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# IMMUNOCHEMICAL ANALYSIS OF SELECTED CYTOPLASMIC PROTEINS OF NEISSERIA GONORRHOEAE

BY

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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## ABSTRACT

The cytoplasm of clonal type 1 and type 4 _Neisseria gonorrhoeae_, obtained after ultrasonication of the bacteria and differential centrifugation, was subjected to analytical disc-electrophoresis and isoelectric focusing. Studied by both methods and by the intravenous toxicity test in 11-day old chicken embryos, the type 4 cytoplasm consisted of ten protein components whereas the type 1 cytoplasm contained eleven proteins of which one of them (β+t) was a unique toxic biopolymer. This toxic protein consisted of 16 amino acids, lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine, occurring in the molar ratios of 4 : 1 : 4 : 9 : 5 : 4 : 11 : 3 : 10 : 10 : 7 : 2 : 5 : 7 : 2 : 3.

After a further purification by the preparative polyacrylamide gel electrophoresis, a highly purified, non-toxic β(-t) protein was obtained. It exclusively consisted of a protein which amounted to 99.0% of its total dry weight. Only 7 amino acids were found to be present, lysine, aspartic acid, threonine, serine, glutamic acid, glycine, and alanine, combined at molar ratios of 1 : 1 : 1 : 3 : 2 : 7 : 1. This β(-t) biopolymer elicited a strong primary and secondary humoral as well as a cell-mediated response in the rabbit. Preliminary studies revealed that the immunity conferred by the detoxified β(-t) biopolymer was effective in the protection of rabbits against infection with _Neisseria gonorrhoeae_, as examined by injection of live gonococci into the anterior chamber.

TABLE OF CONTENTS

PAGE

ACKNOWLEDGEMENTS . . . . . . . . . . . . . . . . . . . . . . . . . . i

ABSTRACT . . . . . . . . . . . . . . . . . . . . . . . . . . . . . ii

LIST OF TABLES . . . . . . . . . . . . . . . . . . . . . . . . . . iii

LIST OF FIGURES . . . . . . . . . . . . . . . . . . . . . . . . . . iv

INTRODUCTION . . . . . . . . . . . . . . . . . . . . . . . . . . . 1

LITERATURE REVIEW

ANATOMICAL STRUCTURE . . . . . . . . . . . . . . . . . . . . . 2

CHEMICAL STRUCTURE . . . . . . . . . . . . . . . . . . . . . . 3

The Bacterial Cell . . . . . . . . . . . . . . . . . . . . 3
Cytoplasm . . . . . . . . . . . . . . . . . . . . . . . . 7
Pili . . . . . . . . . . . . . . . . . . . . . . . . . . . 9
Extracellular Materials . . . . . . . . . . . . . . . . . 9

ANTIGENIC STRUCTURE . . . . . . . . . . . . . . . . . . . . . 10

Surface Antigens . . . . . . . . . . . . . . . . . . . . . 10
Antigens Extracted with Chemicals . . . . . . . . . . . . . 11
Cytoplasmic Antigens . . . . . . . . . . . . . . . . . . . 15
Antigen Groups and Types . . . . . . . . . . . . . . . . . 18
Immunodiagnosis . . . . . . . . . . . . . . . . . . . . . 23

VIRULENCE OF NEISSERIA GONORRHOEAE . . . . . . . . . . . . . . 29

Intact Organism . . . . . . . . . . . . . . . . . . . . . 29
Endotoxins . . . . . . . . . . . . . . . . . . . . . . . . 36
Exotoxins . . . . . . . . . . . . . . . . . . . . . . . . 40

THE QUESTION OF IMMUNITY IN GONORRHEA . . . . . . . . . . . . 43

Humoral Response . . . . . . . . . . . . . . . . . . . . . 44
Cellular Response . . . . . . . . . . . . . . . . . . . . 46

MATERIALS AND METHODS

THE ORIGIN AND CULTIVATION OF NEISSERIA GONORRHOEAE . . . . . . . 48

PREPARATION OF CYTOPLASM . . . . . . . . . . . . . . . . . . . 48

CHEMICAL EXAMINATION . . . . . . . . . . . . . . . . . . . . . 51

PAGE

MATERIALS AND METHODS (continued)

Qualitative Determination of Polymer-Categories by Disc-Electrophoresis . . . . . . . . . . . . . . . . . . . . 51
Preparative Separation and Purification of the Different Polymer-Categories . . . . . . . . . . . . . . . . 52
Gel Filtration . . . . . . . . . . . . . . . . . 52
The Isoelectric Focusing Technique . . . . . . . 53
Preparative Polyacrylamide Gel Electrophoresis . 53

The Chemical Analysis . . . . . . . . . . . . . . . . . . 54
Dry Weight Determination . . . . . . . . . . . 54
Ash Value Determination . . . . . . . . . . . . 54
Lipid Extraction . . . . . . . . . . . . . . . . 55
Phosphorus . . . . . . . . . . . . . . . . . . . 55
Phospholipid . . . . . . . . . . . . . . . . . . 55
Phosphorus in Nucleic Acids . . . . . . . . . . 55
Nucleic Acids . . . . . . . . . . . . . . . . . 55
Deoxyribonucleic Acid . . . . . . . . . . . . . 56
Ribonucleic Acid . . . . . . . . . . . . . . . . 56
Ribose in RNA . . . . . . . . . . . . . . . . . 56
Pentose . . . . . . . . . . . . . . . . . . . . 56
Pentose in Carbohydrate . . . . . . . . . . . . 56
Carbohydrate . . . . . . . . . . . . . . . . . . 56
Protein . . . . . . . . . . . . . . . . . . . . 56
Amino Acid Analysis . . . . . . . . . . . . . . 56
Determination of Physicochemical Characteristics . . . . . . . . . . . . . . . . . 57

SEROLOGICAL EXAMINATION . . . . . . . . . . . . . . . . . . 58

Sources of Hyperimmune Rabbit Sera and Human Sera . . . . 58
Immunodiffusion . . . . . . . . . . . . . . . . . . . . 58
Agarose -gel Immunoelectrophoresis . . . . . . . . . . . 60
Absorption . . . . . . . . . . . . . . . . . . . . . . . 60
Radial-Immunodiffusion . . . . . . . . . . . . . . . . . 60
The Homogeneity Determination . . . . . . . . . . . . . 61
The Immunogenicity Assays . . . . . . . . . . . . . . . 61

BIOLOGICAL EXAMINATION . . . . . . . . . . . . . . . . . . 62

The Toxicity Assay . . . . . . . . . . . . . . . . . . . 62
Neutralization Assay . . . . . . . . . . . . . . . . . . 63
The Assay of the Immuno-Protective Power . . . . . . . . 63

PAGE

RESULTS

I. PHYSICOCHEMICAL PROPERTIES . . . . . . . . . . . . . . . . . . . . 65

Qualitative and Quantitative Separation of Different Polymer-Categories . . . . . . . . . . . . . . . . . . . . . . 65

Comprehensive Disc-Electrophoresis Pattern . . . 65

The Sephadex G-200 Gel Filtration Pattern . . . 65

Purification of the Unique Cytoplasmic Biopolymer. . . . . 65

Isoelectric Focusing . . . . . . . . . . . . . . 65

Preparative Polyacrylamide Gel Electrophoresis . 71

Chemical Analyses of the β(+t) and β(-t) Biopolymers . . . 71

II. IMMUNOLOGICAL PROPERTIES . . . . . . . . . . . . . . . . . . . . 75

Immunodiffusion . . . . . . . . . . . . . . . . . . . . . . 75

Immunoelectrophoresis . . . . . . . . . . . . . . . . . . . 81

Immunogenicity of the β(+t) and β(-t) Biopolymers . . . . 85

III. BIOLOGICAL PROPERTIES . . . . . . . . . . . . . . . . . . . . 89

Toxicity Assay . . . . . . . . . . . . . . . . . . . . . . 89

Physicochemical Properties of the Toxic Biopolymers . . . 91

Neutralization with Hyperimmune Rabbit Sera . . . . . . . 91

Neutralization with Human Sera . . . . . . . . . . . . . . 91

Immunoprotection Assay . . . . . . . . . . . . . . . . . . 94

DISCUSSION . . . . . . . . . . . . . . . . . . . . . . . . . . . 100

CONCLUSION . . . . . . . . . . . . . . . . . . . . . . . . . . . 107

BIBLIOGRAPHY . . . . . . . . . . . . . . . . . . . . . . . . . . 109

APPENDIX

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis . . . . . . . . . . . . . . . . . . . . . . . 121

# LIST OF TABLES


| TABLE | | PAGE |
|---|---|---|
| I | Chemical composition of organic materials of *Neisseria gonorrhoeae* endotoxins | 8 |
| II | Comparison of different gonococcal antigens prepared by chemical extractions | 16 |
| III | Serological differentiation of *Neisseria gonorrhoeae* | 24 |
| IV | Serological diagnosis for uncomplicated gonorrhea | 30 |
| V | Differences between virulent and avirulent types of *Neisseria gonorrhoeae* | 37 |
| VI | Biological activities of endotoxins of *Neisseria gonorrhoeae* | 41 |
| VII | Morphological characteristics of type 1 and type 4 colonies of *Neisseria gonorrhoeae* | 49 |
| VIII | Procedure for the preparation of cytoplasm | 50 |
| IX | Amino acids detected in the cytoplasmic proteins of *Neisseria gonorrhoeae* | 74 |
| X | The toxicity of α and β(+t) fractions for chicken embryos | 90 |
| XI | Physicochemical properties of *Neisseria gonorrhoeae* cytoplasm and its biopolymers | 92 |
| XII | Neutralization with hyperimmune rabbit sera | 93 |
| XIII | Neutralization with human sera | 95 |
| XIV | Protection of rabbits injected with β(-t) and β(+t) biopolymers against anterior chamber infection with *Neisseria gonorrhoeae* | 99 |

LIST OF FIGURES


FIGURE PAGE

1 Proteinaceous materials separated by the disc-electrophoresis from type 1 cytoplasm and type 4 cytoplasm . . . . . . . . . . . . . . . . . . . . . 66

2 Contours of the proteinaceous fractions separated by the disc-electrophoresis from type 1- and type 4 cytoplasm, revealed by scanning in the Chromoscan . . . . . 67

3 Proteinaceous fractions separated by Sephadex G-200 gel filtration . . . . . . . . . . . . . . . . . . . . . . . 68

4 Distribution of proteinaceous fractions separated by the isoelectric focusing of the leading fraction of type 4 cytoplasm, eluted from Sephadex G-200 column . . . . . . . . . . . . . . . . . . . . . . . . . . 69

5 Distribution of proteinaceous fractions separated by the isoelectric focusing of the leading fraction of type 1 cytoplasm, eluted from Sephadex G-200 column . . . . . . . . . . . . . . . . . . . . . . . . . . 70

6 Distribution of proteinaceous fractions separated by the isoelectric focusing of the β(+t) biopolymer . . . . 72

7 The profile of β(-t) protein on the preparative polyacrylamide gel electrophoresis . . . . . . . . . . . . . 73

8 Precipitin bands formed on reaction between the rabbit antiserum produced against type 1 cytoplasm and the cytoplasms of *Neisseria gonorrhoeae* . . . . . . . . . 76

9 Precipitin bands formed on reaction between the rabbit antiserum produced against type 4 cytoplasm and the cytoplasms of *Neisseria gonorrhoeae* . . . . . . . . . 77

10 Precipitin bands formed on reaction between the absorbed anti-type 1 cytoplasm serum and the cytoplasms of *Neisseria gonorrhoeae* . . . . . . . . . . . . . . 78

11 Precipitin bands formed on reaction between the rabbit antiserum produced against type 1 cytoplasm and the toxic biopolymers of *Neisseria gonorrhoeae* cytoplasms . . . . . . . . . . . . . . . . . . . . . . . . . . 79

12 Precipitin bands formed on reaction between the rabbit antiserum produced against type 1 cytoplasm and the antigens prepared from *Neisseria gonorrhoeae* cytoplasms . . . . . . . . . . . . . . . . . . . . . . . . . . 80

LIST OF FIGURES (CONTINUED)

FIGURE PAGE

13 Precipitin bands formed on reaction between the rabbit antiserum produced against the β(-t) biopolymer and the cytoplasms of Neisseria gonorrhoeae ....82

14 Precipitin bands formed on reaction between the hyperimmune rabbit antiserum and human sera and the cytoplasms of Neisseria gonorrhoeae . . . . . . . . .83

15 Immunoelectrophoresis of the toxic α biopolymers of Neisseria gonorrhoeae cytoplasms . . . . . . . . . . . . . .84

16 Immunoelectrophoresis of the non-toxic β(-t) biopolymer of type 1 cytoplasm of Neisseria gonorrhoeae . .86

17 Profile of antibody response of the β(+t) and β(-t) proteins, as shown by radial-immunodiffusion . . . . . . .87

18 Patterns of dermal hypersensitivity response to the β(-t) and β(+t) biopolymers . . . . . . . . . . . . . . . . . . .88

19 The cornea of a rabbit immunized with the β(-t) biopolymer and challenged with live type 1 Neisseria gonorrhoeae . . . . . . . . . . . . . . . . . . . . . . .96

20 The cornea of a rabbit pre-injected with the β(+t) biopolymer and challenged with live type 1 Neisseria gonorrhoeae . . . . . . . . . . . . . . . . . . . . . . .97

21 Molecular weight determination of the β (-t) biopolymer by SDS-polyacrylamide gel electrophoresis . . .123

# INTRODUCTION

The aim of this research project has been the elucidation of the biological role played by toxic and non-toxic immunogenic biopolymers present in the cytoplasm of Neisseria gonorrhoeae.

This study had been originated from the postulates, proposed in 1969 and published in 1974 (Kwapinski and Cheng, 1974), that (1) the immunogenicity of Neisseria gonorrhoeae depends upon a single immunochemical component present in the interior of the bacterium; (2) that the immunogenicity of this component is normally 'masked' by a co-existing, non-immunogenic, toxic component of the bacterial cell; and (3) that the removal of the latter constituent would free the gonococcal immunogen.

In order to test the above hypotheses, a step-wise approach had been adopted for this research. At the initial stage, an overall analysis of the cytoplasmic components (clonal types 1 and 4) of Neisseria gonorrhoeae had been carried out. This was followed by the separation and purification of immunochemical constituents and by the determination of their antigenic properties. Whenever applicable, the findings derived from the analyses above had been compared with the biologically active materials obtained by different methods and reported by other investigators. This information, gathered from the scientific literature, had been studied and critically evaluated before and during the period of the research. The literature review will be presented under the following headings: anatomical structure, chemical structure, antigenic structure, virulence, and host response.

# LITERATURE REVIEW

ANATOMICAL STRUCTURE

The cell of Neisseria gonorrhoeae is composed of a wall, a cytoplasmic membrane with mesosomes of a simple form, and cytoplasm. The cell wall is a gently undulating, triple-layered component overlaying a very thin but dense and taut layer. This latter feature is missing in the large and lysing forms, in the L-forms, and in those gonococcal organisms altered by penicillin (Murray et. al., 1963). These findings have been confirmed by electron microscopy studies (Ovchinnikov et. al., 1968) with the additional demonstration of granules and a ribosomal apparatus within the cytoplasm, a nucleus with deoxyribonucleic acid threads, and mesosomes forming a continuation of the cytoplasmic membrane. Between the external wall and the cytoplasmic membrane there is a granular mass participating in cellular metabolism.

The cells of Neisseria gonorrhoeae divide by a combination of pinching and septation involving a simple type of membranous fold. Mesosomes are present in the cell periphery away from the plane of division. In spite of the separation of daughter cells by the membranous process, the completion of the cell wall often requires more time than membrane development. It is this delay in separation of the already divided cell which accounts for the high proportion of apparently dividing duplex forms seen in cultures of this organism (FitzJames, 1964).

Electron microscopic examination of negatively stained Neisseria gonorrhoeae also reveals that pili, with a diameter of 80-85 Å and a length of 0.5-4.0 μ, are present on the surfaces of types 1 and 2 virulent gonococci but not on avirulent types 3 and 4 (Swanson et. al., 1971; Jephcott et. al., 1971). Freeze-etch and freeze-fracture studies

have confirmed that the pili, of which there are about ten per cell and which may occur anywhere on the surface of the organism, maintain a close anatomical relationship with the cell wall (Swanson et. al., 1972).

Although it has been claimed that Neisseria gonorrhoeae is encapsulated (Israeli, 1921; Almaden, 1938; Bernstein, 1941), this remains controversial. Casper (1972) has argued that the capsule is a carbohydrate which disappears with in vitro cultivation. On the other hand Reyn (1949 c) has not been able to demonstrate capsular swelling, and electron microscopic studies have not shown a capsule (FitzJames, 1964; Ovchinnikov & Delektorskii, 1968). Perhaps the 'capsules' demonstrated by Casper and others are artifacts arising from the fixation or staining processes.

## CHEMICAL STRUCTURE

The whole cell of Neisseria gonorrhoeae and its extracellular products have been analyzed chemically but less thoroughly than the endotoxins. Other components of the cell, including cytoplasm and pili, have only been studied with the availability of more sophisticated separation methods.

### The Bacterial Cell

Microchemical analyses of six different strains of Neisseria gonorrhoeae grown in Douglas' broth (Stokinger et. al., 1944 a) reveal the dry mass of the gonococcus consists of an average of 60-65% nucleoprotein, 5-9% carbohydrate, and 10-14% lipid. Volatile and non-volatile matter constitutes an additional 13-18%. The protein portion consists of at least two major fractions: a relatively insoluble complex

containing approximately 25% bound lipid, and a minor, lipid-free, nucleoprotein which constitutes the major part of the gonococcal cell. The lipid recovered is highly complex and is separable into several crystalline substances which have been identified as lecithin, cephalin, and sphingomyelin.

By gas chromatography Moss et. al. (1970) and Yamakawa & Ueta (1964) have shown that the gonococcus contains the following major cellular fatty acids: lauric, myristic, palmitic, palmitoleic, hexadecenoic, octadecenoic, beta-hydroxylauric, and beta-hydroxymyristic. Glucose, galactose, and glyceromannoheptose are the major carbohydrates and alanine, methionine, glutamic acid, and phenylalanine occur as the major amino acids.

Neisseria gonorrhoeae elaborates several enzymes. A phosphatase is present in washed lyophilized gonococcal cells from broth cultures, but not in the supernatant (Leahy, Stokinger and Carpenter, 1940). A manometric oxygen uptake study of Neisseria gonorrhoeae (Tonhazy & Pelczar, 1953) in the presence of citric acid cycle compounds has demonstrated the presence of an oxaloacetic acid decarboxylase and a glutamic-aspartic transaminase, but not glutamic-alanine transaminase. The existence of a potent reduced diphosphopyridine nucleotide oxidase and a diphosphopyridine nucleotide-linked lactic dehydrogenase has been detected (Tauber & Russel, 1962) using the Thunberg technique with tetrazolium salt as the electron acceptor. This technique also has been useful in detecting the presence of a cyanide sensitive, aerobic, cysteine oxidase, and a cysteine desulfhydrase in the gonococcus. It has been shown that carbonic anhydrase is an enzyme produced by Neisseria gonorrhoeae (Veitch & Blankenship, 1963; Sanders & Maren, 1967) and that bicarbonate

is a growth requirement for Neisseria gonorrhoeae strains that contain carbonic anhydrase. Furthermore, the anti-bacterial activity of the sulfonamide carbonic anhydrase inhibitors results from their ability to inhibit bacterial carbonic anhydrase, reducing the rate of bicarbonate formation, and thus impeding the hydration of carbon dioxide.

Several investigators have reported on the isolation of different endotoxins from Neisseria gonorrhoeae when the cells are treated with various chemicals, including alkali, phenol-water, and aqueous ether. Extraction with alkali, followed by alcohol precipitation, yields a polysaccharide endotoxin containing 4.2% nitrogen (Miller & Boor, 1934). If cold acetone is incorporated with the alkali, a nucleoprotein endotoxin with a composition of 14.24% nitrogen and 1.08% phosphorus, is produced (Tauber & Garson, 1957). However, if the alkali treatment is followed by ion-exchange chromatography a pure acidic glycoprotein, known as the beta-antigen, is obtained (Apicella, 1973). This antigen has a $S_{20}W$ of 8.55 and contains four major amino acids (alanine, glutamic acid, glycine, and proline). Chemical analysis reveals its composition to consist of 11.2% nitrogen, 61.2-63.7% protein, 27.6-29.8% carbohydrate, and negligible amounts of phosphorus, pentose, methylpentose, KDO, and lipid. It has been suggested that the paucity of aromatic amino acids or the high carbohydrate content may be the cause of a negative color reaction with Folin-Ciocalteau reagent (up to 1 mg/ml antigen). The purified beta-antigen so obtained is sensitive to pronase treatment, whereas periodation and trypsin has little effect on the antigenic determinant. Furthermore, this antigen loses its ability to adsorb directly to erythrocytes as the crude antigen does, probably due to the loss of a portion of the molecule containing a lipid or carbohydrate

moiety; or to the loss of other charged groups. The location of this antigen has not been elucidated. However, it has been suggested that this is not likely a component of endotoxin due to the absence of 2-keto 3-deoxy sugar acids and heptoses.

Phenol-water extraction of Neisseria gonorrhoeae cells, on the other hand, yields a protein-free endotoxin with a nitrogen content of 8.5% consisting of polysaccharide (52%) and nucleic acid (53%) (Tauber & Garson, 1958). In contrast, Maeland and Kristofferson (1971) have isolated a lipopolysaccharide by the phenol-water method containing 35.6% lipid, 13.1% neutral sugars, 9% hexosamine, 4.4% nitrogen, 0.9% phosphorus, 8.1% 2-keto-3-deoxy-octulosonic acid (KDO), and 12.5% protein. This lipopolysaccharide can also be obtained by a hydrolytic extraction with phenol-water mixture, dialysis and differential centrifugation (Tauber & Garson, 1959). This fraction consists of 28.4% lipid, 13.8% hexosamine, 43% saccharide, 3.84% phosphorus, 3.3% nitrogen, and only 0.3% protein. The combination of phenol-water, dialysis and differential centrifugation appears to be superior to using phenol-water alone as less protein contamination is present in the latter procedure. It may be that dialysis removes the degraded protein product from the preparation. Further investigation has shown that the lipopolysaccharide endotoxin is a phosphoric acid ester, as demonstrated by its acidic property, high phosphorus content, and the release of phosphoric acid upon hydrolysis. Paper chromatographic analysis of this lipopolysaccharide endotoxin shows the presence of glutamic acid, glycine, alanine, ethanolamine, phosphoethanolamine, and trace amounts of $\alpha,\varepsilon$-diaminopimelic acid as amino acids; D-glucosamine, glucose, and galactose occur as the only carbohydrate (Tauber & Russel, 1961).

In contrast to the production of a lipopolysaccharide endotoxin by extraction with phenol-water, aqueous ether-extracted endotoxin consists mainly of protein, and much less lipid and polysaccharide. Chemical analysis of this endotoxin reveals 82-88% protein, 1.4-2.1% neutral sugars, 0.7-1.1% hexosamine, 2.6-6.3% lipid, 12.6-14% nitrogen, and 0.3-0.4% phosphorus. This protein fraction contains all the commonly occurring amino acids, except cysteine, cystine, and diaminopimelic acid. Among the 16 amino acids detected, lysine, arginine, aspartic acid, glutamic acid, alanine, valine, and leucine appear to be the major components. Since diaminopimelic acid is absent, it would indicate that mucopeptide linkage is not present in the endotoxin preparation (Maeland, 1969 b).

The reason for the differences in chemical composition between the various endotoxin preparations is still largely unexplained. It may be that different chemicals attack different parts or layers of the outer cell structure of the gonococcus thus releasing endotoxins of different chemical structure. The chemical composition of the endotoxins extracted from Neisseria gonorrhoeae by the methods detailed above are summarized in Table I.

Cytoplasm

Fractionation of the gonococcal cells into cytoplasm and cell wall components by Ribi's pressure cell fractionator produces two endotoxins (Martin et. al., 1969), one derived from the cell wall, the other from the cytoplasm. The cytoplasmic endotoxin may be recovered as Fraction I by the Sephadex G-200 filtration method (Peacock & Schmale, 1969), and as the 0.4M fraction by the ion-exchange chromatography method (Schmale et. al.,

TABLE I

CHEMICAL COMPOSITION OF ORGANIC MATERIALS OF *NEISSERIA GONORRHOEAE* ENDOTOXINS

| Method of Extraction | Major Chemical Entity | Percentage of Organic Materials | | | | | | Author |
|---|---|---|---|---|---|---|---|---|
| | | Protein | Lipid | Carbohydrate | Nucleic Acid | $N_2$ | $P_2$ | |
| Phenol-water | Polysaccharide and nucleic acid | * | * | 52 | 53 | 8.5 | * | Tauber & Garson (1958) |
| Phenol-water | Lipopolysaccharide | 12.5 | 35.6 | 22.1 | * | 4.4 | 0.9 | Maeland & Kristofferson (1971) |
| Phenol-water, dialysis & differential centrifugation | Lipopolysaccharide | 0.3 | 28.4 | 57 | * | 3.3 | 3.8 | Tauber & Garson (1959) |
| Alkali + alcohol | Polysaccharide | * | * | * | * | 4.2 | * | Miller & Boor (1934) |
| Alkali + cold acetone | Nucleoprotein | * | * | * | * | 14.2 | 1.08 | Tauber & Garson (1957) |
| Alkali + ion-exchange chromatography | Glycoprotein | 62.5 | * | 28.7 | * | 11.2 | * | Apicella & Allen (1973) |
| Aqueous ether | Protein | 85 | 4.5 | 2.7 | * | 13.4 | 0.35 | Maeland (1969 b) |

* Not reported

1969). It has been suggested that the cytoplasmic endotoxin has a different chemical composition from that in the cell wall as it is insoluble in the aqueous phase of the phenol extract although the cell wall endotoxin is soluble in this fraction; and that the aqueous phase of the phenol extract of the cell wall contains a lipopolysaccharide. However, no chemical analysis has been reported.

Pili

Purified pili have a molecular weight of 24,000 and a pI of 4.0. Sensitivity of pili to trypsin and inhibition of pilus formation by an inhibitor of protein synthesis prove their protein nature (Buchanan, 1972 and 1973).

Extracellular Materials

Gas chromatography of the fatty acids found in whole culture extracts of 3 strains of *Neisseria gonorrhoeae* shows that they are a homogeneous group. They can be distinguished from *Neisseria meningitidis* on the basis of production of acetic acid, failure to use propionic acid, and production of only a moderate amount of alpha-keto-isocaproic acid (Brooks *et. al.*, 1971). This confirms Barron and Miller's (1932) and Barron's (1936) findings that *Neisseria gonorrhoeae* oxidizes pyruvate to acetate, and Tonhazy and Pelczar's (1953) finding that the gonococcus is unable to oxidize propionate.

The culture filtrate of *Neisseria gonorrhoeae*, purified by precipitation with ammonium sulphate, appears to contain a protein degradation product derived from the nucleoprotein of the cells. It consists of 15.7-16% total nitrogen, 0.70% amino nitrogen, 0.13% total phosphorus, 0.81% total sulphur, and approximately 2.8% carbohydrate

(Stokinger _et_. _al_., 1944 a). The virulent type 1 gonococcus grown on supplemented Frantz medium, extracted first with EDTA (ethylenediamine-tetraacetic acid) and then with aqueous ether, yields a fraction which is devoid of endotoxic activity and produces 3-5 immunoprecipitation bands with the homologus antiserum (Foster _et_. _al_., 1973). Pronase and trypsin treatments eliminate 4 and 2 bands, respectively, whereas RNase and lipase have no apparent effect. The chemical composition of this fraction is 34.6% protein, 3.5% carbohydrate, 20% lipid, and 1-4% RNA. A similar preparation from type 4 _Neisseria gonorrhoeae_ contains less protein and more carbohydrate (18.3%). Whether this difference in chemical composition is associated with the basic virulence characteristic of types 1 and 4 gonococci is still unknown.

## ANTIGENIC STRUCTURE

_Neisseria gonorrhoeae_ contains a number of antigens: some are located on the surface, some in the cytoplasm, while others are obtainable only through chemical extraction.

### Surface Antigens

The first evidence of the existence of surface antigens is Chanarin's (1954) report that preparations obtained from freshly isolated strains of the gonococcus are adsorbed by red blood cells thus rendering them agglutinable by specific antibodies. This phenomenon as suggested by Chanarin, is due to a polysaccharide complex present on the surface of the smooth phase gonococcus which is lost when it undergoes the smooth-rough change.

Investigations, using a direct fluorescent staining and agglutination