1980

Confirmation of Neisseria gonorrhoeae by reversed passive hemagglutination and by pyrolysis-gas-liquid chromatography

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CONFIRMATION OF *NEISSERIA GONORRHOEAE* BY REVERSED PASSIVE HEMAGGLUTINATION AND BY PYROLYSIS-GAS-LIQUID CHROMATOGRAPHY

by

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B. A., University of Montana, 1963

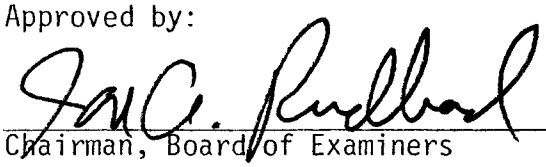
Presented in partial fulfillment of the requirements
for the degree of

Master of Science

UNIVERSITY OF MONTANA

1980

Approved by:



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Kelly, Judith R. Seerup, M.S., June, 1980 Microbiology

Confirmation of Neisseria gonorrhoeae by Reversed Passive Hemagglutination and by Pyrolysis-Gas-Liquid Chromatography

Director: Jon A. Rudbach

Accurate differentiation of Neisseria gonorrhoeae from other neisserial isolates is a continuing problem for clinical microbiology laboratories. Carbohydrate utilization methods now used are cumbersome and require 24-72 hr for completion. Also, confirmation of N. gonorrhoeae is often delayed because some strains fail to produce detectable amounts of acid in glucose-containing media.

Two alternate methods for confirmation were tested in this study; reversed passive hemagglutination (RPHA) and pyrolysis-gas-liquid chromatography (PGLC). These methods were chosen because both use simple procedures that can be performed on bacterial isolates taken from primary isolation media. Results can be obtained with either methods within an 8 hr day.

The RPHA method used was developed by Abbott Laboratories, Diagnostic Division, North Chicago, IL, and designated GONO-cell (Abbott Laboratories, registered trademark). This primary research and clinical trial of the GONO-cell test showed it to be simple, easy to read, and very sensitive. All 246 N. gonorrhoeae isolates tested were GONO-cell positive. Only 2 of 110 non-neisserial organisms and 1 of 41 non-pathogenic neisseria showed any immunological cross-reactivity in the test. All 4 of these isolates could have been eliminated from testing due to non-lysis during the lytic step of the RPHA procedure. Thirteen of 69 Neisseria meningitidis and Neisseria lactamica isolates lysed completely and were also GONO-cell positive. These 13 isolates were all cultured from respiratory specimens. Five non-gonococcal neisseria were isolated from a group of 1746 cultures from genital sources. All 5 were correctly eliminated by the GONO-cell test. The low frequency of isolation of non-gonococcal neisseria from genital sources made the GONO-cell test acceptable, despite the observed cross-reactivity, as a confirmatory test for neisserial isolates from genital sources.

Inconclusive results were obtained by PGLC analyses. The method did not appear to be practical for use in an average clinical laboratory since expensive equipment is required and apparatuses now available do not allow simultaneous analyses of several specimens.

ACKNOWLEDGEMENT

I wish to thank Dr. Jon Rudbach for his guidance and patience during all phases of this project.

I would also like to thank Dr. Fred Shafizadeh and the several workers in the Wood Chemistry Laboratory for their invaluable advice, repair work and equipment loans during the PGLC analyses.

I am grateful that Dr. Al Armstrong and Abbott Laboratories trusted me to do the RPHA clinical trial. I thank Linda Hedstrom of the Missoula City-County Health Department and Lou Colbo of the State of Montana, Department of Health and Environmental Science Microbiology Laboratory Bureau for providing me with needed neisseria isolates. I thank the Infectious Disease Center for use of laboratory facilities, and especially Liz Clemans who made the media used.

Finally, I would like to thank my family - my husband, Bob, my sons, Jim and Sean and my mother-in-law, LaVerne for their patience, understanding and toleration of the domestic upset caused by my pre-occupation with this project.

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ABBREVIATIONS

BBL	Baltimore Biological Laboratories, Cockneysville, MD
C	Degrees centigrade
CDC	Center for Disease Control, Atlanta, GA
CPPA	Cysteine proteose-peptone agar
CTA	Cysteine tripticase agar
FA	Fluorescent antibody
GC-C	Thayer-Martin medium containing no antibiotic inhibitor; i.e. enriched GC agar base with hemin added
GC agar	GC agar base with 1% BBL IsoVitaleX enrichment added
IDC	Infectious Disease Center
LPS	Lipopolysaccharide
M-H	Mueller-Hinton agar
NYC	New York City medium
ONPG	Ortho-nitrophenyl beta-D-galactopyranoside
PGLC	Pyrolysis-gas-liquid chromatography
RBC	Red blood cell
RPHA	Reversed passive hemagglutination
R-TM	Thayer-Martin medium made with 5% sterile defibrinated rabbit blood substituted for bovine hemoglobin
SBA	Sheep blood agar made using tripticase-soy agar base with 5% sterile defibrinated sheep blood added
TM	Thayer-Martin medium
TSA	Tripticase-soy agar

CHAPTER I

INTRODUCTION

Historical Statement

The differentiation of Neisseria gonorrhoeae from related oxidase-positive, Gram-negative diplococci poses a delicate problem for the clinical microbiologist. Misidentification of an isolate may allow an individual with gonorrhea to remain untreated; or it may lead to emotional or marital problems for an individual who was not infected with gonococci.

Progress made during the last decade in the development of better primary isolation media and collection and transport systems have made it likely that isolation of N. gonorrhoeae is routine in small clinical laboratories. More lawsuits are being filed against laboratories and their personnel. A laboratory worker no longer can assume that a genital or rectal neisserial isolate is N. gonorrhoeae.

The development of a rapid, reliable, inexpensive method for the accurate diagnosis of gonorrhea in an infected individual would be a major epidemiological achievement. Coventional methods used to collect, culture and identify an isolate as N. gonorrhoeae consume 72-96 h or longer. During this interval, an infected person may spread the infection to several more persons.

In the average United States clinical microbiology laboratory, specimens are cultured for gonococci from genital, rectal and oral

sources. Enriched, selective media developed during the 1960s and 1970s allow isolation of gonococci from specimens heavily contaminated by normal flora. Primary isolation media most often used are Thayer-Martin medium (T-M) (122), modified T-M medium (79) or NYC medium (39). Inoculated media are incubated at 35 C for 24 to 48 h in an atmosphere enriched to contain 6-10% CO_2. The CO_2-enriched atmosphere may be obtained through use of a candle-extinction jar, a CO_2 incubator or a sealed, self-contained system such as the JEMBEC (81) plate. Media are examined after 24-, 48-, and possibly 72-h incubation for the presence of colonies of oxidase-positive Gram-negative diplococci. If such colonies are found, they may be present in small numbers and may be contaminated by other bacteria or yeast. In these instances, sub-culturing on to antibiotic-free media is necessary to multiply and/or purify the isolate. This step consumes an additional 24 h. An oxidase-positive Gram-negative diplococcus isolated on the primary media listed above might be N. gonorrhoeae, N. meningitidis, N. lactamica, Branhamella catarrhalis, Moraxella spp. or occasionally another Neisseria sp.

Isolates from genital sources are most probably gonococci, but may be meningococci. Faur et al. (41) recorded the isolation of 32 strains of N. meningitidis and N. lactamica from genital and rectal sites in a large gonorrhea screening program in New York City. Chapel et al. (22) isolated genital N. meningitidis from 7 of a group of 157 homosexual men.

Isolates from oral sources are probably but not necessarily N. meningitidis. N. gonorrhoeae is not uncommonly isolated from oral sources (14, 27, 43, 49, 117), especially from homosexual men (59).

The next step, in the clinical laboratory, therefore, is to differentiate positively N. gonorrhoeae from related organisms. The most widely used method is the demonstration that the isolate can oxidize glucose, but not maltose, sucrose or lactose in CTA (3), a medium developed by Vera (126). Heavily inoculated media must be incubated 24-48 h before discernable acidity is produced in the tubes. Acidity produced by N. gonorrhoeae in CTA glucose may be observable only after 4-6 h of incubation.

Most newly isolated gonococci are of colony type 1, which were found by Kellogg et al. (63) to oxidize glucose more slowly than gonococci of colony types 2, 3 and 4. Baron and Saz (9) noted that in CTA medium, even when heavily inoculated, the "weak" or "glucose-negative" N. gonorrhoeae strains still failed to metabolize glucose.

N. meningitidis typically oxidizes glucose and maltose but not lactose or sucrose. Strains have been isolated that fail to utilize maltose (67, 88). These strains, using the CTA method, would be misclassified as N. gonorrhoeae.

Numerous media have been proposed for use as alternatives to CTA. Some use alternative bases such as enriched GC agar base (9, 42, 16), modified CTA (120, 132), modified NYC medium (40), a modified antibiotic-free TM medium (108), modified Mueller-Hinton agar (130), and a liquid fermentation base developed in England (50). Kellogg and Turner (64) and Brown (16) developed a non-growth 4-h carbohydrate utilization test. Reddick (98) and Morse and Bartenstein (89) adapted the Minitek system (Baltimore Biologicals Laboratories registered trademark) for use as a rapid "fermentation" procedure. Yong and Prytula developed another

rapid micro-carbohydrate test (136). Carbohydrate-disk plate techniques have also been used (113, 125).

Part of the difficulty with carbohydrate media has been that gonococci metabolize glucose using a combination of the Entner-Doudoroff and pentose-phosphate pathways during aerobic growth, producing small amounts of acetic and lactic acids (90). The small amounts of acid produced may not cause a definite color change of a pH indicator and may be further metabolized as growth becomes limiting or glucose is depleted as the organism's tricarboxylic acid cycle becomes operational (53).

A 3-h radiometric method, which required a relatively expensive instrument, BACTEC (Johnston Laboratories, Cockneysville, MD registered trademark), was adapted for neisserial confirmation by Cox et al. (31) and tested by Strauss, et al. (118). This method bypasses the acid detection problem. The $^{14}CO_2$ evolved as a metabolic product of labeled carbohydrate utilization was measured. A similar technique, described by Slifkin and Pouchet (111), detected evolved CO_2 as a $BaCO_3$ precipitate formed in a saturated solution of $Ba(OH)_2$.

The use of growth-requiring carbohydrate media for differentiation of the pathogenic neisseria has other inherent drawbacks. The isolate to be speciated must be in pure culture. Substitutes for CTA are not commercially available. The "rapid" carbohydrate methods also require specially made media and very pure carbohydrates. The Minitek and BACTEC methods require a substantial investment in equipment. As both Minitek and BACTEC have other applications, this kind of investment could be feasible for a large clinical laboratory.

A novel biochemical confirmation method was reported by D'Amato et al. (32). This group employed 48 chromogenic substrates to profile N. gonorrhoeae, N. meningitidis, related species of neisseria, Moraxella spp. and Acinetobacter spp. They selected 10 substrates as most differential and reproducible. The final battery of substrates detected production of beta-galactosidase, acid phosphatase, valerate esterase and 7 different aminopeptidases. This method, developed for Analytab Corporation, has not been marketed.

Carifo and Catlin (21) investigated the nutritional requirements of 325 strains of N. gonorrhoeae. They used growth or no growth on a series of 11 chemically defined media as criteria to separate strains of N. gonorrhoeae into auxotypes. This technique has promising epidemiological but probably not taxonomic applications.

Bacterial species with active autolytic enzyme systems make good candidates for transformation. Bawdon et al. (11) identified 69 of 71 strains of N. gonorrhoeae by detection of the ability of the DNA from each gonococcal strain to transform a nutritionally deficient gonococcal mutant, allowing the transformed mutants to grow on a specific defined medium. Unfortunately, this technique used procedures unfamiliar to the average microbiologist.

Several serological methods have been developed, or are in development for the identification of N. gonorrhoeae. The continuing problem with serological identification has been that antisera raised against N. gonorrhoeae whole cell antigens also react with the antigenically closely-related N. meningitidis.

A serological technique, to be sufficiently specific for N. gonorrhoeae confirmation must:

1) present the cellular antigens to the antiserum in a manner such that antigens common to N. gonorrhoeae, N. meningitidis and other Neisseria spp. are either not present or are not available for reaction; or

2) use an antiserum that has been prepared from N. gonorrhoeae cell fragments which contain antigens unique to all N. gonorrhoeae strains, or

3) use an antiserum which has been adsorbed, or otherwise treated, to remove all crooss-reacting antibodies. This antiserum must remain sensitive enough to react strongly with N. gonorrhoeae cells or antigens.

Several techniques have been used to visualize gonococcal antigen-antibody reactions, including direct and indirect fluorescent antibody stains, macroscopic slide agglutination, counter-immunoelectrophoresis, and coagglutination with antibody-coated Staphylococcus aureus.

Deacon et al., (34) of the Center for Disease Control (CDC) announced the application of a direct fluorescent antibody (FA) technique to the detection of N. gonorrhoeae in 1959. Gonococci were detected in urethral exudate which had been stained with a fluorescein-labeled antigonococcal antiserum. Gonococci are unlikely to be as numerous in a female infection as in a male infection. Detection of sparse gonococci is complicated by the fact that phagocytic white blood cells non-specifically react with the fluorescein-conjugated antibody, staining as brightly or more brightly than the gonococci (104). The direct smear FA technique

therefore was inadequate for diagnosis of gonorrhea in women.

The group at CDC (35, 36) modified the direct FA technique for diagnosis of females. A clinical specimen was inoculated onto a chocolate agar slant and incubated in a candle-extinction jar for 16 to 20 h. A smear was prepared from material taken from the slant surface, stained and examined. Multiplication of N. gonorrhoeae on the slant increased the sensitivity of this "delayed-direct FA" technique. N. gonorrhoeae was detected both by culture and delayed-direct FA in 29 of 50 women who were listed as contacts by men diagnosed as having gonorrhea. Only 13 of the 50 were positive by the direct FA method (35). The results of 15 studies comparing the culture and FA techniques for the diagnosis of gonorrhea in women were summarized by Hare in 1974 (52). In general, the delayed-direct FA technique was found more sensitive than either of the other procedures. Use of the delayed-direct FA technique never became widespread. After the development of TM medium, reports of its use disappeared from the literature.

Use of the direct FA technique has continued for diagnosis of extragenital, non-oral gonorrhea, because N. gonorrhoeae is culturable with difficulty from body fluids. The direct FA has successfully been applied to the detection of gonococci in skin lesions, in white blood cells from venous blood and in synovial fluid (113, 65, 10). The specificity of the method is in doubt. Tronca et al., (123) said that the commercial FA conjugate they used was not adsorbed to remove antibodies which did cross-react with N. meningitidis. Disseminated meningococcal disease may be symptomatically similar to disseminated gonococcal disease. The isolation of both gonococci and meningococci from one person with

apparent disseminated neisserial disease has been reported (133).

Peacock et al., (93) in 1968, applied the FA technique to the confirmation of oxidase-positive Gram-negative diplococci grown on TM as N. gonorrhoeae.

FA conjugates marketed prior to 1974 for diagnosis or confirmation of N. gonorrhoeae exhibited troublesome cross-reactivity. Conjugates also brightly stained certain strains of Staphylococcus aureus. In 1974, Abbott Laboratories introduced an FA test kit, GONO-tect (Abbott Laboratories, registered trademark), which featured a gonococcal antibody conjugate with improved specificity. In a clinical trial, the GONO-tect conjugate specifically stained all 90 N. gonorrhoeae strains tested and none of 110 strains of related neisseria. It did not react with 40 strains of non-neisserial bacteria, including S. aureus (83). Unfortunately, this excellent conjugate is not now marketed.

Recently, an FA conjugate was prepared and tested which used hen fluorescein-labeled gonococcal lipopolysaccharide antibody (7). This conjugate, when adsorbed with N. meningitidis, Groups B and C, stained all N. gonorrhoeae strains tested adequately (3+ to 4+) and all strains of N. meningitidis less brilliantly (1+ to 2+). It cross-reacted with certain streptococci. No reports of a clinical trial of this preparation have been published.

A slide agglutination test, which used anti-gonococcal antibody absorbed to cells of S. aureus as a reagent, was reported by Danielsson and Kronvall (33). It has been marketed by Pharmacia Diagnostics as the Phadebact (Pharmacia Diagnostics registered trademark) Gonococcus test. The S. aureus strain used in this coagglutination test has a

large amount of Protein A in its cell walls. Protein A binds the F_c portion of IgG antibody to the staphylococcal cell surface (44). This test and several others recently developed (23, 69) exploit the ability of Protein A to absorb antibody protein. (Protein A is also the substance which causes certain strains of staphylococci to nonspecifically react in FA stains.)

For the performance of the Phadebact coagglutination test, colonies of oxidase-positive Gram-negative diplococci were taken from culture media, smeared on a defined area of a microscope slide and allowed to dry. A drop of gonococcal antibody-coated staphylococci was added to the smear and the slide was rocked while the drop was observed for macroscopic coagglutination of N. gonorrhoeae with the coated S. aureus cells (33).

Conflicting reports concerning the specificity of this test have been published. Menck (84) found all 53 strains of N. gonorrhoeae gave positive coagglutination. About half showed pseudo-coagglutination, or agglutinated with staphylococci with no antibody coating. These strains showed only true coagglutination if the dried bacterial smear was treated with trypsin before addition of the staphylococcal reagent. All 68 non-gonococcal neisseria tested gave negative coagglutination if treated with trypsin, except 4 N. lactamica strains and one B. catarrhalis strain which persistently pseudo-coagglutinated. Barnham and Glynn (8) found 98% of 140 N. gonorrhoeae strains gave clearly positive results, but 75% of 10 N. meningitidis strains were also positive.

A simple antiserum-bacterial cell slide agglutination test would be the ideal diagnostic tool in a clinical laboratory. The major reason, other than lack of specific antisera, that this type of test has not been pursued more avidly is that many newly isolated neisseria are auto-agglutinable. Colonies grown on TM were found to be generally auto-agglutinable (131). Pretreatment of the cells with trypsin (84) helped to solve the problem for the coagglutination test. Other treatments of bacterial cells used have included boiling (1), glycerol (131), use of only very young cultures (2), use of nucleases (2), growth on serum-free media (84) and growth on NYC medium (131). If autoagglutinability was overcome, an antiserum which reacted only with a unique gonococcal antigen would meet the specificity requirement, and could be used in a slide agglutination test.

A group of Canadian researchers, Perry et al. (95), isolated and characterized N. gonorrhoeae lipopolysaccharide (LPS). They found the core LPS, that portion of LPS possessed by all gonococci whether S-type or R-type, was essentially identical for all N. gonorrhoeae strains. The R-type organisms had only the core LPS. Hens, injected with pure R-type LPS produced good antibody titers (131). The LPSs of N. meningitidis and N. lactamica have been found to be immunologically different though biochemically similar to N. gonorrhoeae LPS (94). Hen anti-LPS looked as if it might meet the specificity requirement.

Primary testing of hen anti-LPS antiserum as a slide agglutination reagent was done by Wallace et al. (131). A total of 251 N. gonorrhoeae strains were tested. Primary isolates of 249 were agglutinated by the antiserum and all 251 were agglutinated after subculture. None of 24

N. meningitidis strains reacted. None of the organisms tested were agglutinated in the normal hen serum control. The antiserum was submitted to CDC, where it was examined by Arko et al. (2). They found it necessary to add ribonuclease and deoxyribonuclease to colony suspensions to aid in eliminating false positive reactions by N. meningitidis, N. sicca, B. catarrhalis and Corynebacterium vaginale. They also had technical difficulties with the hen anti-LPS.

One further method of visualizing the gonococcal antigen-antibody reaction has been reported. Kwapinski et al. (71) used a counter-immunoelectrophoresis procedure to identify N. gonorrhoeae antigens.

As may be discerned after reading this summary, there is no confirmatory diagnostic test, now commercially available, which will distinguish N. gonorrhoeae rapidly and reliably from all other Gram-negative diplococci. A laboratory worker may, from experience, "know" that an isolate is a gonococcus, but when confirmatory tests are equivocal, the nagging doubts persist.

A previously untried method for visualizing serological reactions might work. Reversed passive hemagglutination (RPHA) had not been tried. It also seemed reasonable that the physical technique, pyrolysis-gas-liquid chromatography (PGLC), as it had been shown sensitive enough to detect differences between strains of bacteria within species (25, 51, 101), might differentiate between the closely related neisseriae.

Boyden (13) in 1951 reported a passive hemagglutination technique. Treatment of erythrocytes with tannic acid was found to alter the surface properties of the cells, possibly by changing the surface from a hydrophilic to a hydrophobic state. The "tanned" erythrocytes were found

able to adsorb proteins, when mixed under the appropriate conditions of cell and protein concentration, pH and temperature. The protein used had to be available in a relatively pure state and in relatively high concentration per ml. In Boyden's trials, protein sensitized cells were added to a test tube containing antiserum diluted in 1:100 normal rabbit serum in normal saline. If no specific antibody was present, the cells settled in the center of the tube bottom in a small button. If specific antibody was present, the erythrocytes agglutinated and the cell clumps settled, covering the whole bottom of the tube with a thin layer of erythrocytes.

Standardization of the passive hemagglutination procedure was studied by Stavitsky (115, 116). He emphasized that minor impurities in the antigens or antisera used to sensitize the tanned erythrocytes were capable of causing hemagglutination and confusing results.

A serious drawback to the wider use of the procedure was that fresh sensitized red blood cells (RBCs) were relatively unstable. Cole and Farrell (24) found formalin-preserved, benzidine-treated RBCs much more stable and still able to absorb proteins. Butler (20) successfully sensitized formalinized erythrocytes which had been treated with either tannic acid or bis-diazotized benzidine. Cook (28) was able to preserve sensitized formalin-preserved tanned erythrocytes by lyophilization. Cook's method for estimation of tetanus toxins and antitoxins used a reversed passive hemagglutination technique in which antibody protein was used to sensitize the cells rather than antigen protein.

Hirata and Brandriss (55), in 1967, investigated in depth the conditions of pH, antigen concentration and duration of antigen-cell contact

necessary for optimal RBC sensitization. Erythrocytes they used were stabilized by either formaldehyde or pyruvic aldehyde or both. The cells were sensitized with protein with no further chemical treatment. Their work led to the development of Abbott Laboratories' Duracytes, (Abbott Laboratories, registered trademark). Duracytes are erythrocytes stabilized by pyruvic aldehyde followed by formaldehyde (6).

Microhemagglutination techniques which used multiwell plastic plates in place of test tubes and microliter amounts of reagents aided in visualization and interpretation of hemagglutination reactions (46).

Passive hemagglutination or RPHA has been used to detect and/or quantify many proteins, including staphylococcal enterotoxin (109), Treponema pallidum antibody (30), diptheria antibody (45), Clostridium perfringens enterotoxin (124), tetanus toxins (28), Mycobacterium tuberculosis antibody (20), diptheria toxin (60), trench fever antibody (29), antibody to Group A streptococcal polysaccharide (57), Hepatitis B virus antigen (56) and Rubella antibody (4).

Sensitized formalin-stabilized erythrocytes were used to detect anti-gonococcal antibody by Maeland et al. (77). A saline gonococcal extract was the sensitizing agent for the RBCs. Hirschberg and Harper (58) employed a similar saline extract in an unsuccessful attempt to detect gonococcal antibody in persons culturally positive for gonorrhea. Logan et al. (75) tried untreated erythrocytes sensitized with an alkaline extract of type 1 gonococci and tanned erythrocytes sensitized with a sonicate of type 1 gonococci to detect antibody in persons with uncomplicated gonorrhea. The superior, tanned, sonicate-sensitized erythrocytes agglutinated with sera from 88% of infected females and

with sera from 18% of culturally negative females. In order to achieve this specificity level, the sonicate was adsorbed with one strain each of N. sicca and N. flava. Reimann and Lind (99) sensitized formalin-treated tanned sheep RBCs with gonococcal pili. They found their test to be more sensitive and specific than the complement-fixation test then in use in Europe to detect gonococcal antibody in human sera.

Investigators in the Diagnostic Division of Abbott Laboratories were the first to apply the RPHA technique to N. gonorrhoeae confirmation. They possessed a good specific anti-gonococcal antibody preparation. This column-purified antibody to whole N. gonorrhoeae cells had been conjugated with fluorescein and used as the reagent in the GONO-tect FA test. Used as a fluorescein-labeled reagent, it brightly stained 100% of all N. gonorrhoeae strains tested, and did not stain any of the N. lactamica, N. meningitidis or others of a series of neisserial organisms and other bacteria capable of growth on TM (74). As an FA conjugate, this antibody preparation was not successfully marketed. It appeared that if this antibody were used in an immunological confirmation procedure which required no instrumentation, it might be successful.

Abbott Laboratories was commercially producing Duracytes. Rabbit-raised, column-purified anti-gonococcal antibody was used to sensitize human type-O Duracytes and employed in an RPHA procedure to react with lysed N. gonorrhoeae cells (6). Armstrong et al. (6) used a panel of 11 Duracytes, each sensitized with purified anti-gonococcal antibody to a different N. gonorrhoeae strain, to react with alkaline lysates of several N. gonorrhoeae strains. They were able to distinguish between gonococcal strains, based on the pattern of hemagglutination observed.

Each of the 11 antibodies used were absorbed with 3 strains of N. meningitidis. The anti-gonococcal antibody sensitized Duracytes did not cross-react with alkaline-lysates of several non-gonococcal neisseria strains. The results of these primary laboratory tests were promising. It appeared that this RPHA test might be used to distinguish N. gonorrhoeae from other neisserial organisms. This paper reports a primary clinical trial and a field test of the RPHA procedure, designated GONO-cell (Abbott Laboratories, registered trademark).

The second technique explored in this paper was PGLC. With this technique, whole desiccated bacterial cells or cell fragments are pyrolyzed under carefully controlled conditions of time, temperature and atmosphere. During pyrolysis of the macromolecular mixture, which is the cell, chemical bonds are broken and rearranged. The volatile products which result are swept by an inert carrier gas (such as N_2 or He_2) directly onto the column of a gas chromatograph. This bolus of pyrolysis products is separated during passage through the column. Each product component has a different boiling point and is soluble to a different degree in the liquid material coating the gas chromatograph column. The products are quantified by a detector as they leave the column and are graphically recorded as a chart or pyrogram. Products may be identified by mass spectrophometry. Each species or strain of microorganism is chemically unique. This uniqueness is reflected in its pyrogram..

The PGLC technique had not been used by a microbiologist as an aid for the identification of Neisseria spp. Meuzelaar and In't Veld (86), used N. meningitidis and N. sicca strains to test the performance

of their pyrolysis system. Their pyrograms of N. meningitidis, Group A and N. meningitidis, Group X showed only minor differences. Pyrograms of N. sicca, however, showed more differences from both N. meningitidis strains.

Several microbiologists have employed methods other than pyrolysis to extract biochemical mixtures from neisseriae and have used gas chromatography to separate those mixtures into components. All were analyses of products released by neisseriae into culture media during growth.

A group at CDC has published a series of papers. Brooks et al. (15) extracted organic acids from liquid culture media in which neisseriae had been grown. Distinctive chromatograms were prepared from the derivatized extracts for 14 species of neisseriae. Morse et al. (87) used electron capture gas chromatography to quantify acetylmethylcarbinol produced by several Neisseria spp. This research was extended to the identification of neisseriae by more extensive analyses of spent culture media extracts (89). Reproducible metabolic chromatograms were obtained. Non-pathogenic neisserial strains fell into 6 distinct groups. The 23 N. gonorrhoeae strains, 10 N. meningitidis strains and 3 N. lactamica strains each formed a distinguishable group. An attempt (66) was made to use this method to reveal unique metabolic products released by N. gonorrhoeae during growth in human knee fluid.

Jantzen et al. (62) used gas-liquid chromatography to screen monosaccharides produced by the genera Neisseria, Moraxella and Acinetobacter. The extracted carbohydrates were derivatized and segregated chromatographically. Chromatograms produced were used for classification.

PGLC was developed as one of a family of techniques for the study of pyrolytic processes (107). Reiner (100) in 1965 was the first to apply PGLC to the taxonomic differentiation of microorganisms. He collected pyrograms of many strains of Gram-negative rods of the Enterobacteraceae family. He was able to differentiate between genera and even strains within genera by visually comparing pyrograms (101). Reiner also employed PGLC for the speciation of mycobacteria (102).

Cone and Lechowich (25) used PGLC to separate types of Clostridium botulinum. PGLC has been successfully applied in studies of the taxonomic status of Aspergillus spp. (119, 127, 128), dermatophytes (105), Penicillum spp. (70), Sporothrix spp. (121) and algae (112). PGLC analyses have been a tool to aid classification of streptococci (61, 114), Vibrio cholerae (48), mycoplasmas (97), Bacillus spp. (92) and Salmonella spp. (38, 51, 103).

Simmonds (110) used PGLC coupled with mass spectrophotometry to identify pyrolysis products unique to microorganisms which would therefore not be found in sterile soil. The results of this experiment were intended to aid in interpretation of PGLC-mass spectrophotometry data collected by Viking I analysis of Martian soil.

As more investigators began to work with PGLC, several common problems began to emerge. There are many variables in each portion of the procedure. PGLC may be partitioned into: growth of the microorganism; preparation of the microbial sample for pyrolysis; pyrolysis of the sample; injection of the sample onto the gas chromatograph column; chromatographic separation of product components; detection, quantification and recording of components separated; and analysis of graphic

data collected.

Some researchers made careful attempts to control variables in each stage. Several papers have been published which report solutions for particular problems (25, 51, 86, 91, 97, 106). Investigation outlines in other reports do not include quality control procedures. PGLC has been slow to gain popularity because intra-laboratory reproducibility was difficult and inter-laboratory reproducibility did not occur. Use of PGLC has meant that each laboratory must develop its own library of pyrograms of known organisms for comparison with unknown isolates (97). In the next several paragraphs, work toward PGLC standardization is summarized.

The chemical composition of microbial cells to be pyrolyzed is affected most by the composition of the growth medium (25, 51, 92) and by the age of the culture (119). Pigments, for example, may be produced on one medium and not another. Older cultures may lose cellular constituents by autolysis. Contamination of cells to be pyrolyzed by growth medium may obscure bacterial components (51). Various methods have been used to obtain cells free from growth media. These include growth of the culture in a dialysis sac (85), and growth on either cellulose memebranes (106) or membrane filters (91) placed on the surface of solid growth media.

The majority of investigators have taken organisms grown on solid media or in liquid media, washed the cells and dried the samples either by lyophilization or in a desiccator. Weight of samples in some studies was carefully standardized (86, 97). In others, only an approximate sample weight was recorded. Levy (73) stated that sample size affected

the pyrolysis process qualitatively as well as quantitatively. Secondary reactions between radicals and fragments formed when primary bonds were ruptured are affected by quantity of material pyrolyzed.

Levy also stated that the rate of decomposition of material pyrolyzed is dependent on film thickness. This variable was not controlled in early experiments. Meuzelaar and in't Veld (86), who used a sophisticated Curie point pyrolyzer, obtained uniform film thickness by suspending a weighed amount of dried bacteria in a measured amount of carbondisulphide (CS_2). Thin pyrolyzer filaments could be evenly coated with drops of suspended bacterial cells. The CS_2 then evaporated - and due to its chemical nature, would not have been detected by chromatography had any residue not volatilized.

Pyrolysis products obtained are affected by rate of temperature rise, final pyrolysis temperature and residence time of material in the reactor (25). Rate of temperature rise was an uncontrollable variable until after the development of the Curie point pyrolysis method (86). This method involved the high-frequency induction heating of thin ferromagnetic filaments coated evenly with the material to be pyrolyzed. Quinn (97) showed that as the heating rate increased from 60 C to 75 C to 100 C per second that the amounts of lower molecular weight products increased. The amounts of higher molecular weight components tended to become more consistent.

Effects of various pyrolysis temperatures on pyrolysis products have been investigated. Hanson (51) obtained better resolution of pyrolysis products of two Escherichia coli strains after pyrolysis at 350 C than after pyrolysis at 500 C. Quinn (97) tried final pyro-

lysis temperatures of 400 C to 900 C in 100 C increments. He observed major pyrogram changes between 400 C and 500 C with only minor decreases in higher molecular weight compounds between 500 C and 900 C. He chose a final pyrolysis temperature of 600 C. His major interest was, however, in standardization, not differentiation.

Most reports do not mention an attempt to control residence time of material in the pyrolyzer.

The bolus of pyrolyzed products has in general been swept directly onto the gas chromatograph column by inert carrier gas. This same stream of carrier gas also carries products through the column as they are separated. Pyrolysis products of higher molecular weight--tars--condense on the walls of the pyrolyzer. These tars will, if allowed to remain on the walls, slowly break down and add additional peaks to later run pyrolyses (114). Walls of the pyrolyzer and the path of the products to the gas chromatograph must be heated to avoid excessive condensation and loss of product (51, 97).

The longer the path from pyrolyzer to gas chromatograph column, the more product will be lost by condensation. Sekhon and Carmichael (106) experienced plugging of their heated line from pyrolysis furnace to column due to condensation, and therefore mounted their pyrolyzer directly on the inlet port of the gas chromatograph.

Higher molecular weight compounds which reach the column will move slowly into the column. They build up in the first section of the column necessitating replacement of either the first section of the column (114) or the entire column. Quinn (97) found it necessary to hold the column at its highest operating temperature overnight to

clear the column of those slow-moving contaminants. Quinn periodically replaced the first 15 feet of his 500 foot capillary columns.

Two types of columns have been used for the chromatographic separation of pyrolysis products. Short columns prepared from tubing of 2-4 mm in diameter and 6-20 feet in length were used by all but 3 investigators. These short columns were packed with various treated diatomaceous earths coated with a polyethylene glycol. The polyethylene glycol most often used was 20M carbowax (Applied Science Laboratories, Inc.). The 20M carbowax served as the liquid stationary phase which acted to separate the volatilized compounds. The second type of column used was the capillary column (61, 86, 97, 110). These columns were coiled from tubing 0.1 - 0.5 mm in diameter and were 150-500 feet in length. The polyethylene glycol was coated directly onto the interior surface of the tubing. Polyethylene glycols were used as the stationary phase as this non-polar compound has been shown to efficiently separate the nitriles, aldehydes, ketones, furans, pyrolles, alkenes and alkanes which have been shown to be the products of pyrolysis of microorganisms (110).

For the reported separations, the temperature of the chromatograph oven during separation of pyrolysis components was programmed from an initial temperature of 35 C to 70 C, to increase at a rate of 5 C to 12 C per minute to a final temperature of 145 C to 240 C. The initial and final temperatures were dictated by the properties of the pyrolysis components and of the polyethylene glycol used. As was reported by Hanson (51), the temperature program is generally determined by trial and error.

Quinn (97) stated that the freezing point of 20M carbowax was 63-65 C, and that the material tended to behave anomalously around this point, leading to reversal of component elution. Quinn was the only investigator to show this concern. Several other chromatographic problems, with solutions, are summarized by Quinn (97).

Initial and final temperatures, rate of temperature rise, column length and diameter are all variables in the resolution of components. Experimenters who used columns that were shorter and of greater diameter were able to resolve 16-60 peaks (101, 103). The high efficiency capillary columns were able to resolve up to 200 pyrolysis components (97). The high efficiency columns may not be necessary to achieve the degree of separation needed to produce distinguishable pyrograms.

The packing and coating of columns is an art, and like all arts tends to result in a unique product. Sekhon and Carmichael (106) lamented that their new columns did not produce pyrograms comparable to those produced by their old columns, although they were identically prepared. They felt uncontrolled variation introduced by different columns to be the biggest drawback to the use of PGLC for taxonomic studies.

All investigators used flame ionization detectors and recorded the detector signals graphically.

Most pyrograms were interpreted by visual inspection. Retention times (distance of an individual peak from a common reference peak at or close to the origin), the presence or absence of specific peaks and the ratio of peak heights to each other were used in pyrogram interpretation. Various calculations were made from the measured values

to obtain distinctive differentiations of organisms.

Vincent and Kulik (127) designated a peak close to the origin, present on all pyrograms, as a reference peak. They measured the retention times of each peak and considered two peaks, on different pyrograms similar if their retention times were within 1% of each other. They then used a formula to arrive at similarity values for comparison of unknown pyrograms to reference pyrograms of known origin, or to compare two reference pyrograms to aid in determination of taxonomic placements.

$$\%S = N_s / N_s + N_d \times 100$$ where

S = similarity value

N_s = number of similar peaks present in any pyrogram pair

N_d = number of peaks present in the first pyrogram but not the second plus the number of peaks present in the second but not the first.

Emswiler and Kotula (118) modified the formula to include comparison of peak areas also. They considered peaks similar if peak areas were within 25% of each other and peak retention times were within 2.5% of each other.

Menger et al. (85) compiled a library of known pyrograms composed of retention times and peak heights for six Salmonella serotype groups. A computer program was written which directed the computer to read peak heights and retention times of an unknown organism pyrogram, search the library of knowns and assign a serotype identification to the unknown. Nine of 10 unknowns were identified correctly.

Simmonds (110) coupled PGLC to mass spectrophotometry and was thus able to identify the compounds represented by 70 pyrogram peaks

by comparison with library reference spectra.

MacFie et al. (83) used sophisticated statistical methods--cononical variate analysis--to compare pyrograms. They felt this method would improve the usefulness of low resolution PGLC.

Statement of Problem

The objective of this research was to test the two methods, PGLC and RPHA, for differentiating N. gonorrhoeae from other Gram-negative diplococci. These methods, as tested, involved simple direct procedures which could be performed on bacterial colonies taken directly from primary isolation media. Results could be obtained rapidly since neither method required subcultures or isolate purification. The RPHA microtiter test could be set up and read within 4 h after colonies were first observed. The PGLC procedure would be able to deliver results almost as quickly.

Because of the failure of one instrument, differentiation of neisseria isolates by PGLC was attempted using two different pyrolysis furnace - gas-liquid chromatograph combination. The instruments, pyrolysis temperatures and chromatography conditions differed enough that the two sets of results obtained were different radically and were not comparable. In both instances, an attempt was made to obtain reproducible pyrograms from a single strain of N. gonorrhoeae; then pyrograms were produced for other N. gonorrhoeae strains and non-gonococcal neisserial species. The pyrograms were examined visually for major differences in peak height and area. Peak height ratios were calculated from the ratios of the heights of all peaks of a pyrogram to the height of a chosen reference peak. Retention times

(distance of individual peaks from the reference peak) were calculated.

The RPHA procedure, hereafter designated GONO-cell (Abbott Laboratories registered trademark) was developed at Abbott Laboratories by the Microbiology Laboratories in the Diagnostic Division. The work reported in this study was a primary clinical trial of the procedure. All necessary reagents were supplied by Abbott Laboratories in the anticipated marketable form. All organisms tested were received by the Infectious Disease Center (IDC), an independent clinical laboratory located in Missoula, Montana. Isolates tested were collected over a 10-month period.

The GONO-cell procedure employed stabilized human erythrocytes (DURACYTES, Abbott Laboratories, registered trademark) which had been coated with purified N. gonorrhoeae antibody (rabbit). These sensitized cells, when mixed with a lysed bacterial suspension, will form a complex with N. gonorrhoeae antigens present. The procedure may be performed with microliter amounts of antibody-coated DURACYTES and prepared bacterial antigen. Complexes formed will settle in a disperse pattern on the bottom of a V-shaped microtiter plate well. If no N. gonorrhoeae antigen is present, uncomplexed antibody-sensitized DURACYTE cells will roll down the well sides to settle in a sharp button at the bottom of the V-well.

All isolates tested with the GONO-cell or PGLC procedures were classified taxonomically by standard laboratory methods. Neisserial isolates, for example, were identified by Gram-stain reaction, cytochrome-oxidase production, carbohydrate fermentation, ability to reduce nitrate and to grow on unsupplemented media, and degree of observed fluorescence after staining with fluorescein-conjugated anti-gonococcal antibody.

CHAPTER II

MATERIALS AND METHODS - GONO-CELL TRIAL

Media

The plated medium used to isolate potentially pathogenic neisseria and to maintain stock strains of neisseria was prepared as described by Thayer and Martin (122). Briefly, GC agar base (BBL) was rehydrated and autoclaved. Before it was poured into sterile plates, the medium-base was supplemented with 1% bovine hemoglobin (BBL), 1% IsoVitaleX enrichment (BBL), and VCN (BBL) antibiotic inhibitor. This medium will be referred to as TM medium.

Several modifications of TM medium were used. For the PGLC phase of this project, it was felt that a simpler growth medium was less likely to add confusing peaks to the pyrolysis effluent elution patterns. Therefore, during most of this study, GC agar base with only 1% IsoVitaleX was used. This medium will be designated GC agar. Pyrolysis elution patterns were also obtained from colonies grown on Rabbit-TM medium (R-TM), which was GC agar base with 5% defibrinated sterile rabbit blood substituted for the 1% bovine hemoglobin in TM medium.

Most of the GONO-cell tests were performed with colonies taken from TM medium. However, during the initial stages of the study two modifications of TM were used. One lot of commercially prepared, modified TM medium was purchased as prepoured plates from BBL. This modi-

fied TM medium contained 1.15% glucose rather than 1% and 2% agar rather than 1.5% agar (80). Also, the first few clinical isolates were grown on R-TM.

For purification of Neisseria isolates that were to be tested for fermentative properties, GC-C or TM without VCN antibiotic inhibitor was used as the growth medium.

Non-pathogenic neisserial strains and non-neisserial organisms used in this study were isolated either on TM, or if they were incapable of growth on TM, were isolated on sheep blood agar plates (SBA), plates prepared using Tripticase Soy Agar (TSA) (BBL), enriched with 5% sterile defibrinated sheep blood. The organisms unable to grow on TM were subcultured to GC-C plates before GONO-cell tests were performed.

The following media and reagents were among those used for identification of isolates.

1. Plain TSA plates were used to determine whether neisserial isolates were able to grow at room temperature on an unsupplemented media.
2. Indol-nitrate agar (BBL) deep tubes were used to detect nitrate reduction.
3. Taxo-N discs (BBL), which are filter paper discs impregnated with paraamino-dimethylanaline were used to detect cytochrome oxidase production.
4. CPPA agar, which is a modification of CTA prepared from supplemented proteose-peptone, purchased from Prepared Media Laboratory of Tualatin, Oregon was used to determine the ability of the neisserial isolates to ferment carbohydrates.

CPPA agar tubes were used with 1% glucose, lactose, sucrose or maltose added. On occassion, CTA agar (BBL) with 1% fructose prepared at IDC was employed.

5. ONPG (BBL) discs, which are filter paper discs impregnated with orthonitrophil-beta-D-galactopyanoside, were used to detect the production of beta-galactosidase by neisseriae other than N. gonorrhoeae.
6. Amies Transport Medium with activated charcoal (Difco Laboratories, Detroit, MI) was the transport medium in which genital specimens were received at IDC. It was prepared in 13 x 100 mm screw-top test tubes.
7. Various media, most prepared from dehydrated ingredients purchased from BBL or Difco at IDC, were used in the identification of non-neisserial organisms. Media and methods used were those widely employed in clinical microbiology laboratories.

Except as noted above, all media used were rehydrated, sterilized and poured at IDC. With few exceptions, all media were used within a week after preparation.

Stock Cultures

Two reference cultures obtained from the Microbiology Laboratory Bureau, Division of Health and Environmental Sciences, Helena, Montana were used as daily controls for the duration of the study. These cultures were a CO_2-dependent strain of N. gonorrhoeae, 7040-1972 and a N. meningitidis strain, serotype Slaterus X, CDC-660. These cultures were maintained by daily transfer on TM. Incubation of these cultures,

and of all other neisserial organisms isolated, was at 35 C in an incubator with an internal atmosphere maintained at 6-10% CO_2 (monitored twice daily).

The group of non-neisserial organisms included a series taken from the stock culture collection of IDC. The identity of the majority of these had been verified by laboratories other than IDC, and all had been streaked for purity and had been checked biochemically during the 6 months prior to use in this study.

Sources of Clinical Isolates

Clinical isolates were divided into two groups. These were neisserial or non-neisserial (or contaminant) organisms.

A neisserial (or neisseria-like) organism, for this study, was defined as an oxidase-positive, Gram negative diplococcus. Non-neisserial organisms were all other bacterial aerobes and yeasts.

All non-neisserial organisms that were not taken from stock cultures were isolated at IDC from clinical specimens submitted to the laboratory. They were isolated from specimens of either genital or throat sites inasmuch as these types of specimens could be cultured for N. gonorrhoeae.

Non-neisserial organisms may cause problems in N. gonorrhoeae confirmation tests. They could be confusing contaminants on growth media, or media that were used for biochemical tests. Furthermore, these bacteria could cross-react in immunological assays. The oxidase-positive organisms, expecially those able to grow on TM, may be misidentified by an inexperienced technician as presumptive N. gonorrhoeae. These

organisms were divided into four groups:

1. cytochrome-oxidase positive, TM growth positive;
2. cytochrome-oxidase positive, TM growth negative;
3. cytochrome-oxidase negative, TM growth positive;
4. cytochrome-oxidase negative, TM growth negative.

Colonies of organisms of group "1" are often mistakenly selected as possible N. gonorrhoeae colonies, since the average technologist performs an oxidase test from a primary isolation plate before Gram-staining; also, bacterial morphology from the Gram-stain may be misread. Colonies of organisms from groups "1" and "3" most often create problems by contaminating media used for metabolic tests.

Neisserial isolates were obtained from three laboratory sources. Differences in techniques of specimen collection, transport, inoculation, and cultivation were reflections of the varying clientele of the three laboratories. These differences add a certain breadth to this study. Pertinant methods used by each laboratory are discussed separately below.

Infectious Disease Center. Clinical specimens were sent to this laboratory from two small hospitals, a large out-patient clinic, the Student Health Service of the University of Montana, an abortion clinic, and various private physician's offices in the Missoula area. It is a centralized facility in which patients are not seen directly. Specimens cultured for N. gonorrhoeae were received in Amies Transport Medium within 6 h after collection. Soon after they were delivered, the swab-specimens were inoculated onto TM, and incubated at 35 C in the CO_2 incubator. Plates were examined for the presence of oxidase-positive

Gram-negative diplococci daily for 3 days, after about 16, 40 and 64 h of incubation. From specimens treated in this manner, colonies of neisseriae generally did not grow to visible size until after 30 to 40 h of incubation. Growth was recorded as "48 hours old" when initial colonies were observed on these primary cultures, but the colonies had not begun to autolyze as colonies on a true 48 h old Neisseria secondary culture would have done. Probably, residence of the neisseriae in Amies Transport Medium exaggerated the lag phase of growth. Anatomical sites cultured for N. gonorrhoeae included urethra, urine sediment, cervix, vagina, rectum and oropharynx.

In accordance with the request of the patient's physician, specimens taken from the listed anatomical sites were cultured for N. gonorrhoeae only or were inoculated onto other media for the isolation of other potential pathogenic bacteria and fungi. A series of throat and sputum specimens primarily inoculated onto TM and SBA was used as a source of strains of Neisseria species other than N. gonorrhoeae. The neisseriae isolated included both strains which were able to grow on TM and strains which could not grow on TM.

Missoula City-County Health Department. This facility, among its other responsibilities, conducts venereal disease clinics. All patients in the screening program are cultured for N. gonorrhoeae. All women attending Planned Parenthood Clinics in Missoula also are cultured in the same laboratory. Specimens are collected on swabs, inoculated directly onto TM (prepared by IDC) and are incubated at 35 C in candle jars. These specimens that are inoculated directly often grow N. gonorrhoeae colonies within 24 h. Specimens were taken from

urethral, cervical, rectal and oropharyngeal sites. Urine sediment was cultured from males that had scanty amounts of urethral discharge.

Microbiology Laboratory Bureau. This laboratory is a part of the Department of Health and Environmental Sciences, State of Montana and located in Helena, Montana. The state laboratory supplies JEMBEC plates, (a special plastic petri dish, rectangular in shape, which has a hole built in it to receive a sodium bicarbonate pellet; after the plate is sealed in a plastic carrier envelope the pellet will generate and maintain its own enriched with CO_2 atmosphere) which are poured with modified TM in that laboratory, to cities and towns across Montana. After they are inoculated, the JEMBEC plates are mailed to Helena, subcultured to TM, incubated and observed for growth of N. gonorrhoeae. All strains were cloned and were identified to species, by carbohydrate fermentation tests, before being supplied to IDC for this study.

At the state laboratory, colonies, usually from a 24-h-old first or second subculture plates, were picked up by swab, placed in Amies Transport Medium, supplied by IDC, and were mailed to Missoula. Upon receipt at IDC, swabs were inoculated onto TM and were incubated as usual. About three-fourths of the isolates sent from Helena in this manner were viable. These strains, probably because they were secondary rather than primary cultures, usually grew colonies of usable size in 24 h.

Specimen Data Records

A separate data sheet was prepared for each clinical isolate.

GONO-cell test results for the organisms drawn from stock cultures were recorded on charts in the project log book. As each specimen was received from any of the three laboratories, it was assigned a project number, and identifying patient information was recorded. Project numbers 1 through 299 were reserved for neisserial isolates, and project numbers 300 and over were assigned to non-neisserial organisms.

A reference number was recorded for each organism tested. This reference number allowed checking of the original clinical report in case a problem arose. Examples of the referencing system are as given below.

1. 2000-78. An isolate that originated from IDC as routine specimen 2000 in 1978.
2. GC-200-78. An isolate that was taken from IDC gonorrhea screening as specimen 200, received in 1978.
3. HD-A. B. The HD stands for Health Department and the A. B. are the patient's initials. This information combined with the original culture date, referenced a specific report.
4. State-300-78. This isolate was sent from the Microbiology Laboratory Bureau in Helena and was specimen number 300 that laboratory received for gonorrhea culture in 1978.

On the data sheets, the date of the original culture was recorded; this was the date that the specimen was collected from the patient, with the exception of the state laboratory strains, wherein the date was that on which the strain was received at IDC. The age and sex of the patient were recorded, if known, as was the anatomical site of the specimen.

Samples of the data sheets that were used are included on the following pages, as Figure 1 and Figure 2.

As a normal procedure, the primary isolation medium and the date the plate was poured were recorded; all tests performed from that plate were listed. The age of the culture at the time of the tests and of preparation of subcultures were also listed.

The tests were performed on colonies from the primary isolation medium on the day that growth was first observed. On that day, colonies were examined visually, Gram-stained, tested for oxidase production, stained with a direct fluorescent-antibody procedure specific for N. gonorrhoeae (conjugate used was that marketed by Abbott Laboratories in the GONO-tect kit), and tested by the GONO-cell procedure. Isolated colonies also were subcultured to GC-C medium for purifying the culture.

After overnight incubation, growth from the GC-C subculture was again Gram-stained and examined visually for culture purity. Colonies from this plate were then used as inocula for carbohydrate fermentation tests. Other identification procedures, if any, were performed on colonies from this subculture. GONO-cell tests were performed from the subculture rather than the primary isolation plate if primary colonies were more than 48 h old.

Performance of Auxillary Identification Tests

All isolates were Gram-stained by Burke's (26) modification of the procedure. Slides were prepared and stained from primary and subculture plates, and for the neisserial isolates, also Gram-stains were performed on bacteria from the carbohydrate fermentation tubes.

Organism project number _____ Date of confirmatory test _____

Specimen reference number _____ Original culture date _____

Patient data:

Age _____ Specimen site _____
Sex _____ Other information _____

Primary culture data:

Primary isolation medium _____
Lot # of medium or date of preparation _____
Tests done from original plate _____
Total age of culture when tests done _____

Sub-culture data:

Sub #1. Date _____ Medium _____ Date of prep. _____
Tests done _____ Age when tests done _____

Sub #2. Date _____ Medium _____ Date of prep. _____
Tests done _____ Age when tests done _____

Supplemental identification tests:

Gram stain _____ Sugars: glucose ____ sucrose ____
Cytochrome oxidase _____ maltose ____ lactose ____
fructose ____ ONPG ____
GONO-tect FA _____ Other: nitrate __ BHI___ 25 C ____

GONO-cell results:

#1. Medium _____ Date _____ Lysis? _____ Titer plate # _____

	plate loc.	rdr. 1	rdr. 2	rdr. 3
High sens. cell				
Low sens. cell				

#2. Medium _____ Date _____ Lysis? _____ Titer plate # _____

	plate loc.	rdr. 1	rdr. 2	rdr. 3
High sens. cell				
Low sens. cell				

Organism identifcation _____

Comments (GONO-cell) _____

Fig. 1. Example of the data sheet used for GONO-cell evaluation of neisserial organisms.

Organism project number _____ Date of GONO-cell test_____

Specimen reference number _____ Original culture date _____

Patient data:

Age _____ Specimen site _____
Sex _____ Other information _____

Primary culture data:

Primary isolation medium _____
Lot # of medium or date of preparation _____
Tests done from original plate _____
Total age of culture when tests done _____

Sub-culture data:

Sub #1 Date _____ Medium _____ Date of prep. _____
Tests done from _____ Age when tests done _____

Sub #2 Date _____ Medium _____ Date of prep. _____
Tests done from _____ Age when tests done _____

Identification tests:

Gram stain _____ Other:
Cytochrome oxidase _____

GONO-cell results:

Medium _____ Date _____ Lysis? _____ Titer plate#____

	plate loc.	rdr. 1	rdr. 2	rdr. 3
High sens. cell				
Low sens. cell				

Organism identification _____

Comments (GONO-cell) _____

Any repeat testing results _____

Fig. 2. Example of the data sheet used for the GONO-cell evaluation of contaminant or non-neisserial organisms.

Morphology and staining reactions were recorded.

Oxidase tests were performed touching BBL Taxo-N discs directly on the colonies to be tested.

Carbohydrate utilization tests were carried out, as stated, from the GC-C subculture plates at 24 h except in the case of the state laboratory strains. Those strains were most often in pure culture, so carbohydrate utilization studies were set up from the original IDC TM subculture.

Carbohydrate tubes were inoculated very heavily, with a loopful of growth scraped from the agar surface. Tubes were incubated at 35 C in an ambient air incubator. Any color change indicating a more acid pH than the uninoculated medium was recorded as positive. Tubes were examined for acidity after 6 h and 24 h growth. If all tubes were negative after 24 h incubation a second set of carbohydrate tubes were inoculated from a fresh sub-culture plate. This repeated procedure was necessary relatively often, as many strains of N. gonorrhoeae do not produce detectable acidity from glucose until subcultured repeatedly. Since the tubes were inoculated individually, each represented a separate culture, and it was necessary to verify, by Gram stain, that each tube contained only Gram-negative diplococci.

For neisserial isolates other than N. gonorrhoeae, and for all isolates from other than a genital source, ONPG tests were set up. These were done by placing an ONPG disc in 0.2 ml of sterile saline in a sterile 13 x 100 mm test tube, inoculating heavily with isolated colonies and incubating at 35 C in an ambient air incubator. These tests were often positive after 2 to 4 h; however, negative tubes were

held for 24 h before being discarded.

Neisserial isolates taken from respiratory sources, which would not grow on TM, were tested for ability to reduce nitrate, to grow at 25 C on tripticase-soy agar and to ferment fructose in CTA. Colony type and pigmentation were also recorded. Neisseria and Moraxella species were classified according to the scheme on Table 1 (17, 72). Each of the neisserial organisms was tested with reagents supplied by Abbott Laboratories. This direct fluorescent antibody test, GONO-tect (FA), employed purified anti-gonococcal fluorescein-labeled antibody. The procedure outlined by Abbott Laboratories in the GONO-tect (FA) package insert was followed. Briefly, sufficient colonies from the primary TM isolation plate, or in the case of the neisseriae which could not grow on TM, from the GC-C agar subculture, were transferred to 0.5 ml of buffer (Abbott Laboratories, organism suspension medium - OSM) to give a suspension visually equal to about a McFarland ½ turbidity standard. A slide was prepared with 5 µl of the suspension dropped into a circle inscribed on a microscope slide. Positive and negative control slides were prepared similarly with each set of unknown isolates; control suspensions were supplied by Abbott, and colonies were taken from 24 h cultures of the stock N. gonorrhoeae and N. meningitidis strains. All slides prepared were air-dried, fixed by immersion for 5 minutes in acetone and again air-dried. Fixed slides were placed on moist paper towels and a 20 µl drop of GONO-tect (FA) conjugate was spread over the area of each smear. The slides were covered with an inverted plastic tray and incubated at room temperature for 30 min. The conjugate was rinsed from the slides with distilled

Table 1. Differentiation of Gram-negative diplococci isolated from human sources.

Genus and Species[1]	Cell Shape	TM Growth[2]	Carbohydrate Utilization in CTA Base					Reduction		Nutrient Agar-25 C
			Glucose	Maltose	Sucrose	Lactose ONPG[3]	Fructose	NO_3	NO_2	
Neisseria gonorrhoeae	coccus	+	+	-	-	-	-	-	-	-
Neisseria meningitidis	coccus	+	+	+	-	-	-	-	±	-
Neisseria lactamica[4]	coccus	+	+	+	-	+	-	-	+	±
Neisseria sicca	coccus	-	+	+	+	-	+	-	+	+
Neisseria subflava[4]	coccus	-	+	+	±	-	±	-	+	+
Neisseria mucosa[4]	coccus	-	+	+	+	-	+	+	+	±
Neisseria flavescens[4]	coccus	-	-	-	-	-	-	-	+	+
Branhamella catarrhalis	coccus	±	-	-	-	-	-	+	+	±
Moraxella osloensis	rod	±	-	-	-	-	-	+		
Moraxella non-liquifaciens	rod	±	-	-	-	-	-	+		
Moraxella phenylpyruvica	rod	-	-	-	-	-	-	+		

[1]All organisms listed are cytochrome oxidase-positive

[2]Thayer-Martin agar

[3]Ortho-nitrophenyl-beta-D-galactopyranoside

[4]May have yellow pigment

water, the slides were again air-dried, mounted in glycerol-buffer, and examined using a halogen-bulb equipped fluorescent microscope. A 2+ to 4+ fluorescence by diplococcal organisms was considered a positive test. The positive controls consistently stained 3+ to 4+, and the negative controls stained with no more than ± fluorescence. A positive test identified an isolate as N. gonorrhoeae.

Non-neisserial organisms were identified using accepted laboratory methods. Many of the organisms drawn from the stock cultures at IDC had been either acquired from or had their identity verified by the Montana Microbiology Laboratory Bureau. Others had been procured from the College of American Pathologists proficiency survey specimens.

The GONO-cell Reversed Passive Hemagglutination Procedure

Reagents

The reversed passive hemagglutination microtiter plate test, referred to by its Abbott Laboratory trademark, GONO-cell, for confirmation of neisserial isolates, was performed as outlined in the protocol provided by the Microbiology Laboratories, Abbott Diagnostics Division. A brief description of the procedure follows.

The GONO-cell test was designed to be used only to test oxidase-positive Gram-negative diplococci. However, other organisms were tested in this study; they were surveyed for possible immunological cross-reactions or other test interference.

The reagents used in the GONO-cell test were supplied by Abbott Diagnostics Division as follows.

1. Lyophilized Duracytes (Abbott Laboratories stabilized human

erythrocytes), sensitized with antibody to gonococci, were supplied in 25-test vials. At the beginning of this study, 2 Duracyte-gonococcal antibody preparations were used, a "high sensitivity" sensitized Duracyte preparation (20-20 cell) and a "low sensitivity" cell (15-15 cell) preparation. Later in the study, only the 15-15 cell preparation was used. To each cell preparation, 0.01% thimerosal was added as a preservative. Just prior to daily use, cells were reconstituted with intravenous quality distilled water to give a 0.1% cell suspension.

2. A positive control was provided, which was a N. gonorrhoeae protoplasm, preserved with 0.01% thimerosal. The control was run in two dilutions with each set of GONO-cell tests. One gave a "strong positive" reaction and the other gave a "weak positive" reaction. Also, each time a set of unknown isolates was tested, positive and negative controls were set up with bacterial suspensions obtained from 24 h cultures of the stock strains of N. gonorrhoeae and N. meningitidis. The stock cultures provided a pair of controls that were treated identically to the unknowns. A cell control was also set up with each batch of unknown GONO-cell tests.
3. Specimen dilution buffer was provided by Abbott Diagnostics Division. It was used as a diluent in the microtiter plate portion of the procedure. It was 0.1 M phosphate buffer, with protein and ion stabilizers added, and was preserved with 0.1% sodium azide.

4. Sodium hydroxide, 1 N, in distilled water was used to lyse bacterial suspensions.
5. A turbidity control was used as an aid to standardize bacterial suspensions. It was a suspension of heat-killed Escherichia coli.

Preparation of Bacteria for the GONO-cell Test

Colonies of 24-to 48-h old cultures, grown on either TM or GC-C media, were used. Two to 6 isolated colonies were removed from the agar surface with a sterile nichrome loop, and these were suspended in 1.0 ml of distilled-deionized water in a new, clean disposable 10 x 75 mm glass tube. Suspensions were mixed by flicking the tube with a finger until the suspension was viewed as homogeneous under a fluorescent lamp. Colonies from areas of heavy or confluent growth were unsuitable for testing due to premature autolysis. Also, homogenous suspensions could not be made from these partially autolyzed colony areas.

Some strains of N. gonorrhoeae, especially from primary isolation plates, formed colonies that were rather adherent to the agar, and generally were difficult to disperse into a homogeneous suspension. This problem was noted less often with other species of neisseriae. Adjusting the turbidity was difficult in some cases due to small floating pieces of bacterial colonies. Several attempts were made to solve the dispersion problem.

In the case of isolate number 39, which dispersed poorly, three suspending media were used: (1) distilled/deionized water; (2) organism suspension medium from the Abbott GONO-tect (FA) kit; and (3) 0.85% NaCl

in distilled-deionized water. This isolate, which confirmed as N. gonorrhoeae, dispersed no better in the organism suspension medium or in the saline than in the water.

Several different tools were tried as substitutes for a nichrome loop in making the suspensions. Dacron or cotton swabs soaked up too much of the suspending medium. Plain wooden applicator sticks did not easily pick up neisserial colonies. Calcium alginate, wire-shafted nasopharyngeal swabs functioned quite satisfactorily. These swabs were small enough that they did not soak up a large amount of liquid and did not change the volume of the suspension medium. However, since the swabs were relatively expensive, they were used only when problems were encountered. In all cases, the supending medium, the particular plate, and the date of the test were recorded at the time the suspension was made.

Suspensions were compared visually with the E. coli turbidity control by placing the tubes side by side in a single-row test tube rack and looking through the suspensions at a piece of printed paper. The test suspensions were considered acceptable if the print clarity through the test suspension was observed as equal to the print clarity through the turbidity control. If a suspension was too light, more organisms were added. If a suspension was too heavy, distilled water was added until the turbidity was correct, and then 1 ml of the diluted specimen was transferred to a new 10 x 75 mm tube in order to obtain the correct volume.

The standardized suspensions were then lysed with 20 µl of 1 N NaOH. Each tube was flicked with a finger several times to mix the NaOH thor-

oughly with the bacterial suspension. The tubes were observed while the suspensions lysed. Each lysing tube was compared with the N. gonorrhoeae stock positive control tube, which always lysed to sparkling clarity within 3 to 5 sec. Suspensions which did not clear to distilled water clarity, or did not lyse as rapidly as the known N. gonorrhoeae were noted as potentially nongonococcal. The degree and rapidity of lysis were recorded on the data sheet in the following manner.

(1) Rapid and complete lysis: Rapid, total clearing of the suspension resulted in a rather viscous-appearing, clear solution within 10 sec.

(2) Slow but complete lysis: The suspension lysed to clarity, but complete lysis took up to a minute.

(3) Incomplete lysis: Some lysis of the suspension took place, but the suspension remained opalescent or cloudy.

(4) No lysis: No change in turbidity was observed.

Specimens which did not lyse completely properly should have been discarded as unsuitable for the GONO-cell test. In this series of tests, the GONO-cell test was carried to completion in instances in which incomplete lysis or no lysis occurred. In practice, if complete lysis did not occur, a recheck should have been made to confirm that the isolate consisted of oxidase-positive, Gram-negative diplococci. If so, a new suspension should have been made, standardized and lysed. If lysis occurred, the test would have been completed; if no lysis occurred, the test should have been terminated.

Lysed suspensions were allowed to set 15 min before proceeding with the microtiter portion of the test. No suspensions were held

longer than 30 min, though lysed suspensions could have been held up to 4 h at 2 to 27 C.

Microtiter Plate Procedure

Microtiter "V"-well plates were wiped across the top and bottom with a damp paper towel to remove static charge. Plates were then labeled. Duplicate wells were marked for the "strong-positive" control, the "weak-positive" control, the known N. gonorrhoeae and N. meningitidis specimens, and each of the unknown isolate. Two wells also were reserved as cell controls.

The amount of sensitized Duracyte cells needed for that day's tests was reconstituted with distilled water, mixed by swirling gently and set aside until needed.

Labeled plates were placed on a black countertop to aid in visualizing V-well interiors. With a 25 µl disposable dropper, one drop of specimen dilution buffer was added to each labeled well. Specimen dilution buffer was spread evenly over the bottom of each well by tapping the microtiter plate. The plate was held at an angle of about 45° to the countertop and the lower edge tapped on the countertop. Each plate edge was so tapped. The microtiter plate wells containing buffer were examined for air bubbles that could interfere with the cell settling patterns; this was done by holding the plates under a fluorescent lamp over the black countertop; and any bubbles seen were dislodged with a clean toothpick.

With a micropipette equipped with disposable tips, 5 µl of lysed, unknown suspensions or control suspensions were added to the appropriately

labeled wells. Nothing was added to the 2 cell control wells. The plate was held in the palm of one hand and tapped repeatedly on each edge with a finger to mix thoroughly the buffer with the specimen.

The reconstituted Duracyte cells were remixed gently to assure a homogeneous suspension. Then 25 µl of sensitized Duracytes, dispensed from a 25-µl disposable calibrated dropper, was added to each test and control well. The pipette tip was wiped carefully with lens paper, and the pipette was checked for correct drop formation, before dispensing the cells into the wells. The used portion of the plate was sealed with wide transparent plastic tape.

Plate edges were tapped repeatedly with the index finger of one hand while holding the plate in the other hand, to mix thoroughly all of the reactants. Care was taken not to splash reagents onto the plastic sealing tape.

Plates were incubated on a foam rubber cushion on a non-vibrating shelf for 3 to 24 h.

Reading and Recording of Test Results

GONO-cell test results were read initially at 3 h and after overnight (about 12 to 16 h) incubation. Especially with the 15-15 cell preparation, maximal settling of the cells was obtained after overnight incubation. After only 3 to 4 h, it was difficult to distinguish weak-positive from negative results. Overnight settling also allowed the GONO-cell tests to be set up in late afternoon or early evening and then read early the following morning. Most primary gonococcal isolates were not examined until early afternoon and GONO-cell readings in the

late evening would have been inconvenient.

The microtiter V-plate was removed carefully from its cushion and placed on the rack of microtiter test reading mirror. Care was taken not to jar or agitate the plate. A cover, made from a piece of white typing paper, was placed over the plate and a fluorescent lamp was positioned about an inch above the plate. The negative cell controls were read first and recorded as satisfactory if the cells had settled in a sharp, compact, dark-red button with entire edges in the cone of the V-well. Positive and negative controls were read next. The N. meningitidis negative control was considered satisfactory if it appeared the same as the cell control. The N. gonorrhoeae positive controls had to show a disperse cell settling pattern. Cells in the "weak-positive" control wells settled as an enlarged, lighter-colored button with irregular edges. The cells in the "strong-positive" and N. gonorrhoeae stock control wells settled as a diffuse film over the entire bottom of the V-wells.

Figure 1 is a photograph, provided in the GONO-cell protocol, of negative, "weak-positive" and "strong-positive" GONO-cell results with both the high-sensitivity (20-20) cells and the low-sensitivity (15-15) cells.

Test wells were then read and the results recorded as follows.

1. s+ = "strong-positive". Very strong agglutination of the formed cell-complex resulting in a diffuse film of cells covering over half of the entire bottom surface of the V-well.
2. + = positive. Agglutination of the formed cell-complex resulting in a disperse cell settling pattern, resulting in an enlarged light-red colored button of cells with a somewhat irregular edge

Figure 3. Photograph of positive and negative GONO-cell tests. Test specimens and controls set up in the microtiter plate wells in the photograph below are:

well A-1	positive test
well A-2	cell control, negative
well A-3	unknown isolate, negative
well B-1	cell control, negative
well B-2	weak positive test
well B-3	positive control, strong positive

Low sensitivity cells

(15-15 cells)

on the bottom of the V-well.

3. w+ = "weak-positive". Slight formation and agglutination of cell-complex resulting in a cell button slightly larger and lighter in color than the negative and cell controls.
4. neg = negative. No cell-complex formation. The cell button appeared as does the negative and cell controls. Cells settled in a sharp, compact, deep-red button with an entire periphery.

Most results, except the first few, were read and recorded independently by 2 or 3 persons. This was done since it was felt that a person who read the GONO-cell results and who was aware of the culture source, colonial appearance, FA-test results and any completed biochemical tests, might not be totally objective. The GONO-cell test was designed to stand as the sole confirmatory test for a presumptive N. gonorrhoeae isolate. Therefore, of the people reading each GONO-cell test, only 1 reader each time knew the biochemical species identifications of the unknowns. During the test series, 6 different individuals were used as readers, as they were available.

The RPHA tests became easier to read after the readers' eyes were trained. Problems in distinguishing weak-positive from negative results tended to diminish as the readers gained experience.

In the cases of isolates before number 90, the low-sensitivity (15-15) cell wells were set up just below the high-sensitivity (20-20) cell wells. GONO-cell tests performed with the low-sensitivity cells were not as clear-cut nor as easily read as were tests set up with the high-sensitivity cells. It was felt that some of the 19 "weak-positive" N. gonorrhoeae (15-15) GONO-cell tests may have been read

as weak because of the design of the test pattern. After this subjective difficulty was realized, the pattern of GONO-cell test wells was altered on the microtiter plates. High (20-20) and low (15-15 sensitivity cell wells were separated spatially.

CHAPTER III

RESULTS--GONO-CELL TRIAL

Two separate series of GONO-cell tests were run. The GONO-cell pre-clinical trial was completed between January 1978 and April 1978. The clinical investigation was completed between September 1978 and December 1978. All GONO-cell and FA reagents, for each trial, were from a single lot, and the reagents were shipped by air freight to IDC, in Missoula.

For the pre-clinical trial, all the N. gonorrhoeae strains which IDC had received during the first week of January 1978, plus two stock neisserial cultures and several non-neisserial organisms were tested with the GONO-cell procedure as soon as fresh 24 h sub-cultures were available. These preliminary tests, although they were "procedure shakedown" tests, were included in the tabulated results.

It was at this point that new data sheets, as included in the materials and methods chapter, were devised in order to facilitate record-keeping and allow easy recording of readings by the several readers.

Starting with culture 13, all GONO-cell tests were set up from colonies taken from the primary isolation plates for the neisserial isolates received from IDC and from the Missoula City-County Health Department, or from TM sub-culture plates of cultures received from the Montana State Health Department.

Cultures were treated identically in the two test series.

Results from Contaminated Suspensions

Several times during the course of this study, primary N. gonorrhoeae cultures were encountered that were heavily contaminated. On these plates, there were very few colonies of neisseriae and very many contaminants. No difficulty was encountered in confirming these neisseriae as N. gonorrhoeae with the FA technique. However, several sub-cultures and often several sets of carbohydrate media were inoculated and incubated before a truly pure culture was obtained and its identity could be confirmed by standard procedures. Inasmuch as only the carbohydrate media fermentation results could be used legally at IDC for confirmation of identity, up to a week was sometimes needed before the patient's physician could be told that the isolate was indeed N. gonorrhoeae.

On Table 2 are shown the GONO-cell test results obtained by removing colonies from the original contaminated TM plates. Unavoidably, suspensions made (for lysis) were contaminated. In all cases the neisseriae present were confirmed as N. gonorrhoeae despite the presence of the contaminating organism. All were retested in pure culture with little change in reaction strength. The pure cultures of the contaminating bacteria were always negative in the GONO-cell test.

Lysis Problems

Workers in Abbott Diagnostics Division reported that N. gonorrhoeae strains lysed completely under the conditions specified in the GONO-cell test procedure. Other bacteria may or may not lyse completely, but only those that did lyse to clarity of water could be tested. With

Table 2. Confirmation of Neisseria gonorrhoeae from contaminated primary cultures using the GONO-cell test.

Culture number	Results and observations of primary TM culture plate				Pure culture results
	Contaminant	FA	GONO-cell	Contamination degree	GONO-cell
36	Staphylococcus	4+[a]	positive	light	positive
65	Pseudomonas	3+	positive	light	strong positive
72	Candida	3+	strong positive	light	strong positive
139	Bacillus	4+	strong positive	light	not done
140	Bacillus	4+	positive	light	not done
150	Streptococcus Group D	4+	weak positive	heavy	positive

[a]4+ - Brilliant fluorescence of cell wall of stained organism; crisp cell outline; dark, unstained cell interior.

3+ - Bright fluorescence of cell wall of stained organism; slightly hazy cell outline; dark, unstained cell interior.

2+ - Distinct fluorescence of cell wall of stained organism; hazy cell outline; hazy cell interior.

1+ - Dull fluorescence of stained organism; hazy cell wall; hazy cell interior.

± - Faintly distinguishable organism.

the distilled-deionized water prepared at IDC, it appeared that some TM-positive strains of neisseriae species other than N. gonorrhoeae lysed completely under the specified conditions. However, other non-gonococcal isolates gave incomplete or no lysis. An organism was recorded as having lysed completely if its lysed suspension was comparable in clarity with the N. gonorrhoeae stock culture, which was set up as secondary positive control with each set of GONO-cell tests. On Table 3 are tabulated the degrees of lysis observed for each of the neisseria strains tested in the pre-clinical study, after suspension in IDC water. All strains of N. gonorrhoeae lysed rapidly and completely, usually within 3 to 5 sec. However, 6 N. meningitidis, 7 N. lactamica, 1 N. flavescens, and 1 Moraxella strain were also observed to lyse completely, producing a viscous solution of crystal clarity. All N. meningitidis strains which lysed completely gave negative results in the GONO-cell test. Six of the 7 N. lactamica strains which lysed completely gave weak-positive to positive GONO-cell results. However, 10 N. lactamica strains which did not lyse completely were also GONO-cell positive. The 1 N. flavescens strain that lysed completely gave a weak-positive GONO-cell test as did the 1 N. flavescens strain that lysed incompletely. The Moraxella strains were all GONO-cell negative.

One set of isolates, which had been retained and carried in the laboratory by daily transfers, was tested to see if one factor affecting lysis might be age of the culture. Some isolates in the preceding experiment had been tested after 24 h growth, and some had been tested after 48 h growth. Cultures of neisseriae are prone to autolysis as

Table 3. Neisserial organisms: Degree of lysis by NaOH. Rapid, complete lysis by definition occurred within 10 sec producing a crystal-clear viscous solution. Slow complete lysis occurred within one minute. Incomplete or partial lysis resulted in a slightly clouded to translucent solution.

Organism	Total isolates	Total in lysis group/GONO-cell positives in group			
		Degree of lysis			
		rapid complete	slow complete	incomplete or partial	no lysis
Neisseria gonorrhoeae	142	142/142	0/0	0/0	0/0
Neisseria meningitidis	26	6/0	10/2	10/4	0/0
Neisseria lactamica	27	7/6	6/2	14/8	0/0
Neisseria flavescens	4	1/1	0/0	2/1	1/0
Neisseria subflava	5	0/0	0/0	4/0	1/0
Neisseria sicca	18	0/0	0/0	3/0	15/0
Branhamella catarrhalis	7	0/0	1/1	6/3	0/0
Moraxella osloensis	5	1/0	0/0	3/0	1/0

they age, and autolysis may affect the reactivity of the cells and their antigens. Results of this single experiment are give in Table 4. It was shown that the N. lactamica and N. meningitidis strains lysed more readily as the cultures aged, especially after 72 h. It also appeared that reactivity of these cultures with GONO-cell reagents increased with culture age. This was true for several of the N. lactamica and N. meningitidis strains. Ordinarily, the cultures would have been tested only at 24 h, at which time slow or incomplete lysis of those gonococcal strains would have eliminated them from further testing.

Some concern was raised that different sources of distilled water may affect the lysis of the cultures and the subsequent outcome of the GONO-cell test. Therefore, pyrogen-free distilled water supplied by Abbott Laboratories was compared with IDC's distilled water. A set of subcultured isolates, which included 6 of the same organisms tested in the preceding lysis experiment was set up. Four different lytic conditions were used. All of the cultures were 24 h old. Abbott water used was distilled water processed in the Abbott Laboratories in Chicago, IL. IDC's distilled-deionized water is Missoula, MT well water distilled in a stainless steel still, collected in a glass container, deionized by filtration and stored in a polyethylene jug at room temperature. Water used in this study was also autoclaved in pyrex glass bottles before use.

Four sets of suspensions were set up from each culture. Three were in IDC water and one was in Abbott Water. Two sets were lysed normally with 20 µl of 1 N NaOH; 1 set was lysed with 5 µl of NaOH; and 1 set was not lysed. Otherwise the GONO-cell tests were performed

Table 4. The effect of age of culture on suspension lysis by NaOH and on GONO-cell results. Low sensitivity (15-15) GONO-cell Duracytes used. A single TM plate culture was used at three ages for each isolate.

Organism		Age of culture					
		24 hr		48 hr		72 hr	
#	Name	lysis	GONO-[a] cell	lysis	GONO-[a] cell	lysis	GONO-[a] cell
5	M. osloensis	none	neg	none	neg	none	neg
13	N. lactamica	slow complete	w+	slow complete	w+	rapid complete	+
42	N. meningitidis	slow complete	neg	slow complete	w+	rapid complete	w+
93	M. osloensis	partial	neg	partial	neg	none	neg
98	N. lactamica	slow partial	w+	slow complete	w+	rapid complete	+
99	N. lactamica	slow partial	w+	slow complete	w+	rapid complete	w+
104	N. lactamica	slow partial	w+	slow partial	w+	rapid complete	+
105	N. gonorrhoeae	rapid complete	s+	rapid complete	+	rapid complete	s+
117	N. meningitidis	slow partial	neg	slow partial	w+	rapid complete	w+
108	N. meningitidis	slow complete	neg	slow complete	w+	rapid complete	+
109	N. meningitidis	slow complete	neg	slow complete	w+	rapid complete	+
121	N. meningitidis	partial	neg	partial	+	rapid complete	+

[a] s+ - strong-positive agglutination w+ - weak-positive agglutination
+ - positive agglutination neg - no cell-complex formation

as usual. The results are given in Table 5.

Four of the organisms lysed more quickly in the IDC water than in the Abbott water when 20 µl of 1 N NaOH were added. GONO-cell reactions were stronger for 5 of the 11 organisms lysed in the IDC water than the Abbott water. Four of the 5 were the same organisms which had lysed more completely in the IDC water. When 5 µl of the 1 N NaOH were used as the lytic agent, only 2 N. gonorrhoeae and 2 of the N. lactamica strains still gave positive GONO-cell results. Interestingly, in the set of suspensions which were unlysed, 1 N. gonorrhoeae and 1 N. lactamica strain still gave positive GONO-cell reactions.

With this set of isolates, when the lytic step was performed in injection quality distilled water from Abbott Laboratory, lack of rapid and complete lysis would have eliminated the non-gonococcal neisseriae from further testing. Nevertheless, other workers in the IDC laboratory felt the ability to distinguish between incomplete and complete lysis was learned and the distinction a fine one.

GONO-cell Duracyte Preparation Stability: Observations

Concurrent tests by scientists in the Microbiology Laboratories at Abbott had indicated that vials of GONO-cell test Duracytes cells retained at Abbott Laboratories, of the same lot as sent to IDC, appeared to be losing reactivity. For example, high sensitivity (20-20) cells were giving the reactivity originally shown by the low sensitivity (15-15) cells.

From similar tests set up at IDC, it appeared that there was very little diminished reactivity of the Duracyte cells. However, it was

Table 5. The effects of various concentrations of NaOH on neisserial suspensions and the GONO-cell test; effects of two types of water on the degree of lysis and the GONO-cell test results. High sensitivity (20-20) GONO-cell Duracytes used.

Organism		IDC distilled/deionized water suspension						Abbott H_2O suspension	
		Lytic agent						Lytic agent	
Culture No.	Name	20µl 1N NaOH		5µl 1N NaOH		no lysis		20µl 1N NaOH	
		lysis?	GONO-cell[a]	lysis?	GONO-cell[a]	lysis?	GONO-cell[a]	lysis?	GONO-cell[a]
5	M. osloensis	none	neg	none	neg	none	neg	none	neg
13	N. lactamica	slow complete	w+	partial	neg	none	neg	partial	neg
42	N. meningitidis	slow complete	w+	partial	neg	none	neg	partial	neg
93	M. osloensis	slow partial	w+	none	neg	none	neg	slow partial	neg
98	N. lactamica	slow complete	s+	slow partial	s+	none	+	slow partial	+
99	N. lactamica	rapid complete	+	slow partial	s+	none	neg	slow partial	+
177	N. gonorrhoeae	rapid complete	s+	rapid complete	s+	none	neg	rapid complete	s+
183	N. sicca	none	w+	none	neg	none	neg	none	neg
184	N. sicca	none	neg	none	neg	none	neg	none	neg
8	N. gonorrhoeae (stock culture)	rapid complete	s+	rapid complete	s+	none	s+	rapid complete	s+
9	N. meningitidis (stock culture)	slow complete	neg	slow complete	neg	none	neg	slow partial	neg

[a] s+ - strong-positive aggultination w+ - weak-positive agglutination
+ - positive agglutination neg - no cell-complex formation

observed there was definite diminished reactivity of the gonococcal protoplasmal-suspensions that were used as w+ and + controls. During these tests, the stock N. gonorrhoeae strain was run as a secondary positive control at IDC each time a set of GONO-cell tests was set up. This stock strain did not appear to have diminished in reactivity. Therefore, it appeared that the positive control antigen was losing activity, not the sensitized-Duracytes.

The remainder of this chapter was compiled by tabulation of the data sheets prepared during the 8 months this study was conducted.

Auxillary Identification Tests

The fluorescein-conjugated antibody to N. gonorrhoeae provided by Abbott Diagnostics performed superbly in the direct fluorescent-antibody confirmation test. All 142 N. gonorrhoeae isolates stained with a 2+ fluorescence or better. In fact, 135 of the 142 stained 3+ or better. Six of the 26 N. meningitidis isolates stained very faintly with a + fluorescence. One of the 27 N. lactamica isolates gave a + fluorescence. All other neisserial isolates did not fluoresce at all.

The carbohydrate fermentations, as anticipated, gave no problems with non-gonococcal neisseria. Problems, which necessitated setting the fermentation tubes up more than once, were noted with 18 of the 142 strains of N gonorrhoeae.

High Sensitivity (20-20) Duracyte Cell Results

The high sensitivity (20-20) Duracyte cells gave clear-cut results in the GONO-cell test with N. gonorrhoeae isolates. These sensitized cells also appeared to show rather high cross-reactivity with several

strains of non-gonococcal neisseriae. N. lactamica strains showed the strongest cross-reactions. The results with these high-sensitivity antibody-coated Duracytes are shown in Table 6.

It was decided on the basis of the above results that the 20-20 cell was too sensitive, and its use was discontinued after isolate #107. After this point, duplicate wells were set up using the low-sensitivity 15-15 Duracyte preparation.

Low Sensitivity (15-15) Duracyte Cell Results

GONO-cell tests using the low sensitivity (15-15) cells did not have the immediate clear-cut readability as did tests performed with the high sensitivity cell. However, RPHA tests became easier to read after a reader's eyes were trained to watch for nuances in the cell buttons.

Table 7 gives the tabulated results for all primary GONO-cell tests set up with the 15-15 Duracyte preparation. Results of repeated tests are given below the chart. The initial tests were tabulated as if the GONO-cell test was the only confirmatory method available in a laboratory. That is to say, the technologist performing the test would probably accept the initial result, especially if it were a clear-cut positive or negative. In practice, all w+ GONO-cell tests would be repeated. In this series, seven of the w+ N. gonorrhoeae tests were not repeated inasmuch as they were duplicates of 20-20 high sensitivity cell tests in which the 20-20 cell result was stronger.

All 142 N. gonorrhoeae strains gave initial positive GONO-cell tests. Several non-gonococcal Neisseria strains were still positive with the

Table 6. Test results with GONO-cell high sensitivity (20-20) Duracyte reagent.

Organism	Total isolates	GONO-cell test results			
		strong+	positive	weak+	negative
N. gonorrhoeae	71	60	8	3	0
N. meningitidis	12	0	0	5	7
N. lactamica	12	2	3	5	2
N. flavescens	1	0	1	0	0
N. subflava	3	0	0	0	3
B. catarrhalis	2	0	0	1	1
M. osloensis	3	0	0	1	2
Total tested	104				

Results of repeated tests:

N. gonorrhoeae: Of the 3 weak+ : 2 retested strong+
1 retested +
13 of the strong+ or + reacting isolates were retested with no result changes. The remaining strains were not retested.

N. meningitidis: Of the 5 weak+ : 1 retested negative
Of the 7 negative : 1 retested weak+
Remaining isolates were not retested

N. lactamica: Of the 2 strong+ : 2 retested +
Of the 3 + : 1 retested strong+
2 were not retested
Of the 5 weak+ : 1 retested weak+
2 retested strong+
1 was not retested
2 retested negative
Of the 2 negative: 2 retested negative

Table 7. Test results with GONO-cell low sensitivity (15-15) Duracyte reagent. The number of isolates which lysed completely, either rapidly or slowly is given in parentheses after the tabulated GONO-cell results.

Organism	Total isolates	GONO-cell test results			
		strong+	positive	weak+	negative
N. gonorrhoeae	142	71 (71)	48 (48)	19 (19)	4 (4)
N. meningitidis	26	0	0	6 (2)	20 (14)
N. lactamica	27	0	6 (3)	10 (5)	11 (3)
N. flavescens	4	0	0	2	2 (1)
N. subflava	5	0	0	0	5
B. catarrhalis	7	0	1	3 (1)	3
M. osloensis	5	0	0	0	5 (1)
N. sicca	18	0	0	0	18
Total isolates	234				

Results of repeated tests:

N. gonorrhoeae: Of the 4 initially negative: 1 retested strong+
1 retested positive
2 retested weak+
(those 2 were contacts)
Of the 19 initially weak+: 8 retested weak+
2 retested strong+
2 retested +
7 were not retested

N. meningitidis: Of 6 weak+: None were retested
Of 20 negative: 6 retested negative

N. lactamica: Of 6 initially positive: 3 retested +
2 retested weak+
1 was not retested
Of 10 initially weak+: 1 retested weak+
1 retested +
8 were not retested

lower sensitivity cell. These cross-reactions will be discussed in more detail later on.

The N. gonorrhoeae GONO-cell results were tabulated according to institution of origin of the culture. The tabulations in Table 8 show differences in strength which may be attributable to culture age and to sub-culturing. All Montana Microbiology Laboratory Bureau isolates were sub-cultures. This set of isolates contained a higher percentage of s+ reactors. Most of the Missoula City-County Health Department cultures were 24-h old and were all primary cultures. This set of cultures shows the lowest percentage of initial s+ reactors and the highest percentage of w+ reactors. The IDC isolates, which were mainly 48-h old primary cultures, fell between the previous two.

Tables 9 and 10 show the effects of culture age and of sub-culturing on GONO-cell results. Degree of lysis for all cultures is given. Generally, GONO-cell tests appeared more reactive after sub-culture. Cultures either 24-h or 48-h old seemed equally suitable for GONO-cell testing.

GONO-cell Test Reproducibility

After isolate #107, all GONO-cell tests were set up in duplicate. Two or three readers independently recorded the results for each test well. There were 133 isolates tested in duplicate, most only once. However, the majority of the tests were read by 2 or 3 individuals, so there were 346 readings made. Of the 346 readings, duplicate wells were read in complete agreement 318 times. There were then, 28 disagreements on readings made of duplicate wells. Overall, readers read dupli-

Table 8. Neisseria gonorrhoeae tested by GONO-cell: Initial results with the low sensitivity Duracytes tabulated by institution.

Institution of origin of isolate	Total	GONO-cell test results							
		strong +		positive		weak +		negative	
		#	%	#	%	#	%	#	%
Infectious Disease Center	38	18	47	13	34	6	16	1	3
Missoula Co. Health Department	32	12	38	6	19	11	34	3	9
Montana Microbiology Laboratory Bureau	72	41	57	29	40	2	3	0	0

Table 9. The influence of culture age and of subculturing on GONO-cell results. Any degree of GONO-cell positivity, weak positive or greater, was recorded as GONO-cell positive.

Organism	Type and age of culture							
No. of isolates/ total in set	Primary 24 hr		Subculture 24 hr		Primary 48 hr		Subculture 48 hr	
No. in set which lysed completely	GONO-cell pos.	GONO-cell neg.	GONO-cell pos.	GONO-cell neg.	GONO-cell pos.	GONO-cell neg.	GONO-cell pos.	GONO-cell neg.
Neisseria gonorrhoeae	$^{18}/_{20}$	$^{2}/_{20}$	$^{75}/_{76}$	$^{1}/_{76}$	$^{44}/_{45}$	$^{1}/_{45}$	$^{6}/_{6}$	$^{0}/_{6}$
	20		76		45		6	
Neisseria meningitidis	$^{0}/_{7}$	$^{7}/_{7}$	$^{4}/_{10}$	$^{6}/_{10}$	$^{3}/_{7}$	$^{4}/_{7}$	$^{1}/_{4}$	$^{3}/_{4}$
	4		6		5		3	
Neisseria lactamica	$^{5}/_{9}$	$^{4}/_{9}$	$^{17}/_{20}$	$^{3}/_{20}$	$^{1}/_{5}$	$^{4}/_{5}$	$^{1}/_{3}$	$^{2}/_{3}$
	5		6		4		2	

Table 10. The effect of subculturing, regardless of culture age, on GONO-cell results.

Organism	Type of culture							
No. of isolates/ total in set	Primary Culture				Subculture			
No. in set which lysed completely	GONO-cell results[a]				GONO-cell results[a]			
	s+	+	w+	neg.	s+	+	w+	neg.
Neisseria gonorrhoeae	$34/_{63}$	$16/_{63}$	$10/_{63}$	$3/_{63}$	$39/_{82}$	$34/_{82}$	$8/_{82}$	$1/_{82}$
	all 63				all 82			
Neisseria meningitidis	$0/_{14}$	$0/_{14}$	$3/_{14}$	$11/_{14}$	$0/_{14}$	$0/_{14}$	$5/_{14}$	$9/_{14}$
	-	-	1	8	-	-	1	8
Neisseria lactamica	$0/_{14}$	$3/_{14}$	$3/_{14}$	$8/_{14}$	$0/_{23}$	$8/_{23}$	$10/_{23}$	$5/_{23}$
	-	3	3	4	-	2	5	1

[a] s+ - strong-positive agglutination
+ - positive agglutination
w+ - weak-positive agglutination
neg - no cell-complex formation

cate wells the same 92% of the time. Most disagreements in readings were either between w+ and + or between w+ and negative.

Subjectivity: Reader Variations

Most of the GONO-cell tests after isolate #19 were read by more than 1 reader. In the compilation below, each well read was considered as a separate test. No distinction was made between low and high sensitivity Duracyte preparation tests. Wells read by 2 readers and those read by 3 readers were tabulated separately. Again, for readings to be considered in agreement, the strength of the positive reactions had to be read identically (s+, + or w+). Therefore, finer distinction was made among the reactions than a simple positive or negative. Results are shown in Table 11.

Twenty-seven readings were disagreements between w+ and negative. The remainder of the 98 disagreements were in degree of positivity. However, reader disagreement became less frequent as reader experience increased.

Epidemiological Data for the Neisserial Isolates

Table 12 lists the institutional source of the neisserial isolates. All non-pathogenic neisseria, other than 1 N. lactamica isolate, were taken from organisms isolated at IDC.

Table 13 summarizes the epidemiological data collected for all of the neisseria and moraxella isolates. Also listed on the table is information on whether or not the isolate could grow on TM; TM-growth-negative neisseriae which cross-reacted in the GONO-cell test would be unlikely sources of difficulty in the GONO-cell test. Only 2 of

Table 11. Reader variability in recording GONO-cell reactions. Each well of a microtiter plate was read as a separate test. All readings were made and recorded independently. No distinction was made between low and high sensitivity Duracyte preparation tests.

Reader variation: Tests for which 3 readers were employed

Total readings: 317

Agreement of 3 readers: 235

Agreement of only 2 readers: 81

Disagreement by all 3 readers: 1

Percent agreement of all 3 readers: 74%

Percent agreement of 2 of 3 readers: 99.6%

Readers variation: Tests for which 2 readers were employed

Total readings: 133

Agreement between readings: 117

Disagreement between readings: 16

Percent of agreement: 88%

Table 12. The institutional sources of the neisserial isolates.

Organism	Total isolates	Institutional origin of isolates		
		Infectious Disease Center	Missoula Health Dept.	Montana State Laboratory
N. gonorrhoeae	142	38	32	72
N. meningitidis	26	19	5	2
N. lactamica	27	26	1	0
N. flavescens	4	4	0	0
N. subflava	5	5	0	0
N. sicca	18	18	0	0
B. catarrhalis	7	7	0	0
M. osloensis	5	5	0	0

Table 13. Anatomical source and age of patient from whom isolated for the neisserial isolates. Ability of isolates to grow on TM agar.

Organism	Total isolates	Anatomical source			Age of patient			TM growth	
		genital	respiratory	other	adult	child	not known	yes	no
N. gonorrhoeae	142	131	3	8	101	0	41	142	0
N. meningitidis	26	2	20	4	17	7	0	26	0
N. lactamica	27	0	27	0	5	22	0	27	0
N. flavescens	4	0	4	0	2	2	0	4	0
N. subflava	5	2	3	0	3	2	0	0	5
N. sicca	18	0	18	0	10	8	0	0	18
B. catarrhalis	7	0	7	0	1	6	0	4	3
M. osloensis	5	1	4	0	4	1	0	5	0
Total isolates	234							208	26

the 26 TM-growth-negative isolates were found to be even weakly-positive in the GONO-cell test.

Of the 142 N. gonorrhoeae isolates, 101 were recorded as having been isolated from adults. For the 41 patients for whom age data was not available, it may be assumed with high probability that these materials were adult. (These data were subsequently tabulated with "adults"; they were considered to be 14 years of age or older).

Three of the N. gonorrhoeae isolates were cultured at the Missoula Health Department from respiratory specimens. No respiratory isolates were reported from IDC or the Montana State Laboratory; in this study, one cross-reacting neisseria was isolated from a genital source. This 1 non-gonococcal neisseria and the 2 N. gonorrhoeae respiratory isolates represent the cross-reaction problem in this tested population.

An age breakdown of the sources of the Neisseria isolates is interesting. The major cross-reaction problem was seen with N. lactamica isolates. Sixteen of the 27 N. lactamica isolates gave at least a w+ GONO-cell test, and 6 of the GONO-cell tests were +. Fifteen of those 16 isolates originated in children's specimens. A total of 21 of the 28 cross-reacting Neisseria isolates were taken from children's cultures. Roughly half of the respiratory specimens were taken from children. In this particular sample of respiratory specimens then, most of the cross-reactivity was observed in specimens taken from the age group least likely to have gonorrhea.

GONO-cell Results from Tests Performed with "Contaminant" Organisms

The "contaminant" isolates tested were placed in 4 groups by use

Table 14. Population sampled: Infectious Disease Center specimens.

A. Total number of respiratory specimens cultured on TM plates for neisseria and moraxella in the period 2/1/78 to 3/30/78.	692
Number of specimens from which neisserial organisms were isolated.	80
Number of specimens positive for N. gonorrhoeae.	0
Number of non-gonococcal TM-positive neisserial isolates giving false positive GONO-cell tests.	28
B. Approximate number of the 592 respiratory specimens obtained from children.	286
Total number of the 80 TM-positive non-gonococcal neisserial organisms isolated from children.	48
Number of the above 48 isolates which were GONO-cell positive.	21
C. Approximate number of the 592 respiratory specimens obtained from adults.	306
Total number of the 80 TM-positive non-gonococcal neisseria isolated from adults.	32
Number of the above 32 isolates which were GONO-cell positive.	7
D. Number of genital specimens screened for TM-positive neisseria and moraxella from 1/1/78 to 4/15/78.	1746
Number of N. gonorrhoeae recovered.	36
Number of cultures from which non-gonococcal, TM-positive neisserial organisms were isolated.	5
Number of the above 5 isolates which were GONO-cell positive.	1

of the 2 characteristics of ability to grow on TM and ability to produce cytochrome oxidase.

Those organisms able to grow on TM constituted the most important contaminant group. All of these organisms would be eliminated as "gonococcus possibilities" by a competent technologist by the Gram-stain reaction, microscopic observations of bacterial morphology or the oxidase test. Of the group of 53 TM-growth positive isolates tested, only 2 gave false positive GONO-cell results. Both were Group D streptococci. However, neither isolate lysed rapidly or completely.

Organisms of the TM-growth negative group tested were also almost totally non-reactive in the GONO-cell test. Of the 57 isolates tested only 2 gave w+ GONO-cell results. Both were S. aureus. One of the 2 was a "CAMP" staph. On a repeated assay in the GONO-cell test, it was negative. Two of the 14 S. aureus strains tested gave 2+ fluorescence in a direct FA reaction with a conjugated antiserum directed against Streptococcus pyogenes, Group A. Both of these staphylococci could, therefore, have been carrying Protein A antigen in their cell wall and could have reacted non-specifically with the N. gonorrhoeae antibody. Both isolates were GONO-cell negative.

Clinical Investigation Results

A second series of neisserial isolates was tested with a second lot of GONO-cell reagents during the period from September 1968 to December 1968. GONO-cell tests and auxillary identification tests were performed exactly as described for the first series of isolates. The sensitized Duracytes were "low sensitivity" cells similar to the 15-15

Table 15. GONO-cell results for TM-growth positive "contaminant organisms".

Organism	Number of isolates tested			
	GONO-cell negative	GONO-cell positive	lysed by NaOH	cytochrome oxidase pos.
Pseudomonas stutzeri	2	0	0	2
Pseudomonas sp. - colistin resistant	23	0	0	23
Flavobacterium sp.	1	0	0	1
Bacillus sp.	1	0	0	1
Torulopsis sp.	1	0	0	0
Candida albicans	6	0	0	0
Candida tropicalis	1	0	0	0
Candida sp.	1	0	0	0
Lactobacillus sp.	3	0	0	0
Corynebacterium sp.	5	0	0	0
Streptococcus - alpha hemolytic, not Group D	2	0	0	0
Streptococcus - Group D	3	2(weak +)	1[a]	0
Acinetobacter calco-aceticus var. anitratus	1	0	0	0
Pseudomonas maltophilia	2	0	0	0
Staphylococcus epidermidis	7	0	0	0
Proteus spp. (P. mirabilis, P. vulgaris, P. morganii)	4	0	0	0

[a]This Streptococcus lysed slowly and incompletely. It was one of the 2 w+ GONO-cell tests.

Table 16. GONO-cell results for TM-growth negative "contaminant organisms".

Organism	Number of isolates tested			
	GONO-cell negative	GONO-cell positive	lysed by NaOH	cytochrome oxidase pos.
Pseudomonas aeruginosa	1	0	0	1
Aeromonas hydrophila	1	0	0	1
Pasteurella multocida	1	0	0	1
Corynebacterium sp.	2	0	0	0
Listeria monocytogenes	1	0	0	0
Streptococcus pneumonia	1	0	0	0
Streptococcus viridans	1	0	0	0
Streptococcus - beta, one each, Groups A,B,C,D,G	5	0	0	0
Haemophilus influenzae	5	0	5	0
Escherichia coli	7	0	0	0
Enterobacter cloacae	1	0	0	0
Klebsiella pneumoniae	1	0	0	0
Citrobacter freundii	1	0	0	0
Yersinia enterocolitica	1	0	0	0
Arizona hinshawii	1	0	0	0
Serratia marcescens	1	0	0	0
Salmonella enteriditis- Groups A,B,C_1,C_2,D,E)	6	0	0	0
Shigella sp.-(dysenteriae sonnei, boydii, flexneri)	4	0	0	0
Staphylococcus aureus	14	2(weak+)[a]	0	0

[a]Of the 2 GONO-cell positive staphylococci, one repeated negative. Two strains stained 2+ positive with the Abbott GONO-tect FA conjugate. Both were GONO-cell negative.

cells used in the pre-clinical study.

GONO-cell results and lytic results were as recorded on Table 17. All gonorrhoeae strains gave solidly positive GONO-cell results. Again, cross-reactivity was observed for N. meningitidis and N. lactamica strains. Two strains of N. meningitidis gave s+ GONO-cell results. One of these 2, strain #130, lysed completely and was the only non-gonococcal isolate of the two series to give questionably positive FA results (2+). Unfortunately, no antisera were available to serotype this isolate. Eleven of the 18 non-gonococcal neisseria gave positive GONO-cell results and 6 of those isolates lysed completely. Intravenous quality distilled water supplied by Abbott was used for lysis of all suspensions.

A series of GONO-cell reproducibility studies was done, as outlined by Abbott, with a selected set of isolates. Six separate suspensions from colonies on a single 24-h old TM sub-culture plate were made for each organism listed on Table 20. GONO-cell tests done from the multiple suspensions showed only minor variation. On Table 19 are recorded the results of 3 series of 25 GONO-cell tests. One set of tests, for each isolate, were set up from a single suspension taken from a TM subculture plate. Again, reproducibility was very good. Isolate #107, a N. meningitidis strain, was deliberately chosen for testing as it gave w+ results when initially tested. Not surprisingly, isolate #107 results were read w+ for 19 wells and negative for 6 wells. Distinguishing between a w+ and negative hemagglutination tests involves reader subjectivity.

Table 17. Clinical investigation: Results of GONO-cell tests and tabulation of lysis of organism suspensions by NaOH.

Organism	GONO-cell test results[a]				Suspension lysis by NaOH		
	s+	+	w+	neg.	clear	hazy	none
N. gonorrhoeae	73/104	31/104	0/104	0/104	103/104	1/104	0/104
N. meningitidis	2/11	2/11	5/11	2/11	5/11	6/11	0/11
	37,130[b]	95,20	57,68,91 107,115	82,75	20,68,75 107,130	37,57,82 91,95,115	
N. lactamica	0/5	1/5	1/5	3/5	2/5	3/5	0/5
		6[b]	69	60,81,86	69,86	6,60,81	
M. osloensis	0/1	0/1	0/1	1/1	0/1	1/1	0/1
B. catarrhalis	0/1	0/2	0/1	1/1	0/1	0/1	1/1
H. influenzae	0/2	0/2	0/2	2/2	2/2	0/2	0/2

[a]GONO-cell test results:
s+ - strong-positive agglutination
+ - positive agglutination
w+ - weak positive agglutination
neg.- no cell-complex formation

[b]Specimen numbers are written in beneath ratios to allow individual specimen comparisons of GONO-cell and lysis results. Point of culture numbers is to point out non-correspondence of lysis with GONO-cell positivity.

Table 18. GONO-cell test reproducibility. Six separate suspensions were made from colonies grown on a single TM culture plate, lysed and tested for each organism listed on this table.

Culture No.	Organism name	Results of 6 separate GONO-cell tests	Lysis of all 6 suspensions
91	N. meningitidis	5 tests - weak-positive 1 test - could not read	hazy, not complete
95	N. meningitidis	6 tests - positive	slow complete
96	N. gonorrhoeae	5 tests - strong-positive 1 test - positive	rapid, complete
107	N. meningitidis	5 tests - weak-positive 1 test - negative	rapid, complete
105	N. gonorrhoeae	6 tests - strong-positive	rapid, complete
90	P. mirabilis	6 tests - negative	none
87	Pseudomonas sp.	6 tests - negative	none
104	M. osloensis	6 tests - negative	hazy, not complete
112	S. epidermidis	6 tests - negative	none

Table 19. GONO-cell test reproducibility. A series of 25 GONO-cell tests were done from a single suspension for each of the three organisms listed. All three cultures were grown on TM for 24 hr.

Culture No.	Organism name	Results of 25 GONO-cell tests from a single suspension	lysis of all 25 suspensions
105	N. gonorrhoeae	24 tests - strong-positive 1 test - positive	rapid, complete
107	N. meningitidis	19 tests - weak-positive 6 tests - negative	rapid, complete
90	P. mirabilis	25 tests - negative	none

Table 20 reports the results of GONO-cell tests performed on serial subcultures. The 2 stock strains of neisseriae, laboratory cultures which had been transferred hundreds of times, showed minor variations. The N. gonorrhoeae stock strain varied from s+ to + in no pattern. This implied human variation in test performance. The same type of variability was seen with the other N. gonorrhoeae strain and the N. lactamica strain serially subcultured and tested. The 2 N. meningitidis isolates tested became less reactive after serial subculture. Possibly these 2 isolates gradually lost a cross-reacting antigen. All strains were 24 h old TM subcultures when tested. Each day, before the GONO-cell tests were performed, each culture was Gram-stained and tested for cytochrome-oxidase production. All plates were visually pure cultures of oxidase-positive Gram-negative diplococci.

Effects of Growth Medium on GONO-cell Tests

This series of tests was run using GONO-cell reagents supplied for the clinical investigation. On one day, subcultures were made of 10 neisserial isolates which had already been tested with the GONO-cell procedure. Each isolate was subcultured onto the following 5 media.

(1) TM - Thayer-Martin medium;

(2) R-TM - Thayer-Martin medium made by substituting 5% defibrinated rabbit blood for the 2% bovine hemoglobin;

(3) SBA - Sheep blood agar, consisting of TSA enriched with 5% defibrinated sheep blood;

(4) GC-C - Chocolate agar, a TM medium with the antibiotic inhibitor omitted;

Table 20. GONO-cell test reproducibility. Serial subcultures of several isolates were made on TM. GONO-cell tests were done using colonies from each 24 h old subculture of each isolate. Numbers given in parentheses after the GONO-cell test results refer to the number of the serial subculture.

#	Organism name	Results of GONO-cell test[a] done from serial subcultures	Lysis of all suspensions
N/A	N. gonorrhoeae (stock culture)	18 tests: 9 - strong + 9 - + no pattern of results	rapid, complete
N/A	N. meningitidis (stock culture)	18 tests: 17 - negative 1 - could not read	rapid, complete
20	N. meningitidis	10 tests: 4 - + (1,2,3,8)[b] 6 - weak + (4-7,9,10)	rapid, complete
37	N. meningitidis	10 tests: 1 - strong + (1)[b] 3 - + (2,3,4) 6 - weak + (5-10)	hazy, not complete
36	N gonorrhoeae	11 tests: 7 - strong + 4 - + no pattern of results	rapid complete
6	N. lactamica	6 tests: 4 - strong + 2 - + no pattern of results	hazy, incomplete

[a] s+ - strong-positive agglutination
+ - positive agglutination
w+ - weak-positive agglutination
neg - no cell-complex formation.

[b] Numbers given are the serial subculture number. These numbers are provided to show the pattern of the GONO-cell results.

(5) M-H - Mueller-Hinton agar from BBL Mueller-Hinton agar, according to the manufacturer's directions, and lacking enrichments.

All cultures were incubated at 35 C in an atmosphere of 4-10% CO_2 for 24 h. GONO-cell tests were performed from selected colonies. Results are reported on Table 21. The medium on which the neisseriae were grown did not appear to affect the GONO-cell test.

Table 21. The effect of growth of several isolates on various media on the GONO-cell test. All original cultures and original GONO-cell tests from TM. Result code is: s+ - strong-positive agglutination, + - positive agglutination, w+ - weak-positive agglutination.

Culture No.	Organism name	Primary TM GONO-cell result	GONO-cell result from suspensions made from subcultures grown on listed media				
			TM	R-TM	SBA	GC-C	M-H
92	N. gonorrhoeae	s+	+	+	+	+	+
93	N. gonorrhoeae	s+	s+	s+	s+	s+	s+
94	N. gonorrhoeae	s+	+	+	+	+	+
95	N. meningitidis	+	w+	w+	w+	w+	w+
96	N. gonorrhoeae	s+	+	+	+	+	+
97	N. gonorrhoeae	s+	+	+	+	s+	+
98	N. gonorrhoeae	s+	s+	s+	s+	s+	no growth
99	N. gonorrhoeae	s+	+	+	+	+	no growth
100	N. gonorrhoeae	s+	s+	s+	s+	s+	s+
107	N. meningitidis	w+	bubble (no result)	w+	w+	w+	w+

CHAPTER IV

MATERIALS AND METHODS--PYROLYSIS GAS-LIQUID CHROMATOGRAPHY

Media

Media used to isolate, identify and maintain the neisseria strains used in the pyrolysis gas-liquid chromatography (PGLC) portion of this project were described in Chapter II. Briefly, primary isolates were cultured on TM, R-TM or sheep blood agar (SBA). Cultures were maintained and grown for use in the PGLC procedure on either GC agar or R-TM.

The identity of stock cultures was confirmed and the identify of primary isolates was determined as described in Chapter II.

Stock Cultures and Clinical Isolates

Two stock strains of N. gonorrhoeae were maintained by daily transfer on GC agar. These strains were F-62, which was obtained from D. S. Kellogg, Jr., Ph.D., CDC, Atlanta, GA; and Montana Microbiology Laboratory Bureau strain 7040-1972. The F-62 strain was maintained in two colony phases, T-1 and T-3,4. Kellogg's method of colony type selection (63) was used. The second N. gonorrhoeae strain, 7040-1972 was a stock culture of T-3,4 colony type.

Cultures to be pyrolyzed were grown on GC agar or occasionally on R-TM for 18-24 h in a candle-extinction jar. Seven to 10 isolated colonies were carefully removed from the agar surface with a nichrome wire loop. Isolated colonies were used, as neisseria colonies growing in confluent areas are very difficult to remove without disturbing the

agar surface.

Samples were placed in preweighed aluminum-foil pyrolysis boats (Perkin-Elmer 29-410) and dried overnight in a vacuum desiccator over $CaCl_2$. This amount of bacterial paste resulted in samples of about 1.0 mg, as weighed after desiccation. Sample weight was rounded to the nearest 0.001 mg. Desiccated samples were pyrolyzed within 1-3 days after preparation, and were stored continuously in a vacuum desiccator until pyrolyzed.

Pyrolysis and Gas-Liquid Chromatography

Initially, the same equipment assembled by Hanson (51) in his work differentiating enteric bacillis was used for pyrolysis and gas-liquid chromatography.

The power source for the pyrolysis furnace was an F & M Scientific Pyrolyis unit (F & M Scientific Model 80). Varying amounts of current could be delivered to the furnace, which consisted basically of furnace wire wrapped around a Pyrex tube and insulated with asbestos. The distal end of the tube furnace was connected, via a 6-inch piece of teflon tubing, to a syringe needle which was inserted into one injection port septum of the gas chromatograph. The area of the furnace, from the furnace wire to the injection port was wrapped with heating tape. The tape was heated during operation of the system to about 300 C. The proximal end of the furnace tube could be opened to allow introduction of the aluminum boat containing the desiccated bacterial paste to be pyrolyzed.

It shortly became necessary to rebuild the furnace as its electrical

connections had deteriorated. The new furnace was built with the considerable aid of Tom Aanerud, a chemist employed by the Wood Products Laboratory, Chemistry Department, University of Montana.

The furnace as rebuilt, had two major improvements. One was an internal rather than an external thermocouple. This enabled more direct measurement of sample pyrolysis temperature. The second was a sample pan constructed in a manner such that the sample-containing pyrolysis boat could be moved from the heated to the unheated portion of the furnace without venting the system to atmosphere. This allowed better control of the time of pyrolysis. One boat at a time could be placed in the silver foil carrier and pushed into the unheated portion of the furnace. The furnace was then purged with N_2 carrier gas. The carrier pan was moved into the furnace area, which had been preheated to 350 C. After 30-90 sec the charred sample remains were moved back into the unheated furnace section. During pyrolysis, the N_2 carrier gas swept the pyrolysis products onto the column of the gas chromatograph. After 5 minutes, using the valving system devised by Hanson, (51), the N_2 carrier gas was shunted directly onto the column, by-passing the furnace.

The gas chromatograph was a Packard Model 409 fitted with dual columns and flame ionization detectors. The columns used were prepared by Hanson. The column solid support material was Gas-Chrom Q 100-120 mesh (Applied Science Laboratories, Inc.) and the liquid phase was 20M carbowax (Applied Science Laboratories, Inc.). The ratio of carbowax to Gas-Chrom Q was 1:10. The carbowax-coated support material was packed into 8' stainless steel tubing which was 1/8" (.085") in diameter.

The volatile products which resulted from sampled pyrolysis were swept onto the sample column. The second column was used as a reference column. The dual column detectors were operated with their outputs connected in series but with opposite polarities. Operated in this way, the negative signal from the reference column detector would cancel out any identical positive signal from the sample column. Changes in both columns due to temperature changes would thus cancel out.

N_2 carrier gas flow rates were fixed by Hanson and checked by myself so the detectors would give identical responses to 1 μl injections of methanol. The N_2 flow rates were, at 35 C, 28 pounds per square inch gauge (psig) (16 ml/min) for the sample column and 14 psig (5.5 ml/min) for the reference column. Gas flow rates to the detectors were 17 psig (25 ml/min) for hydrogen and 21 psig (250 ml/min) for air.

The oven temperature of the gas chromatograph was programmed in the manner Hanson had found to give the best resolution of the volatile products of pyrolysis. Five minutes after pyrolysis, when the carrier gas was shunted directly onto the column, the oven was closed. The temperature was maintained at 35 C for 5 additional minutes. The oven temperature was then programmed to rise at a rate of 7.5 C/min to 160 C. The oven was then operated isothermally until all pyrolysis products had been eluted from the column.

Injection port temperatures were maintained at 280 C and the detectors were operated at 320 C. The sensitivity of the detector was set at 2.0×10^{-10} amperes. The signal attenuation was varied from 2 to 8. Detector output was recorded by a Honeywell Electronik Recorder, Model 194, operated at a speed of 5 min/chart inch.

Differential thermal analysis and thermal gravimetric analysis determinations were made with desiccated samples of N. gonorrhoeae, strain F-62, again with the aid of Tom Aanerud. The intent of these measurements was to determine whether the thermal decomposition of gonococcal cell material differed markedly from that of the lippopolysaccharide (LPS) pyrolyzed by Hanson (51). The gonococci, being chemically much more complex than the LPS gave less well-defined, but similar results. It was therefore decided to retain the 350 C pyrolysis temperature used by Hanson.

Feasibility Study

An attempt was made to produce characteristic, reproducible pyrograms of N. gonorrhoeae strain F-62 and N. gonorrhoeae strain 7040-1972. strains of several other neisseria species were also grown, sampled and pyrolyzed. Pyrograms were made of 1 mg desiccated slivers of GC agar medium to see what contribution inadvertantly included medium would have made to the pyrograms.

At this point, it was decided to analyze all pyrograms produced to see whether differentiation of neisseriae by PGLC appeared feasible. Pyrograms were not entirely comparable as some were made before and others after the furnace was rebuilt.

No further pyrograms were made for 6 months. When work on the project was resumed, the gas-chromatograph was found to be non-functional. Numerous attempts were made to repair the apparatus. The oven programmer was found to be defective--and as there was no money available to repair it--it was decided to use another apparatus.

Second Series of PGLC Analyses

The second PGLC apparatus used was quite similar to the first. The two pyrolysis furnaces differed in that the path pyrolysis products traveled to reach the gas chromatograph injection ports was 6" for the first apparatus and 30" for the second.

The power source for the pyrolysis furnace was an F & M Scientific Pyrolysis unit and the gas chromatograph was an F & M Scientific-Hewlett-Packard Research chromatograph series 5750 equipped with flame ionization detectors. It was set up to process relatively large samples, and as others were using it, no major adjustments could be made. The 1 mg samples of desiccated bacterial cells were just about the smallest samples that could produce detectable amounts of volatile products.

Initial attempts to use this apparatus employing the same pyrolysis, gas flow and temperature conditions used with the first apparatus resulted in total failure. A flat baseline was the pyrogram--no pyrolysis products reached the detector. Quadrupling of the sample size, to 4 mg, did not result in the detection of volatile pyrolysis products.

After consultation with workers in the Wood Chemistry Laboratory, several conclusions were reached. Pyrolysis at 350 C, under the conditions imposed by the physical arrangement of the second apparatus would always result in an insufficient amount of volatiles reaching the detectors. It was hypothesized that pyrolysis at 350 C resulted in a predominance of higher molecular weight volatiles which condensed on the walls of the 30" teflon tubing which connected the furnace with the injection port of the gas chromatograph. A shift to larger amounts of lower molecular weight products was found by Quinn (97) with an increase in

pyrolysis temperature.

The concern was expressed that a final column temperature of 160 C would result in the deposition of "tars" onto the column. These "tars" would travel very slowly through the column and would bleed off onto the detectors at an unwanted moment. The final column temperature was set at 210 C to avoid this occurrance. The injection port and the detectors were also to be left on, at operating temperature at all times to aid in stabilizing the system.

Table 22 compares the conditions of pyrolysis and gas chromatography used for the two apparatus. Inspection of the differences in the physical conditions shows it is not likely that pyrograms produced could be comparable.

Table 22. A comparison of the pyrolysis and gas-liquid chromatography conditions for the two apparatuses employed.

Operation Conditions

Parameter	first apparatus	second apparatus
Sample size	1 mg	1-2 mg
Pyrolysis temperature	350 C	550 C
Pyrolysis time	30-90 sec	uncontrolled
Path length from furnace to injection port	6 in	30 in
Columns:		
number	2 - sample and ref.	1 - sample only
solid support	Gas Chrom Q: 100-120 mesh	Gas Chrom Q: 80-100 mesh
liquid phase	20 M carbowax	20 M carbowax treated with TPA
Chrom Q: carbowax	10:1	10:1
column length	8 ft - stainless steel	12 ft stainless steel
column diameter	1/8 in	1/4 in
Constant chromatograph temps.		
injection ports	280 C	240 C
detectors	320 C	250 C
Oven programmed temp. rise		
preprogram	5 min at 35 C	5 min at 35 C
isothermal protime	5 min at 35 C	5 min at 50 C
programmed temp. rise	7.5 C/min	6 C/min
final temperature	160 C until all peaks eluted	210 C until all peaks eluted
Gas flow rates		
N_2 sample carrier gas	16 ml/min	40 ml/min
N_2 reference carrier gas	5.5 ml/min	none
H_2 to detectors	25 ml/min	24 ml/min
air to detectors	250 ml/min	38 ml/min
Detector signal amplification sensitivity setting	2.0×10^{-10} amp	1.0×10^{-3} amp
Signal attenuation	2 to 8	1

CHAPTER V

RESULTS--PYROLYSIS GAS-LIQUID CHROMATOGRAPHY

First Apparatus: PGLC Analyses for Two N. gonorrhoeae Strains

The entire PGLC apparatus was checked out, using the methods and operating conditions outlined by Hanson (51). Several needed repairs were made. The columns were reconditioned by holding the temperature of the gas chromatograph oven, in which they were installed, at 200 C for 48 h. Each column was tested using 1 μl amounts of injected methanol, to make certain the detectors were giving identical responses and that the retention time of methanol was nearly the same for each column.

Several desiccated samples of the 2 N. gonorrhoeae strains, F-62 and 7040-1972, were pyrolyzed to see if reproducible pyrograms could be made. The results were not good--but promising. Eight of this collection of pyrograms were chosen,4 for each of the 2 strains. Each pyrogram must have been traced on a different day; using the following criteria. This would give representative examples of the expected daily variation. Bacterial samples and PGLC conditions must have been as nearly identical as possible. Conditions of pyrolysis and chromatography must have been as similar as possible for all pyrograms selected.

Partially with the intent of evaluating the evident reproducibility problem, retention times and peak height ratios were calculated for the 8 selected pyrograms. One hundred-percent (100%) retention time

was defined as the distance between the point at which the tracing of the inital peak left the baseline and the point at which the final recorder tracing returned to the baseline after the final peak. Distances from the initial point to the center of each peak in each pyrogram were measured. These distances were recorded as a % of the total retention time:

$$\frac{\text{peak x}}{\text{total retention time}} = \text{\% retention time for peak x}$$

Each pyrogram had 25 to 27 individually recognizable peaks. Each peak on the selected 8 pyrograms was assigned a number by visual inspection, retention time comparisons and pattern comparison. Proposed % retention time values were calculated by averaging the calculated % retention times for each of the numbered peaks on each of the 8 pyrograms. It was intended that peaks on pyrograms of other neisseria (or other bacteria) could be compared with the selected N. gonorrhoeae pyrograms using the calculated standard % retention times. A peak could be considered as representing the same pyrolysis product if:

(1) its % retention time was within 3% of the calculated standard % retention time; and/or

(2) the peak visually appeared to be in "similar sequence" even if the retnetion time, due to gas flow changes, was deviated more than 3% from the standard retention time.

Culture growth and pyrolysis details for the 8 pyrograms are given on Table 23. Results of the retention time calculations are given on Table 24. As is painfully obvious, the similar sequence criterion had to

Table 23. Growth, sample preparation and pyrolysis information for the 8 Neisseria gonorrhoeae pyrograms chosen for calculation of average percent retention times and average peak height ratios. All cultures were grown on GC agar for 24 h. Each removed, desiccated colony paste was held 24 to 72 h before pyrolysis.

Run No.	Organism	Sample weight in mg	Pyrolysis		
			Date pyrolyzed	Furnace used	Pyrolysis time in minutes
1	N. gonorrhoeae 7040-1972	1.27	2/12/76	original	uncontrolled
2	N. gonorrhoeae 7040-1972	1.85	2/21/76	original	uncontrolled
3	N. gonorrhoeae 7040-1972	1.0	3/17/76	new	1.5
4	N. gonorrhoeae 7040-1972	.91	4/29/76	new	1.5
5	N. gonorrhoeae F-62	1.08	2/11/76	original	uncontrolled
6	N. gonorrhoeae F-62	1.0	3/19/76	new	1.5
7	N. gonorrhoeae F-62	1.0	3/24/76	new	1.5
8	N. gonorrhoeae F-62	1.0	3/22/76	new	1.5

Table 24. Average percent retention times (%RT) calculated from 8 *N. gonorrhoeae* pyrograms described in Table 23.

Peak No.	*N. gonorrhoeae* 7040-1972 run 1	run 2	run 3	*N. gonorrhoeae* F-62 run 4	run 5	run 6	run 7	run 8	Average % RT	Acceptable range: ± 3% of average %RT
1	20.9	24.8	22.4	19.8	23.6	23.3	24.3	24.6	23.0	20.0-26.0
2	24.4	27.9	25.4	22.7	28.4	27.7	28.0	26.9	26.4	23.4-29.4
3	26.2	29.3	26.3	24.2	30.1	29.3	29.1	28.3	27.8	24.8-30.8
4	29.7	31.3	28.1	27.9	32.5	31.7	31.4	30.8	30.4	27.4-33.4
5	33.6	-	29.9	-	-	33.7	32.8	-	32.5	29.5-35.5
6	34.6	33.8	30.8	30.9	35.9	34.3	33.4	33.3	33.4	30.4-36.4
7	36.9	36.6	33.2	33.9	38.7	37.0	36.1	37.1	36.2	33.4-39.4
8	39.4	38.5	34.7	35.8	40.4	39.3	37.8	40.2	38.2	35.2-43.2
9	40.5	38.9	-	37.6	41.4	-	-	42.1	40.1	36.5-42.5
10	40.9	39.7	36.0	39.4	42.5	40.7	39.4	43.1	40.2	37.2-43.2
11	42.5	41.0	37.5	40.6	44.0	42.0	40.5	45.2	41.7	38.7-44.7
12	43.5	42.2	38.7	44.2	44.9	43.0	41.9	46.4	43.1	40.1-46.1
13	45.2	43.8	40.1	46.1	46.2	44.7	43.9	48.3	44.8	41.8-47.8
14	45.7	-	40.5	48.8	46.9	45.3	44.9	48.9	45.9	42.9-48.9
15	46.8	44.5	41.4	51.5	48.6	46.0	46.9	50.5	47.0	44.0-50.0
15a	47.8	45.9	42.3	50.7	-	-	-	-	47.2	44.2-50.2
16	49.8	48.4	45.3	54.5	51.0	50.0	48.3	53.1	50.1	47.1-53.1
17	53.2	51.2	48.6	57.3	54.7	53.0	50.3	56.0	53.0	50.0-56.0
17a	55.8	53.8	50.8	59.7	57.0	54.7	-	58.6	55.8	52.8-58.8
18	58.1	56.9	53.7	62.4	59.9	58.0	53.7	61.1	58.0	55.0-61.0
19	61.1	59.0	54.1	64.8	63.0	61.7	60.1	64.5	61.0	58.0-64.0
20	63.5	61.4	56.5	67.3	66.1	-	-	66.9	63.6	60.6-66.6
21	67.1	64.8	59.5	73.9	70.5	-	69.6	-	67.6	64.6-70.6
22	74.1	72.0	71.0	77.3	75.7	74.3	73.0	76.3	74.2	71.2-77.2
23	81.4	79.6	79.8	84.5	83.6	83.0	81.8	84.1	82.1	79.1-85.1
24	91.4	89.3	90.6	91.5	93.8	93.7	92.2	93.5	92.0	89.0-95.0
25	94.4	92.8	94.9	95.1	96.9	97.0	95.6	96.9	95.4	92.4-98.4

be applied at times to compare even the chosen 8 pyrograms. In retrospect, it seems probable that the programmer module of the gas chromatograph was malfunctioning at the time, contributing to the variability of the pyrolysis patterns.

Once peaks were identified on the chosen 8 pyrograms, peak height ratios were calculated using one of the initial peaks, arbitrarily designated peak 1, as a reference peak. The peak height ratios were calculated as:

$$\text{peak height ratio} = \frac{\text{peak x (height in mm)}}{\text{peak 1 (height in mm)}}$$

Comparison of peak area would probably have been preferable, but the results did not warrant the analysis. Table 25 gives the results of peak height ratio calculations for 6 pyrograms, three each of the 2 N. gonorrhoeae "standard" strains. These are 6 of the same 8 pyrograms used for the retention time measurements. As a general observation, peaks 2-14 are higher for F-62 than for 7040-1972. Two of the 6 pyrograms analyzed on Tables 24 and 25 are reproduced as Figures 4 and 5.

Pyrograms of Other Neisseria Isolates

Before an attempt was made to standardize the pyrograms for the two standard strains, it was decided to collect pyrograms for an assortment of neisseriae to see if there would be gross differences in the pyrograms of the different Neisseria spp. Eight pyrograms were selected and compared with the pyrograms of the 2 standard N. gonorrhoeae strains. Retention times were measured, percent retention times were calculated and peak numbers were assigned. Peak height ratios were calculated and comparisons were made between this second set of 8 pyrograms and

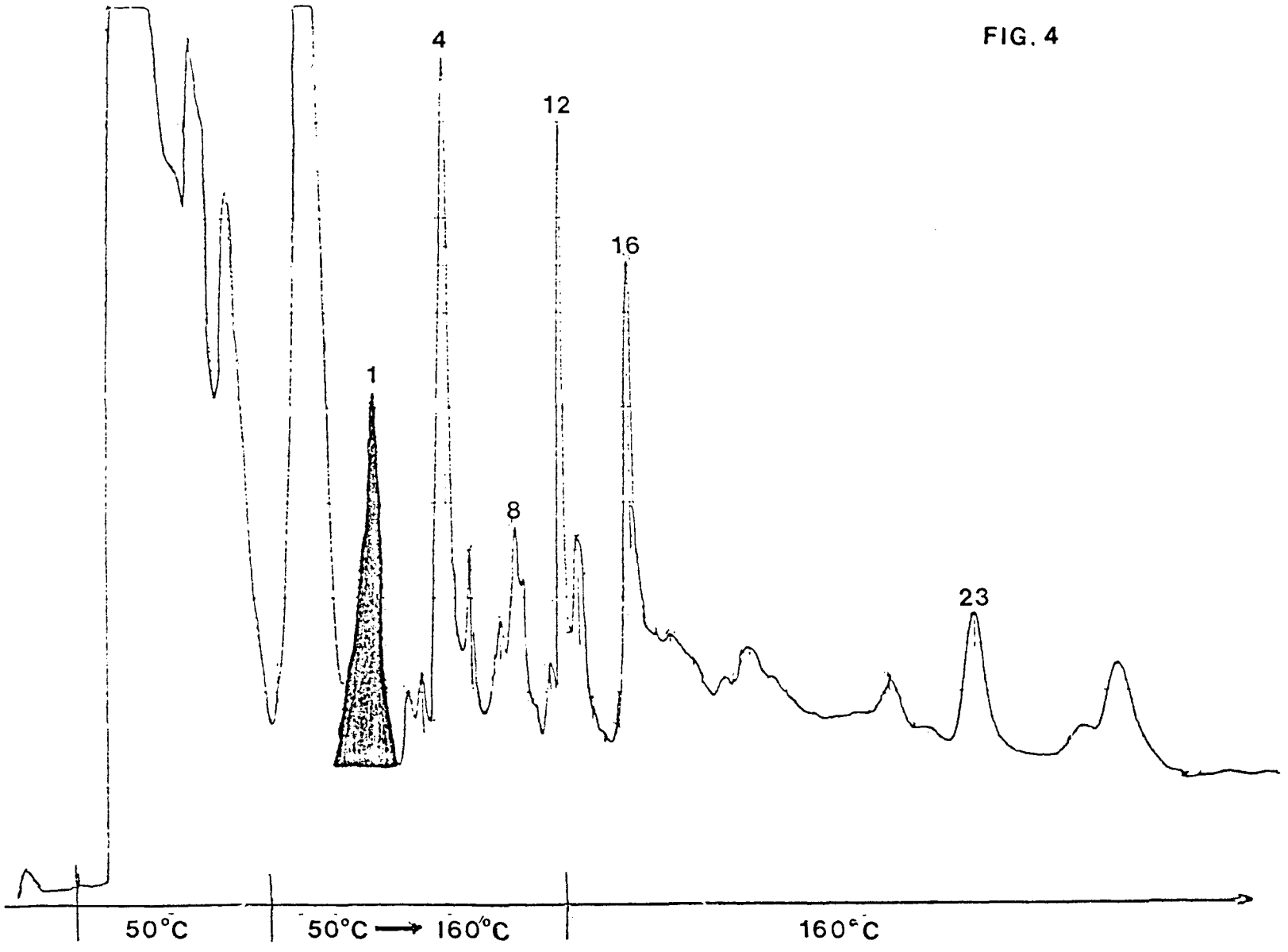
Table 25. Peak height ratios calculated for 6 of the 8 N. gonorrhoeae pyrograms given in Tables 23 and 24. Peak height ratios were calculated as:

$$\text{peak height ratio} = \frac{\text{peak x (height in mm)}}{\text{peak 1 (height in mm)}} .$$

	N. gonorrhoeae 7040-1972					N. gonorrhoeae F-62				
	Peak height ratio			Aver-	Peak ht.	Peak height ratio			Aver-	Peak ht.
Peak No.	run 1	run 2	run 4	age ratio	ratio range	run 5	run 6	run 8	age ratio	ratio range
1	1.00	1.00	1.00	1.00	-	1.00	1.00	1.00	1.00	-
2	.33	.35	.21	.30	.21- .35	.44	.36	.41	.40	.36- .44
3	.29	.39	.22	.30	.22- .39	.35	.38	.46	.40	.35- .46
4	1.03	1.72	1.18	1.31	1.03-1.72	1.58	1.76	1.88	1.74	1.58-1.88
5	.35	-	-	-	-	-	.64	-	-	-
6	.32	.64	.39	.45	.39- .64	.59	.58	.77	.65	.58- .77
7	.35	.49	.31	.38	.31- .49	.49	.48	.66	.54	.48- .66
8	.78	.68	.45	.64	.45- .78	.88	1.08	1.01	.99	.88-1.08
9	.43	.57	.29	.43	.29- .57	.55	-	.76	.66	.55- .76
10	.44	.30	.14	.29	.14- .44	.49	.50	.59	.53	.49- .59
11	.50	.35	.20	.35	.20- .50	.60	.68	.72	.67	.60- .72
12	1.55	1.55	.63	1.24	.63-1.55	1.74	1.85	2.38	1.99	1.74-2.38
13	.70	.65	.23	.53	.23- .70	.73	.66	1.03	.81	.66-1.03
14	.66	-	.43	.55	.43- .66	.75	.68	1.01	.81	.68-1.01
15	.44	.28	.35	.36	.28- .44	.59	.60	.62	.60	.59- .62
15a	.44	.21	.33	.33	.21- .33	-	-	-	-	-
16	1.29	1.24	1.15	1.23	1.15-1.29	1.87	2.15	1.79	1.94	1.79-2.15
17	.29	.42	.35	.35	.29- .35	.37	.34	.39	.37	.34- .39
17a	.24	.36	.23	.28	.23- .36	.36	.26	.28	.30	.26- .36
18	.19	.31	.29	.26	.19- .31	.20	.19	.20	.20	.19- .20
19	.46	.38	.27	.37	.27- .46	.21	.20	.26	.22	.20- .26
20	.18	.31	.21	.23	.18- .31	.14	-	.10	.12	.10- .14
21	.04	.22	.08	.11	.04- .22	.16	-	-	-	.16
22	.19	.29	.32	.27	.19- .32	.15	.16	.20	.17	.15- .20
23	.46	.40	.52	.46	.40- .52	.34	.40	.50	.41	.34- .50
24	.06	.13	.10	.10	.06- .13	.05	.08	.09	.07	.05- .07
25	.33	.26	.39	.33	.26- .39	.21	.17	.30	.23	.17- .30

Figure 4. Pyrogram of N. gonorrhoeae 7040-1972, run 2. This pyrogram was 1 of the 8 chosen to be analyzed. Results of analysis are given on Tables 24 and 25. The shaded area is the reference peak. Several other peaks are numbered to aid peak orientation. Chromatography temperatures are given below the baseline.

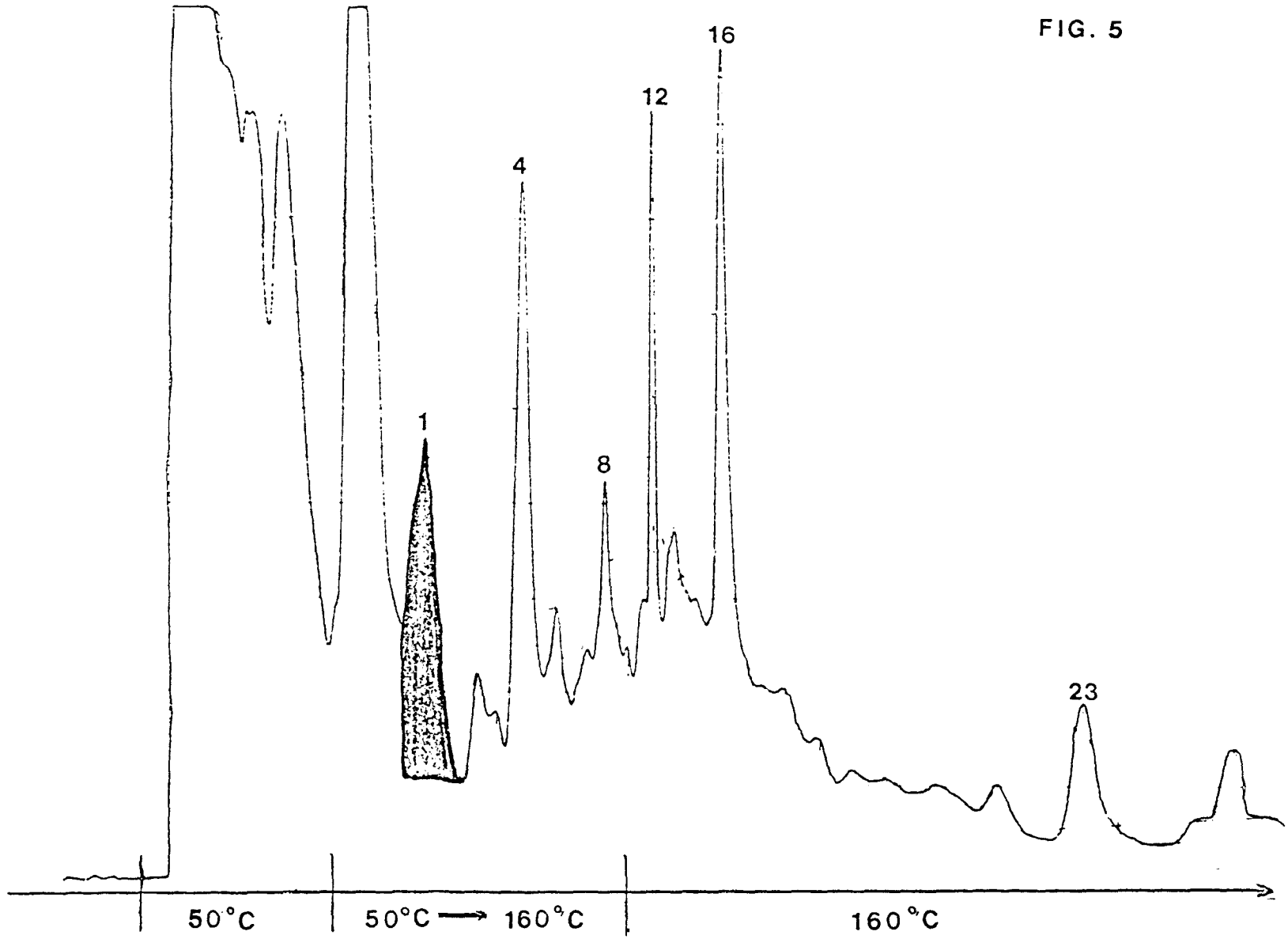
FIG. 4



100

Figure 5. Pyrogram of N. gonorrhoeae F-62, run 5. This pyrogram was 1 of 8 chosen to be analyzed. Results of the analysis are given on Tables 24 and 25. The shaded area is the reference peak. Several other peaks are numbered to aid peak orientation. Chromatography temperatures are given below the baseline.

FIG. 5



the average peak height ratios calculated for N. gonorrhoeae F-62 and 7040-1972. Identifying data for the 8 pyrograms are given on Table 26. Retention time and peak-height ratio calculations are given on Tables 27 and 28. Observed differential calculations are given on Table 29. Figures 6 and 7, which follow the tables, illustrate the pyrograms analyzed. A pyrogram of GC agar is also included as Figure 8. The agar was pyrolyzed to see which peaks would be most affected if agar were inadvertently picked up with the colonies.

Research was discontinued for 6 months. When operation of the gas chromatograph was resumed, the apparatus malfunctioned. It could not be repaired as there were no funds available.

Second Apparatus: PGLC Analyses

As the PGLC procedure showed some promise, a decision was made to try the same procedure using a different pyrolysis furnace--gas-liquid chromatography apparatus. Dr. Shafizadeh generously offered time on an apparatus located in the Wood Chemistry Laboratory at the University of Montana. Operational differences between the two apparatuses have been listed on Table 22 in Chapter IV.

The results obtained after pyrolysis of bacterial cells, using the second apparatus, were disappointing. They were not at all comparable with the first set of pyrograms. Pyrograms of different Neisseria spp. were very similar. Figure 9 illustrates 4 of this group of pyrograms. No attempt was made at analysis, except to number the peaks by visual examination. The only distinctive pyrogram was produced by a strain of Moraxella non-liquifaciens. This strain produced equal amounts

Table 26. Growth, sample preparation and pyrolysis information for pyrograms produced after pyrolysis of several strains of neisseriae. All cultures were grown on GC agar for 24 h. Each removed, desiccated colony paste was held for 24 to 72 h before pyrolysis. A sample of GC agar was pyrolyzed for comparison with neisseriae pyrograms.

Chart no.	Organism	Sample weight in mg	Pyrolysis	
			Date pyrolyzed	Pyrolysis time in min
9	sterile GC agar	1.0	3/24/76	1.5
13	N. subflava 3046	1.0	3/23/76	1.5
14	N. sicca 3045	1.0	3/25/76	1.5
10	N. gonorrhoeae CoH 5	1.0	3/19/76	1.5
11	N. gonorrhoeae 956	1.0	3/18/76	1.5
42	N. sicca 3044	.86	5/9/76	1.5
38	N. lactamica 648	.78	5/7/76	1.5
40	N. lactamica 731	.86	5/7/76	1.5
43	N. lactamica 641	.92	5/20/76	1.5

Table 27. Percent retention times calculated for pyrograms of several Neisseria spp. and of sterile GC agar compared with the acceptable range for corresponding peaks identified on the pyrograms of the 2 N. gonorrhoeae strains listed in Table 23.

Peak No.	Percent retention times (% RT) Chart numbers of organisms pyrolyzed[a] 9	13	14	10	11	42	38	40	43	Acceptable %RT range from Table 24
1	23.0	23.8	25.4	22.2	22.1	21.2	21.0	18.3	20.1	20.0-26.0
2	24.7	27.7	25.6	26.4	25.8	25.0	22.0	25.2	26.4	23.4-29.4
3	26.3	29.4	29.5	27.6	27.4	26.7	23.5	27.0	27.8	24.8-30.8
4	-	30.4	30.8	29.2	29.8	30.4	29.6	26.9	30.3	27.4-33.4
5	-	33.0	32.5	-	31.8	32.0	31.0	-	31.6	29.5-35.5
5a	-	-	33.5	-	-	-	-	-	-	-
6	34.7	36.6	34.9	31.9	32.4	32.6	31.6	29.3	32.6	30.4-36.4
7	-	38.3	36.2	35.2	34.8	35.2	34.2	31.2	34.7	33.4-39.4
7a	-	-	-	-	-	36.2	35.3	33.3	35.7	-
8	37.3	39.9	39.6	37.5	37.1	37.2	36.7	34.6	37.3	35.2-43.2
9	-	40.6	-	-	-	-	37.6	35.5	39.0	36.5-42.5
10	-	42.6	39.0	39.8	38.8	38.8	37.6	40.2		37.2-43.2
11	-	44.6	40.3	40.8	40.1	40.1	42.2	40.0	40.7	38.7-44.7
12	44.3	45.9	42.0	42.2	41.1	41.5	44.8	41.9	42.1	40.1-46.1
13	-	47.9	43.4	43.2	42.5	42.5	48.8	45.9	43.4	41.8-47.8
14	-	49.2	43.7	44.2	43.1	42.8	48.8	45.9	43.4	42.9-48.9
15	-	49.5	45.4	45.2	43.8	44.8	50.9	-	46.1	44.0-50.0
15a	-	50.5	46.8	-	45.2	45.4	52.0	50.5	-	44.2-50.2
15b	-	-	-	-	-	-	52.9	-	-	-
16	50.7	52.1	48.1	47.5	47.5	47.4	54.3	53.5	48.1	47.1-53.1
17	-	54.4	50.9	50.8	51.2	50.6	57.4	-	50.5	50.0-56.0
17a	-	55.4	52.2	54.2	53.2	53.6	57.5	56.3	53.2	52.8-58.8
18	-	58.7	56.6	55.8	55.8	55.9	59.8	58.7	56.2	55.0-61.0
19	61.0	60.3	58.0	57.8	58.2	58.4	62.3	62.1	57.6	58.0-64.0
20	-	62.7	61.0	59.8	61.2	60.8	-	63.0	66.3	60.6-66.6
21	69.7	65.7	67.8	-	-	68.0	-	-	67.0	64.6-70.6
22	-	76.2	72.2	70.4	70.9	71.2	75.0	76.8	71.7	71.2-77.2
23	-	83.2	79.7	78.1	79.6	79.1	84.1	84.1	78.1	79.1-85.1
24	-	85.1	90.1	87.7	90.3	90.5	93.1	93.6	87.2	89.0-95.0
25	-	98.0	92.9	91.0	94.0	92.2	95.4	96.9	92.9	92.4-98.4

[a]Names of organisms identified only by chart number are:

9	sterile GC agar	10	N. gonorrhoeae	38	N. lactamica
13	N. subflava	11	N. gonorrhoeae	40	N. lactamica
14	N. sicca	42	N. sicca	43	N. lactamica

Table 28. Peak height ratios calculated for neisseriae other than the 2 standard N. gonorrhoeae strains. Other information about these other neisseria are in Tables 27 and 28. Peak height ratios were calculated as:

$$\text{peak height ratio} = \frac{\text{peak x (height in mm)}}{\text{peak 1 (height in mm)}}$$

Peak No.	Peak height ratios — Chart numbers of organisms pyrolyzed[a]									Peak ht. ratio range for both stnd.strains
	9	13	14	10	11	42	38	40	43	
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2	.29	.26	.31	.27	.35	.34	.10	.15	.17	.21- .44
3	.30	.35	.34	.35	.39	.34	.12	.14	.19	.22- .46
4	-	1.00+	1.01+	1.49+	1.95+	1.78	.76	1.17	1.43	1.03-1.88
5	-	.41	.54	-	-	.52	.24	-	.41	.35- .64
5a	-	-	.36	-	-	-	.14	-	-	-
6	.31	.24	.25	.60	.55	.50	.23	.29	.36	.39- .77
7	-	.21	.33	.50	.51	.48	.13	.19	.19	.31- .66
7a	-	-	-	-	-	.59	.12	.16	.21	-
8	.08	.28	.74	.80	1.00	1.04	.16	.24	.88	.45-1.08
9	-	.26	-	-	-	-	.17	.23	.38	.29- .76
10	-	.18	.32	.50	.57	.45	.06	.06		.14- .59
11	-	.25	.41	.58	.65	.66	.14	.13	.66	.20- .72
12	.03	1.00	1.01+	1.49+	2.09+	1.79	.23	.43	1.96	.63-2.38
13	-	.54	.47	.80	.97	.80	.20	.16	.80	.23-1.03
14	-	.55	.44	.79	.94	.81	.20	.16	.80	.43-1.01
15	-	.33	.28	.61	.63	.56	.13	-	.57	.28- .62
15a	-	.34	.38	-	.61	.73	.15	.13	.70	.21- .33
15b	-	-	-	-	-	-	.27	-	-	-
16	.04	.68	.69	1.31	2.09+	1.42	.69	1.24	1.34	1.15-2.15
17	-	.23	.22	.27	.33	.39	.16	-	.36	.29- .39
17a	-	.21	.20	.24	.25	.39	.12	.17	.37	.23- .36
18	-	.16	.15	.21	.22	.24	.22	.06	.22	.19- .31
19	.07	.15	.24	.27	.35	.39	.12	.13	.56	.27- .46
20	-	.26	.16	.21	.30	.26	-	.06	.44	.10- .31
21	.34	.17	.07	-	-	.23	-	-	.30	.04- .22
22	-	.17	.16	.19	.23	.27	.11	.14	.21	.15- .32
23	-	.46	.47	.33	.59	.77	.18	.27	.47	.34- .52
24	-	.10	.11	.12	.15	.16	.03	.18	.12	.05- .13
25	-	.24	.24	.20	.35	.54	.10	.33	.39	.17- .39

[a]Names of organisms identified only by chart number are:

9	sterile GC agar	10	N. gonorrhoeae	38	N. lactamica
13	N. subflava	11	N. gonorrhoeae	40	N. lactamica
14	N. sicca	42	N. sicca	43	N. lactamica

Table 29. Differential observations made after comparison of the peak-height ratios on Table 28. A plus (+) is entered if the ratio was greater than for any of the standard N. gonorrhoeae pyrograms, a minus (-) is entered if the ratio was lesser. For the 2 N. gonorrhoeae pyrograms IR (in range) or not IR (not in range) is entered.

	Identification of Organism pyrolyzed						
Peak	N. gonorrhoeae		N. perflava	N. sicca		N. lactamica	
no.	10	11	13	14	42	43	38
1[a]							
2	IR	IR				-	-
3	IR	IR				-	-
4	IR	IR					-
5	no peak	no peak	+	+	+	+	-
5a				peak			peak
6	IR	IR	-	-		-	-
7	IR	IR	-			-	-
7a					peak	peak	peak
8	IR	IR	-				-
9	no peak	no peak	+	-	-	+	-
10	IR	IR				+	-
11	IR	IR					
12	IR	IR					
13	IR	IR				13 and 14 are	
14	IR	IR				equal ht.	
15	IR	IR					
15a	no peak	not IR	-	-	-	-	-
16	IR	IR	-	-			-
17	IR	IR	-	-			-
17a	IR	IR					-
18	IR	IR					
19	IR	IR	-	-			
20	IR	IR					no peak
21	not IR	not IR	+	+	+	+	no peak
22	IR	IR					
23	IR	IR					
24	IR	IR					
25	IR	IR		+	-		

[a]Reference peak

Figure 6. Pyrogram of N. sicca, clinical isolate 3045. This pyrogram was 1 of 8 pyrograms of neisseriae compared with the pyrograms of the 2 standard N. gonorrhoeae strains. The reference peak is shaded in. Chromatography temperatures are given below the baseline. The analysis of this pyrogram was given on Tables 27, 28 and 29.

FIG. 6

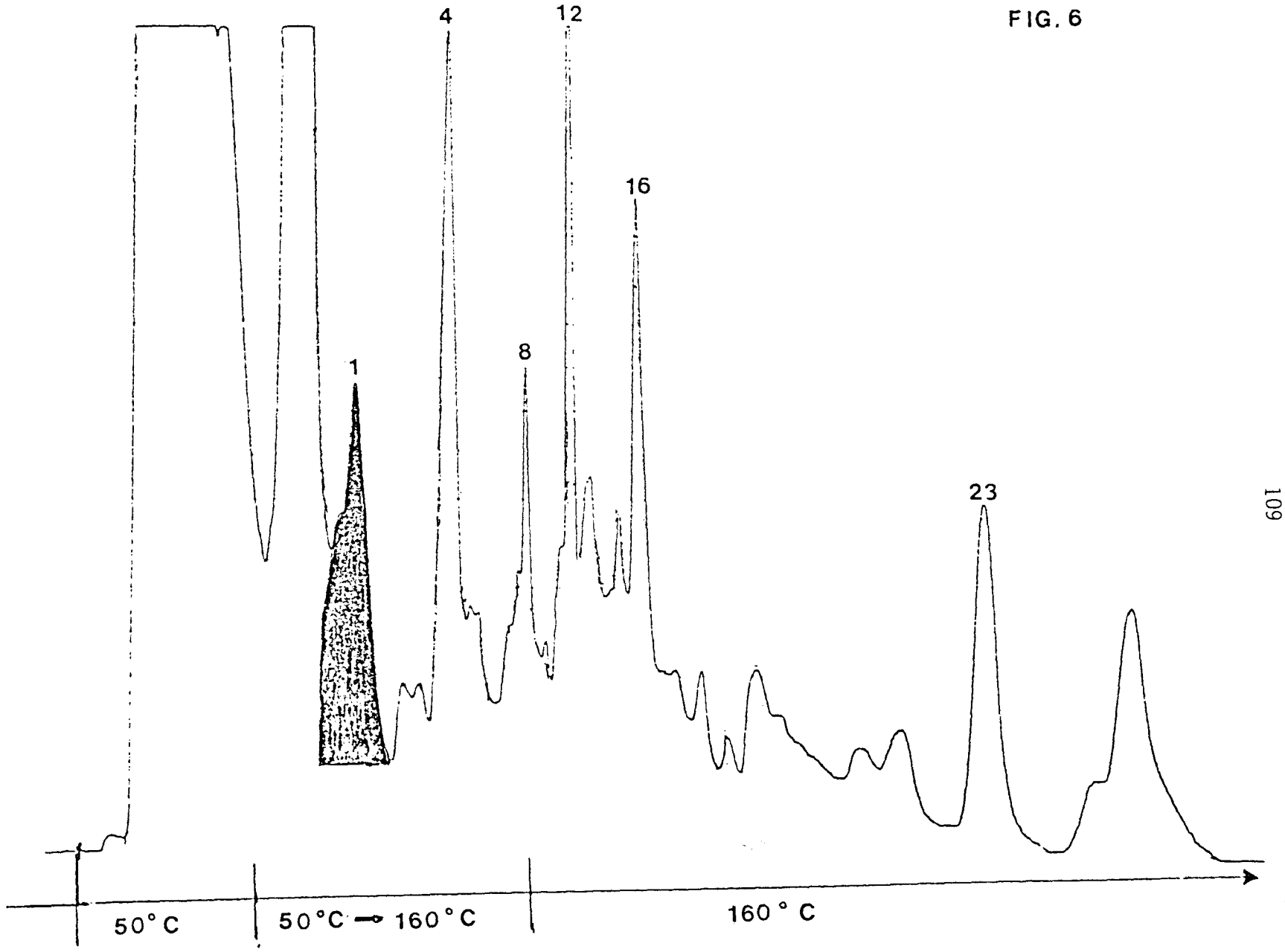


Figure 7. Pyrogram of N. lactamica, clinical isolate 648. This pyrogram was 1 of 8 pyrograms of neisseriae compared with the pyrograms of the 2 standard N. gonorrhoeae strains. The reference peak is shaded. Chromatography temperatures are given below the baseline. The analysis of this pyrogram was given on Tables 27, 28 and 29.

FIG. 7

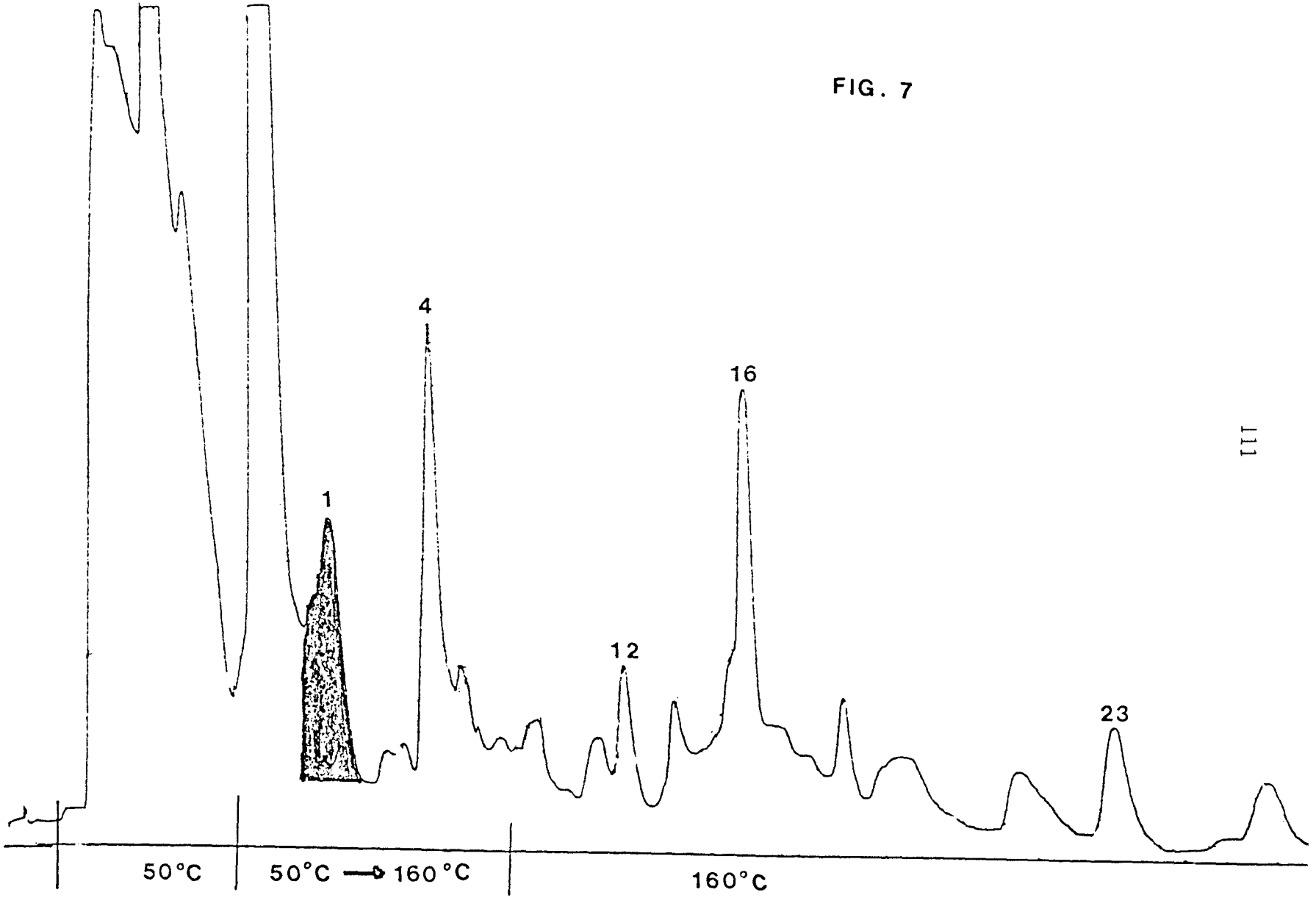


Figure 8. Pyrogram of Sterile GC Agar. This pyrogram is included as it shows the apparently minimal effect that a small amount of agar picked up with the colonies of bacteria might have had with this series of pyrograms. The reference peak is shaded and several other small peaks are numbered. Chromatography temperatures are given below the baseline.

FIG. 8

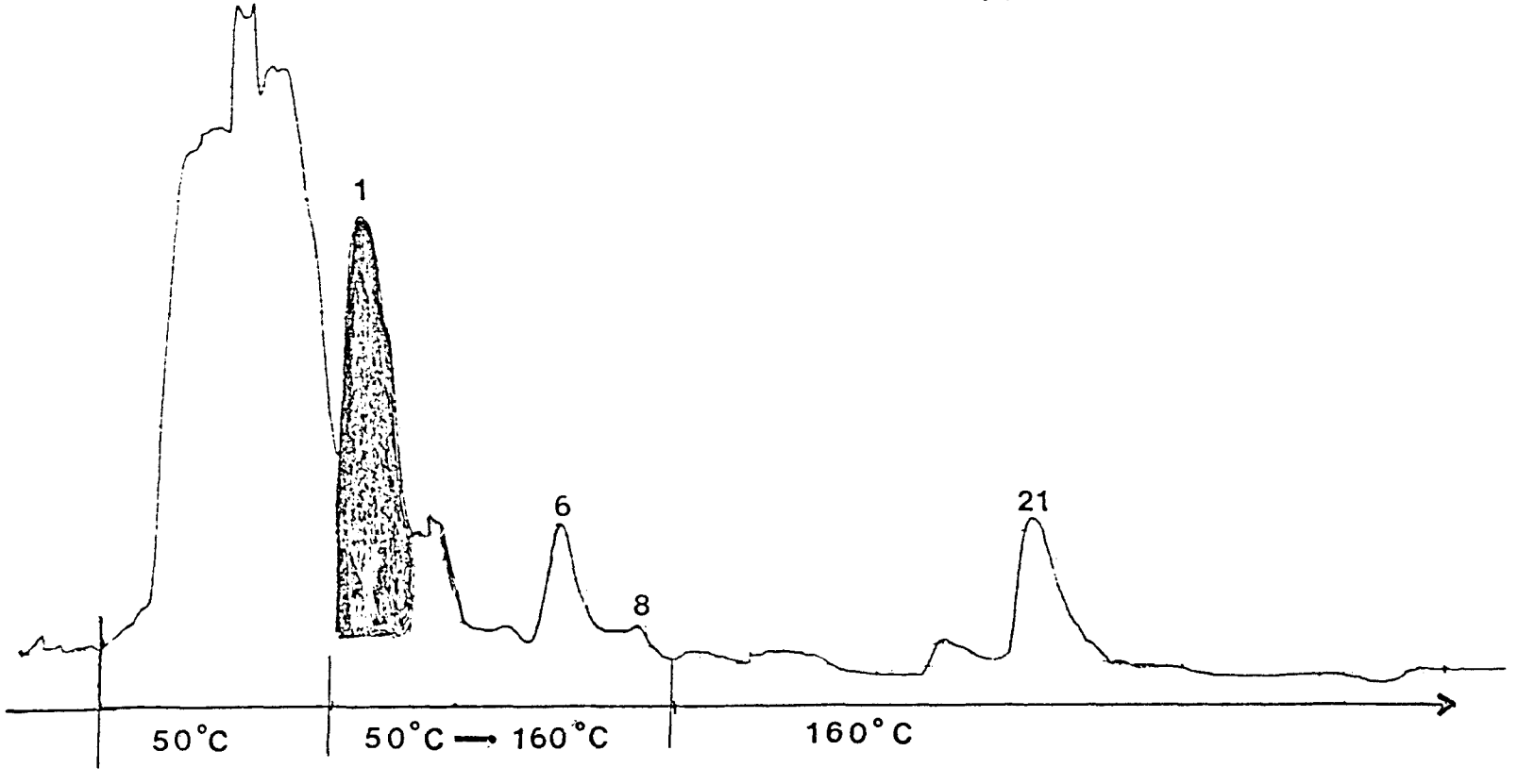
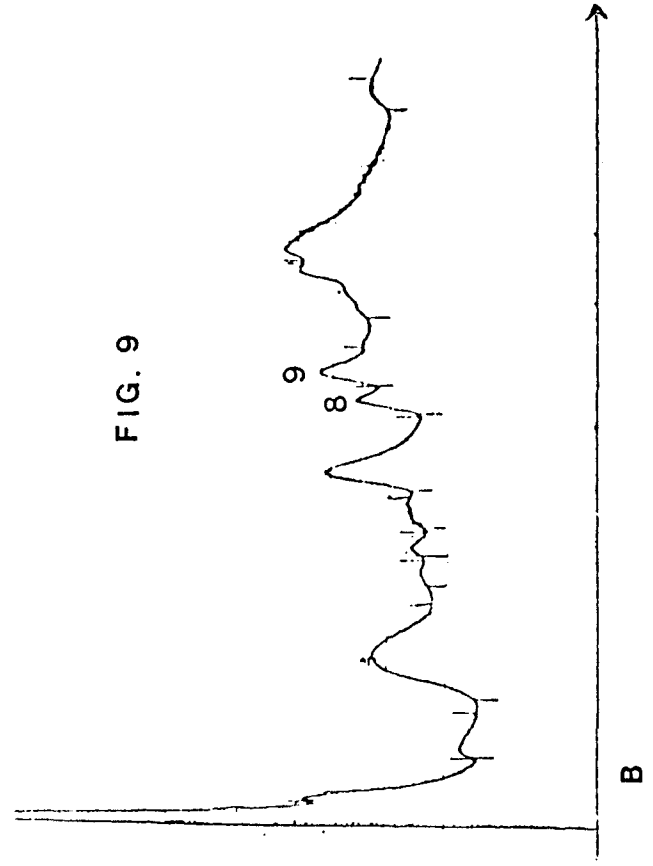
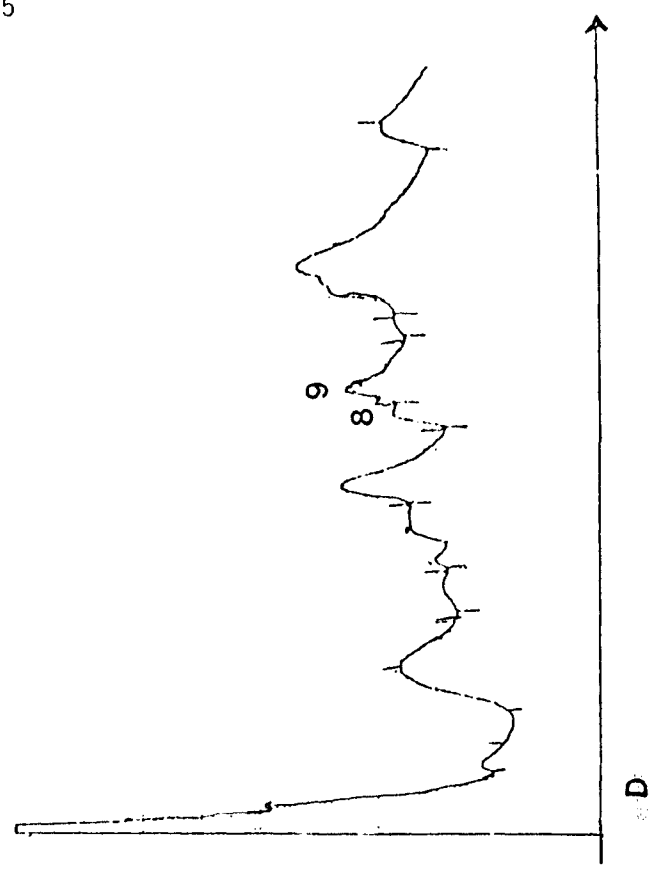
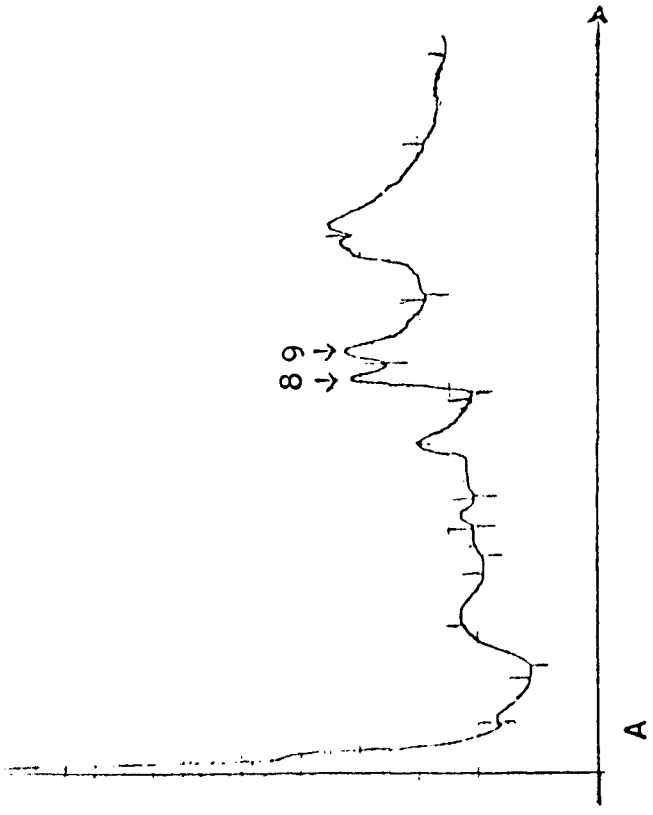


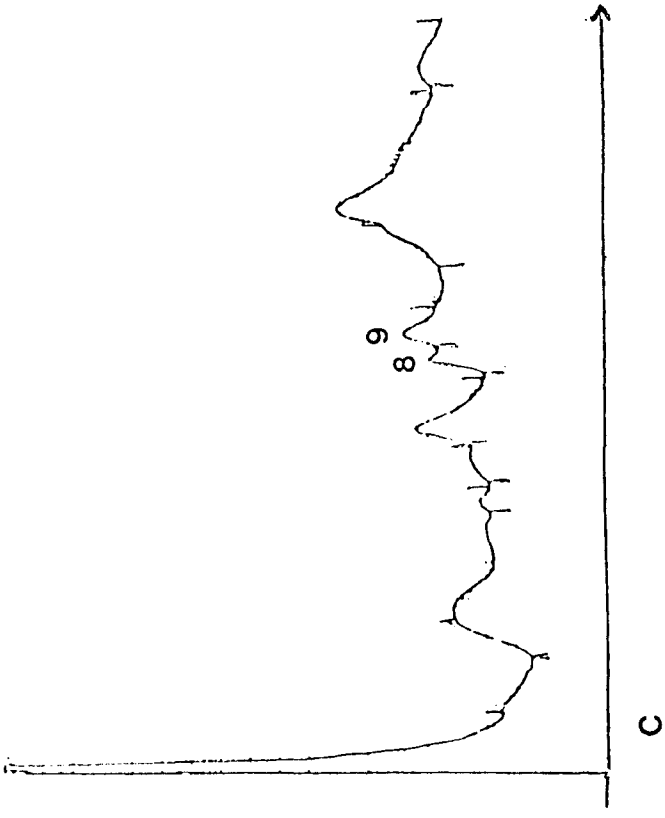
Figure 9. Four sample pyrograms produced by the second pyrolysis furnace-gas-liquid chromatography apparatus. Pyrogram A resulted from the pyrolysis of M. non-liquifaciens cells, clinical isolate 3872; B from cells of N. gonorrhoeae 7040-1972; C from cells of N. lactamica, clinical isolate 4484; and D from cells of N. sicca, clinical isolate 4407. These 4 pyrograms illustrate the general similarity of all neisserial pyrograms produced by this apparatus. The broad peaks indicate incomplete separation of pyrolysis effluent components. Only pyrogram A is clearly different. The arrows designate equal central peaks found only in pyrograms of this isolate.

FIG. 9









of pyrolysis products which eluted as peaks 8 and 9. Pyrograms of the other neisserial organisms showed peak 8 smaller than peak 9.

The broad peaks of these pyrograms showed incomplete separation of the pyrolysis products. The 1/4 in diameter columns used could not separate the complete mixture of products produced by the pyrolysis of bacterial cells.

CHAPTER VI

DISCUSSION

Observations Relating to the Specificity of the GONO-cell Test

The reversed passive haemagglutination (RPHA) technique, as developed by Abbott Laboratories, showed a good deal of promise as a confirmation technique for definitive identification of presumptive N. gonorrhoeae isolates. The procedure is simple to perform, rapid and very sensitive. All of the N. gonorrhoeae isolates tested in this study were positive with the GONO-cell RPHA test. The 4 gonococcal isolates which tested negative initially, gave positive repeat tests. Minimal training of personnel is necessary for the performance of the test. After a short period of experience, all readers who participated in this study were able to distinguish between positive and negative results and to grade the positive results.

The major difficulty with the GONO-cell test was an unacceptable level of cross-reactivity. Anatomical site of isolation is not a reliable criterion for separation of the pathogenic neisseriae. As was documented in Chapter I, N. meningitidis and N. lactamica may be isolated from genital sites and N. gonorrhoeae may be isolated from oral sites. Of the 208 TM-positive neisserial organisms tested in the primary clinical trial portion of this study, 3 of the 66 non-gonococcal neisseriae were from genital sites. Two of the TM-negative neisseriae, both N. subflava, were genital isolates. Three of the 142 N. gonorrhoeae

strains were grown from oral specimens. Only 1 of the non-gonococcal isolates of genital origin gave a positive GONO-cell test. This is nonetheless an acceptable level of cross-reactivity.

The GONO-cell test must be made more specific or the cross-reacting organism must be easily and definitely ruled out prior to GONO-cell testing.

An attempt was made to exclude the possible cross-reactors prior to GONO-cell testing. As an experienced clinical microbiologist knows, N. meningitidis and N. lactamica colonies generally appear sooner than do N. gonorrhoeae colonies. N. gonorrhoeae colonies are non-pigmented and non-hemolytic. The other 2 species may be both pigmented and hemolytic. These colonial differences become more pronounced as the colonies age. If these criteria, however, were dependable differential characteristics, no confirmatory testing would be necessary for speciation.

The lytic portion of the GONO-cell testing procedure appeared to be a point at which the non-gonococcal neisseria might be eliminated. Workers in the Abbott Laboratories (5) had noted that all N. gonorrhoeae strains in their collection lysed completely after 20 µl of 1N NaOH were added to a 1 ml suspension of the bacteria. Non-gonococcal strains in the Abbott collection did not lyse under those conditions. Therefore, elimination of all incompletely lyzed suspensions from further testing would effectively eliminate cross-reacting neisseriae. This criterion was not found to be dependable for the two series of neisseriae tested in this study.

Early in the first series of GONO-cell tests, N. lactamica and N. meningitidis strains were found which appeared to lyse completely under the specified conditions. As was shown on Table 3, 16 of 26 N. meningitidis strains in the first series of tests lysed completely. Two of these isolates were GONO-cell positive. Thirteen of the 27 N. lactamica isolates lysed completely. Eight of the 13 were GONO-cell positive.

There did not appear to be any correlation between those strains which lysed completely and those which cross-reacted in the GONO-cell test. Examination of Tables 3, 7 and 17 showed that a total of 69 N. lactamica and N. meningitidis strains were tested in the two series. Thirty-three of the 69 strains were GONO-cell weak-positive or stronger. Of these 33, 15 lysed completely and 18 did not. Or, as tabulated for lytic activity, 36 of the 69 N. lactamica and N. meningitidis strains lysed completely. Of these, 15 were GONO-cell positive and 21 were GONO-cell negative.

During the first set of tests, it was decided after consultation with Dr. Armstrong at Abbott Laboratories, who is one of the investigators who developed the GONO-cell test, to determine why the lytic results observed at IDC differed from the results observed at Abbott Laboratories. The series of tests reported in Table 4 showed that cultures 72 h old lysed more completely than cultures 24 or 48 h old. This was as expected, because neisseriae are autolytic. The information was not pertinent since GONO-cell tests were not to be run from cultures older than 48 h. On Table 4, 3 of the 12 isolates tested were more reactive at 48 h old than at 24 h old. An attempt was made to analyze the results of the

GONO-cell test run in the first series to see if GONO-cell results were substantially stronger with 24 or 48 h old cultures. Results are shown on Table 9. There was no discernible difference in reactivity. It therefore appeared that use of younger (24 h) cultures would not change either lytic or GONO-cell test results substantially.

It seemed possible that the Abbott Laboratories distilled/deionized water might differ from IDC's distilled/deionized water. A set of 11 isolates was GONO-cell tested using both IDC and Abbott-supplied water. Results were as reported on Table 5. With that set of isolates, it did appear that IDC's water enhanced lysis. Three of the 8 non-gonococcal neisseriae lysed less completely in the Abbott-supplied water. The series of isolates tested in the clinical investigation series was done, therefore, with Abbott-supplied intravenous-quality distilled/deionized water. Of the 18 non-gonococcal neisseriae tested in this second series, 7 lysed completely. Five of the 7 were GONO-cell positive. Standardization of the water did not solve the problem.

One final reason why incomplete lysis by NaOH could not be used to eliminate cross-reactors in the GONO-cell test was that observation of the difference between the clear, slightly viscous solutions which resulted from complete lysis and the opalescent, slightly hazy solutions which resulted from partial lysis was subjective. Other workers in the IDC laboratory expressed frustration when asked to differentiate complete from partial lysis.

It appeared that cross-reactivity had to be eliminated from the GONO-cell test itself. No serious attempt was made in this study to investigate cross-reactivity. Several problem strains of neisseriae

were sent to Abbott Laboratories. Dr. Armstrong of Abbott Laboratories stated that the cross-reactivity shown by a N. lactamica strain sent could not be adsorbed out of the GONO-cell antibody preparation (5).

Inferences, however, may be drawn concerning cross-reactivity from the data of this study. Two interesting observations may be made from Table 5. The amount of 1 N NaOH used for suspension lysis was varied to see if less NaOH would result in complete lysis of the gonococci and yet not promote complete lysis of the non-gonococcal neisseriae. When the amount of NaOH reagent added was cut from 20 µl to 5 µl, 5 of the 8 non-gonococcal neisseriae lysed more slowly. The 2 N. gonorrhoeae strains tested still lysed rapidly and completely. GONO-cell reactivity of those 2 N. gonorrhoeae strains was unaffected. Reactivity of 4 of the 5 non-gonococcal neisseriae which were lysed less completely by 5 µl of NaOH was weaker. Reactivity of the fifth, a N. lactamica strain, was stronger. If this latter result is excluded, the implication is that with more complete lysis some cross-reacting antigen(s), possibly protoplasmic, was liberated. The second interesting observation was that 1 N. gonorrhoeae strain and 1 N. lactamica strain were GONO-cell positive when unlysed.

Results of testing with the fluorescent-antibody (FA) conjugate, GONO-tect, supplied by Abbott Laboratories, appeared to substantiate the inference that antibody-mediated cross-reactivity of a non-surface bacterial antigen or antigens could have been involved. The purified antibody used to sensitize the GONO-cell Duracytes was essentially the same purified antibody preparation labeled with fluorescein for the GONO-tect FA test (5). The GONO-tect conjugate did not cross-react

to a significant extent with any of the non-gonococcal neisseriae tested. One N. meningitidis strain stained 2 + (on a scale of 1 + to 4 + with 4 + being the strongest). That strain also gave a strong-positive GONO-cell result, but biochemically was definitely N. meningitidis. It was rechecked and found to be a pure culture. All the remaining 109 non-gonococcal neisserial isolates stained FA ± or weaker. One N. gonorrhoeae isolate stained 2 +; the remaining 245 N. gonorrhoeae strains stained 3 + or 4 + in the GONO-tect FA test.

An essential difference between the two serological procedures was that the FA reaction involved only cell wall and/or capsular antigens, as intact cells were stained. The GONO-cell RPHA test used lysed neisserial cells. The RPHA test may have involved cell wall, capsular and cytoplasmic antigens. The RPHA test could continue to exhibit cross-reactivity as long as lysed cells were employed. Antibody-coated erythrocytes will not sediment dependably with a particulate antigen. The reasonable solution would be another antibody preparation, perhaps specific for an isolated cell fraction such as the hen anti-gonococcal lipopolysaccharide antibody employed in the slide agglutination test by Wallace et al. (131).

A second solution to the antigenic cross-reactivity problem would be the use of the existing antibody preparation in a test which involved only intact cells. The FA test, GONO-tect, did not sell, possibly because it required the use of relatively expensive equipment, an FA microscope, not then possessed by all clinical laboratories. Also, the FA test was badly reviewed by eminent individuals such as Holmes (59), who stated "Sugar fermentation tests are less cumbersome and more reliable means

of identifying neisseriae." Everyone working in one clinical laboratory, IDC, would disagree with Dr. Holmes.

Kellogg (65) stated that, "FA staining as an identification-detection procedure is low or not very efficient; i.e., there is a relatively high personnel time-cost for a low rate of detection or identification." The major thrust of Dr. Kellogg's quote may have been directed against direct specimen smear FA stains, but the prejudice is there.

The Phadebact coagglutination test appears to suffer from cross-reactivity problems, as well as non-suspension problems (8). Use of the Abbott Laboratory produced anti-gonococcal antibody in a similar coagglutination test might be very effective. Procedures are also in use which employ latex particles as the antibody carriers (76). Latex particles might also be employed as the carriers for the excellent Abbott anti-gonococcal antibody available.

The second observation taken from Table 5 was that 1 N. gonorrhoeae and 1 N. lactamica strain were GONO-cell positive when unlysed. This result could be ascribed to the fact that particulate matter may create false-positive RPHA test results. However, in this study, many GONO-cell tests were done using organism suspensions of unlysed organisms, both neisserial and non-neisserial, with no visible disturbance of the negative settling-pattern of the Duracytes. See Tables 3, 15 and 16.

A second explanation is that the sensitized Duracytes were non-specifically agglutinated. Duracyte agglutination may have been pili mediates. It has been reported by several investigators, Punsalang and Sawyer (96), Koransky et al. (68) and Buchanan et al. (18), that T_1 and T_2 piliated gonococci cause human erythrocytes to hemagglutinate.

This hemagglutination did not occur with RBCs from other animal species (68). Waitkins observed agglutination of tanned fowl and human Group O erythrocytes by T_1 and T_2 gonococci (129). Gonococci of colony types T_1 and T_2 are always piliated (63). Wistreich and Baker (135) demonstrated the presence of pili on cells of N. catarrhalis, N. perflava and N. subflava. Pili which persist on subculture were found on a meningococcal strain by DeVoe and Gilchrist (37). They also noted that usually pili of meningococcal strains were lost on subculture. Table 20 showed a gradual loss of GONO-cell reactivity by two strains of N. meningitidis after serial subculture. The loss of reactivity could have been due to either antigen loss or loss of pili. Henrichsen et al. (21) reported isolation of stable piliated Moraxella spp. strains. Pili could be responsible for apparent GONO-cell reactivity which persisted after serial sub-culture of non-gonococcal neisserial strains.

Buchanan and Pearce (19) demonstrated that isolated pili could cause hemagglutination. Buchanan et al. (18) found that pili dissolved at pH 8 in 0.01 M Tris buffer.

The 1.0 ml bacterial suspensions used in the GONO-cell test were made in distilled water and then lysed by the addition of 20 µl of 1N NaOH. The pH of the lysed suspensions was certainly 8.0 or higher. After 5 µl of lysed suspension was added to 25 µl of 0.1M phosphate buffer at pH 7.2 and 25 µl of sensitized Duracytes suspended in 0.1M buffer at pH 7.2, the pH of the system should have returned to near neutrality. Unfortunately, no pH measurements were made at IDC. Possibly suspensions of pili treated with NaOH would be able to cause hemagglutination as the pH was returned to neutrality.

If some of the N. lactamica and N. meningitidis strains which cross-reacted in the GONO-cell test were piliated, the isolated and possibly intact pili present may have caused hemagglutination of the sensitized Duracytes, as those Duracytes were made from human type O erythrocytes. In this case, the cross-reactivity problem could be solved by the use of Duracytes made from non-human erythrocytes. This supposition could have been tested by the use of non-sensitized Duracytes or Duracytes sensitized with a non-gonococcal antibody as a negative control. This, however, was not done.

The GONO-cell test would be very useful, with no change, as long as it was used in conjunction with properly performed sugar utilization tests. This combination would not be unusual, as it is generally suggested that biochemical tests be performed in conjunction with serological tests in the definitive identification of an organism.

Munro and Mallon (90a) have published the results of a field study of the GONO-cell test. This test series appears to have been done according to the same protocol outlined by Abbott Laboratories for the field study which was the second series of GONO-cell tests reported in this investigation. In their hands the GONO-cell test gave positive results for 418 of 422 N. gonorrhoeae strains, for a false-negative rate of 1%. They reported a false-positive rate of 1.2%. However, their series of isolates included only 7 non-gonococcal neisseriae, 5 of which gave positive GONO-cell results. They conceded that the GONO-cell test did not distinguish well between N. gonorrhoeae and other neisseriae, but nonetheless felt that the GONO-cell test had advantages of speed of results, and ease of interpretation over conventional methods

of N. gonorrhoeae identification.

Thoughts Regarding PGLC

Pyrolysis-gas-liquid chromatography (PGLC) investigations were more frustrating than rewarding. PGLC involves many variables, as were documented in Chapter I. A single PGLC apparatus complete with all components should be marketed. This apparatus would have to include a pyrolyzer, preferably Curie-point or better, a gas chromatograph with information directing programming and all temperature settings, and carefully standardized commercially-packed columns. It should preferably be computer operated from the time a desiccated bacterial sample was coated onto the pyrolyzer filaments until an identification was printed. The computer would make the identification by comparison of a newly-produced pyrogram with a bank of known pyrograms. This type of operation would eliminate many variables--and most operator error. It would enable interlaboratory reproducibility. The cost of such an apparatus would prohibit its purchase by all but the largest laboratories. The use of PGLC would be clinically limited unless the gas chromatograph component had multiple columns and detectors, so allowing simultaneous analyses. As PGLC is now performed, one hour each day would be needed to check and standardize the apparatus operation and 6 or 7 separate analyses could be performed in the remaining hours of a work-day.

The two sets of pyrograms included in Chapter V show how non-reproducible results from apparatus to apparatus may be. No decision can be reached regarding the utility of PGLC for the differentiation of neisseria from the results given in Chapter V. The project was abandoned

before completion.

It is discouraging that Meuzelaar and In't Veld (86) found only minor differences between the pyrograms of 2 serotypes of Neisseria meningitidis. These investigators were using capillary columns capable of separting pyrolysis effluent into 80 to 100 component peaks. The 1/8-in packed columns used with the first apparatus employed in this study distinguished 25 to 27 component peaks and the 1/4-in packed columns used with the second apparatus distinguished only 15 to 16 component peaks.

As neisserial speciation must be correct nearly 100% of the time, and error-free for TM-positive genital isolates, PGLC would not appear to be the method of choice at this time.

CHAPTER VII

SUMMARY

Two methods for definitive identification of N. gonorrhoeae were investigated, RPHA and PGLC. The RPHA method used was developed by Abbott Laboratories and designated GONO-cell (Abbott Laboratories registered trademark). Reagents and materials necessary for performance of the GONO-cell test were provided by Abbott Laboratories to IDC. All organisms tested were patient culture isolates identified using standard biochemical tests at IDC, which is a functional clinical microbiology laboratory.

The GONO-cell test was performed in a microtiter plate well, and therefore required only drops of reagents and, if necessary, only a single isolated bacterial colony. Colonial growth was taken directly from TM medium. TM is the enriched-selective medium most commonly used in the United States for growth and isolation of N. gonorrhoeae. A drop of stabilized human erythrocytes coated with column-purified anti-gonococcal antibody was added to an alkaline lysate of suspected gonococci. Within 3 to 4 h, erythrocytes agglutinated by reaction with antigen in the bacterial lysate settled in a disperse pattern in the well bottom. Non-agglutinated erythrocytes rolled into a small discrete button in the center of the well bottom.

The GONO-cell test proved to be very sensitive, as it gave positive results for all N. gonorrhoeae isolates tested. Test cross-reactivity

was seen with 13 of 69 N. lactamica and N. meningitidis strains. These 2 neisseriae, which also grow on TM medium, are unfortunately the species which must be differentiated from gonococci. No significant cross-reactivity was recorded in a series of 35 neisserial organisms, most of which could not grow on TM medium. Also essentially negative were series of 53 TM-growth-positive isolates of 18 different species and of 57 isolates of 19 different genera which were not capable of growth on TM.

It was postulated that the observed reactivity of N. meningitidis and N. lactamica was due either to liberation of cytoplasmic antigen which was antigenically similar to that of N. gonorrhoeae or to non-specific agglutination of the sensitized erythrocytes by pili of the non-gonococcal neisseria.

It was found that age of the culture when tested, up to 48 h, had little effect on GONO-cell test results. Test results were not affected by culture growth on 5 different media. Serial sub-culturing of tested isolates had little effect on the reactivity of N. gonorrhoeae isolates tested. Two N. meningitidis isolates appeared to become less reactive after repeated sub-culture. The GONO-cell test proved to be very reproducible, as tests done using a series of colonies taken from a single plate showed only small differences in degree of reactivity.

The GONO-cell test proved to be simple to perform, easy to read and rapid. The test, with no change, could be used as a gonococcal confirmation procedure if accompanied by tests for carbohydrate utilization.

Clinical neisserial isolates were also analyzed using a PGLC technique. Two different pyrolyzer - gas-chromatograph apparatuses were used. Results with the first apparatus were promising and appeared to show some differentiation between neisserial isolates tested. Results of a second series of PGLC analyses, using the second apparatus, showed no differentiation of the pyrolyzed strains. PGLC of whole bacterial cells, as a procedure for speciation of neisseriae, did not appear to be feasible with the apparatus(es) used in this study.

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