing capsule (p.30). The 1985 stock was labeled JS1 N and JS3 N,

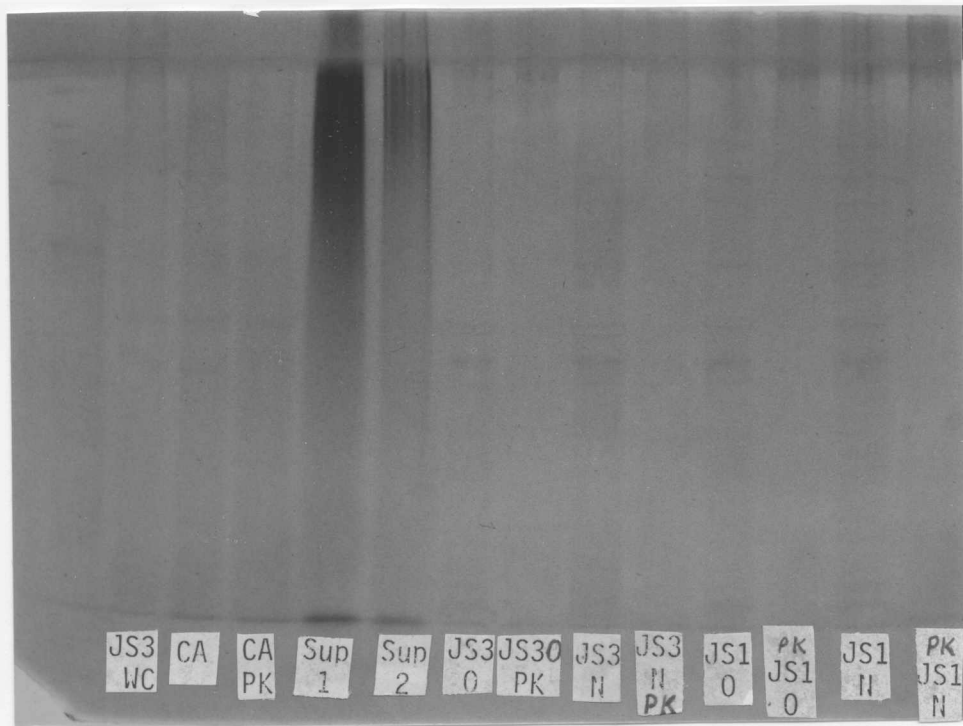


Fig A. 12.5% SDS-Polyacrylamide gel displays electrophoretic patterns of 10 ug samples of CA, Supe 1 (10 ug), Supe 2 (10 ug), JS3 N, JS3 0, JS1 N and JS1 0. The gel was stained with Stains - All. Polysaccharide stains red in this procedure.

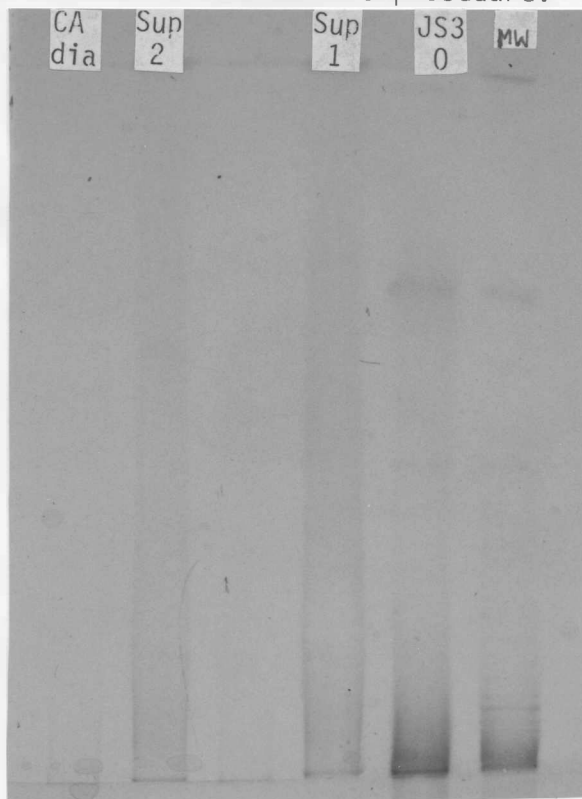


Fig B. 12.5% SDS-polyacrylamide gel containing JS3 WC lysates (10 u1), Supe 1 and 2, and dialyzed CA (2 u1) by Stains - All.

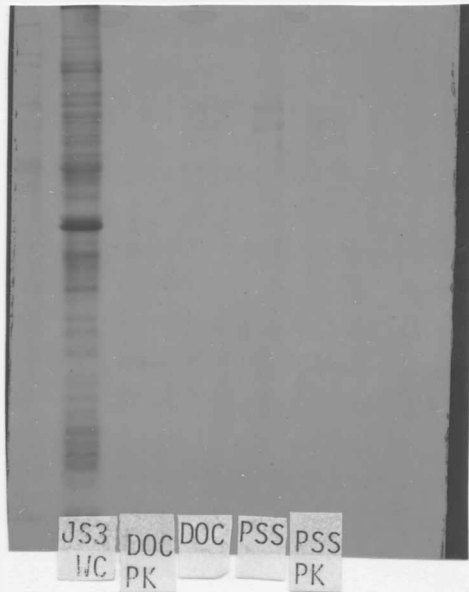


Fig C. 12.5% SDS-Polyacrylamide gel stained with Coomassie Brilliant Blue containing JS3 WC, PSS CA, DOC CA lysates and PK digested lysates. (Bacterial lysate was incompletely digested by Proteinase-K.)

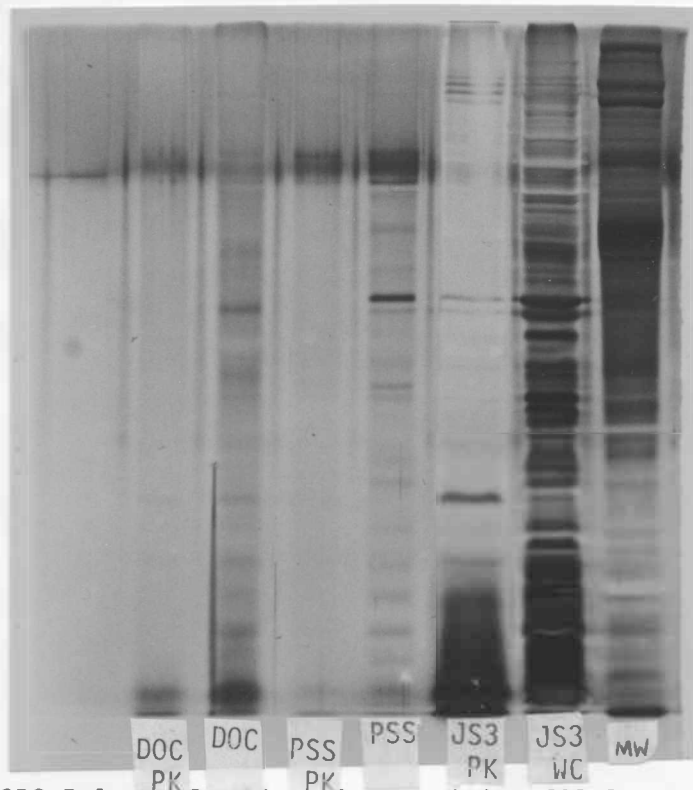


Fig D. 12.5% SDS-Polyacrylamide gel containing JS3 lysates (WC and PK digested), and CA extracts stained by Ag-LPS and protein staining procedure (19). Densely stained upper portion may reflect capsule mobility.

and the 1981 cultures were referred to as JS1 0 and JS3 0. A positive correlation between capsule production and electrophoretic staining patterns was not observed.

### Scanning Electron Microscopy

It has been suggested that prolonged in vitro cultivation of N. gonorrhoeae can substantially decrease its virulence (40). It is postulated that repeated subculturing may lead to the production of non-encapsulated variants.

Strains JS1 and JS3 were incubated for 42h (37°C, 5% CO<sub>2</sub>) on modified, clear typing medium. They were refrigerated (4°C) for 24h and processed as previously described with a 1h glutaraldehyde fixation period. Extracellular material was stabilized on colonies of JS1 (Fig.1, p.31) and JS3 (Fig.3, p.32) compared to colonies processed without prior refrigeration (Fig. 2 & 4, p.31,32). JS3 colonies are piliated (39).

In order to compare the effects of repeated subculturing on capsule production, frozen gonococcal suspensions were thawed and cultured on modified, clear typing agar. Strains JS1 (P-,0-) and JS3 (P-,0-) that had been frozen at -70°C on May 1981 were used. Five groups of each strain processed under variable refrigeration periods and glutaraldehyde fixation times as follows:

group 1-no refrigeration, glutaraldehyde for 1h

group 2-no refrigeration, glutaraldehyde for 4h

group 3-7h at 4°C, glutaraldehyde for 18h at 4°C

group 4-23h at 4'C, glutaraldehyde for 1h (room temperature)

group 5-48h at 4'C, glutaraldehyde for 1h (room temperature)

All five groups contained colonies from JS1 N and JS3 N, as well as JS1 0 and JS3 0. The bacterial colonies were incubated for 24h at 37'C and 5% CO<sub>2</sub>. The individual groups were processed as previously described.

Examination of scanning electron micrographs revealed a significant increase in capsule production by the organisms that were refrigerated prior to fixation (Fig.5, 6, 11 & 14,p.33-35). A longer refrigeration period seemed to stabilize the capsular material (Fig.11-14, p.34-35). The presence of artifacts on the colonies can be correlated to the period of fixation in glutaraldehyde (Fig.7 & 8,p.33) (artifacts appeared as star-shaped, crystalline structures). The JS1 0 and the JS3 0 colonies were observed to have a greater percentage of encapsulated bacteria than the JS1 N and JS3 N colonies (Fig.11,12,13, 14, p.34-35).

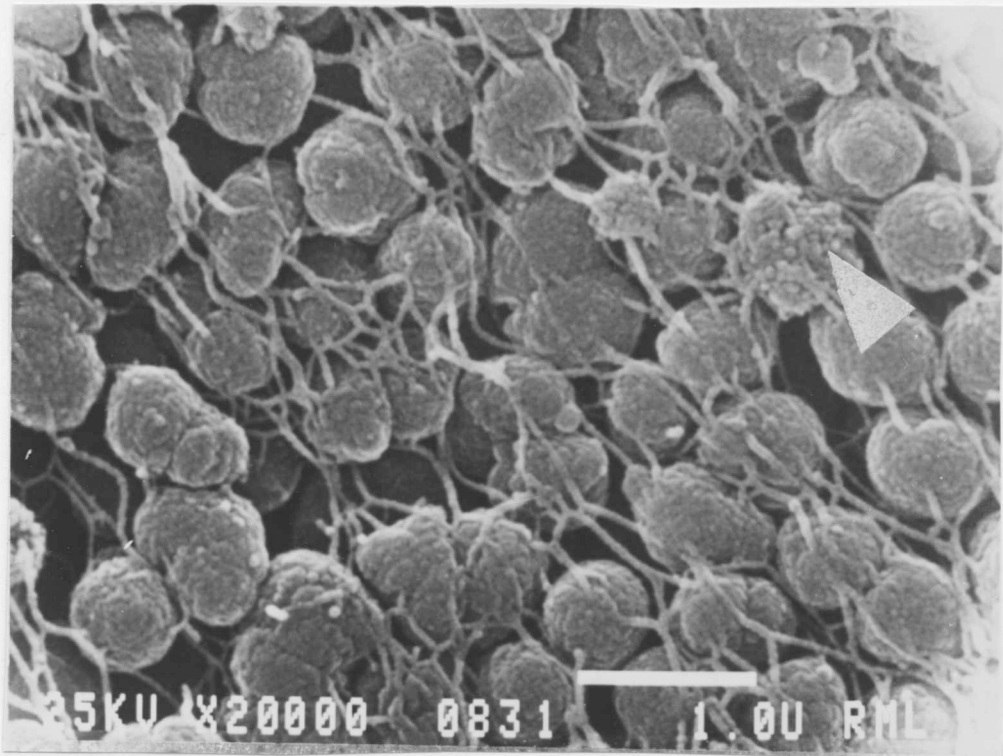


Fig. 1. JSI colony refrigerated for 24h and fixed in glutaraldehyde for 1h at room temperature. A white arrow points to capsular material.

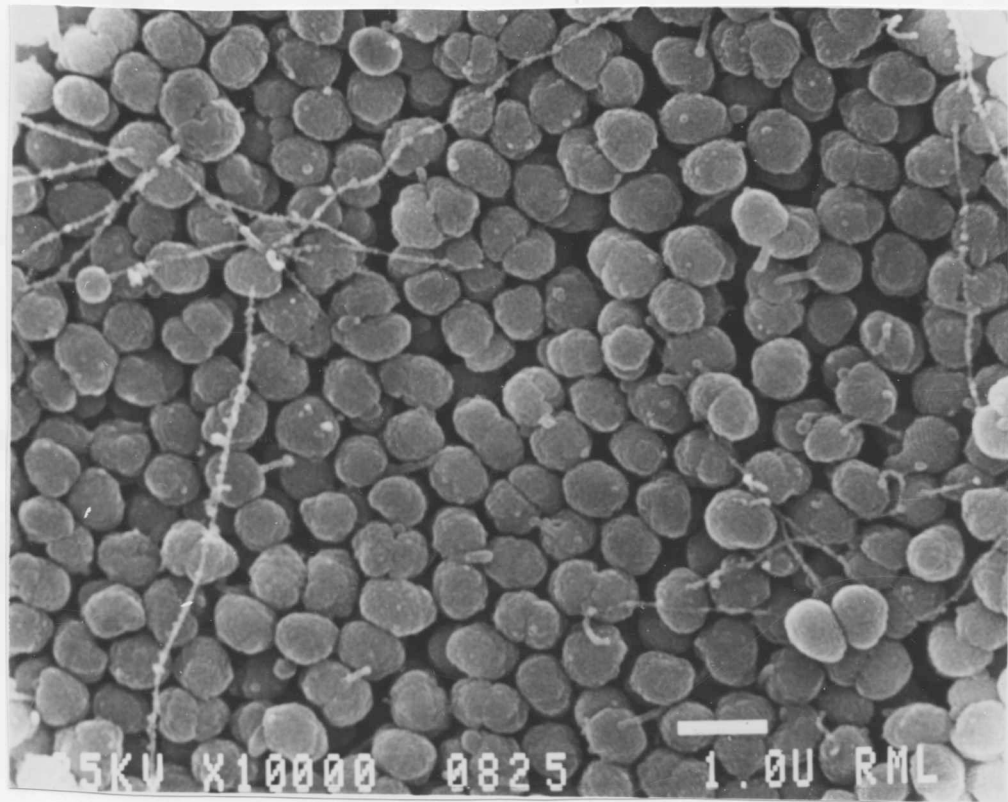


Fig.2. JSI colony fixed in glutaraldehyde for 1h at room temperature without prior refrigeration.

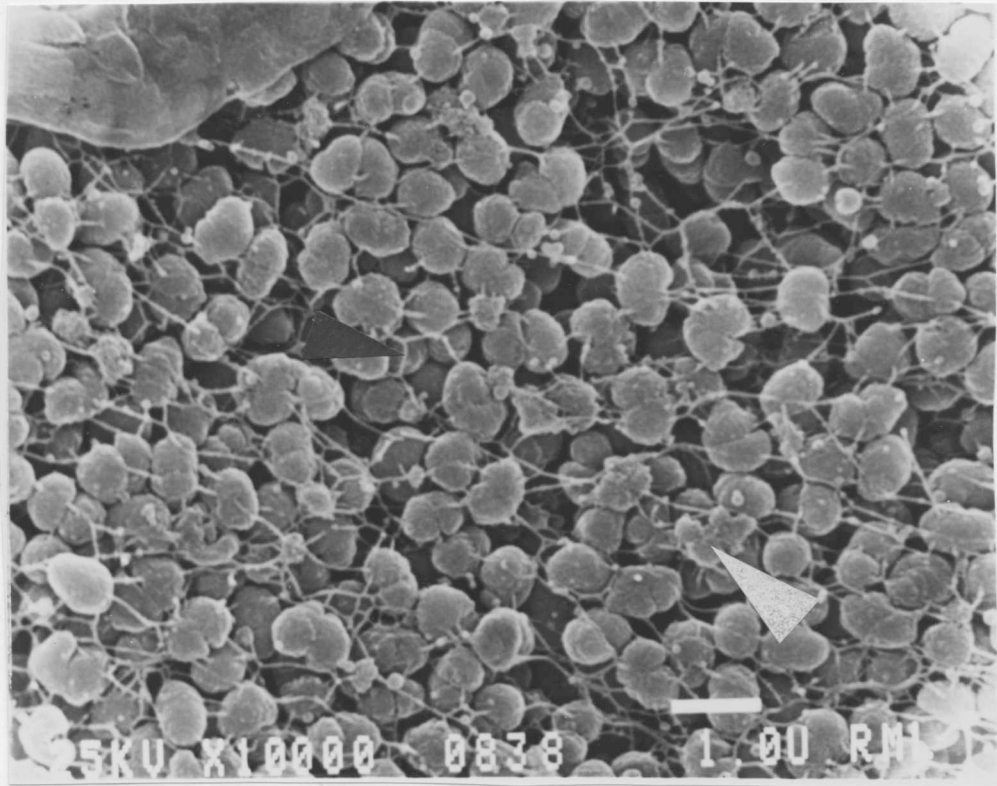


Fig.3. JS3 colony refrigerated for 24h before glutaraldehyde fixation (1h, room temperature). A white arrow points to capsular material. Black arrow points to pili.

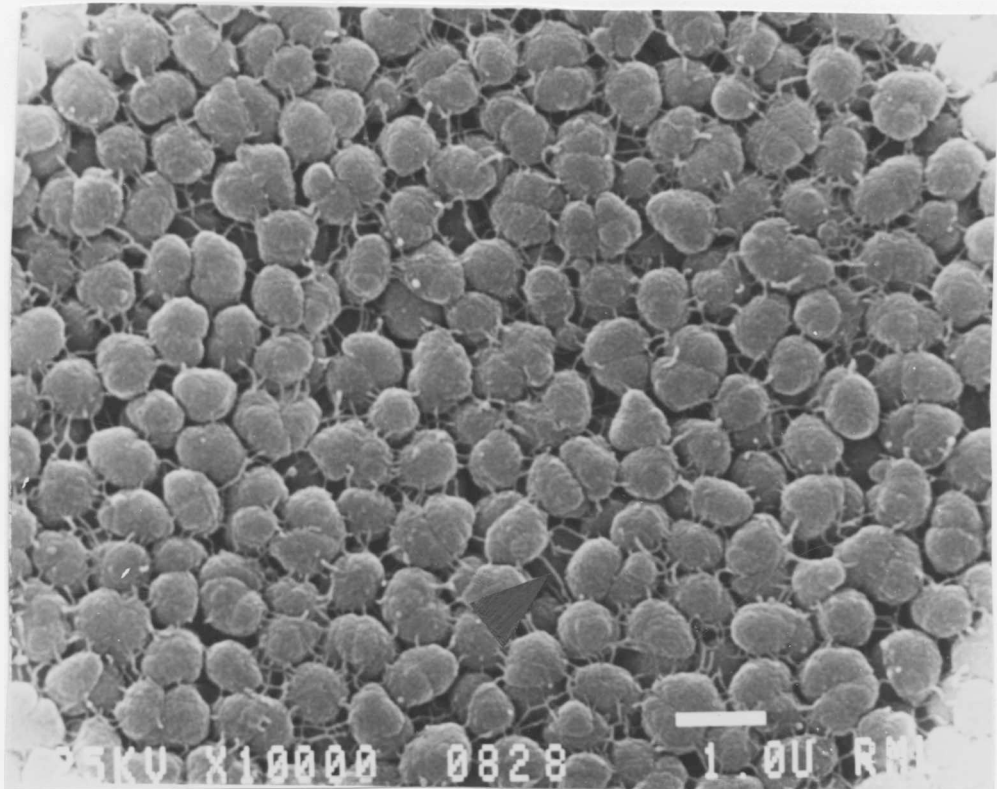


Fig.4. JS3 colony fixed in glutaraldehyde for 1h without prior refrigeration. Black arrow points to pili.

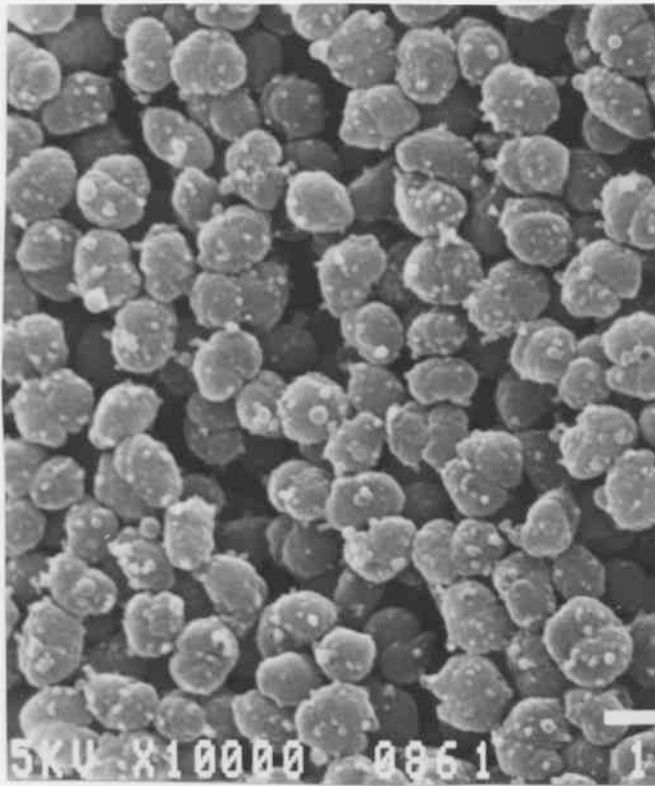


Fig.5. Group 1, strain JS1 O.

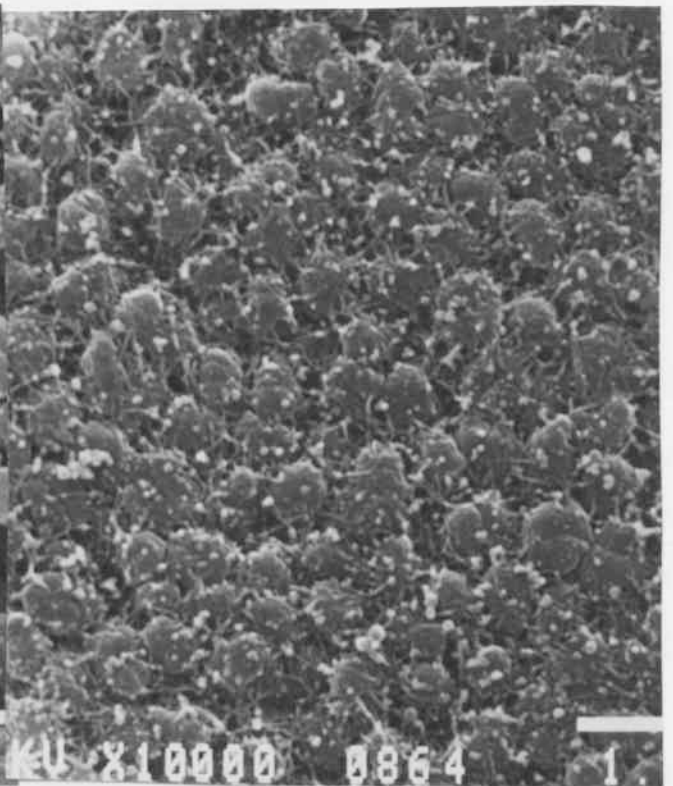


Fig.6. Group 2, strain JS3 N.

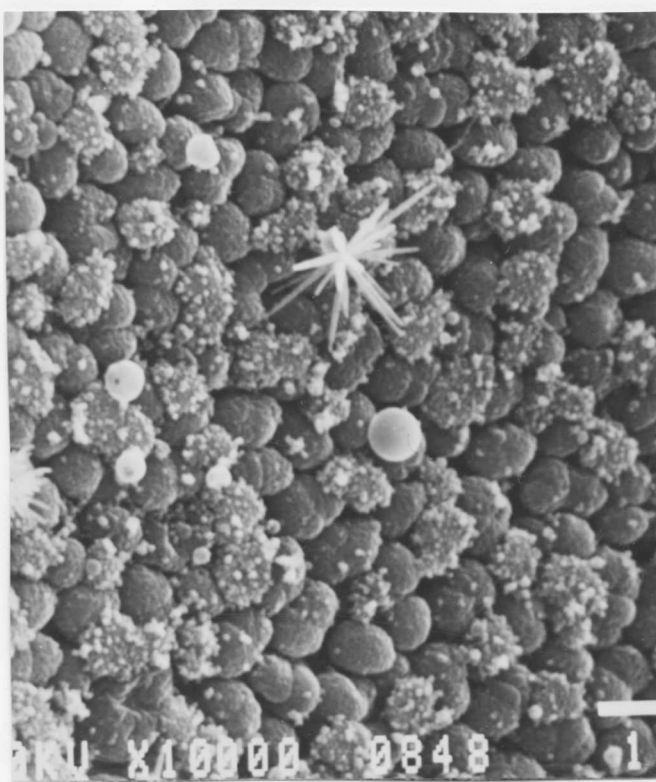


Fig.7. Group 3, strain JS1 O.

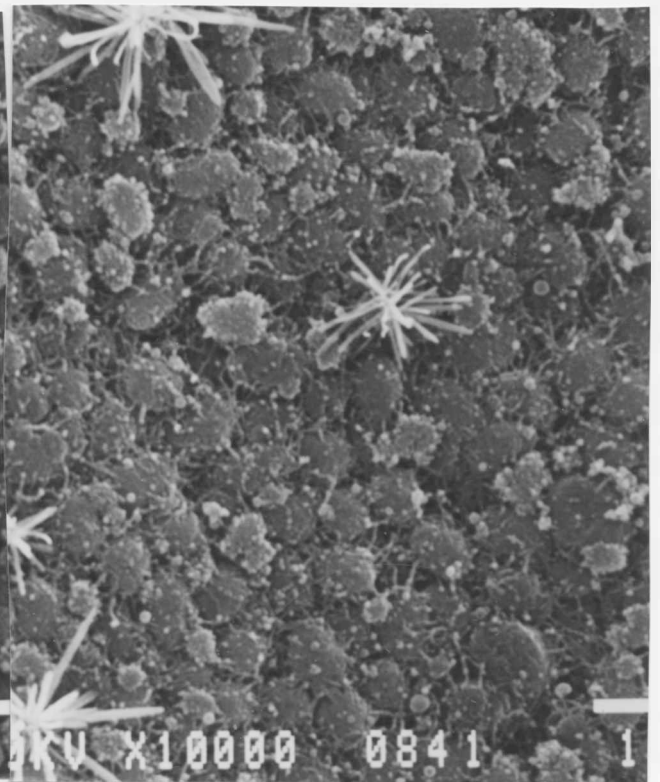


Fig.8. Group 3, strain JS3 N.

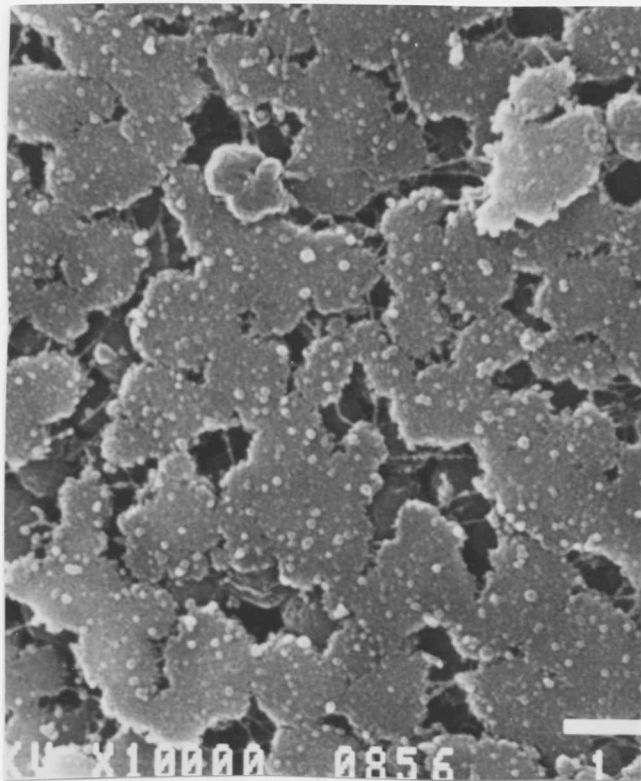


Fig.9. Group 4, strain JS1 O.

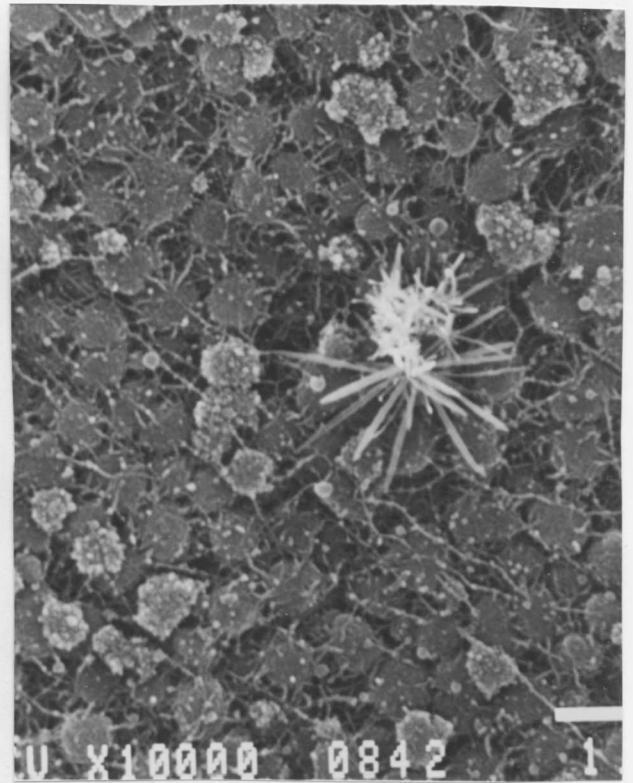


Fig.10. Group 4, strain JS3 N.

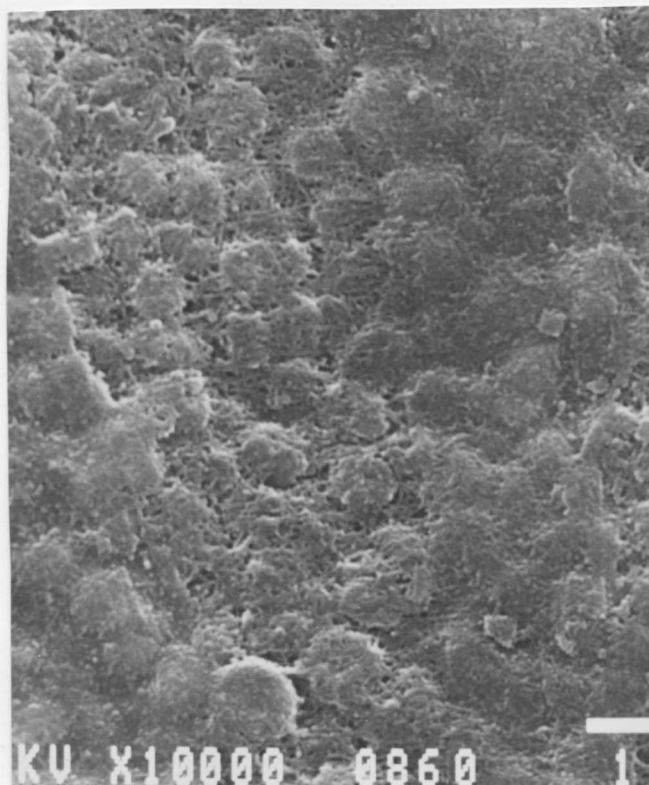


Fig.11. Group 5, strain JS3 N.

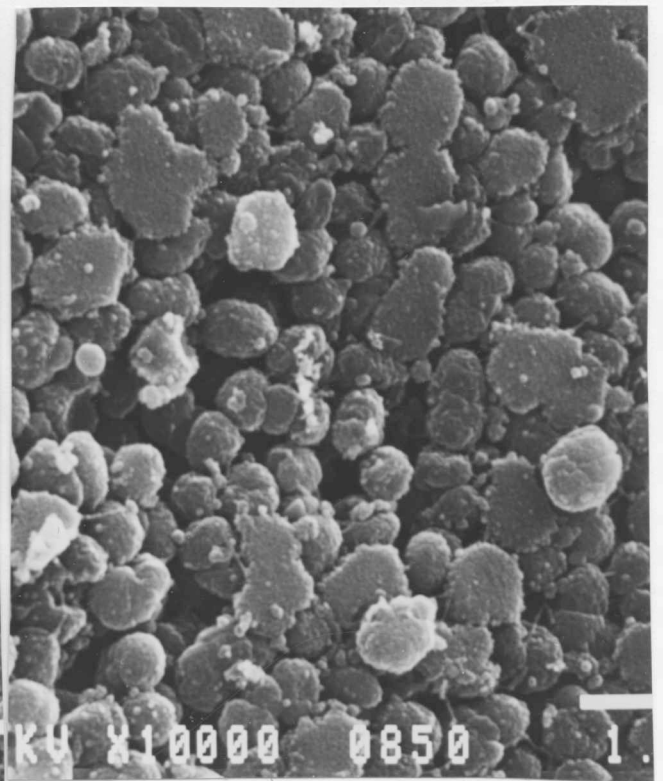


Fig.12. Group 5, strain JS1 N.

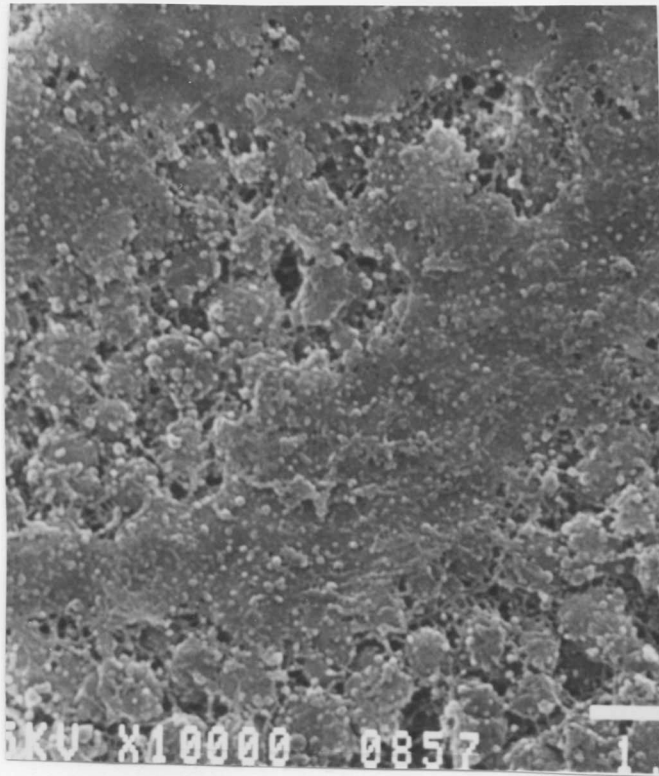


Fig.13. Group 5, strain JS3 0.

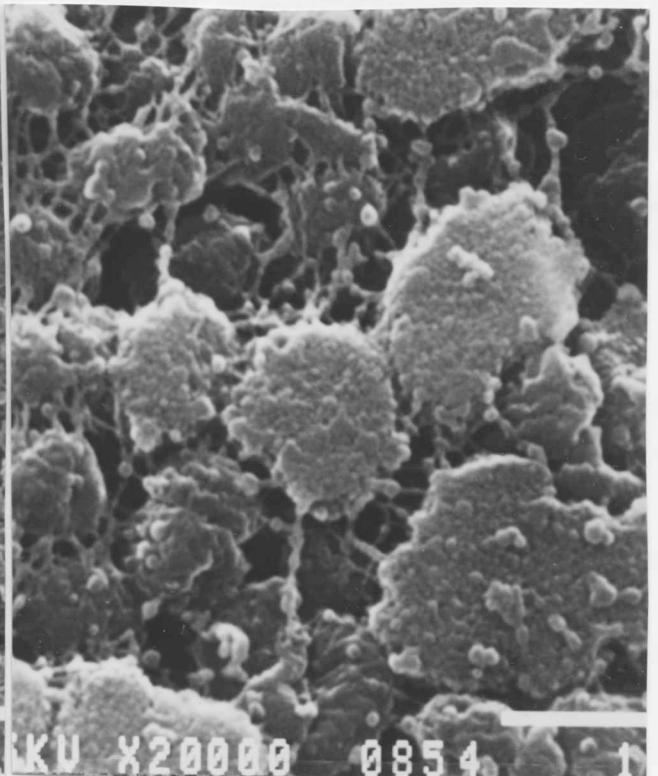


Fig.14. Group 5, strain JS1 0.

## DISCUSSION AND CONCLUSION

Gonococcal capsule was extracted according to the methods of Dr. Wolfgang Casper (4). The extracts were analyzed biologically and biochemically and were determined to contain primarily polysaccharide with some nucleic acid, protein and lipopolysaccharide. The degree of contamination in the capsule extracts can be attributed to physiological changes resulting from lyophilization before processing and to difficulties in clearly separating the centrifuged products.

The first capsule extraction, referred to as Casper's Antigen (CA), contained 36% protein as determined by spectrophotometric scans and Lowry protein assays. SDS-PAGE gels of CA show the presence of major surface proteins. Analysis of the specific carbohydrate components has been inhibited by the presence of these proteins.

Other constituents in the Casper's Antigen extract included substantial amounts of ribonucleic acids, and minute quantities of phosphorus and 2-keto-3-deoxyoctonate.

Two additional substances were examined as byproducts of the first capsule extraction. Supernatants 1 and 2 were found to contain polysaccharide upon re-extraction. Supernatant 1 had a higher concentration of carbohydrate than did Casper's Antigen, but it was more heavily contaminated with RNA. Supernatant 2 also contained a higher percentage of carbohydrate but large quantities of ribonucleic acids and lipopolysaccharide were present. The higher yields of polysaccharide may be a result of refrigerating the supernatant fluid for a long

period before processing. The refrigeration time could facilitate the precipitation of capsule from solution.

A second extraction performed on freshly cultured bacteria proved to be successful in yielding a more carbohydrate rich product. The cultures were harvested from the agar medium and suspended in physiological saline solution. The bacterial suspension was centrifuged, and the bacterial pellet and the supernatant fluid were processed. The bacterial pellet yielded capsular material that was referred to as DOC CA. The products of the supernatant fluid were labeled PSS CA, and PSS CA insol. All of the extracts contained higher concentrations of polysaccharide than Casper's Antigen, however they contained other constituents of varying degrees.

In pursuit of acquiring a gonococcal vaccine, as proposed by Dr. Casper, it is essential to know the amount of bacterial lipopolysaccharide in the preparations. The Limulus Amebocyte Lysate assay and the Chick Embryo Lethality (LD<sub>50</sub>) test were used. The Limulus assay identified the presence of lipopolysaccharide in CA at concentrations above 10 ng, and lipopolysaccharide in Supernatant 2 above 1 ng concentrations. All other capsule extractions were free of detectable endotoxins. The results of the Chick Embryo Lethality test were inconclusive because it could not be determined with certainty whether the embryos were killed by endotoxin or if their deaths were due to hemorrhages in the blood vessels of the chorio-allantoic membrane at the time of injection. The antigenic properties of the capsule will require further investigation.

The electrophoretic properties of the Casper's Antigen extracts

cannot be interpreted with certainty. Electrophoresed staining patterns indicate that there are proteins and some lipopolysaccharides in the extractions. It is expected that the Casper's Antigen extracts are high molecular weight polymers of polysaccharide and that upon staining it migrates within the upper quarter of the separating gels. The production of a monoclonal antibody to the extracts in immunoelectroblotting assays will subsequently confirm or disprove the present interpretation of the preferential staining patterns.

It has been determined that extended in vitro cultivation of N. gonorrhoeae can inhibit capsule production (16, 18, 38), and it is estimated that approximately ten percent of the cells in a colony actively produce capsule. Scanning electronmicroscopy confirmed that a 1981 bacterial culture, which had undergone few serial passages, was producing greater quantities of capsule than a 1985 culture that was passed daily.

Our experiments have proven that refrigeration of bacterial cultures before processing them for electron microscopy stabilized capsular material. The PSS bacterial suspension and the specimens used for electron microscopy were refrigerated before they were utilized. Longer refrigeration periods seemed to maximize capsule stability however extended fixation times created microscopic artifacts.

An extract comprised of carbohydrate, protein and nucleic acid was isolated from cultures of Neisseria gonorrhoeae following a protocol first published by Wolfgang Casper in 1937. The presence of capsule was visualized by scanning electron-micrographs and cold incubation of the colonies before processing appeared to stabilize the material. The exact

biochemical composition of the extract and the role it may have as a protective antigen remains to be determined.

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