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# Characterization of Corynebacterium Vaginale Soluble and Cellular Antigens

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# Characterization of *Corynebacterium vaginale* Soluble and Cellular Antigens

by

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| 1. | EDTA | Ethylenediamine tetraacetic acid |
| 2. | Min | Minute |
| 3. | TCA | Trichloroacetic acid |
| 4. | $(NH_4)_2SO_4$ | Ammonium sulfate |
| 5. | HCl | Hydrochloric acid |
| 6. | psi | Pounds per square inch |
| 7. | CDC | Communicable Disease Center |
| 8. | NCTC | National collection of type cultures |
| 9. | PSD | Peptone-starch-dextrose |
| 10. | IEP | Immunoelectrophoresis |
| 11. | PAGE | Polyacrylamide gel electrophoresis |
| 12. | ATCC | American type culture collection |
| 13. | C | Centigrade |
| 14. | rev/min | Revolutions per minute |
| 15. | h | Hour |
| 16. | ml | Milliliter, $10^{-3}$ liter |
| 17. | $H_2O_2$ | Hydrogen peroxide |
| 18. | $Na_2HPO_4$ | Sodium phosphate dibasic |
| 19. | $NaH_2PO_4$ | Sodium phosphate monobasic |
| 20. | x g | Times gravity |
| 21. | M | Molar |
| 22. | w/v | Weight per volume |
| 23. | g | Gram |
| 24. | PBS | Phosphate buffered saline |
| 25. | nm | Nanometer, $10^{-9}$ meter |
| 26. | Tris | Tris-(hydroxymethyl)-aminomethane |
| 27. | cm | Centimeter, $10^{-2}$ meter |
| 28. | DEAE cellulose | Diethylaminoethyl cellulose |
| 29. | CM cellulose | Carboxymethyl cellulose |
| 30. | KCl | Potassium chloride |
| 31. | $KH_2PO_4$ | Potassium phosphate monobasic |
| 32. | CNBr | Cyanogen bromide |
| 33. | $NaHCO_3$ | Sodium bicarbonate |
| 34. | NaCl | Sodium chloride |
| 35. | OD | Optical density |
| 36. | Bld | Blood |
| 37. | Di | Diphasic |
| 38. | BE | Broth extract |
| 39. | AB | Antibody against |
| 40. | Ag | Antigen |
| 41. | F | Fraction |
| 42. | P | Precipitate |
| 43. | SC | Subcutaneous |
| 44. | ID | Intradermal |

LIST OF ABBREVIATIONS CONTINUED

| | | |
|---|---|---|
| 45. | IM | Intramuscular |
| 46. | IV | Intravenous |
| 47. | FTA | Fluorescent treponemal antibody |
| 48. | mm | Millimeter, $10^{-3}$ meter |
| 49. | mamp | Milliampere |
| 50. | Temed | N:N:N:N-tetramethyl-1:2 diamino-ethane |
| 51. | $H_3PO_4$ | Phosphoric acid |
| 52. | ug | Microgram, $10^{-6}$ gram |
| 53. | $HIO_4$ | Periodate |
| 54. | LiOH | Lithium hydroxide |
| 55. | $CaCl_2$ | Calcium chloride |
| 56. | $MgCl_2$ | Magnesium chloride |
| 57. | $MnCl_2$ | Manganese chloride |
| 58. | $C_2H_5OH$ | Ethanol |
| 59. | $V_{E-D}$ | Exclusion volume of blue dextran |
| 60. | $V_{E-S}$ | Theoretical exclusion volume of sample |
| 61. | $V_o$ | Void volume |
| 62. | NRS | Normal rabbit serum |

# I. INTRODUCTION

## A. REVIEW OF MICROBIAL ANTIGENS

Serological methods are commonly used to classify and identify bacteria. In a clinical laboratory, these antigens are often detected in order to identify the bacteria. However, these bacterial products are, in fact, produced because they are essential for the organism's existence. Studies of these bacterial antigens have demonstrated that they have a wide range of chemical compositions and have provided information which has led to a more in-depth knowledge of the architecture of many organisms. Many studies have attempted to determine the chemical composition and location of those antigens in order to: (a) determine the role of various antigens in the successful existence of the organisms, (b) develop more precise classification systems, and (c) gain an insight into their role in the mechanisms of pathogenicity.

The structural diversity present among bacterial organisms has made it difficult to isolate and characterize their antigenic determinants. Therefore, various extraction procedures have been developed in an attempt to circumvent these problems. The purpose of this literature review is to discuss: (a) the manner in

which studies of antigenic structure may be used to demonstrate similarities and differences among organisms, (2) the factors which determine the specificity of the bacterial antigens, (3) the methods commonly used for extraction of bacterial antigens and the characteristics of the antigens obtained, and (4) the manner in which studies of the bacterial antigens have helped to clarify our classification of bacteria and our knowledge of their pathogenic mechanisms.

1. Cellular Antigens

a. Peptidoglycan

Both gram positive and gram negative organisms possess a peptidoglycan backbone of alternating N-acetyl glucosamine joined to N-acetyl muramic acid by β-1,4 linkages. The diameter of the peptidoglycan layer is greater in gram positive organisms (200-800 Å) than gram negative organisms (20-30 Å). Among gram positive organisms, the carboxyl group of the muramic acid is attached to a tetrapeptide and this tetrapeptide is linked by a peptide bridge, consisting of several amino acids, to a tetrapeptide of neighboring polysaccharide chain. In contrast, some gram negative organisms have a direct peptide bond between the neighboring tetrapeptides. Other variations observed in this layer, especially among gram positive organisms are taxonomically useful (44).

Lysozyme acts on peptidoglycan and breaks the β-1,4 linkages between N-acetyl glucosamine and N-acetyl muramic acid, and lysozyme is commonly used to release antigenic compounds useful in the study of the architecture of the cell wall(44). However, gram negative organisms possess additional layers on their surface which tend to interfere with the action of lysozyme. Ethylenediamine tetraacetic acid (EDTA), which chelates metal ions, can sensitize certain gram negative bacteria to the action of lysozyme (48).

Lysostaphin is also used to disrupt the peptidoglycan layer. Lysostaphin is a peptidase which degrades the penta-glycine bridges in the cell wall of Staphylococcus aureus (63).

b. Protein antigen A.

Lyosostaphin digestion of Staphylococcus aureus has been used to obtain protein antigen A which is covalently linked to the peptidoglycan (109). Protein A is also excreted into the culture media by certain methicillin-resistant strains of Staphylococcus aureus which do not incorporate it into their cell wall. Commercial preparations of this antigen are prepared from this culture medium by affinity chromatography (65). Depending upon the extraction method employed, variations in the properties of protein A are observed; that is, affinity columns isolate an antigen which has a 13,000 molecular weight and does not absorb at 280 nm, while lysostaphin

digestion releases an antigen with a molecular weight of 42,000 containing 4 tyrosine residues (65, 108). Protein A has been shown to be antiphagocytic. The mechanism of action lies in (a) the ability of protein A to bind to the Fc region of IgG and (b) the affect of protein A on the complement system (23, 38, 39).

The specific interaction of protein A with the Fc region of IgG molecules of subclasses 1, 2, and 4 has been applied in cytochemical and immunochemical studies. For instance, protein A is: (a) coated on RBC which are used in rosette assays to study the surface of lymphoid cells, (b) tagged with fluorescent dyes and used in indirect assays to demonstrate antibodies bound to tissue antigens, and (c) bound to Sepharose to provide affinity columns (94).

c. Teichoic acids

Beside the differences in the peptidoglycan layer, additional distinctions exist between the architecture of gram positive and gram negative organisms which serve to differentiate them, i.e., gram positive organisms have teichoic acids while gram negative organisms tend to have lipopolysaccharides. Due to the chemical nature of these differences certain extraction procedures have been devised which have become classical methods for the isolation of antigens generally present in these two groups of organisms.

Characteristic major surface antigens of many gram positive organisms are composed of teichoic or teichuronic acids (i.e., Staphylococcus, Streptococcus, Bacillus, Lactobacillus, Micrococcus) (62). Teichoic acids are polymers of either ribitol or glycerol residues with phosphodiester linkages which are attached to the N-acetyl muramic acid of the peptidoglycan backbone. Ribitol polymers are found only in the wall while glycerol polymers are also present as intracellular polymers. Teichoic acids are commonly extracted by cold 10% TCA. However, the side chains of teichoic acids are labile to pH extremes and even dilute alkali will split the ester linkage of α-alanine to teichoic acid (123a).

The various substitutions (i.e., sugars, amino sugars, choline, D-alanine) attached to the teichoic acids provide the specific antigenic determinants. For example, the species specific determinant of Staphylococcus aureus is N-acetyl glucosamine linked either alpha or beta to polyribitol phosphate. In contrast, Staphylococcus epidermidis contains alpha-glucose as the specific determinant which is linked to polyglycerol phosphate. Other examples are the antigens of group D and group N Streptococci which are intracellular teichoic acids commonly extracted by dilute alkali after cell disruption. The specificity appears to be determined by substituted Kojibiose and Kojitriose in the group D antigen and by galactose phosphate in the group N antigen (123b). Also, unsubstituted glycerol phosphate has been suggested

as the compound characterized as the Rantz antigen which is detected by passive hemagglutination in many gram positive organisms (96).

d. Characterization of endotoxin

Gram negative cell walls do not contain teichoic acids but instead contain a layer composed of lipoprotein and lipopolysaccharide. The lipoprotein is generally attached to the peptidoglycan by covalent bonds. The lipopolysaccharide or endotoxin is an essential component in the classification of these organisms and can act as a mechanism of pathogenicity. The lipid is the toxic moiety of the molecule while the polysaccharide is the antigenic component (13).

The extraction methods most widely used to isolate endotoxin are those described by: (1) Boivin et al. (89), in which bacteria are disrupted in the presence of 5% cold trichloroacetic acid (TCA) or (2) Westphal et al. (89), in which the bacteria are mixed with 45% phenol at 78° C for 10 min. Although several other methods exist, e.g. those which employ acid, alkali, pyridine, ethylene glycol, urea or ether, these have not been widely used (123c).

The method used to extract the endotoxin determines the nature of the antigen released. Only the TCA procedure releases the whole antigen which can act as an immunogen in the rabbit. In contrast, the phenol extraction procedure

yields a partially degraded antigen which is not immunogenic. Acid or alkali treatment results in release only of the polysaccharide. In addition, the acid procedure yields a smaller molecule which, in contrast to the antigen released by either TCA or phenol extraction, neither fixes complement nor binds to RBC (123c).

The polysaccharide component of endotoxin, designated the "O" antigen, is composed of a core whose composition varies only slightly among the genera of Enterobacteriaciae. The core contains the unique sugars L-glycerol-D-mannoheptose and 2-keto-3-deoxy-D-mannooctonoic acid. The side chains determine the "species" specificity and contain common sugars (mannose, rhamnose, etc.) in addition to sugars unique to the Enterobacteriaciae, e.g., 3,6 dideoxyhexoses, i.e., colitose, abequose, paratose, and tyvelose (76).

The Kaufmann-and-White classification of the Salmonella is based on the major antigenic determinants found on the polysaccharide side chains, on the flagella, and on the capsule. The subtleness of the variations which provide antigenic distinction are especially emphasized in these studies. In general, the composition and sequence of a trisaccharide unit determines the specificity of the "O" side chains. The differences within these trisaccharide determinants that have been observed to cause variation include: (a) changes of position of glycosidic linkages, i.e. 1-4 vs

1-6; (b) altered anomeric configuration, i.e. α vs β linkage; (c) attachment of additional monosaccharides such as glucose; (d) presence or absence of acetyl groups; and (e) deletions or substitutions of the monosaccharides. Although there is usually one sugar in the trisaccharide unit that is immunodominant and can define the determinant, the additional residues of the repeating trisaccharide unit contribute considerably to affinity for binding to the corresponding antibodies (76).

e. Flagellar antigens

Flagellar antigens, designated "H" antigens, are important in the classification of bacteria, the most notable example being in the Kaufmann-White scheme. In this classification scheme, minute variations in Salmonella can be detected. A given strain may form at different times, one of 2 different "H" antigens. This shift in antigen composition, referred to as phase variation, can be induced by long term incubation or by growing the cells in antisera specific for one of the phases. Flagellar antigens can interfere with determination of the specific "O" antigen. This interference can be eliminated by denaturing the flagella by heat, acid or alcohol (35).

f. Antigens found on specific organisms, e.g. streptococci

The extraction procedures previously mentioned are methods which can be applied to several genera to obtain antigens which vary slightly in their chemical composition. These antigenic variations provide the means for differentiating the various organisms and give an insight into the basis of their antigenic distinctiveness. However, each genus studied to date also possesses unique antigens and thus extraction procedures have evolved which are specific for the particular antigen under study. The Streptococci have been particularly well characterized and will serve as the typical example for purposes of this discussion.

(1) Streptococcal protein antigens

Hot acid extraction of streptococci releases both a group carbohydrate antigen and a type specific M protein antigen. While proteins are normally destroyed by heating over 60° C (67a), M protein can be extracted by hot 10% TCA and this acid, in comparison to HCl, privides a more homogeneous M protein, as indicated by polyacrylamide gel electrophoresis (PAGE) (113).

The M protein or the type specific antigens of group A streptococci are found on fimbriae which project from the cell surface. The M proteins are readily accessible antigens and, unlike the somatic antigens of Enterobacteriacea, the

M antigens are not blocked by the capsule (40). The M proteins act as a bacterial defense mechanism by preventing phagocytosis (64) and may be the means by which the bacteria adhere to specific cells (45). The importance of the M protein antigen in disease is emphasized by studies which have implicated specific M types, especially type 12, in the pathogenesis of acute glomerulonephritis (83).

M-typing is time consuming and several factors are involved in obtaining satisfactory results. The preparation of potent adsorbed antisera is, in many instances, a very difficult task. Usually, typing is performed using the precipitin reaction with acid extracts serving as the source of M antigen. Acid extraction is used because it destroys T antigens which may cause interference. The type of growth media has been shown to affect M-antigen concentration. Todd-Hewitt medium is recommended because its low glucose concentration prevents proteinase formation (101).

Streptococci also possess two other cell surface protein antigens designated "T" and "R"; neither of which is important in virulence. Since "M" antigen cannot always be detected with the available antisera, M typing alone is not reliable. A second typing method, the T agglutination system, provides a means of labeling strains that cannot be M-typed. Disadvantages of the T-typing system are: (a) a single T antigen can be shared among a number of M

types, and (b) a single strain can synthesize a number of T proteins (81).

The "R" antigens, like the "M" antigens, are found in several streptococcal groups (70). Basically, two types of "R" antigens exist which are differentiated on their sensitivity to various enzymes; that is, antigen 3R is destroyed by trypsin or pepsin while 28R is destroyed only by pepsin (69).

(2) Streptococcal carbohydrate antigens

Many extraction procedures have been published for the group carbohydrates of streptococci and for the type specific carbohydrates of group B and D streptococci. These include the use of: (a) hot HCl, (b) 10% TCA, (c) enzymes obtained from Streptomyces albus, (d) autoclaving, and (e) formamide (101). The 10% TCA method is preferred over that of Lancefield in extraction of the group O carbohydrate (85). The specificity of the group carbohydrates appears to involve either rhamnose or N-acetylglucosamine (64, 85).

In addition to the group and type specific carbohydrate antigens, streptococci also possess polysaccharides termed C polysaccharides. N-acetyl glucosamine has been identified in the C-polysaccharide of Group A streptococci and been implicated as the major determinant of the endotoxin-like effect first ascribed to the C-polysaccharide (105). Later studies

by Schwab (102) revealed that the mucopeptide extracted with the carbohydrate is responsible for the toxicity of Group A C-polysaccharide. The C-polysaccharide plays a dual role of protecting the mucopeptide from tissue lysozyme and masking the toxic property. Thus, the mucopeptide-polysaccharide complex can persist in tissue in a relatively innocuous state and as the polysaccharide is gradually removed by tissue enzymes, the peptidoglycan is exposed to produce chronic irritation (102). One of the most dramatic effects of the peptidoglycan is the extensive carditis which occurs 3 days after IV administration of as little as 10-80 µg in rabbits. It is not known whether this toxicity is due to an intrinsic toxicity of the molecule or to acquired hypersensitivity immunity (64).

The C-polysaccharides are also found in other bacteria (i.e., Streptococcus penumoniae), fungi (i.e. Aspergillus fumigatus, Epidermophyton floccosum) and parasites (i.e. nematodes (121a). The C-polysaccharides antigens precipitate Beta globulins in serum which are termed C-reactive proteins. The C-reactive proteins are produced by the liver during an inflammatory response (121b).

## 2. Exoantigens

Exoantigens comprise many bacterial products of immunological importance which occur extracellularly and include such substances as enzymes, exotoxins and exopolysaccharides. Generally, toxins and enzymes are secreted in low concentration and are quite labile to heat, pH extremes, and oxygen (121c, 123d). In contrast, the exoantigens which are predominantly polysaccharide in nature, are present in higher concentration and are generally heat stable. The exopolysaccharides are usually capsules or slime layers and form an important group of substances because of their existence on the cell surface which is in direct contact with the host's defense mechanisms.

### a. Identification of capsular antigens

Capsules appear as loose-gel-like structures surrounding the organism. Generally, capsules can be demonstrated as distinct layers by negative staining in India ink suspension. However, some "capsules" or slime layers are not readily visualized and appear to diffuse into the surrounding media (123e). A clearer concept of their structure is gained by using electron microscopy after fixation of the cells with osmium tetroxide and staining with ethidium red (61, 111).

b. Methods for extraction of capsular antigens

Capsules are readily extracted from the bacterial cells by dilute acid or alkali or by washing the bacteria with water or saline (67b, 123e). They are also recovered from the growth media by decreasing their solubility with the addition of alcohol or salts (67c, 89, 100). Alcohol, a weakly polar solvent, precipitates the capsular antigens by causing a reduction of the dielectric constant of the solution of a polar solute in a polar solvent. Salts increase the ionic strength of the media and cause a concomitant decrease in solubility of the solute. Precipitation by salt involves less problems than does alcohol, since alcohol can disrupt the tertiary structure of proteins (84a).

c. Role of capsular antigens in pathogenicity

The experiments of Griffith with Streptococcus pneumoniae (49) demonstrated the importance of capsules in pathogenicity. Capsules protect the organism from phagocytosis. The most accepted theory at present attempts to explain this phenomenon on the basis of hydrostatic interactions. Phagocytic membranes, containing surface lipids, have a hydrophobic character which is repelled by the hydrophilic nature of the bacterial capsule. Early reports suggest that the capsules are composed exclusively of carbohydrates with the minor exceptions of Bacillus anthracis which has a polypeptide capsule and Bacillus megatarium whose capsule is composed of protein and carbohydrate (18a).

d. Characterization of exopolysaccharides found in specific organisms

(1) Pseudomonas

Recent reports suggest a more complex chemical composition of capsules and give evidence for another mechanism of pathogenicity. Liu et al. (74) found that the slime layer of Pseudomonas aeruginosa is toxic for mice. Although hemolysins and extracellular enzymes contribute to the lethality of the organism, the slime layer appears to be the most important factor in its pathogenicity. Sensakovic and Bartell (106) found the slime to be two to three times more toxic than lipopolysaccharide, and antigenically and chemically distinct from lipopolysaccharide. Antigenic and chemical differences were also detected among strains. Generally, the slime contains hexoses, uronic acids, amino sugars and fatty acids (106). Removing the protein by phenol-heat extraction did not reduce the biological properties of the slime. The slime also has the ability to bind to cells. After intraperitoneal injection, the slime was found bound to peripheral erythrocytes. This ability of the slime to bind to cells may play an essential role in the pathogenicity of the organism (21).

(2) Streptococcus

Schiffman et al. (104) characterized the capsular polysaccharide of Streptococcus pneumoniae and compared it with the C-polysaccharide present in the wall of the organism. They found that the capsular polysaccharide is a high molecular weight compound composed of teichoic acid and mucopeptide. Previously, Brundish and Baddiley (9) characterized the C-polysaccharide structure as a complex teichoic acid with a molecular weight of 26,400. Differences and similarities exist between the capsule and C-polysaccharides of a single strain and among the capsule and C-polysaccharide of different strains. The differences appear to be due to the peptidoglycan portions of the molecule, while the cross reactions are due to the teichoic acid moieties (104).

Similar cell wall polysaccharides and capsular polysaccharides were isolated from various strains of alpha streptococci (58,60). The cell wall polysaccharides of the alpha streptococci cross react with the cell wall and capsular polysaccharides of Streptococcus pneumoniae. The cross reactions appear to be due to the teichoic acid moieties. These studies (59) also suggest that the cell wall polysaccharide contains the group specific antigen while the type specific antigen is present in the capsular polysaccharide.

The polysaccharides (glucans) released by Streptococcus mutans are important factors in dental carries. These extracellular polysaccharides enable the organism to adhere to teeth and to each other resulting in the formation of dental plaque (50, 125). Vaccination of monkeys with Streptococcus mutans has prevented dental carries (72). Possible protective mechanisms sited include prevention of growth and/or adherence of the streptococci (72). Burckhardt et al. (11) agree with these views and suggest that antibody directed against glucosyltransferase, which cross links sucrose to form glucans, inhibits adherence.

(3) Neisseriae

The polysaccharides of Neisseria meningitidis are important since they are responsible for group specificity, and they have been used as the immunogen in effective vaccines (41). If the group C-polysaccharide is extracted with chloroform-butanol (120), the extract also contains appreciable quantities of mucopeptide which may have toxic effects. In contrast, extraction with Cetavolon or with $(NH_4)_2SO_4$ precipitation followed by alcohol precipitation yields only C-polysaccharide (47, 100).

e. Characterization of exoantigens found to cause cross reactions

Cross reactions among capsular antigens do occur and

attempts have been made to use these cross reactions advantageously. Streptococcus penumoniae capsular antigens serve to subgroup this organism into more than 80 serological types; however, pneumococcal capsular antigens also cross-react with polysaccharide antigens present on alpha hemolytic and non-hemolytic streptococci, Salmonella, and Friedlander's bacillus (18b). Another example in which cross-reacting antigens may play a role is in the case of the polysaccharides isolated from the genus Bacillus which cross react with capsular antigens of Streptococcus penumoniae type III, Haemophilus influenzae type b and Neisseriae meningitidis group A. It is suggested that the Bacillus, which may be part of the normal flora, serves as the source of the "natural" serum antibody in adults to pneumococcus, meningococcus, and Haemophilus influenzae (88). Similarly, capsular antigens of Haemophilus influenzae type b cross react with E. coli K antigens. Feeding E. coli to neonatal rabbits has been found to accelerate the development of "natural" serum antibody to Haemophilus and may be a method of immunization (87).

In summary, the literature previously mentioned demonstrates that capsular antigens are important because they (a) provide the means to identify an organism and to classify it as belonging to a specific group or type, and (b) play a role in protection against infections.

3. Additional Procedures Used to Extract Bacterial Antigens

Many extraction procedures other than those previously mentioned have been utilized to study the antigenic structure of organisms. These methods include treatment with ether, with alkali, and with phenol-acetic acid-water. Phenol-acetic acid-water has been used to liberate hydrophobic proteins from Mycoplasma (97, 116). Ether-water extraction is preferred over other methods for the extraction of immunogens from Pasteurella tularensis (90). Ether has been used to liberate the type specific antigens from rumen Bacteroides and the lipid group antigen from Chlamydia trachomatis (20). Alkali is frequently used to solubilize fungal cell walls for determination of their chemical composition (14) and to release antigens useful for diagnostic serological testing (36, 121d). Relatively mild alkaline conditions have been shown to disrupt 1-3 glycosidic linkages while leaving the 1,3 glucan present in cell walls of Blastomyces dermatidis intact (14).

Organic solvents such as phenol, ether, alcohol, and formamide disrupt electrostatic interactions (84b), and are frequently used to dissociate cell wall antigens and precipitate antigens in the growth media. A similar effect is observed with EDTA, a chelating agent, which binds metal ions and disrupts bonds. Salts can also be used to isolate antigens; that is, excess salt is added to "salt out" or precipitate antigens while conversely, small quantities of salt are added to solubilize or "salt in" antigens (84a).

Cells may be disrupted by various methods to yield soluble antigens. For example, sonication, which employs ultrasonic waves, disrupts cells by producing streams of microscopic bubbles from gases dissolved in the media. The Mickle disintegrater disrupts cells by shearing them with glass beads, while the French press subjects the cells to 13,000 psi (67d). Cell walls are often separated from their cell membranes by means of enzymes or chemical treatment, prior to disruption, to allow a precise delineation of antigen location (67d).

4. Summary

Generally, several extraction procedures, in addition to mechanical shearing or enzymes, are used in combination to isolate and purify any given antigen. Subsequently, column chromatography and/or preparative electrophoresis are used to further purify and isolate desired antigenic components (121c).

If one compares the extraction techniques used and the characteristics of the antigens released, it becomes evident that no single technique is applicable under all circumstances. The diversity observed in the antigens released by acid extraction is a prime example. Acid extraction releases endotoxin from gram negative bacteria; but, in contrast, releases teichoic acid from gram positive bacteria. Also, both the group carbohydrate and type specific protein antigens of group A streptococci are released by acid extraction.

Similarly, alkali extraction is frequently used to extract the polysaccharide antigens from fungi and the polysaccharide moiety of endotoxin. However, not all polysaccharide antigens can be extracted by the alkali method. For example, certain pneumococcal capsules are alkali labile while others are alkali stable (123f).

The method by which antigens are released and isolated affects the chemical and biological properties of the resultant product. Studies in which "harsh" techniques are used may result in homogeneous, small molecular weight compounds which are relatively free of contamination by neighboring compounds but bear no resemblance to the component as it exists in the organism *in vivo*. Studies in which mild extraction procedures are utilized tend to be concerned with the overall architecture of the antigen and in the manner in which molecules which border the antigenic determinants influence both the antigenicity and pathogenicity of the organism. Both approaches have been important in developing systems for classification and subsequent clinical identification and in understanding the mechanisms of pathogenicity and ultimately prevention of disease.

## B. REVIEW OF C. VAGINALE LITERATURE

*Corynebacterium vaginale* (*C. vaginale*) is a fastidious, gram variable, pleomorphic organism which has been implicated as the major cause of "non-specific" vaginitis and urethritis. The

taxonomic position and pathogenic role of C. vaginale has been the subject of much controversy and numerous publications. The purpose of this dissertation was to isolate and immunologically characterize the antigenic components from C. vaginale in an attempt to provide data which can clarify the problems associated with this organism.

## 1. Pathogenicity

In 1953, C. vaginale was first isolated from cases of "non-specific" vaginitis and urethritis, independently by Leopold (73) and Gardner and Dukes (42). The term "non-specific" has been used to characterize those cases in which the symptoms of an infection are present although no etiological agent can be isolated. A role for viruses, mycoplasma and chlamydia in "non specific" infections has also been suggested (56).

In the initial case studies (3, 7, 20, 21) which have been published, C. vaginale was isolated in 12% to 94% of the patients with "non-specific" vaginitis. However, in these studies, the criteria for isolation and identification of C. vaginale as well as the types of population examined have varied. Several of these early studies reported that C. vaginale was the sole or predominant organism cultured from cases of so-called "non-specific" vaginitis or urethritis. Also, it was noted that ampicillin therapy irradicated the organism and symptoms. On this basis, many workers have concluded that C. vaginale is a true pathogen (8, 42, 43, 73, 77).

Others have opposed this view for various reasons, i.e., (a) C. vaginale has been isolated from asymptomatic patients (51), (b) C. vaginale was not isolated from patients with genital infections in some studies (22) and (c) lactobacilli were found to be absent from the vaginal flora in some cases, and the concomitant pH change was suggested as the cause of the abnormalities (12).

Still another view point was raised by a third group of workers who concluded that C. vaginale appears to be similar to Candida albicans; i.e., is an opportunistic pathogen of low virulence (28, 29, 71).

In more recent studies, in which specific criteria for identification of C. vaginale were followed, the results have supported the hypothesis C. vaginale is pathogenic. Platt (95) reported on two of his patients with neonatal C. vaginale infections and also sighted five other cases in which C. vaginale was implicated as a maternal, fetal and/or neonatal pathogen. The identification of the organisms were verified by CDC.

Akerlund and Mardh (1), following the identification criteria outlined by Dunkelberg (32), isolated C. vaginale in 31.4% of 70 patients with lower genital tract infections. The organism was not isolated in patients with a predominance of lactobacilli nor in 28 healthy controls. These workers also tested for the presence of other possible genital pathogens. They cultured Mycoplasma hominis in 44% of the patients, 7.1% of the normal controls of

81.8% of patients with C. vaginale. Trichomonas was isolated in 15.7%, Candida albicans in 7.1% and Neisseria gonorrhoeae in only 1% of the patients.

The cells of C. vaginale have the ability to bind to each other (34, 97) and to various other cell types and in this respect resemble streptococci (50, 125). Gardner and Dukes (42) first noted the diagnostic character of "clue" cells in C. vaginale infections. These "clue" cells are epithelial cells found in the genital exudate which contain many gram negative bacilli either intracellularly or attached to their surface.

Mardh and Westrom (79) tested the adherence of bacteria to vaginal cells in an in vitro system. They reported that Neisseria gonorrhoeae, group B streptococci and C. vaginale adhered to the cells in significant numbers while other bacteria considered to be part of the normal flora in the genital tract, e.g., Lactobacillus, Fusobacterium glutinosus, Bacteroides fragilis, did not adhere in significant numbers.

In summary, the overall importance of C. vaginale as a genital tract pathogen is still questionable. However, recent studies in which strict criteria for the identification of C. vaginale have been followed do strongly favor the pathogenic hypothesis. Moreover, C. vaginale does resemble other genital tract pathogens since it possesses a mechanism for attachment to vaginal epithelial cells. Such a mechanism would appear to be a necessary criteria for subsequent invasion of the host tissue.

2. Taxonomy

C. vaginale was originally designated Hemophilus vaginalis because it appeared to be a gram negative coccobacillus which required blood or other serum factors for growth (3, 42, 73, 114). Subsequent reports noted that the organism stained gram positive (30, 33, 127) and did not require X or V factor for growth. Attempts to categorize the organism on the basis of its growth characteristics, morphology, and biochemical fermentation patterns resulted in suggestions that it be placed in the genus Lactobacillus (4) or Corynebacterium (127) or that a new genus be established (92).

Subsequently, more critical evaluation of the composition of the cell wall and metabolites of C. vaginale have been performed. Gas chromatography studies initially suggested that acetic acid is the major volatile acid produced by the type strain (31). In contrast, Malone et al. (78) recently reported on 9 isolates of C. vaginale, five of which were obligate anaerobes on primary isolation, and noted that they produced a small quantity of lactic acid. She suggested that the organism is related to Bifidobacterium.

Weitzman and Jones (55, 122) reported that C. vaginale produces a citrate synthetase with characteristics typical of those produced by gram positive bacteria. They have suggested that this enzyme can be used as a taxonomic tool and characterized this enzyme in other organisms which have a questionable taxonomic position.

Vickerstaff and Cole (118) analyzed the cell wall of the NCTC (10287) strain of C. vaginale and found the walls contained galactose, mannose, rhamnose and 6-deoxytalose, while diaminopimelic acid was lacking. They concluded that the type strain has a wall composition similar to that which they observed with Actinomyces bovis. However, Kwapinski (66) claims that Actinomyces bovis contains arabinose in its cell wall. Furthermore, C. vaginale has been excluded from the genus Corynebacterium by some workers because it does not possess arabinose in its cell wall nor produces catalase (10).

Electron microscope studies have also resulted in two opposing views. Criswell et al. (15, 16), working with the type strain, reported that C. vaginale has a trilaminar cell wall containing eleven amino acids, a low peptidoglycan content and no teichoic acids. They concluded that the organism is gram negative and probably a member of the genus Hemophilus. Reyn (99) reported that the cell wall structure and septa formation in C. vaginale closely resembles that found in other gram positive organisms. She also noted that the cell wall of Lactobacillus acidophilus differed from that of C. vaginale.

Serological studies have been performed in an attempt to find an antigen which was shared by C. vaginale and organisms from possible related genera. Although Pease and Laughton (93) have reported cross reactions between C. vaginale, C. cervicis and H. influenzae, such cross reactions have not been observed by

others with the genera Lactobacillus (110, 114, 117), Corynebacterium (110, 114, 117), Actinobacillus (117), Streptococcus (mutans) (117), Actinomyces (117) or Nocardia (117).

There is evidence which suggests that a common antigen exists among the C. vaginale isolates which have been isolated to date (98, 110, 114, 117). An antiserum designated anti-14018 Di, which was prepared for the M.S. thesis by this author, was shown to be specific for C. vaginale and also gave positive fluorescent antibody tests with all C. vaginale isolates checked (117). When this antisera was used in studies employing the Ouchterlony technique at least one precipitin band was produced with each C. vaginale isolate tested (110). Therefore, our previous studies provided: (a) a rapid method for clinical identification of the organism and (b) presumptive evidence for a common antigen.

## C. PURPOSE OF THIS RESEARCH

The purpose of this dissertation was to isolate and immunologically characterize antigenic components of C. vaginale in an attempt to: (1) develop a method for the rapid identification of C. vaginale, (2) provide data which might clarify the taxonomic position of C. vaginale and (3) ultimately clarify the role of C. vaginale as a pathogen.

Previous studies have utilized polyvalent C. vaginale antisera to investigate the relationship between C. vaginale isolates and possible related bacteria; however, no attempts were made to isolate these antigens and prepare monospecific antisera (27, 93, 98, 115, 117). Therefore, in order to provide a clearer understanding of the antigenic structure of C. vaginale, it was decided to isolate and immunologically characterize C. vaginale antigens.

## II. MATERIALS AND METHODS

### A. ORGANISMS

Six reference strains of *C. vaginale* were employed in this study. W. E. Dunkelberg supplied three strains (594D, 6488D, and T94), R. E. Weaver forwarded two strains (6488W and 8226), and type strain 594 (ATCC No. 14018) was obtained from ATCC. P. Pease provided *Corynebacterium cervicis* (*C. cervicis*) strain 13. Also obtained from the ATCC were *Corynebacterium diptheriae* (*C. diptheriae*) strain 11913, *Corynebacterium xerosis* (*C. xerosis*) strain 7711, *Lactobacillus acidophilus* (*L. acidophilus*) strain 4356, *Actinomyces bovis* (*A. bovis*) strain 13683, *Nocardia asteroides* (*N. asteroides*) strain 19247, and *Hemophilus influenzae* (*H. influenzae*) strain 9247. *Corynebacterium hofmanii* (*C. hofmanii*) strain 231 was obtained from the National Type Culture Collection. Nine clinical isolates, two obtained from W. E. Dunkelberg (V28 and V44) and seven isolated in our laboratory (144, 359, 1544, 1575, 1637, 6234, and 8315), that gave a positive indirect fluorescence with anti-*C. vaginale* antiserum and morphologically and biochemically resembled *C. vaginale* (117) were included in this study. A vaginal diptheroid-like organism

(8372) that gave a negative fluorescence and biochemically did not resemble C. vaginale was also included.

B. MEDIA

C. vaginale was isolated and maintained on blood agar plates (BBL, Cockeysville, Maryland) incubated under increased carbon dioxide tension in a candle jar at 37° C.

In order to obtain large yields of organisms, C. vaginale was grown either diphasically (117) or in liter flasks of peptone-starch-dextrose (PSD) broth (30) on a rotary shaker (150 to 200 rev/min) (Lab-Line Instruments, Melrose Park, Illinois) at 37° C for 48 to 72 h. To facilitate high yields of organisms in the PSD broth, organisms were collected by swab from five blood agar plates and inoculated into 200 ml of PSD broth which was allowed to grow on a shaker for 48 h. The 200 ml of material was then transferred to the flasks containing 1000 ml of PSD broth which were incubated for 48 h at 37° C.

H. influenzae, L. acidophilus, and N. asteroides were cultivated on media specific for their growth requirements. The Sabouraud agar used for the cultivation of N. asteroides and the chocolate agar used for the cultivation of H. influenzae were prepared commercially (BBL). Tomato juice agar (BBL, prepared by instructions of manufacturer), was used for the cultivation of L. acidophilus. All other organisms were grown on blood agar plates for 24 to 48 h at 37° C.

The media used for biochemical differentiation of possible C. vaginale isolates were previously described (117).

## C. EXTRACTION OF ANTIGEN(S)

Cells were separated from the PSD broth culture by centrifuging 350 ml for one h at 13,700 x g (Sorvall RC 2-B, Newton, Connecticutt). Fifty ml of the liquid phase from inoculated diphasic media was centrifuged at 27,000 x g for 20 min to separate broth from cells and agar particles.

### 1. Cells

The cells were washed four times with 10 volumes of physiological saline (0.85%), followed by a final washing with deionized water. Subsequently, several techniques were tested in an attempt to extract C. vaginale cell wall antigens.

#### a. Ether-water extraction (107)

Cells suspended in 0.15 M NaCl were shaken with excess ether (Mallinckrodt Chemical Works, St. Louis, Missouri). After a 24 to 48 h incubation at 26° C, the aqueous phase was collected, adjusted to pH 7.0 and concentrated by lyophilization (Virtis Research Equipment, Gardiner, New York).

b. Alkali extraction (14)

Cells were suspended in 1 M NaOH (0.4 mg/2 ml) for 3 h at 37° C and then centrifuged for 10 min at 1,800 x g (Sorvall GLC-1, Newton, Connecticutt). The supernatant was dialyzed at 4° C for 48 h against two changes of deionized water at a ratio of 1:10 and concentrated by lyophilization.

c. Phenol-acetic acid-water extraction (97, 116)

Cells were suspended at a concentration of 1 mg/0.2 ml, in phenol, water, and acetic acid (2:1:0.5, w/v/v), left at 37° C overnight, centrifuged, and the supernatant was dialyzed and concentrated as described above in section b.

d. Hydrochloric acid extraction (68)

Cells were suspended in solution (0.4 mg cells/1.2 ml of 0.05 M HCl, 0.15 M NaCl), boiled for 10 min, and centrifuged at 1,800 x g for 10 min. The supernatant was adjusted to pH 7.0 and concentrated by lyophilization.

e. Autoclaving (107)

Cells were suspended in 0.15 M NaCl (0.4 mg/0.2 ml) and were autoclaved for 20 min at a pressure of 10 lb/sq in. The supernatant was obtained by centrifuging at 3,000 rpm for 10 min and then concentrated by lyophilization.

f. 10% Trichloroacetic acid (86)

Lyophilized cells were suspended in 10% trichloroacetic

acid (50 mg/2 ml) and the solution was continuously stirred overnight at 4° C. The material was centrifuged, dialyzed, and concentrated as described above in section b.

g. Lysozyme

In order to protect osmotically fragile cells from lysis and thus eliminate cytoplasmic contamination, cells were suspended in a phosphate-buffered saline, pH 7.3, with a high ionic strength (0.15 M NaCl) at a concentration of 0.1 g per 6 ml. (52). Lysozyme (Worthington Biochemical Corporation, Freehold, New Jersey) at a concentration of 0.01 g/6 ml was added and the mixture was incubated at 26° C for 1 h. The supernatant obtained by centrifuging at 13,000 x g for 20 min was dialyzed and concentrated as described above in section b. Since some workers have suggested that C. vaginale is a gram negative bacillus (3, 42, 73, 114), this procedure was also performed with the addition of the disodium salt of ethylenediamene tetraacetic acid (EDTA) (Sigma Chemical Company, St. Louis, Missouri) at a concentration of 0.12 g per 6 ml. EDTA has been reported to be necessary for the optimal release of cell wall components from typical gram negative bacteria (48).

h. Sodium chloride or water extraction

Unwashed cells (100 mg per ml) were suspended in physiological saline (0.85%) or distilled water. The cells were

dispersed by manual shaking either vigorously, so as to obtain a uniform suspension, or gently, so that clumps of cells were still visible. The suspensions were centrifuged, and the supernatant was dialyzed and concentrated as described above in section b.

2. Cultured Broth Supernatant

Two techniques were investigated when the broth was used as the source of antigen(s).

a. Acid precipitation (53)

The broth was adjusted to pH 3.7 with 4 M HCl and a precipitate was allowed to form overnight at 4° C. The precipitate was recovered by centrifugation at 7,500 x g for 10 min, resuspended in deionized water, and the pH adjusted to pH 10.5 with 4 N NaOH to dissolve the active component. Finally, the pH was readjusted to pH 6.8 with 4 M HCl.

b. Ammonium sulfate precipitation and alcohol precipitation (100)

The 72 h broth supernatant was dialyzed for 48 h against two changes of deionized water at a ratio of 1:10. The material was lyophilized and reconstituted with deionized water to a fifth of its original volume. Ammonium sulfate (51 g per 100 ml) was added gradually with constant stirring

to achieve 80% ammonium sulfate saturation. After overnight incubation at 4° C, the precipitate was collected by centrifugation of 350 ml aliquots at 9,000 x g for 1 h. The precipitate was resuspended in deionized water at a 3:1 (v/v) ratio and dialyzed for 72 h against multiple changes of phosphate buffered-saline (PBS) to remove ammonium sulfate. The material was subjected to alkaline hydrolysis at pH 11.0 for 60 min at room temperature with constant stirring. This was followed by precipitation with 5 volumes of cold absolute alcohol and resuspension in deionized water at a concentration of 0.5 g (wet wt) per ml.

The latter procedure was more critically analyzed to determine conditions specific for purification of C. vaginale antigen(s). The conditions used for precipitation were varied to determine the optimum procedure. Ammonium sulfate was added to aliquots of lyophilized PSD broth culture supernatant so that the final percent saturations of ammonium sulfate were 20, 40, 60, 80 and 100.

Similarly, the alcoholic precipitation of the broth culture supernatant, previously lyophilized and precipitated with ammonium sulfate, was further evaluated. Ethanol was added to the extract so that the final alcohol to extract ratios were: 0.13/1, 0.25/1, 0.5/1, 0.75/1, 1/1, 1.25/1, 1.75/1, 2/1, 2.5/1, 3.0/1, 5.0/1 (v/v). The extract was

adjusted to a specific pH prior to the addition of ethanol. Various pH's, that is: 5.5, 5.7, 6.0, 6.5, were utilized for each ratio tested.

Finally, each step was evaluated for its ability to purify antigen, that is, to exclude material which did not react with antisera prepared against the diphasically grown C. vaginale cells ATCC 14018. The titer before and after each step was tested by the Ouchterlony technique. The various precipitates were placed in neighboring antigen wells to detect patterns of identity, and, in addition, were further tested by placing them in wells bordered by PSD broth extract. This served to enhance the formation of precipitin bands when antigen was present in minimal concentration (26, 110).

## D. PARTIAL PURIFICATION AND SEPARATION OF ANTIGENIC COMPONENTS

Column chromatography was utilized in an attempt to further purify and to isolate C. vaginale antigenic components. The number and identity of the antigenic components were monitored using the Ouchterlony technique. The initial column eluates were monitored for protein at 280 nm and for carbohydrate by means of the Dubois procedure in which micrograms of carbohydrate were estimated by comparing the readings at 590 nm to a standard fructose curve (25).

1. Molecular Sieving Chromatography

Sephadex G75 (particle size 40-200 nm, bed volume per g dry gel: 12-15 ml), Sephadex G200 (particle size 40-400 nm, bed volume per g dry gel: 30-40 ml) and Sepharose 4B (Pharmacia Fine Chemicals, Inc., 800 Centennial Avenue, Piscataway, New Jersey, 08854) were used in these experiments. The gels were allowed to swell and/or equilibrate in the liquid to be used as eluant, that is: G75 Sephadex in PBS (pH 7.3) for 24 h, G200 Sephadex in 0.1 M Tris-HCl (pH 7.7) for 3 days, and Sepharose 4B in 0.1 M Tris-HCl (pH 7.7) overnight. After removal of the fines by suction and de-aeration under vacuum, a gel slurry consisting of a 2:1 ratio of gel to buffer was prepared. Either a small column measuring 2.6 x 40 cm or a large column measuring 10 x 100 cm was used. After removal of air bubbles from the bottom plunger, the column was filled by pouring the slurry down a rod, avoiding splashing. The bed was left to settle (5 min for G75 Sephadex and 15 min for G200 Sephadex and Sepharose 4B) and then flow started at a rate lower than that to be used in the experiments. The flow rate used during experiments was 1.9 ml/min for G75 Sephadex and 0.3 ml/min for both G200 Sephadex and Sepharose 4B. Operating pressure was kept constant by raising the outlet tube to a height slightly below the top of the column. The void volume and uniformity of packing were measured using Blue Dextran 2000 (Pharmacia) at a concentration of 5 mg per ml buffer; one ml of

dye was used for small columns and 4 ml of dye was used for large columns (126a). The small G75 Sephadex column had a bed volume of 210 ml and void volume of 65 ml. The small G200 Sephadex column had a bed volume of 210 ml and void volume of 60 ml. The large G75 Sephadex column had a bed volume of 2 liters and void volume of 800 ml. The large Sepharose 4B column had a bed volume of 800 ml and void volume of 300 ml. Sample size was varied depending upon the size of the column. The sample antigen extracts to be run on the columns contained approximately 70 mg/ml of protein as measured by the Lowry procedure (75). Sephadex G200 columns were run with either 0.5 ml or 1.5 ml of sample. Small Sephadex G75 columns were run with 2.5 ml of sample and large G75 columns with either 10 or 15 ml of sample. Five ml of sample was applied to the Sepharose 4B column. Fractions, ranging from 3 to 5 ml, were collected and subsequently combined as described in the result section, dialyzed, and tested for antigenic activity.

2. Ion-Exchange Chromatography

Anion and cation exchangers were evaluated under various conditions of ionic strength and pH. Diethylaminoethyl (DEAE) cellulose (medium mesh, 0.93 meq/g, Sigma Chemical Company, St. Louis, Missouri) and carboxymethyl (CM) cellulose (medium mesh, 0.60 meq/g, Sigma Chemical Company) were allowed to swell in distilled water overnight. Fines were removed by

suction and excess water was removed by filtering on a Buchner funnel (Whatman no. 541 filter paper). The ion exchange cellulose was regenerated and purified by alternate treatment with alkali (0.5 M NaOH), acid (0.5 M HCl), and alkali (0.5 M NaOH) for 30 min intervals with intermediate water rinses which were continued until no change could be detected in the pH of the rinse water. Prior to use, the ion exchanger was allowed to equilibrate with the respective buffer (0.1 M) to be used in the experiment. However, in those experiments in which the buffering system contained phosphate instead of chloride ions, the ion exchange cellulose was first equilibrated with a 0.1 M solution of the acidic component of the buffer, filtered and then changes of a 0.1 M solution of the starting buffer added until the desired pH was reached. After de-aeration, the columns (1.6 x 40 cm, bed volume 80 ml) were gravity packed and equilibrated with 4 times the bed volume of a 0.01 M buffer.

In initial experiments, linear molarity gradients were used to elute the material from the column. DEAE and CM columns initially equilibrated at pH 5.5, 7.0, or 8.0 were tested. The buffers used were: (1) Tris-hydrochloride at a pH 5.5 or 7.0 and (2) Tris-sodium acetate at a pH 8.0. The starting buffer was passed through the column until all non-adhering material was collected (approximately 100 ml) and then a KCl gradient was started. The maximum molarity was either

0.4 M or 0.8 M and the column was finally purged with saturated KCl. The total volume of the gradient applied was 750 ml. The sample (1.5 ml) in these initial comparative studies was obtained from the same batch of ammonium sulfate fractionated broth. The columns were monitored at 280 nm using a Beckman DB spectrophotometer.

The next series of experiments tested the effect of a stepwise pH gradient elution. DEAE columns were initially equilibrated with a 0.01 M Tris-HCl buffer. Elution was performed using a 0.01 M citric acid-$Na_2HPO_4$ buffer applied in 200 ml aliquots of decreasing pH; that is, pH 7.3, 7.0, 6.5, 5.5, 5.0, 4.5, and 2.5. Both non-lyophilized whole broth and an ammonium sulfate-alcohol extract of lyophilized broth were applied to such columns to re-evaluate the effect of lyophilization and precipitation.

In the final series of experiments, linear pH gradients were used to elute the material. Table 1 lists the alternations in the type of buffer, pH, molarity, as well as the time intervals which were used in the attempts to isolate and purify the antigens. Linear gradients were prepared using the LKB 11300 ultograd gradient mixer and small mixing chamber (1136-1) with the volume set at 0.25 ml. (LKB Instruments, 12221, Rockville, Maryland). At the end of each run, the columns were purged with the final buffer saturated with sodium chloride. In other experiments, several columns were run using the level sensor

TABLE I

Conditions Used in DEAE cellulose columns

| Equilibration of DEAE cellulose | | Conditions of before starting gradient[1] | | | Gradient conditions[2] | | |
|---|---|---|---|---|---|---|---|
| Buffer | pH | Buffer | pH | time (h)[3] | Buffer | pH and molarity range | time (h) |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 7.2 | 1-$\frac{1}{4}$ | 0.01 M Citric acid-$Na_2HPO_4$ | 7.2 →2.8 | 24 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Tris-HCl | 7.7 | 5 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 →6.0 | 24 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Tris-HCl | 7.7 | 1 | 0.01 M Tris-HCl and 0.01 M Citric acid-$Na_2HPO_4$ | 7.7 →4.0 | 8 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 | 1-$\frac{1}{4}$ | 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 →6.0 | 8 |
| 0.067 M $KH_2PO_4$-$Na_2HPO_4$ | 7.7 | 0.067 M $KH_2PO_4$-$Na_2HPO_4$ | 6.5 | 1 | 0.067 M $KH_2PO_4$-$Na_2HPO_4$ | 6.5 →5.0 | 8 |
| 0.0067 M $KH_2PO_4$-$Na_2HPO_4$ | 7.7 | 0.0067 M $KH_2PO_4$-$Na_2HPO_4$ followed by 0.0067 M $KH_2PO_4$-$Na_2HPO_4$ | 7.7<br>6.5 | 4<br>1-$\frac{3}{4}$ | 0.0067 M $KH_2PO_4$-$Na_2HPO_4$ followed by a molarity increase | 6.5 →5.0<br>0.0067 → 0.067 | 8<br>8 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 7.2 | 1-$\frac{1}{4}$ | 0.01 M Citric acid-$Na_2HPO_4$ | 7.2 →2.8 | 8 |

[1] An antigen aliquot was dialyzed against the first buffer indicated. The aliquot was applied to the column and the column was washed with the first buffer for the time indicated.

[2] The gradient was started after the times indicated for the initial conditions.

[3] Time indicates the time for which the designated buffer was run through the column.

TABLE 1 (CONTINUED)

| Equilibration | | Initial conditions | | | Gradient conditions | | |
|---|---|---|---|---|---|---|---|
| Buffer | pH | Buffer | pH | time (h) | Buffer | pH and Molarity range | time (h) |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.5 | 2 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.5 → 5.7 | 8 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 | 1-¼ | Citric acid-$Na_2HPO_4$ | 6.8 → 6.0<br>0.01 M 0.1 M | 8 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.5 | 1-¼ | Citric acid-$Na_2HPO_4$ | 6.5 → 6.0<br>0.1 M 0.05 M | 8 |
| 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 | 1-¼ | 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 → 6.0 | 8 |
| | | | | | Molarity to 0.1 M | 6.0 → | 8 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.5 | 1-¼ | 0.01 M Citric acid-$Na_2HPO_4$ | 6.5 → 6.0 | 8 |
| 0.01 M Tris-HCl | 7.7 | 0.01 M Citric acid-$Na_2HPO_4$ | 6.8 | 1-¼ | 0.01 M Citric acid $Na_2HPO_4$ | 6.5 → 6.0 | 8 |

which maintains the specific buffer mixture entering the column when absorbing material is eluting from the column. Flow rate was regulated by the LKB 12000 peristaltic pump, and for most experiments was maintained at a maximum flow rate of 1.5 ml/min. However, several columns were also run using a decreased flow rate of 0.7 ml/min. Columns were monitored simultaneously at 280 nm and 254 nm using the LKB uvicord III absorptiometer (2089) and LKB 6 channel chopper bar recorder (Typ 6520-5). Fractions were collected using the LKB ultorac fraction collector (7000) with the volume drop/siphon setting at 150 drops per tube. Fractions were combined as described in the result section, dialyzed against two changes of distilled water at a 1:10 ratio, concentrated by lyophilization, reconstituted with 0.3 ml distilled water and tested for antigenic activity by the Ouchterlony technique.

3. Affinity Chromatography

Attempts were made to couple antigenic components to CNBr-activated Sepharose 4B (Pharmacia) and to subsequently isolate antibody directed against those antigens from the coupled Sepharose (7, 17). The dry gel (3 g) was swollen in $10^{-3}$ M HCl to remove the dextran and lactose stabilizers, and resuspended in 12 ml of a pH 8.0, 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl. The sample to be applied to the column consisted of 100 mg of Peak 4 which had been obtained by DEAE ion exchange chromatography.

The sample, which contained two antigens, was dissolved in 2 ml of bicarbonate buffer, mixed with the gel, and the mixture was rotated end over end overnight at 4° C. The mixture was then filtered and the unbound material was washed away with several volumes of bicarbonate buffer. Any remaining CnBr active groups were blocked with 1 M ethanolamine at pH 8.0 for 2 h. Non-covalently bound material was removed by 5 washing cycles. Each cycle consisted of a wash at pH 4.0 (0.1 M acetate buffer containing 1 M NaCl) followed by a wash at pH 8.0 (0.1 M borate buffer containing 1 M NaCl). All washes were dialyzed, lyophilized, and tested for antigenic activity. The gel was resuspended in sodium phosphate buffer (0.1 M, pH 7.5) containing 0.5 M NaCl and a column was packed at 4° C. The antiserum to be coupled to the column was dialyzed overnight against the phosphate buffer at 4° C and was then applied to a water jacketed column (1 x 12 cm) maintained at 4° C. Fractions were collected in 1.1 ml aliquots, dialyzed, lyophilized, and tested for antibody activity against uninoculated and inoculated broth extract. After the antiserum had passed through the column, as indicated by a decrease in OD, the bound antibody was eluted by a pH 2.8, 0.5 M glycine-hydrochloric acid buffer. The eluted material was neutralized 4 h later with 1 N sodium hydroxide, dialyzed, lyophilized, reconstituted to 1 ml, and tested for antibody activity.

## E. ANTISERA

Several different C. vaginale antisera were utilized in this study. Antisera were prepared against (a) washed bacterial cells, (b) soluble antigens released into the PSD broth media during growth of the organism, and (c) immune precipitates.

### 1. Antigen Preparation

When bacterial cells were used as the immunogen, the cells were washed, killed by formalin, and the suspensions adjusted to a density equivalent to a No. 7 McFarland standard (82). Antisera were prepared against the type strain grown on blood agar plates (anti-14018 Bld) and diphasically (anti-14018 Di). Antisera were also prepared against the five reference strains of C. vaginale grown diphasically (anti-T94Di, anti-594 Di, anti-8226 Di, anti-6488 D Di, anti-6488 W Di).

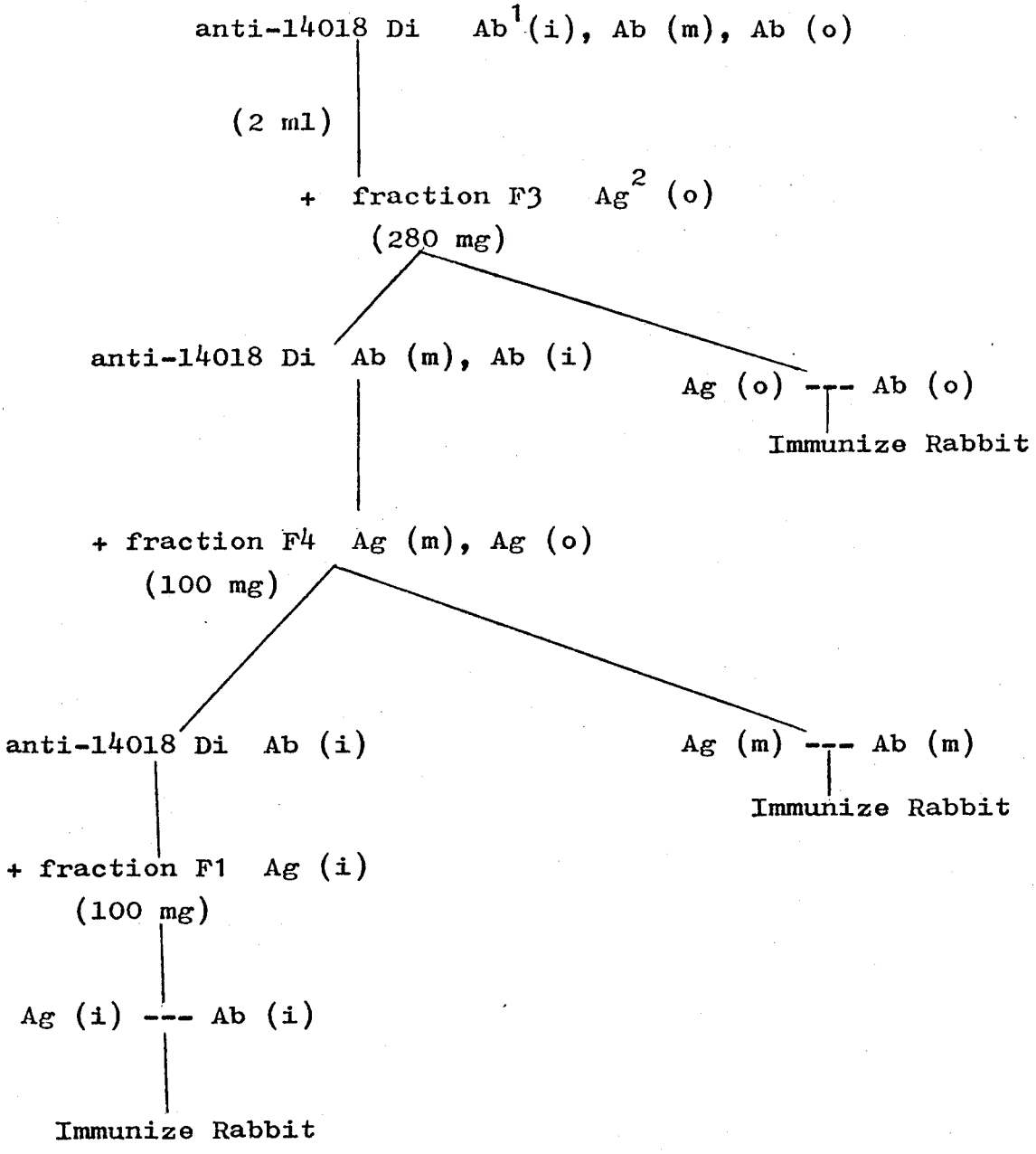
The source of C. vaginale soluble antigens was the PSD broth from a 72 h cultured of the type strain (A TCC 14018). Antisera were prepared against the soluble antigens in the broth and against each single antigen. Prior to use in the immunizations, the broth was dialyzed, lyophilized, and precipitated with ammonium sulfate as previously described (Section C 2b). The extract, reconstituted with distilled water (1 g extract per ml water), contained approximately 5-10 mg protein per 0.1 ml (75) and 20 mg carbohydrate per 0.1 ml (25). Rabbits

were immunized with either 0.4, 0.6 or 1.0 ml aliquots. These antisera were designated anti-BE.

In separate experiments, the broth extract was precipitated by alcohol and the antigens isolated by DEAE column chromatography. Fractions F1 and F3 contained isolated antigens and were used as immunogens. Fraction F1 obtained from twelve columns was combined (1.4 g) and dissolved in 5 ml of distilled water (280 mg per ml). Fraction F3 obtained from twelve columns was combined (0.54 g) and dissolved in 5 ml of distilled water (108 mg per ml). Rabbits were immunized with aliquots of either 0.4 ml or 0.6 ml. The antisera prepared against fraction 1 were designated anti-F1 and the antisera prepared against fraction F3 were designated anti-F3.

Immune precipitates were prepared by sequentially absorbing anti-14018 Di antiserum with column fractions F3, F4 and F1 (Fig. 1). Fraction F3 (280 mg) was added to anti-14018 Di antiserum (2 ml), and the mixture incubated at 45 to 50° C for 2 hr and then overnight at 4° C. After centrifugation at 20,000 rpm for 15 min at 4° C, the precipitate obtained was dissolved in 1.5 ml sterile saline and frozen. This immune precipitate was used to prepare an antiserum which was designated anti-P3. The supernatant of the absorbed anti-14018 Di antiserum was tested against fraction F3 to ensure that all the antibody activity against antigen (o) was removed. This supernatant, remaining after absorption with fraction F3, was next

Figure 1. Formation of immune precipitates used for immunization.

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1 Ab = antibody against

2 Ag = antigen

absorbed with fraction F4 (100 mg) and the precipitate collected was used to prepare an antiserum designated anti-P4. The supernatant following this absorption was absorbed with fraction F1 (100 mg) and an antiserum was prepared against this precipitate which was designated anti-P1.

2. Immunization

New Zealand white rabbits weighing 2 to 2.5 kg were used for the immunizations. The following schedule (Table 2) was used to prepare antisera against the bacterial cells: day 1, 0.1 ml of the cell suspension incorporated in 0.1 ml of Freund complete adjuvant (Difco) was injected intradermally and subcutaneously into several sites on the foot pads and back; day 7, 0.1 ml of the cell suspension incorporated in 0.1 ml of Freund complete adjuvant was injected intramuscularly into the thigh; and day 21, 1.0 ml of the cell suspension was injected intravenously into the ear. The rabbits were bled on day 28.

The schedule followed to prepare antisera against the soluble antigens and immune precipitates is described in Table 2. It is similar to that used to prepare antisera against the whole cells except that when the broth extract was used as the immunogen, injections on day 1 were given only subcutaneously on the back and injections on day 21 consisted of the extract mixed with an equal quantity of Freund complete adjuvant injected intramuscularly into the thigh. When column fractions were

TABLE 2

Immunization Schedule

| | Immunogen (amount and route) | | | |
|---|---|---|---|---|
| Day | Washed cells | PSD broth extract | Column fractions | Immune precipitates |
| 1 | 0.1 ml + 0.1 ml adjuvant SC[1] + ID[2] | 0.4, 0.6 or 1.0 ml + equal amounts adjuvant SC | 0.4 or 0.6 ml + equal amounts adjuvant SC | 0.5 ml + 0.4 ml adjuvant SC + ID |
| 7 | 0.1 ml + 0.1 ml adjuvant IM[3] | 0.4, 0.6 or 1.0 ml + equal amounts adjuvant IM | 0.4 or 0.6 ml + equal amounts adjuvant IM | 0.5 ml + 0.5 ml adjuvant IM |
| 14 | Rest | Rest | Rest | Bleeding |
| 21 | 1.0 ml IV[4] | 0.4, 0.6 or 1.0 ml + equal amounts adjuvant IM | 0.4 or 0.6 ml IV | |
| 28 | Bleeding | Bleeding | Bleeding | |

[1] SC = subcutaneous

[2] ID = intradermal

[3] IM = intramuscular

[4] IV = intravenous

used as the immunogen, injections were given only subcutaneously on day 1. The schedule used to prepare antisera against the immune precipitates also differed in that no injections were given after day 7 and the rabbits were bled on day 14.

## F. IMMUNOLOGICAL PROCEDURES

### 1. Indirect Fluorescent Antibody Staining

The indirect fluorescent antibody procedure developed for C. vaginale identification has been previously described (117). In brief, C. vaginale cells grown on blood agar plates or diphasically were fixed to slides using 95 percent ethanol for 1 min, washed in fluorescent treponemal antibody (FTA) hemagglutinating buffer (pH 7.3) (BBL), and air-dried. The smears were overlayed with the appropriate antiserum or a normal rabbit serum control and incubated in a moist chamber at 37° C for 30 min. Excess antiserum was removed by washing with two changes of FTA hemagglutinating buffer followed by a final wash with deionized water. After air-drying, the slides were overlayed with fluorescein-conjugated goat anti-rabbit globulin (BBL) and the above procedure repeated. The slides were viewed using an AO Spencer microscope equipped with an Osram HBO 200 high-pressure mercury lamp and a dark-field condenser for immersion oil. The following filter combinations were used: Corning 5970 in

combination with a yellow barrier filter or BG12 in combination with a yellow orange barrier filter. The magnification used in microscopy was 10 x ocular lens and 45 x objective lens.

2. Ouchterlony Technique

The specific details of the Ouchterlony technique have been previously described (110). In general, immunodiffusion plates were prepared by pouring 15 ml of agar onto 8.26 by 10.16 cm glass slides or 3.5 ml of agar onto 2.54 by 7.62 cm glass slides. The agar had the following composition (in grams per liter): ion agar, 10; sodium chloride, 8.5; sodium azide, 0.1; glycine, 37.5. Wells were cut in the agar so that the antiserum well measured 6 mm and the antigen well measured 3 mm. The peripheries of the wells were 5 mm apart. Reactions were allowed to proceed for up to 72 h at room temperature in a moist chamber. Slides were immersed in saline for 24 h to stop the reaction, then washed in distilled water for 24 h, and finally air-dried. Slides were stained with a 0.1% solution of naphthalene black 12 B dissolved in methanol-water-glacial acetic acid at a volume ratio of 5:5:2. Slides were destained in the above solvents at a volume ratio of 7:3:1.

The concentration of the material tested for antigenic activity varied depending upon the source and technique employed to obtain the material. In the preliminary studies in which various extraction techniques were compared, the extracts were

reconstituted to 0.1 g per ml. When column fractions were tested, 0.3 to 0.6 ml of deionized water were added depending upon the solubility of the material. Generally, the early fractions eluted from the column were white and fluffy and upon the addition of water became very viscous, probably due to a high concentration of undegraded starch; therefore, they required 0.6 ml of water to become solubilized. Two immunodiffusion patterns were routinely used to test the column fraction. Immunodiffusion systems were prepared in which the fractions were placed in adjacent wells in order to detect patterns of identity. The fractions were also tested in systems in which they were bordered by wells containing broth extract in order to enhance the reactions of antigen(s) present in low concentration and thus detect their presence (26, 110).

Sonicated cell extracts were also utilized as an antigen source. The sonication procedure used for C. vaginale cells has been previously described (110). Washed C. vaginale isolates were sonicated for a total of 30 min. Suspensions were kept cool by baths of dry ice and acetone. The disrupted cells were centrifuged at 27,000 x g for 15 min, and the supernatant lyophilized and reconstituted to a concentration of 1 mg (dry wt) per 0.1 ml of deionized water.

3. Immunoelectrophoresis

Immunoelectrophoresis of the broth extract was carried out at pH 8.6. Slides, measuring 8.26 x 10.16 cm were coated with 20 ml of melted ion agar (1.5% ion agar in 0.05 , pH 8.6 barbitol buffer). The antigen wells measured 0.2 cm and were cut 0.5 cm from the antiserum troughs which measured 0.2 x 8.5 cm. The antigen wells were filled with the broth extract or the control which consisted of normal rabbit serum with bromphenol blue added as the tracking dye. The slides were electrophoresed at 30 mamp per slide for approximately 2 h. The electrophoresis apparatus used was the model no. 3-1014 A purchased from Buchler Instruments (Fort Lee, New Jersey). The agar in the troughs was removed and the troughs were filled with anti-14018 Di antiserum or anti-normal rabbit serum. The plates were allowed to develop at room temperature in a moist chamber for 48 h and stained as described in section F2.

## G. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Various sources of C. vaginale antigens, i.e. broth extracts, column fractions of the broth extract, and saline extracts were electrophoresed. Aliquots of each preparation were layered on five gels. In order to localize the antigens, a gel representative of each antigen source was subsequently tested by immunodiffusion.

The four remaining gels were stained for protein, polysaccharide, lipid, and mucopolysaccharide.

1. Preparation of Gels

Electrophoresis was carried out in glass columns (5 x 85 mm) containing gradient gels (80, 91) composed of 7-½, 6, and 4-½% acrylamide. The pH of the sample gel was 5.5 and the pH of the running gel was 7.4 (19, 124). Table 3 lists the composition of the buffer, the solutions which were prepared fresh daily, and the stock solutions. Chemicals used in the gel preparation were purchased from Eastman Kodak Company, Rochester, New York. Table 4 summarizes the preparation of the gel layers. The first layer, consisting of 7-½% acrylamide, is pipetted and then immediately overlayed with the 6-½% gel. The two layers are immediately overlayed with water and allowed to polymerize for 30 min. The water layer is removed; and the third layer, consisting of 4-½% acrylamide, is added. Water is overlayed on the top of the gel, and it is allowed to polymerize for 30 min. The water layer is removed from the top of the 4-½% gel layer, and the sample gel is added.

The sample gel is prepared as described in Table 4, and the preparation to be electrophoresed is incorporated directly into it (50-100 mg of material and 1 g of dye into 0.25 ml of sample gel). The sample gel is photopolymerized for 45 min directly under a fluorescent light. The gels were electrophoresed

TABLE 3

Composition of Solutions Used in the Preparation and Electrophoresis of Polyacrylamide Gels

| Stock solutions | | Fresh solutions | |
|---|---|---|---|
| A | | C | |
| 1 N HCl | 48 ml | acrylamide | 4.8 g |
| Tris | 6.85 g | bisacrylamide | 0.12g |
| Temed | 0.46 ml | deionized water to | 15 ml |
| (pH 7.5) | | | |
| B | | D-1 | |
| 1 M $H_3PO_4$ | 39 ml | ammonium persulfate | |
| Tris | 4.95 g | 0.14 g per 100 ml | |
| Temed | 0.46 ml | | |
| deionized water to | 100 ml | | |
| (pH 5.5) | | | |
| E | | D-2 | |
| riboflavin | | ammonium persulfate | |
| | 4 mg/100 ml | 0.28 g per 100 ml | |

Electrode buffer

Tris-boric acid, pH 8.9
Tris (0.5 M) 60.57 g
boric acid (0.075 M) 4.63 g
deionized water to 1000 ml

TABLE 4

Procedure for Preparation of Polyacrylamide Gels

| ml/gel | % gel | Formula | Amounts/12 gels |
|---|---|---|---|
| 1.0 | 7-½ | 2 parts A | 5.1 ml |
| | | 2 parts C | 5.1 ml |
| | | 4 parts D-1 | 10.2 ml |
| 0.2 | 6 | 2 parts A | 2.4 ml |
| | | 2 parts C and $H_2O$ | 1.8 ml C |
| | | (3:1 ratio) | 0.6 ml $H_2O$ |
| | | 4 parts D-1 | 4.8 ml |
| Gel 30 min | | | |
| 0.2 | 4-½ | 2 parts A | 1.6 ml |
| | | 2 parts C and $H_2O$ | 0.9 ml C |
| | | (9:7 ratio) | 0.7 ml $H_2O$ |
| | | 4 parts D-2 | 3.2 ml |
| Gel 30 min | | | |
| 0.25 | Sample gel | 1 part B | 1.6 ml |
| | 4-½ | 2 parts C and $H_2O$ | 1.8 ml C |
| | | (9:7 ratio) | 1.4 ml $H_2O$ |
| | | 1 part E | 1.6 ml |
| Gel 45 min | | | |

at 5 mamp per gel for approximately 2 h on a Canalco (Rockville, Maryland) electrophoresis apparatus consisting of a model 300B constant rate source and a model 1200 chamber.

2. Staining

The gels were concurrently stained for protein, carbohydrate, and lipid. Various staining procedures were first compared for their sensitivity and selectivity. The protein stains employed were Coomasie brilliant blue R250 (Colab Laboratories Inc., Glenwood, Illinois), nigrosin (Coleman and Bell Company, Norwood, Ohio), and amido black 10B (Allied Chemical, Morriston, New Jersey). The carbohydrate stains employed were Schiff's reagent (126c) containing basic fuchsin (Harleco, Philadelphia, Pennsylvania) and alpha naphthol (67e). The procedures followed for gel fixation and staining with nigrosin, amido black 10B, and alpha naphthol were as described by Kwapinski (67e). The procedures described by Fairbanks (37) were used when staining with Schiff's reagent and Coomassie brilliant blue.

Gels were also stained with Alcian blue 8 Gx (Canalco, Inc., Rockville, Maryland) which reacts selectively with mucopolysaccharides and glycoproteins (119). After electrophoresis, the gels were immediately immersed in 1% alcian blue dissolved in 7% acetic acid. After 1 h, gels were destained in 7% acetic acid. The acetic acid was changed at hourly intervals,

and a total of 5 or 6 washes was usually sufficient.

Lipids were selectively stained by sudan black (67e). Gels were immersed in 0.7% sudan black B (Fisher Scientific Co., Fair Lawn, New Jersey) dissolved in ethylene glycol. The gels were destained in ethylene glycol for a total time of 2 to 3 h. The ethylene glycol was discarded and fresh ethylene glycol added at hourly intervals. The gels were expanded to original size by immersion in distilled water.

3. Immunodiffusion

Immediately after electrophoresis, the gels were placed on a 3.25 x 4 inch (i.26 x 10.16 cm) glass slide, and 15 ml of cooled (45° C) immunodiffusion agar was added to the slide and allowed to solidify. A trough (0.2 x 8.5 cm) was cut 5 cm from the gel and filled with C. vaginale ATCC 14018 antiserum. The plates were allowed to develop at room temperature for 72 h and stained as previously described in section F2.

## H. PHYSICAL AND CHEMICAL CHARACTERIZATION OF ANTIGENIC DETERMINANTS

1. Heat Stability

The broth extract was heated to 60° C or 100° C for 30 min. Dilutions of the heated and unheated extract were tested for antigenic activity using anti-14018 Di, anti-F1, anti-F3, and anti-P4 antisera.

2. Extractability with Chloroform-Methanol

A 2 g sample of the broth extract was extracted with 30 ml of chloroform:methanol (2:1, v/v). The extraction was performed for 3 h, followed by a second extraction with 10 ml of chloroform:methanol (2:1, v/v) for 30 min. Dilutions of the non-extractable material were tested for antigenic activity using anti-14018 Di, anti-F1, anti-F3 and anti-P4 antisera. An aliquot of the broth extract, not treated with chloroform: methanol, served as the control.

3. Effect of Treatment with Protease

An enzite agarose protease column (Miles Laboratories, Inc., Kankakee, Illinois) was used to test the lability of the C. vaginale antigens to a broad spectrum protease obtained from Streptomyces griseus. Broth extract (100 mg dissolved in 1 ml of a 0.01 M Tris-hydrochloric acid buffer, pH 7.1) was applied to a 3 ml protease column (1.4 x 2.3 cm). After incubation at room temperature for 24 h, the extract was removed from the column, adjusted to 5 ml with deionized water, dialyzed, and a 0.1 ml aliquot was diluted 1 to 10. This solution was read spectrophotometrically at 280 nm and total OD units calculated. The column eluate was subsequently lyophilized, weighed, reconstituted to 0.2 ml with deionized water, and tested for antigenic activity by the Ouchterlony technique. A control sample (100 mg of broth extract per 1 ml of a 0.01 M Tris-

hydrochloric acid buffer, pH 7.1) left at room temperature for 24 h was similarly tested.

4. Effect of Treatment with Urea

Samples consisting of 1 g of broth extract were dissolved in 2 ml of either 4 M urea or 2 M urea. A control sample was dissolved in 2 ml of deionized water. Samples were incubated at room temperature overnight, dialyzed, and titered against anti-14018 Di antiserum by the Ouchterlony technique.

5. Effect of Periodate Oxidation

The effect of periodate on the C. vaginale antigens present in the broth extract was tested by dissolving 200 mg of the broth extract in 5 ml of 0.1 M lithium periodate buffer, pH 7.2 (46). Buffered lithium periodate was prepared by dissolving 2.28 gm of $HIO_4 \cdot 2\ H_2O$ in 50 ml of deionized water; 10 ml of 0.5 M $H_3PO_4$ was added followed by the addition of 20 ml of carbonate-free 1 N LiOH. The solution was diluted to 100 ml. Two-hundred milligrams of broth extract dissolved in 5 ml of distilled water served as a control. After 72 h at room temperature, 0.1 ml of 50% glucose solution was added to decompose the excess periodate (46). The control and test samples were dialyzed, the volumes adjusted to 5 ml, and aliquots from each were diluted and read spectrophotometrically at 280 nm. The samples were lyophilized, reconstituted with deionized water to give a

final concentration of 0.5 mg per ml, and dilutions tested for antigenic activity by the Ouchterlony technique.

6. Ability to Bind to Con A-Sepharose

Con A-Sepharose (Pharmacia) was equilibrated with a pH 6.8, 0.1 M acetate buffer containing $10^{-3}$ M $MgCl_2$, and $10^{-3}$ M $MnCl_2$; deaerated; and packed by gravity into a column measuring 1 x 30 cm. The broth extract was dialyzed overnight against the equilibrating buffer. A 2 ml sample containing 280 mg of broth extract was applied to the column and unbound material was washed from the column using the equilibrating buffer. The flow rate of the column was maintained at 0.3 ml per min throughout the experiment. After 12 h, the material bound to the column was eluated using the equilibrating buffer containing 1 M NaCl; and after 24 h, any material remaining on the column was eluted using the equilibrating buffer containing 0.2 M α-methylglucose (24). The fractions, except for those eluted with the buffer containing α-methylglucose, were tested for carbohydrate using the Dubois technique (25). Subsequently, the fractions were pooled, as described in the Results section, dialyzed, lyophilized, and tested for antigenic activity by the Ouchterlony technique.

# III. RESULTS

## A. EXTRACTION OF ANTIGEN

### 1. Selection of Antigen Source and Extraction Procedure

Extraction procedures that had been used to obtain cell wall antigens from other organisms were applied to C. vaginale. The methods were evaluated for their ability to yield the maximum amount of immunologically reactive antigens. The antiserum used to test the extracts for antigenic activity was anti-14018 Di, which was prepared against cells of the type strain grown diphasically. The Ouchterlony technique was utilized to estimate the number of antigenic components released. Table 5 summarizes the results obtained with the procedures tested. Generally, only a minimal amount of antigen was detected in any of the various cell extracts. In these experiments, all materials (e.g., dialysate, supernatant, etc.) were tested for antigenic activity before being discarded. The extraction procedures employing alkali, hydrochloric acid, or autoclaving did not yield detectable antigen. Variable results were obtained with the extraction techniques employing trichloroacetic acid

TABLE 5

Extraction of *C. vaginale* Antigens[1]

| Source | Technique | Antigen detection[2] |
|---|---|---|
| Whole cells (washed) | Ether-water | +[3] |
| " | 1 N NaOH | -[4] |
| " | Phenol-acetic acid-water | +[5] |
| " | 0.05 N HCl | - |
| " | Autoclaving | - |
| " | 10% TCA | ± |
| " | Lysozyme | + |
| " | Lysozyme - EDTA | + |
| Water or saline extract | ---- | + |
| Cultured diphasic media supernatant | 4 N HCl | + |
| " | 80% $(NH_4)_2SO_4$-5 vol $C_2H_5OH$ | + |
| Cultured PSD broth supernatant | 4 N HCl | ± |
| " | 80% $(NH_4)_2SO_4$-5 vol $C_2H_5OH$ | + |

[1] Each experiment was performed twice

[2] By the Ouchterlony technique

[3] + = At least one antigen detected by the Ouchterlony technique in both tests.

[4] - = No antigens detected by the Ouchterlony technique in both tests

[5] ± = Antigen detected (usually weak precipitin bands) in only one of the two tests

or phenol, acetic acid and water. Extraction of the washed cells with ether or lysozyme, with or without EDTA, did consistantly release antigen but not in large yields, as noted by the weak precipitin bands formed.

Material released from the cells by preliminary washings with 0.9% sodium chloride or deionized water also possessed antigenic activity. Usually 3 antigenic components were detected in the early washes. As the number of washings increased, the quantity and types of antigenic components decreased, but no specific washing procedure could be correlated with the appearance of an unique antigenic component.

A significant amount of antigenic activity was detected in the lyophilized supernatants of C. vaginale diphasic and PSD broth cultures. Therefore, experiments were performed utilizing precipitation with ammonium sulfate and ethanol or hydrochloric acid to determine a method which could be used to concentrate and possibly purify the antigens present in the broth extract. A combination of ammonium sulfate and ethanol was finally utilized since this consistently yielded significant amounts of antigen from both the diphasic and PSD broths. In contrast, antigen could be detected in material precipitated by hydrochloric acid from the diphasic broth but antigen was not always detected when the PSD broth precipitates were tested. PSD broth was preferred as a growth media because of its simpler chemical composition.

Preliminary experiments were also done in which mild alkaline conditions were utilized in an attempt to purify the soluble antigens. Similar techniques have been used by others in the purification of polysaccharide antigens released into broth cultures during bacterial growth (100). Ouchterlony analyses revealed that alkaline treated extract reacted at a titer of 1:2, while untreated extract reacted at a titer of 1:9. Therefore, alkaline treatment was not used.

As Fig. 2 indicates, three precipitin bands formed when the broth extract was tested for antigenic activity. The antigens present in the broth extract were designated according to their location with respect to the antibody well; i.e., the antigen which formed the precipitin band, closest to the antibody well, was designated antigen (i); the antigen which formed the outer precipitin band was designated antigen (o), and the antigen which formed the middle precipitin band was designated antigen (m). When this broth extract was placed in wells adjacent to the water or saline washes or extracts, bands of identity appeared to form between them.

Experiments indicated that the broth extracts when compared to the cell extracts, provided a larger yield of antigen. Also, the procedures used to obtain the antigen present in the broth supernatant were milder than the extraction procedures which had to be employed for whole cells. Therefore, the broth supernatant was chosen as the source of antigen.

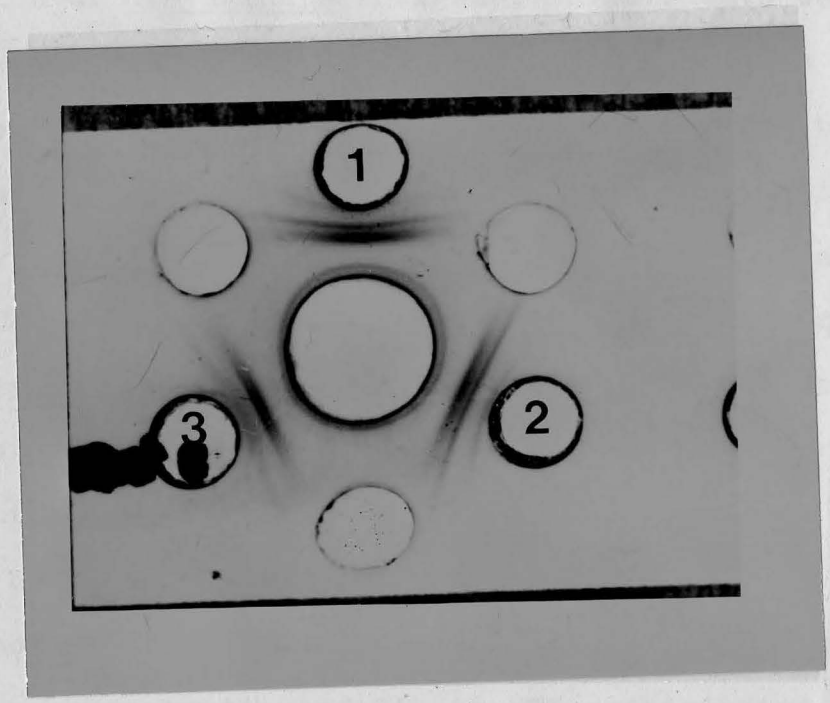



Figure 2. Demonstration of three C. vaginale antigens present in the broth extract of C. vaginale strain 14018. Anti-14018 Di antiserum (center well) was reacted against the broth extract of C. vaginale strain 14018 (wells 1, 2, and 3).

2. Appraisal of Extraction Procedure for Broth Antigens

The yield following each step of the concentration procedures was determined in order to establish the optimum conditions for isolation of C. vaginale antigens. Experiments were performed in which maltose was substituted for starch as the main nutrient source of the PSD broth since maltose could be removed by dialysis. However, the yield of antigen was much lower. Ouchterlony analyses revealed a positive reaction at a 1:9 dilution of the starch broth extract, whereas the maltose broth extract only reacted at a 1:2 dilution. Therefore, starch was used as the main carbon source in all subsequent experiments.

The optimum percentage of ammonium sulfate to be used was determined by varying the concentration of salt added to the broth extract. Ouchterlony analysis revealed that ammonium sulfate at a concentration of 75% to 80% precipitated all the antigenic components. No specific concentration of ammonium sulfate was found which precipitated only one antigenic component.

The volume of ethanol used for precipitation and the pH of the extract were also varied in an attempt to selectively precipitate the antigenic components and to ensure the precipitation of all the antigenic components. None of the various combinations tested resulted in the selective precipitation of any one of the antigenic components. A ratio of 5 volumes of

ethanol to 1 volume of extract was necessary to ensure precipitation of all the antigenic material.

The results from a typical experiment employing ammonium sulfate precipitation followed by alcohol precipitation are presented in Table 6. Each step did result in partial purification of the antigenic components. Initially, C. vaginale was grown in 3 liters of PSD broth. The supernatant before concentration did not produce a precipitin band. The supernatant after dialysis and lyophilization, weighed 29.5 g. When the supernatant was tested against anti-14018 Di antiserum, C. vaginale antigen activity was detected at a dilution of 1:16. Therefore, the total units of activity were 472. After 80% ammonium sulfate precipitation, the weight of the material was 8 g and C. vaginale antigen activity was detected at a dilution of 1:32. Therefore, the total units of activity were 256. This step yielded a 60% recovery and a greater than 32 fold purification of C. vaginale antigens. Five volumes cold ethanol precipitation yielded 7.5 g of material and C. vaginale antigen activity was detected at a dilution of 1:32; therefore, 250 units of activity were recovered. This step yielded a 51% recovery and a greater than 32 fold purification. Although the use of ethanol did not result in significant purification of the material, this step has been included in further experiments since alcohol precipitation has the advantage of eliminating

TABLE 6

Scheme for Partial Purification of C. vaginale Antigens

| Purification step | Weight (g) | Dilution of Antigen detected[1] | Total units of activity[2] | % Recovery[3] | Fold Purification |
|---|---|---|---|---|---|
| Supernatant | | NR[4] | | | 1 |
| Dialyzed, lyophilized supernatant[5] | 29.5 | 16 | 472 | | 16 |
| 80% $(NH_4)_2SO_4$ precipitate | 8 | 32 | 256 | 60 | 32 |
| 5 vol cold ethanol $C_2H_5OH$ precipitate | 7.5 | 32 | 250 | 51 | 32 |

[1] Antigen: 1 g extract per ml deionized water

[2] Total units of activity = dilution of antigen x wt (g)

[3] % Recovery = $\frac{\text{total units of activity of precipitated material} \times 100}{\text{total units of activity of dialyzed, lyophilized supernatant}}$

[4] NR = non reactive

[5] PSD broth culture supernatant before lyophilization produced no precipitin band

salts remaining from prior precipitations (103). Such salts are often difficult to completely eliminate by dialysis alone.

## B. PARTIAL PURIFICATION AND ISOLATION OF ANTIGENIC COMPONENTS

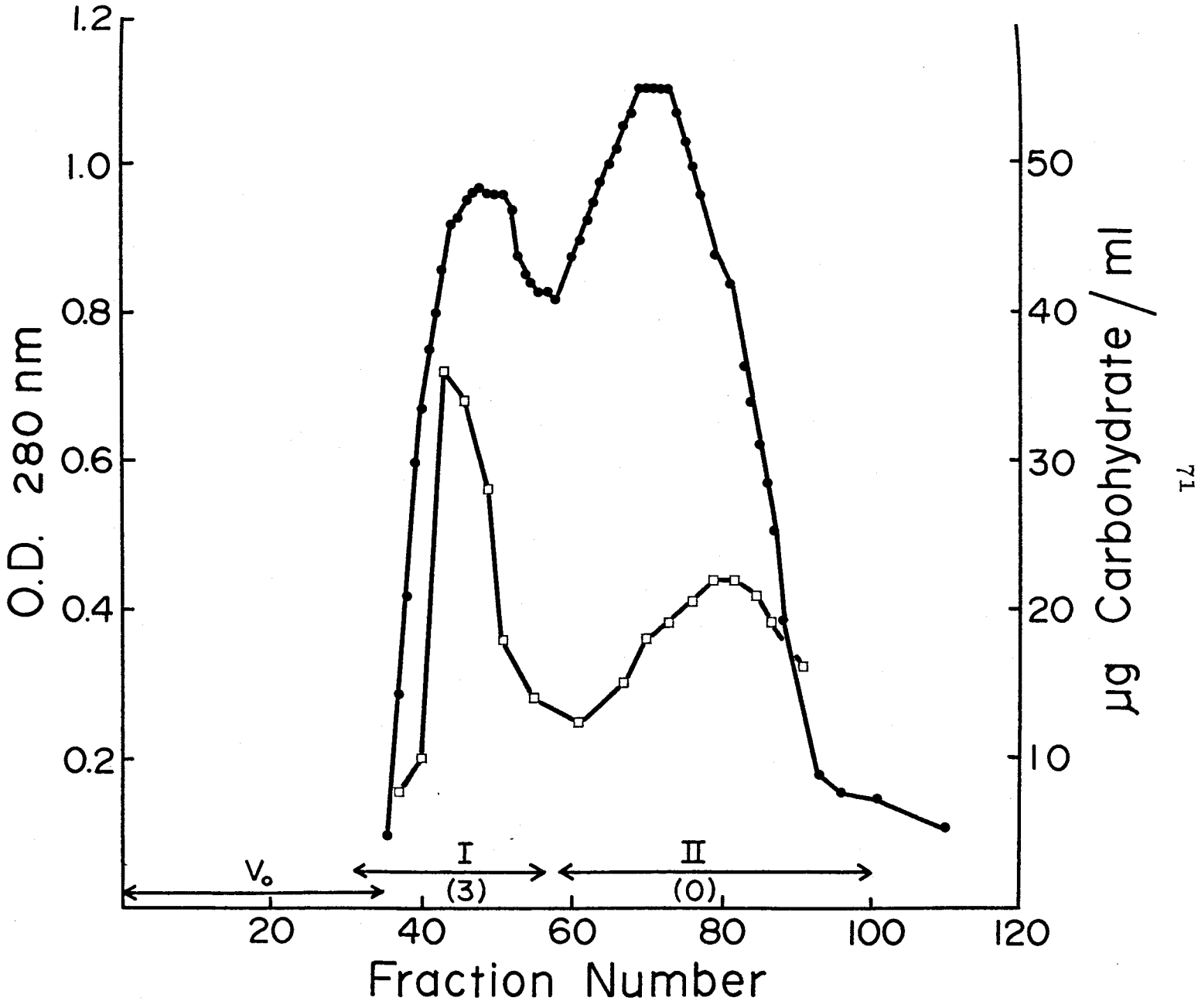
### 1. Molecular Sieving Chromatography

C. vaginale antigenic components present in the broth extract previously precipitated by ammonium sulfate and ethanol could be partially purified by passage through Sephadex G75 columns; however, no isolation was evident (Fig. 3). Two distinct peaks were observed, both of which contained protein and carbohydrate. The fractions from peaks I and II were separately pooled, concentrated, and tested for antigenic activity by the Ouchterlony technique using antisera prepared against C. vaginale whole cells (anti-14018 Di) and the broth extract (anti-BE). Only peak I contained demonstrable antigen activity.

Additional Sephadex experiments (not shown here) were performed in the attempt to obtain a greater yield of antigen. A greater yield was achieved by increasing (a) the column size ten-fold, (b) the sample size four-fold, and (c) the flow rate from 1.5 ml to 1.9 ml per min. Also, experiments were performed in which antigens precipitated from the broth extract by ammonium sulfate but not precipitated by ethanol, were applied to G75

Legend G75 Sephadex

Figure 3. Chromatography of the broth extract of C. vaginale on G75 Sephadex. A 2.5 ml sample of the C. vaginale broth extract was added to a 2.6 x 40 cm column of G75 Sephadex and eluted with a PBS, pH 7.3, , at a flow rate of 1.9 ml/min. The optical density at 280 nm (•) and carbohydrate content (⊖) was determined as described in the Materials and Methods section D. The bar and number designation indicate the manner in which fractions were pooled for further analyses. The parenthesis contain the number of antigens detected in the pooled fractions. $V_o$ indicates the void volume of the column.


1.2
1.0
0.8
0.6
0.4
0.2
O.D. 280 nm
50
40
30
20
10
µg Carbohydrate / ml
V₀
I
(3)
II
(0)
20
40
60
80
100
120
Fraction Number

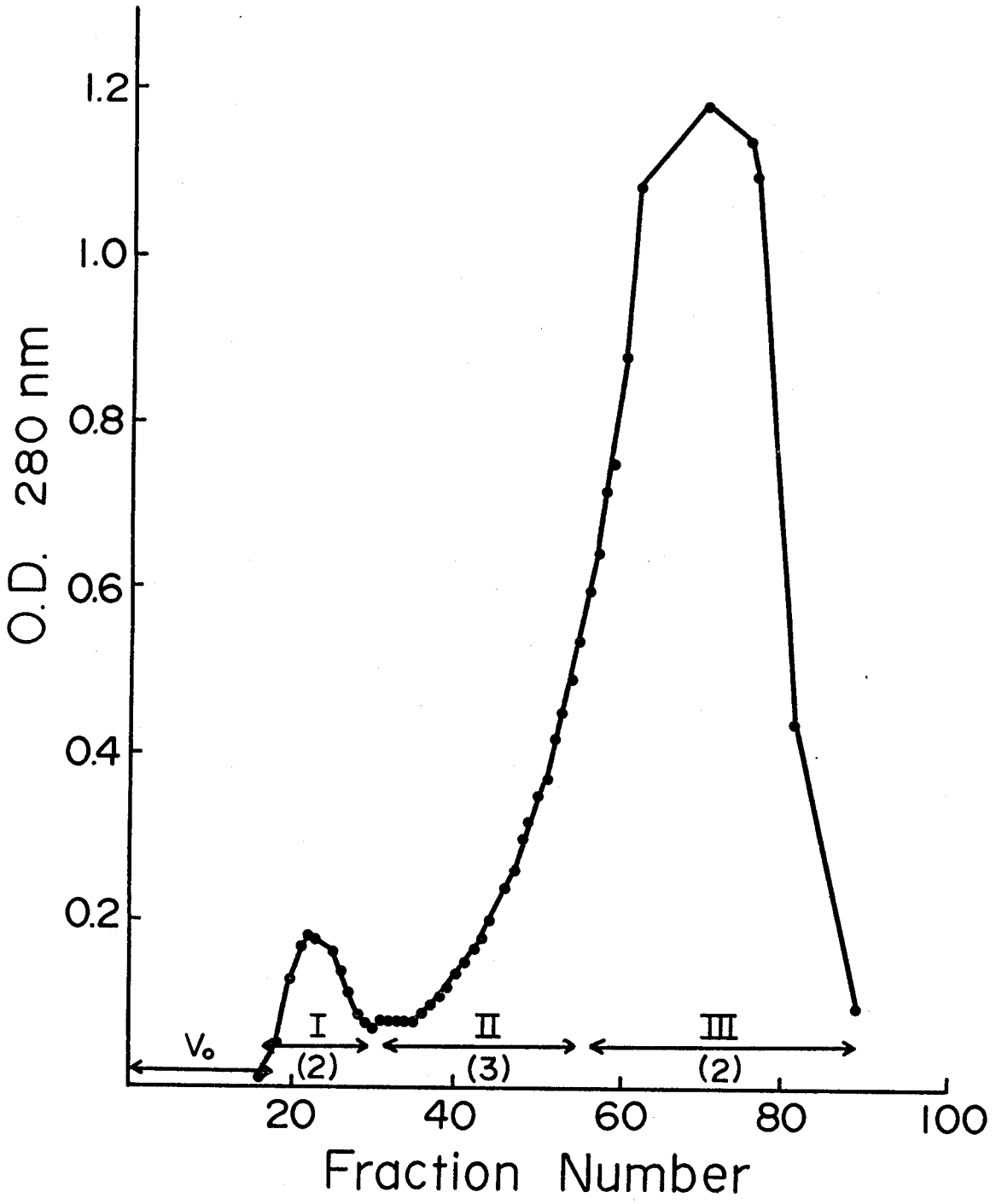
Sephadex columns. In this case, the sample was eluted from the column in one peak and neither purification nor isolation of the antigens was achieved.

Because the antigenic components were eluted near the void volume of the G75 Sephadex columns, and no apparent isolation was obtained, Sephadex G200 columns were next used in an attempt to obtain greater purification and/or isolation of the antigenic components. As shown in Fig. 4, neither purification nor isolation of the C. vaginale antigenic components was obtained when the samples were run on Sephadex G200 columns. The antigenic components were detected in both peaks. The second peak was arbitrarily divided into two fractions on the basis of color (a yellow color was visible in tubes 57 through 90), but this also was not useful in separating the components. The majority of C. vaginale antigenic material was retained by the column.

All the antigenic material was retained by a Sepharose 4B column. Fractions were pooled as indicated in Fig. 5 and concentrated. Subsequently, protein and carbohydrate determinations were performed on each of these fractions (Fig. 5 and Table 7). It appears that the first peak is composed mainly of carbohydrate; while the second peak contained significant quantities of both carbohydrate and protein. Immunodiffusion experiments revealed that Ag (i) appeared to be present in fraction A collected near the void volume, Ag(m) was detected

Legend G200 Sephadex

Figure 4. Chromatography of the broth extract of C. vaginale on G200 Sephadex. A 1.5 ml sample of the C. vaginale broth extract was added to a 2.6 x 40 cm column of G200 Sephadex and eluted with a 0.1 M Tris-HCl buffer, pH 7.7, at a flow rate of 0.3 ml/min. The optical density at 280 nm (•) was determined. The bar and number designated indicate the manner in which fractions were pooled for further analyses. The parenthesis contain the number of antigens detected in the fractions. $V_o$ indicates the void volume of the column.


1.2
1.0
0.8
0.6
0.4
0.2
O.D. 280 nm
Vo
I
(2)
II
(3)
III
(2)
20
40
60
80
100
Fraction Number

Legend Sepharose 4B

Figure 5. Chromatography of the broth extract of C. vaginale on Sepharose 4B. A 5 ml sample of the C. vaginale broth extract was applied to a 10 x 100 cm column of Sepharose 4B and eluted with a 0.1 M Tris-HCl buffer, pH 7.7, at a flow rate of 0.3 ml/min. The optical density at 280 nm (•) was determined. The bar and number designation indicate the manner in which fractions were pooled for further analyses. The parenthesis contain the number of antigens detected in the fractions. $V_o$ indicates the void volume of the column.

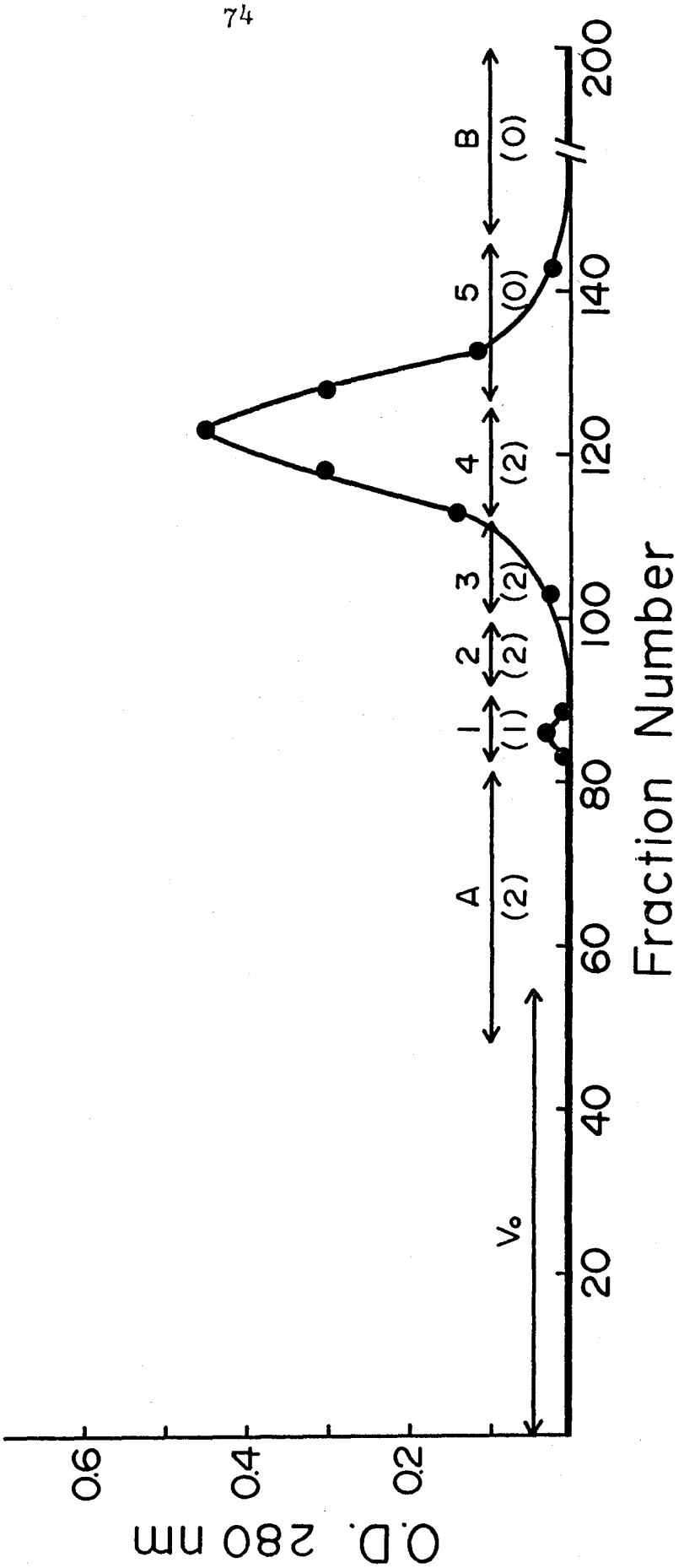

O.D. 280 nm
0.6
0.4
0.2
20
40
60
80
100
120
140
200
Fraction Number
$V_o$
A
(2)
1
(1)
2
(2)
3
(2)
4
(2)
5
(0)
B
(0)

TABLE 7

Analysis of Sepharose 4B Column Fractions

| Fraction Number | Volume[2] | Antigens Detected | Total protein OD units | Total carbohydrate[1] OD units/ml |
|---|---|---|---|---|
| Void | 300 | None | 0 | ND[3] |
| A | 210 | (i), (o) | 6.4 | 241.5 |
| 1 | 65 | (o) | 9.3 | 364.5 |
| 2 | 55 | (m), (o) | 11.4 | 265.5 |
| 3 | 80 | (m), (o) | 18.3 | 151.2 |
| 4 | 90 | (m), (o) | 62.1 | 372.6 |
| 5 | 100 | None | 33.6 | 165.6 |

[1] Dubois procedure (25)

[2] Volumes concentrated to 1 ml for chemical determinations

[3] ND = not done

in fractions 2, 3, and 4; and Ag (o) was detected in all fractions except number 5. This data indicates: (a) antigenic components were not completely separated by the Sepharose 4B column, (b) Ag (o) appears to have a range of sizes, and (c) antigenic components were detected in fractions containing both protein and carbohydrate, and were also detected in fractions composed predominantly of carbohydrate with minimal amounts of protein.

2. Ion-Exchange Chromatography

Experiments were done in which broth extract was examined by immunoelectrophoresis to determine whether a charge difference could be detected among the 3 antigenic components. In comparing the patterns of the broth extract to a normal serum control, it appeared that one antigenic component has a mobility similar to albumin (which has a pI of 5.5) while the other two antigenic components remained at or near the antigen well (and so are comparable to immunoglobulin which has a pI of 8.0).

Anion and cation exchange resins were next utilized under various conditions in an attempt to isolate the antigenic components. Carboxymethyl cellulose columns run at pH 5.5, 7.0 or 8.0 consistently bound one antigenic component. This antigenic component, when compared to the original extract, showed a reaction of identity with the inner (i) precipitin band. Antigen (i) was released from the column by an increasing salt

gradient and appeared early in the elution pattern. The components which were not bound showed bands of identity with the middle (m) precipitin band and outer (o) precipitin band when compared to the original extract.

Diethylaminoethyl (DEAE) cellulose also bound antigenic components, and the initial pH to which DEAE cellulose was equilibrated markedly affected this phenomenon. DEAE cellulose columns initially equilibrated to pH 5.5 bound antigenic components (m) and (o) and a portion of (i). When the DEAE cellulose was initially equilibrated to pH 7.0, none of the antigenic components bound strongly. DEAE cellulose equilibrated to pH 8.0 consistently bound two antigenic components (m) and (o), while antigenic component (i) was detected in the void volume. However, when the extract was dialyzed against a pH 8.0 buffer at $4^{\circ}$ C for 48 h, an apparent decrease in antigenic activity was observed; that is, the extract produced weaker precipitin bands. DEAE cellulose columns were next run in which the cellulose was equilibrated to pH 7.7 but the sample was dialyzed overnight against a pH 7.2 buffer. This procedure minimized antigen degradation and still allowed 2 of the 3 antigenic components to bind to the column.

The first attempts to selectively elute the two bound antigenic components from the DEAE cellulose columns were performed using increasing linear salt gradients. Salt gradients did not selectively elute the antigenic components. A step-wise

pH gradient was next tested in an attempt to selectively elute the antigenic components. Using a DEAE-column initially equilibrated to pH 7.7, a sample equilibrated to pH 7.2, and a stepwise pH gradient, partial separation of the antigenic components was achieved. Antigenic component (i) was detected in the void volume of the column, a fraction of Ag (o) was detected in material eluted by a pH 6.5 buffer, and a pH 6.0 buffer eluted material which contained additional Ag (o) and also Ag (m).

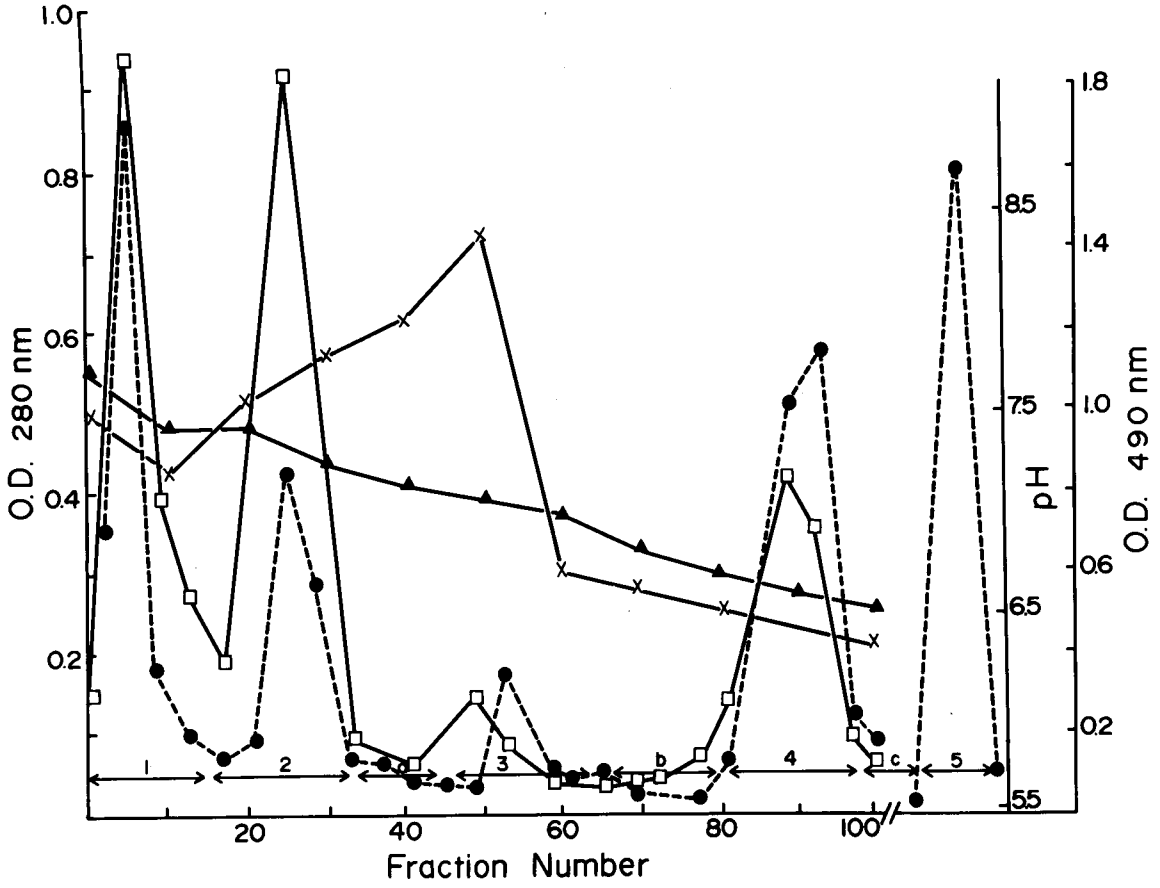
Experiments were next devised in an attempt to (a) circumvent the lyophilization and precipitation of the broth extract and (b) concomitantly, to separate antigenic components (m) and (o). Cultured PSD broth supernatant was applied to a DEAE cellulose column equilibrated to pH 7.7 with 0.01 M Tris-HCl buffer. The broth was neither lyophilized nor precipitated by ammonium sulfate or ethanol but was dialyzed against a 0.01 M citric acid-$Na_2HPO_4$ buffer prior to its application on the column. When the broth extract was not precipitated, 0.8 g of the sample could be applied to the column. In contrast, when the broth extract was partially purified by lyophilization and precipitation, 2.75 g of the extract could be applied to a DEAE cellulose column without column overload occurring. Cultured broth supernatant, which was lyophilized but not precipitated with either ammonium sulfate or ethanol, was applied to a DEAE cellulose column. When fractions obtained when the nonprecipitated material was run on DEAE cellulose were pooled and tested by the

Ouchterlony technique, only antigenic component (i) was detected in the void volume of the column and antigenic components (m) and (o) could not be selectively eluted. Therefore, partial purification of the cultured broth supernatant by lyophilization and precipitation improved subsequent isolation of the antigenic components by column chromatography and all subsequent experiments reported in this dissertation utilized 80% ammonium sulfate -5 vol cold ethanol precipitated PSD broth antigen extracts.

Linear pH gradients were next tested in an attempt to improve the elution profile (Table 1). Linear pH gradients were varied with respect to the type and pH of the buffer and the time and volume of the gradient used. The eluants were examined for antigenic activity. The best separation of antigenic components was achieved when the initial conditions were as follows: (a) the DEAE cellulose was initially equilibrated to pH 7.7 with a 0.01 M Tris-HCl buffer and (b) the sample was equilibrated to pH 7.2 by dialysis against a 0.01 M citric acid-$Na_2HPO_4$ buffer overnight at 4° C. Antigenic component (i) was washed from the column by the equilibrating buffer (0.01 M citric acid-$Na_2HPO_4$, pH 7.2) at a flow rate of 1.5 ml per min. After 70 min, a linear pH gradient ranging from pH 7.2, 0.01 M citric acid-$Na_2HPO_4$ buffer to pH 2.8, 0.01 M citric acid-$Na_2HPO_4$ buffer over a 24 h time interval was started, and antigenic components (m) and (o) were eluted from the column (Fig. 6). Fig. 7 indicates the separation achieved. Antigen (i) was found in peak 1, antigen (m) found in peak 4 and

Legend

Figure 6. Chromatography of the broth extract of C. vaginale on DEAE cellulose. A 5.5 ml sample of the C. vaginale broth extract was added to a 40 x 1.6 cm column of DEAE cellulose and eluted with a pH linear gradient at a flow rate of 1.5 ml/min. The optical density at 280 nm (•), carbohydrate content at 590 nm (□), and pH of the fractions eluted either in the absence of 2 M urea (x) or in the presence of 2 M urea (▲) was determined as described in the Materials and Methods section. The column was purged with the final buffer saturated with NaCl. The bar and letter or number designation indicate the manner in which fractions were pooled for further analysos.


1.0
0.8
0.6
0.4
0.2
O.D. 280 nm
1.8
1.4
1.0
0.6
0.2
O.D. 490 nm
8.5
7.5
6.5
5.5
pH
1
2
3
a
b
4
c
5
20
40
60
80
100
Fraction Number

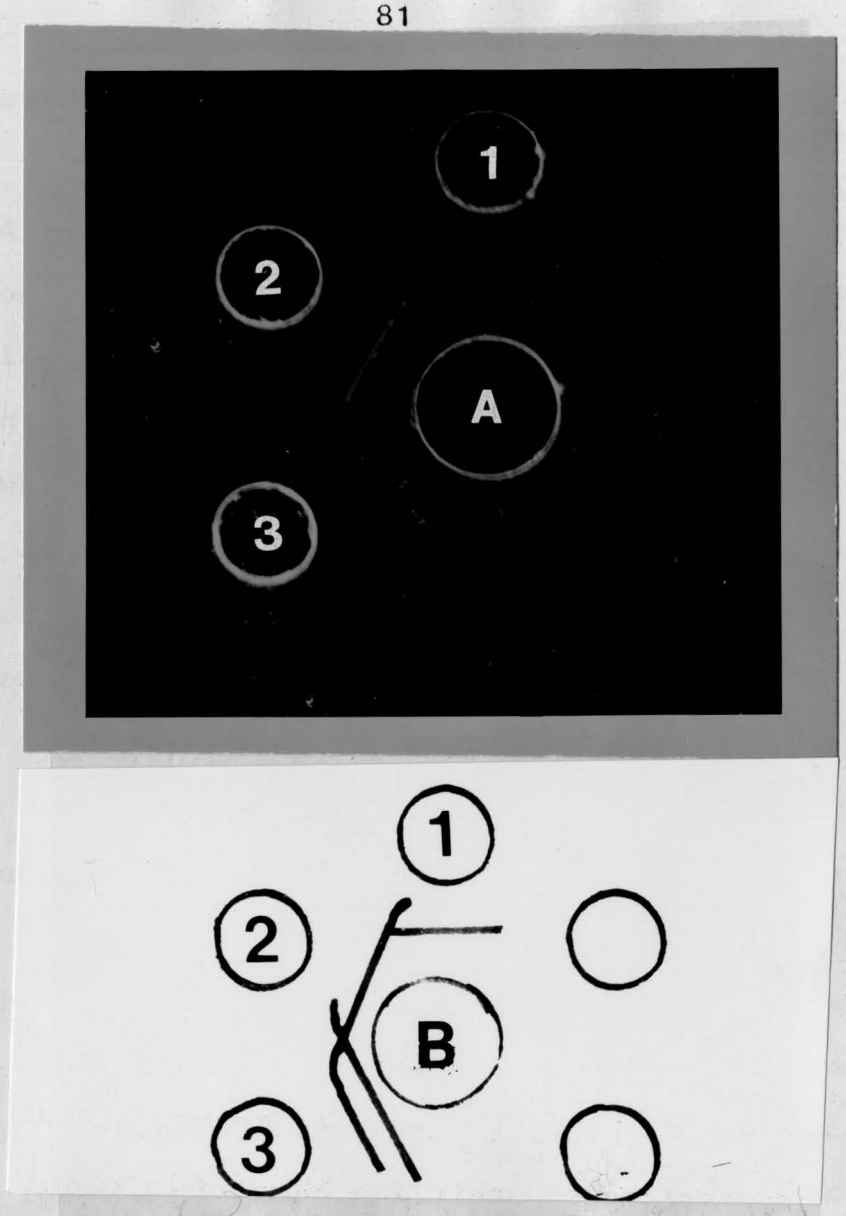



Figure 7. Demonstration of the C. vaginale broth extract antigens isolated by DEAE cellulose column chromatography. Anti-14018 Di antiserum (A, center well) was reacted against the DEAE cellulose column fractions F1 (well 1), F3 (well 2), anf F4 (well 3). B, Line drawing of A.

antigen (o) was found in peaks 3 and 4. Chemical determinations indicated that the carbohydrate peaks coincided with the protein peaks as depicted in Fig. 6.

Although varied conditions of pH, flow rate, time intervals and/or molarity of buffers were utilized, no improvement in the separation of antigenic components (m) and (o) was noted. For example, when phosphate buffers ($NaH_2PO_4$-$Na_2HPO_4$) were employed, a portion of antigenic component (o) was not bound to the column and antigenic components (m) and (o), which did bind to the column, could not be selectively eluted.

When 2 M urea was added to the buffers, the separation of antigenic components (m) and (o) was not improved, but the pH of the elution profile was altered. As Fig. 6 indicates, the pH of the eluted fractions did not rise above pH 7.3 and the pH gradient became apparent at tube 20. Therefore, it appears that urea inhibited the rise in pH under the conditions employed in these experiments; this rise in pH seems to be necessary to obtain at least a partial separation of the antigenic components (m) and (o).

3. Affinity Chromatography

Affinity chromatography was next examined for its ability to separate antigenic components (m) and (o). Attempts were made to selectively bind antigenic component (m) or (o) to CNBr Sepharose. The source of the antigenic components was fraction

F4 obtained from DEAE cellulose columns run under conditions described in Fig. 6. The sample contained a total of 60 OD units and weighed 100 mg. Following addition of the sample, it was found that the material not bound to the column weighed 20 mg after dialysis and contained a total of 21.25 OD units. Therefore, between 66% to 80% of the material appeared to be bound to the column. C. vaginale antigenic components were not detected in the unbound material nor in any of the washes used to remove non-covalently bound material. These results suggest that either: (a) both antigens bound to the column and, therefore, contain protein, or (b) the conditions employed in these experiments resulted in denaturation of the antigens. In any case, the technique could not be used to obtain the isolated (m) and (o) antigenic components.

Attempts were next made to separate the antigenic components (m) and (o) by preparing affinity columns on which antibody directed against antigen (o) was bound. Anti-F3 antiserum directed against antigenic component (o) was first passed through a column to which antigen (o) had presumably bound, in order to concentrate the anti-(o) antibodies. Passage of the anti-F3 antiserum through the column removed antibodies directed against the growth medium but did not remove antibodies directed against C. vaginale antigenic components. Therefore, the column did not absorb and concentrate antibodies directed against C. vaginale. This data suggests that CNBr-Sepharose cannot readily be used to further separate the antigenic components (m) and (o).

4. Final Scheme Selected for Isolation of C. vaginale Antigens

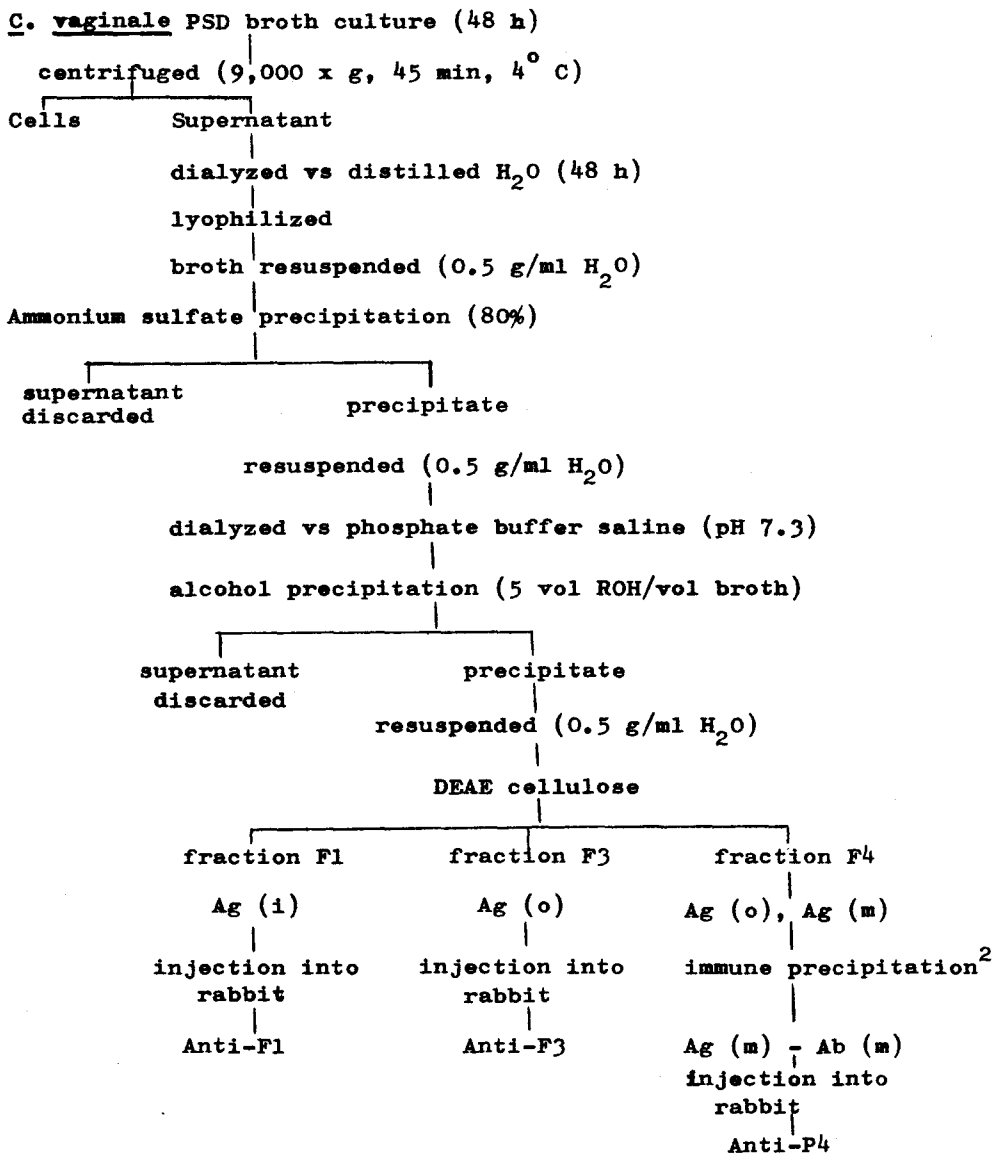
Figure 8 summarizes the final scheme chosen for the isolation of the antigens released by C. vaginale into the broth extract. Broth culture supernatants are dialyzed, lyophilized, and precipitated by ammonium sulfate. This is followed by precipitation with ethanol and the partially purified and concentrated material is then applied to a DEAE cellulose column. Antigenic component (i) is isolated in fraction F1. Antigenic component (o) is isolated in fraction F3. These separated antigenic components were subsequently used to immunize rabbits in an attempt to prepare monospecific anti-(i) and anti-(o) antisera. Antigenic component (m) is found in DEAE fraction F4 along with antigenic component (o). Antigenic component (m) is precipitated with absorbed anti-14018 Di-i, m antiserum which contains antibodies directed against (m) and (i) (further discussion in Section E. 1. of Materials and Methods). Antibodies to (m) are subsequently prepared by immunization of a rabbit with the resultant immune precipitates.

## C. EVALUATION OF THE SPECIFICITY OF ANTISERA

### 1. Antisera Prepared Against Whole Cells and Broth Extract

Previous studies have shown that the anti-14018 Di antiserum prepared against whole cells of C. vaginale ATCC strain

Figure 8. Procedure for separation of antigenic components for use in preparation of specific antisera.




[1]conditions described in Materials and Methods, section B2
[2]conditions described in Results, section E1 and Figure 5

14018 reacts with all C. vaginale isolates tested by the indirect fluorescent antibody and by the Ouchterlony techniques (110, 117). The specificity of this antiserum was further tested by reacting it against heterologous bacteria chosen on the basis of morphology and site of infection. Anti-14018 Di antiserum did not produce a significant reaction with other possible related organisms; i.e. Lactobacillus acidophilus, Nocardia asteroides, Hemophilus influenzae, Actinomyces bovis, Streptococcus mutans nor with other species of Corynebacterium, i.e. C. diptheriae, C. hofmannii, C. xerosis, C. cervicis. Anti-14018 Di antiserum produced three precipitin bands with the homologous broth extract and at least one precipitin band with sonicated extracts and broth extracts of 15 C. vaginale isolates tested. This antiserum was used in our initial search for a source of antigen and to evaluate various isolation procedures.

The extraction studies indicated that the broth extract (BE) could be used as a source of large quantities of antigen. However, it was first necessary to prove that the antigens present in the BE were identical to those found on whole cells. This was done by using 2 distinct approaches. First, antisera were prepared against BE antigens. The reactivity of the anti-BE antiserum was compared to that of anti-14018 Di antiserum in order to demonstrate that the whole cells and BE antigens induce the formation of similar antibodies. Secondly, cross adsorption studies were done to demonstrate that whole cells

could be used to completely remove the antibody activity in anti-BE and anti-14018 Di antisera.

Anti-BE antiserum gave five precipitin bands when reacted against extracts of broth inoculated with C. vaginale and two precipitin bands with extracts of uninoculated broth (Fig. 9A). Anti-BE antiserum absorbed with proteose peptone number 3 no longer reacted with uninoculated broth extract and showed three precipitin bands when tested against extracts of C. vaginale inoculated media (Fig. 9B). The precipitin bands formed when peptone-absorbed anti-BE antiserum was reacted against C. vaginale broth extracts appeared to coalesce with the precipitin bands formed when anti-14018 Di antiserum was tested in adjacent agar wells against the same antigen preparation. In addition, these two antisera produced similar precipitin reaction patterns against each of the organisms utilized in this study.

Later studies did demonstrate some minor differences in the reaction patterns of these two antisera when they were tested against antigens isolated from DEAE cellulose columns. When anti-14018 Di and peptone-absorbed anti-BE antisera were tested against column fractions F1, F2, and F3, similar immunodiffusion patterns developed against the C. vaginale antigens (Fig. 7 and 10). Both antisera produced one precipitin band with fractions F1 and F3, and two precipitin bands with fraction F4. With both antisera, the precipitin band formed with fraction F3 coalesced with one of the precipitin bands formed with fraction F4,

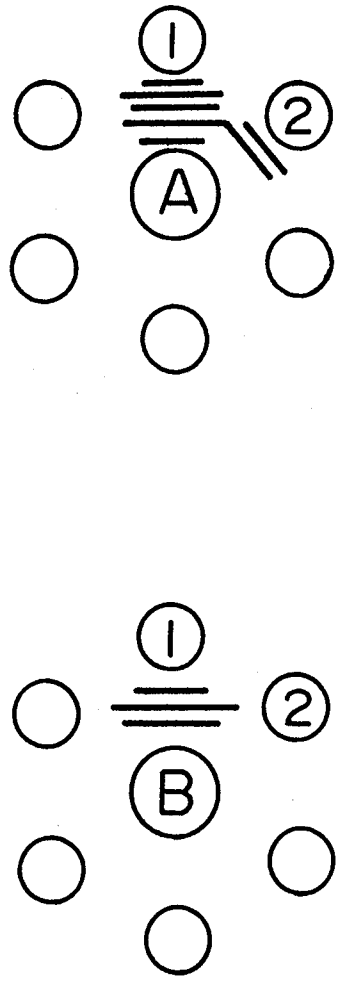



Figure 9. Representation of the reactions which occurred when anti-BE and absorbed anti-BE antisera were tested against an extract of broth inoculated with C. vaginale and with an extract of uninoculated broth extract. Anti-BE (A, center well) and peptone-absorbed anti-BE (B, conter well) were reacted against an inoculated broth extract (well 1) and an uninoculated broth extract (well 2).

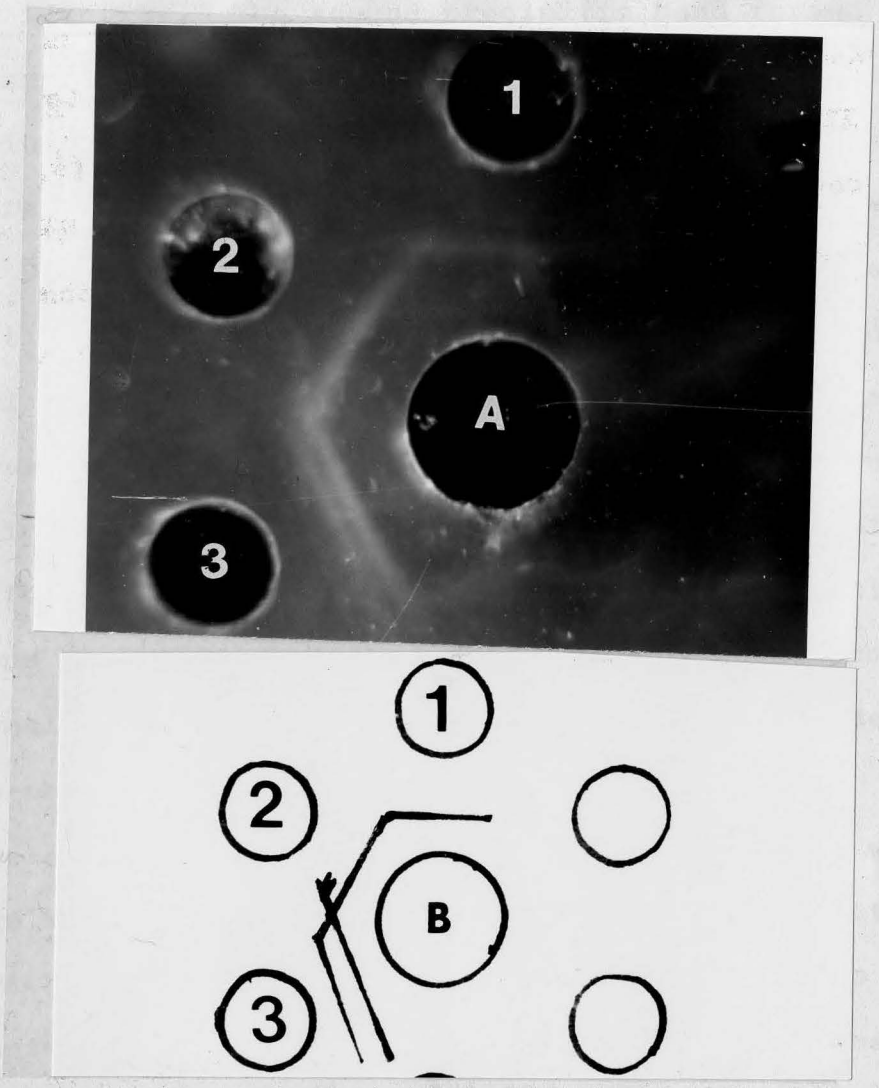



Figure 10. Demonstration of the reactions which occurred when peptone absorbed anti-BE antiserum was tested against C. vaginale broth extract antigens isolated by column chromatography. Peptone absorbed anti-BE (A, center well) was reacted against the DEAE column fractions F1 (well 1), F3 (well 2), and F4 (well 3). B, Line drawing of A.

indicating identity. The second precipitin band formed with both antisera against fraction F4 crossed the precipitin band formed against fraction F3 indicating non-identity. The antisera differed slightly, since the band which developed when anti-14018 Di antiserum was tested against fraction F3 showed a spur (reaction of partial identity) over the band formed against fraction F1 (Fig. 7). In contrast, a reaction of complete identity was seen when peptone-absorbed anti-BE antiserum was tested against these same fractions (Fig. 10). This data suggested that both antisera detect a common determinant present in fractions F1 and F3, but that anti-14018 Di antiserum detects an additional determinant in fraction F3 which is not detected by peptone-absorbed anti-BE antiserum.

The relatedness of the whole cell and BE antigens was further evaluated by absorbing the anti-14018 Di and anti-BE antisera with column fractions in an attempt to prepare monospecific antisera. When anti-14018 Di antiserum was absorbed with column fractions, antisera specific for each antigen were prepared. These antisera were designated as follows: anti-14018 Di-i reacted only with antigen (i) present in fraction F1, anti-14018 Di-o reacted only with antigen (o) present in fractions F3 and F4, and anti-14018 Di-m reacted only with antigen (m) present in fraction F4. Since anti-14018 Di antiserum could be made monospecific for each component by absorption with column fractions from BE, this suggests (a) that each

antigen possess an unique determinant and (b) that the antigens present in the BE are similar, if not identical, to those found on the whole cells.

Anti-BE antiserum was not as readily absorbed with column fractions because prior absorption of the antiserum with proteose peptone number 3, to remove antibodies directed against the growth media, increased the viscosity of the antiserum. After two absorptions of peptone-absorbed anti-BE antiserum with fraction F1 (0.224 g/ml) the antiserum still reacted with fraction F1 and the viscosity of the antiserum prohibited further absorptions. Therefore, this approach to preparing antisera monospecific for either antigen (m) or (o) was prohibited. In addition, when peptone-absorbed anti-BE antiserum was absorbed with fraction F3, precipitin bands still formed against fractions F1 and F4 which suggests that the antigens present in fractions F1 and F4 possess unique determinants not shared by the antigen in fraction F3. Further absorption of this antiserum with either fraction F1 or F4 was prohibited by the viscosity of the antiserum.

In summary, the experiments utilizing anti-14018 Di and anti-BE antisera suggest the following: (a) antigens present in the broth extract detected by anti-14018 Di and peptone-absorbed anti-BE antisera are similar, if not identical, to those present on the whole cells, (b) antigens (i), (m) and (o) each possess an unique antigenic determinant, and (c) antigens

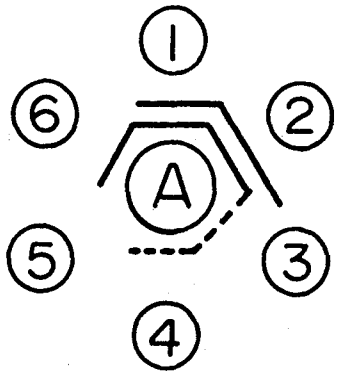
(i) and (o) share a common determinant.

2. Antisera Prepared Against Isolated Antigens

Antisera were prepared against antigens isolated by column chromatography. These antisera were subsequently used to test for the presence of each antigen on 15 C. vaginale isolates. Fraction F1 from several DEAE cellulose columns was used to prepare antisera directed against antigen (i). These antisera were designated anti-F1. Fraction F3 from several DEAE cellulose columns was used to prepare antisera directed against antigen o. These antisera were designated anti-F3.

The specificity of the prepared antisera was assessed using the Ouchterlony and indirect fluorescent antibody techniques. Anti-F1 antiserum at a 1:4 dilution produced a significant fluorescent reaction (2-3+) with C. vaginale ATCC 14018. Normal rabbit serum at a 1:4 dilution did not fluoresce. The titer of anti-F1 antiserum was 20 when tested against the type strain (ATCC 14018). Anti-F3 antiserum could not be shown to produce a significant fluorescent reaction with the type strain and was not utilized in further fluorescent studies.

Using the Ouchterlony technique, anti-F1 and anti-F3 antisera were first examined for antibodies directed against the bacteria and/or growth media (Fig. 11 and 12). Anti-F1 antiserum formed two precipitin bands against an uninoculated broth extract (Fig. 11A). Anti-F3 antiserum formed three precipitin bands against inoculated broth extract and two precipitin bands



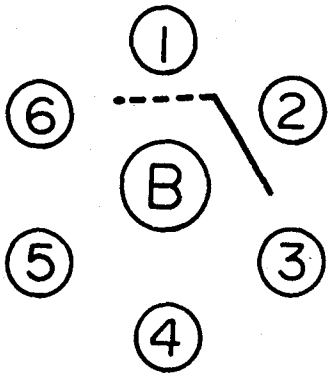




Figure 11. Representation of the reactions which occurred when anti-F1 and absorbed anti F1 (anti-F1-i) were reacted against DEAE column fractions, an extract of PSD broth inoculated with C. vaginale and with an extract of uninoculated PSD broth. Anti-F1 (A, center well) and absorbed anti-F1-i (B, center well) were reacted against an inoculated PSD broth extract (well 1), column fractions F1 (well 2), F3 (well 3), and F4 (well 4 and an extract of uninoculated PSD broth (well 6). Well 5 was left empty.

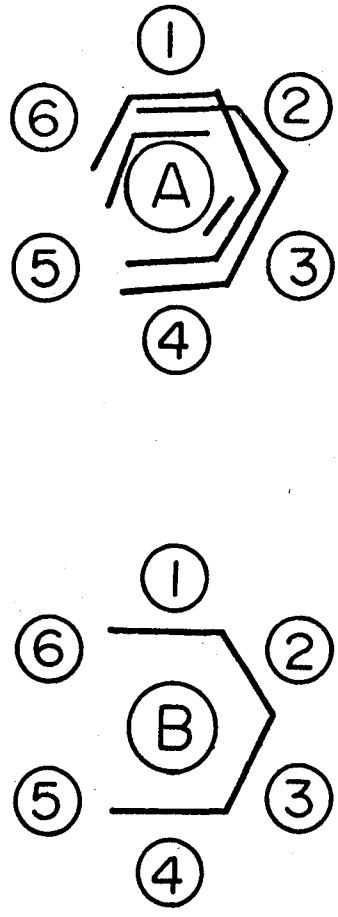



Figure 12. Representation of the reactions which occurred when anti-F3 and absorbed anti F3 (anti-F3-o) were reacted against DEAE column fractions, an extract of PSD broth inoculated with C. vaginale and with an extract of uninoculated PSD broth. Anti-F3 (A, center well) and absorbed anti-F3-o (B, center well) were reacted against an inoculated PSD broth extract (well 1), column fractions F1 (well 2). F3 (well 3), and F4 (well 4) and an extract of uninoculated PSD broth (well 6). Well 5 was left empty.

against an uninoculated broth extract (Fig. 12A). Each of the two antisera were absorbed with proteose peptone number 3 to remove antibodies directed against the growth media. (Fig. 11B and 12B)

The peptone-absorbed antisera were tested against the column fractions F1, F2 and F3. Peptone-absorbed anti-F1 antiserum produced one precipitin band with F1 and no precipitin bands with either F3 or F4. Therefore, peptone-absorbed anti-F1 antiserum appears to be specific for antigen (i) present in fraction F1. Peptone-absorbed anti-F3 antiserum produced a single precipitin band with fraction F3 (containing antigen o), with fraction F4 (containing antigens o and m), and with fraction F1 (containing antigen i). Subsequently, peptone-absorbed anti-F3 antiserum was also absorbed with fraction F1 until no reaction was detected with fraction F1. Aliquots of anti-F3 antiserum absorbed with peptone and anti-F3 antiserum absorbed with peptone and fraction F1 were tested by the Ouchterlony technique against dilutions of fraction F3. The peptone-absorbed anti-F3 antiserum, which had not been absorbed with fraction F1, produced a precipitin band with a 1:3 dilution of fraction F3. In contrast, peptone-absorbed anti-F3 antiserum, absorbed with both peptone and fraction F1, reacted only with undiluted fraction F3. This data suggests that a common determinant is shared by antigen (i) present in fraction F1, and antigen (o) present in fraction F3, but both antigens also possess an unique determinant.

3. Antisera Prepared Against Immune Precipitates

In order to obtain a monospecific antisera against antigen (m), the anti-m antibodies present in anti-14018 Di antiserum were selectively precipitated with antigen (m) present in column fraction F4 (as described in section E. 1. of the Materials and Methods). The resulting immune precipitates were used to produce an antiserum designated anti-P4. The immune precipitates produced when fraction F1, containing antigen (i), was reacted with anti-14018 Di antiserum, and the immune precipitates produced when fraction F3 was reacted with anti-14018 Di antiserum, were also used to immunize rabbits in an attempt to produce additional monospecific antisera, anti-P1 and anti-P3, respectively.

Using the Ouchterlony technique, the antisera were first examined for antibodies directed against C. vaginale and/or the growth media. Anti-P4 antiserum formed two precipitin bands against the inoculated broth extract and one precipitin band against the uninoculated broth extract (Fig. 13A). Anti-P4 antiserum was absorbed with proteose peptone number 3 (0.5 g per ml) to remove antibodies directed against the growth media. When this peptone-absorbed anti-P4 antiserum was tested against the column fractions (F1, F3, and F4) precipitin bands formed against each fraction but the reactions with fractions F1 and F3 were weak (Fig. 13B). In order to produce an antiserum specific for antigen (m), anti-P4 antiserum was further absorbed

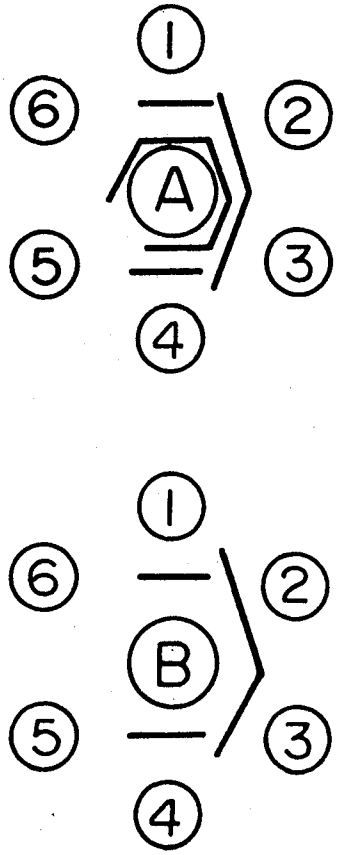



Figure 13. Representation of the reactions which occurred when anti-P4 and absorbed anti-P4 (anti-P4-m) were reacted against DEAE column fractions, an extract of broth inoculated with C. vaginale and with an extract of uninoculated broth. Anti-P4 (A, center well) and absorbed anti-P4-m (B, center well) were reacted against an inoculated broth extract (well 1), column fractions F1 (well 2), F3 (well 3), and F4 (well 4) and an extract of uninoculated broth (well 6). Well 5 was left empty.

with fraction F3. Anti-P4 antiserum absorbed with both proteose peptone and with fraction F3, then reacted only with fraction F4.

Immunization with immune precipitates containing antigens (i) and (o) did not yield antisera which could be used in further studies. Anti-P1 antiserum formed only a weak precipitin band with fraction F1, containing antigen (i). The animal injected with immune precipitates containing antigen (o) became ill shortly after the first injection and died shortly after the second injection. Sera was collected from the animal post mortem and no precipitin bands were visible when the sera was tested against the inoculated broth extract.

Monospecific (absorbed) anti-BE, anti-F1, anti-F3 and anti-P4 antisera were selected for use in further studies to determine the relative distribution of antigens (i), (m), and (o) in the C. vaginale isolates. The specificity of these antisera are summarized in Table 8. In order to compare the reactivity of these antisera, they were tested against various dilutions of the broth extract (Table 9*). Anti-BE-i, m, o antiserum reacted with BE at a dilution of 1:22. Anti-14018 Di and anti-P4-m antisera reacted with BE at a dilution of 1:16. Anti-F3-o antiserum reacted with BE at a dilution of 1:6. Anti-F1-i antiserum reacted with BE at a dilution of 1:2.

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*Anti-14018 Di antiserum was also included in the experiments since preliminary experiments had indicated that it was strongly reactive against C. vaginale soluble antigens.

TABLE 8

Specificity of Selected Antisera Prepared Against Soluble C. vaginale Antigens

| Antisera | Reaction before absorption | Absorping material | Designation after absorption |
|---|---|---|---|
| anti-BE | i, m, o growth media | proteose peptone | anti-BE-i, m, o |
| anti-F1 | i, growth media | proteose peptone | anti-F1-i |
| anti-F3 | o, i, growth media | proteose peptone, F1 | anti-F3-o |
| anti-P4 | m, o, i, growth media | proteose peptone, F3 | anti-P4-m |

TABLE 9

Reactivity of Selected C. vaginale Antisera with the C. vaginale Broth Extract as Detected by the Ouchterlony Technique

| Antisera | Dilution of antigen detected* |
|---|---|
| Anti-Be-i, m, o | 22 |
| Anti-14018 Di | 16 |
| Anti-P4-m | 16 |
| Anti-F3-o | 6 |
| Anti-F1-i | 2 |

*Broth extract: 0.5 g per ml deionized water

Anti-F1 antiserum, when tested against whole cells of the type strain by the indirect fluorescent antibody technique, did produce a significant fluorescence. In contrast, anti-BE, anti-F3 and anti-P4 antisera did not give a significant fluorescent reaction. Therefore, anti-F1 antiserum was evaluated in further studies using both the indirect fluorescent antibody technique and the Ouchterlony technique.

4. Antisera Prepared Against Reference Strains

In later studies, the antisera prepared against the reference strains (T94 Di, 594 Di, 8226 Di, 6488DDi, and 6488W Di) were reacted against the isolated antigens present in the column fractions (F1, F3, and F4). Two of the five antisera, anti-T94 Di and 594 Di, reacted with all three C. vaginale antigenic components (i), (m), and (o). Three of the antisera (anti-8226 Di, anti-6488D Di, and anti-6488W Di) reacted with two C. vaginale antigenic components, (o) and (i).

## D. DETECTION OF A COMMON C. vaginale ANTIGEN

### 1. Antisera Prepared Against Soluble Antigens

Anti-BE, anti-F1, anti-F3 and anti-P4 antisera were evaluated for their ability to react with C. vaginale isolates. When the indirect fluorescent antibody technique was employed, anti-F1 antiserum produced a significant fluorescence with 4 of the 6

reference strains and 4 of the 9 C. vaginale clinical isolates tested (Table 10). Anti-F1 antiserum did not react with possible related organisms including L. acidophilus, H. influenzae, N. asteroides, S. mutans and various species of Corynebacteria, i.e., C. diptheriae, C. xerosis, C. hofmanii, and C. cervicis. The control, which consisted of normal rabbit serum at a 1:4 dilution, did not produce a significant fluorescent reaction with any of the C. vaginale isolates tested. Anti-BE, anti-F3 and anti-P4 antisera did not produce a significant fluorescent reaction with any of the organisms tested.

Using the Ouchterlony technique, anti-F1, anti-F3, anti-P4 and anti-BE antisera were tested against each of the fifteen C. vaginale isolates. The antisera were absorbed as indicated in Table 8 to eliminate non-specific antibodies. The number of isolates reacting with each antisera varied (Tables 11 and 12). Anti-BE-i, m, o antiserum produced at least one precipitin band with all the C. vaginale isolates tested when the source of antigen was either the broth or sonicated extracts. Anti-F1-i antiserum reacted only with the type strain (14018) when the broth or sonicated extracts were tested. Anti-F3-o antiserum reacted with 2 of the 15 C. vaginale isolates tested when the sonicated extracts were tested (Table 11); however, when the broth extracts were tested, all of the 6 reference strains and 3 of the 9 clinical isolates formed precipitin bands (Table 12). Anti-P4-m antiserum reacted with all of the C. vaginale isolates

TABLE 10

Indirect Fluorescent Antibody Reactions of *C. vaginale* Isolates and Anti-Fl[a] Antiserum and Goat Anti-Rabbit Conjugate[b]

| Organism<br>Reference strains | anti-Fl antiserum | NRS[c] |
|---|---|---|
| 14018 | 2-3[d] | 0-1 |
| T94 | 3-4 | 0-1 |
| 594 | 2-3 | 0-1 |
| 8226 | 2-3 | 0-1 |
| 6488D | 0-1 | 0-1 |
| 6488W | 1-2 | 0-1 |
| Clinical isolates | | |
| 359 | 3 | 0-1 |
| 1544 | 2-3 | 0-1 |
| 6234 | 2-3 | 0-1 |
| 8315 | 2-3 | 0-1 |
| V28 | 0-1 | 0-1 |
| V44 | 1-2 | 0-1 |
| 144 | 0-1 | 0-1 |
| 1575 | 1-2 | 0-1 |
| 1637 | 0-1 | 0-1 |
| 8372 | 0-1 | 0-1 |

[a] Anti-Fl used at a 1:4 dilution
[b] Goat anti-rabbit conjugate used at a 1:20 dilution
[c] Abbreviations: NRS, normal rabbit serum
[d] Fluorescent staining intensity rated in degrees from 0 to 4+ with 4+ being maximum intensity

TABLE 11

Immunodiffusion Reactions of Sonicated Extracts of *C. vaginale* Isolates with Antisera Prepared Against Soluble Antigens of *C. vaginale*

| Organism | Antisera | | | |
|---|---|---|---|---|
| | anti-Be-i, m, o | anti-F1-i | anti-F3-o | anti-P4-m |
| Reference strains | | | | |
| 14018 | +[2] | + | + | + |
| 594 | + | - | + | + |
| T94 | + | - | + | + |
| 8226 | + | - | - | + |
| 6488D | + | - | - | + |
| 6488W | + | - | - | + |
| Clinical isolates | | | | |
| V28 | + | - | - | + |
| V44 | + | - | - | + |
| 144 | + | - | - | + |
| 359 | + | - | - | + |
| 1544 | + | - | - | + |
| 1575 | + | - | - | + |
| 1637 | + | - | - | + |
| 6234 | + | - | - | + |
| 8315 | + | - | - | + |

[1] Sonicated extracts prepared as described in Materials and Methods section F2.

[2] + = at least one precipitin band formed

[3] - = no precipitin band formed

TABLE 12

Immunodiffusion Reactions of Broth Extracts of C. vaginale Isolates with Antisera Prepared Against Soluble Antigens of C. vaginale

| Organism | Antisera | | | |
|---|---|---|---|---|
| | anti-BE-i, m, o | anti-F1-i | anti-F3-o | anti-P4-m |
| Reference strains | | | | |
| 14018 | +[2] | + | + | + |
| 594 | + | -[3] | + | + |
| T94 | + | - | + | + |
| 8226 | + | - | + | + |
| 6488D | + | - | + | + |
| 6488W | + | - | + | + |
| Clinical isolates | | | | |
| V28 | + | - | + | + |
| V44 | + | - | + | + |
| 144 | + | - | + | + |
| 359 | + | - | - | + |
| 1544 | + | - | - | + |
| 1575 | + | - | - | + |
| 1637 | + | - | - | + |
| 6234 | + | - | - | + |
| 8315 | + | - | - | + |

[1] Broth extract prepared as described in section C2b
[2] + = at least one precipitin band formed
[3] - = no precipitin band formed

when either the sonicated or broth extracts were tested. Anti-BE-i, m, o, anti-F1-i, anti-F3-o and anti-P4-m antisera did not form precipitin bands with the media controls nor with possible related organisms tested, i.e., L. acidophilus, C. diptheriae, C. cervicis, and the vaginal diptheroid (8372).

2. Antisera Prepared Against Reference Strains

The antisera prepared against the washed cells of five reference strains were tested for their ability to react with C. vaginale isolates. Previously, the indirect fluorescent antibody technique was used to evaluate these antisera (117). All reference strains fluoresced brightly after exposure to the homologous antiserum, but neither they, nor the clinical isolates, fluoresced after exposure to normal rabbit serum (Table 13). Individual differences were noted in the capacity of the strains to react with the same antiserum. The antisera are listed from left to right in order of intensity of reactions, and the number of positive reactions obtained. Three of the antisera were highly reactive. Anti 14018 Di antiserum gave a positive reaction with 15 of the 16 organisms tested, and anti-8226 Di and anti-594D Di antisera reacted with 14 of the 16 organisms tested. The remaining antisera were less reactive; anti-6488D Di reacted with 9 of the 16 organisms tested; anti-T94 Di reacted with 8 of the 16 organisms tested, and anti-6488W Di reacted with 3 of the 16 organisms tested. It can be noted

TABLE 13[a]

Indirect Fluorescent Staining Reactions of C. vaginale Reference Strains and Clinical Isolates Using Anti-C. vaginale Antisera and Goat Anti-Rabbit Conjugate[b]

| Organism | Antisera (1:10 dilution) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 14018Di[c] | 8226Di | 594Di | 6488Di | T94Di | 6488WDi | 14018 Bld[d] | NRS[e] |
| **Reference strains** | | | | | | | | |
| 14018 | 3-4[f] | 3-4 | 3-4 | 2-3 | 3 | 1-2 | 4 | 0-1 |
| 6488D | 3 | 3-4 | 3-4 | 3-4 | 2 | 0-1 | 2-3 | 0-1 |
| 8226 | 2-3 | 3-4 | 3-4 | 3-4 | 1 | 0-1 | 2-3 | 0-1 |
| 594 | 3-4 | 3-4 | 3-4 | 1-2 | 3-4 | 1-2 | 4 | 0-1 |
| T94 | 3-4 | 3-4 | 3-4 | 0-1 | 3-4 | 1-2 | 3-4 | 0-1 |
| 6488W | 3 | 3-4 | 3-4 | 3 | 1 | 3 | 0-1 | 0-1 |
| **Clinical isolates** | | | | | | | | |
| 1575 | 3-4 | 3 | 3-4 | 2-3 | 3-4 | 2-3 | 3-4 | 0-1 |
| V28 | 3-4 | 2-3 | 2-3 | 2-3 | 2-3 | 3-4 | 0-1 | 0-1 |
| 8315 | 2-3 | 3-4 | 3-4 | 2-3 | 3-4 | 1-2 | 0-1 | 0-1 |
| 1637 | 3-4 | 2-3 | 2-3 | 2 | 1-2 | 0-1 | 0-1 | 0-1 |
| 359 | 3 | 3 | 3 | 2 | 0 | 0-1 | 0-1 | 0-1 |
| 6234 | 2-3 | 3 | 3-4 | 0-1 | 1-2 | 0-1 | 0-1 | 0-1 |
| V44 | 3-4 | 2-3 | 2-3 | 0-1 | 2-3 | 1-2 | 0-1 | 0-1 |
| 1544 | 3-4 | 2-3 | 2 | 0-1 | 1-2 | 1-2 | 0-1 | 0-1 |
| 144 | 3 | 1-2 | 1-2 | 0-1 | 1-2 | 0-1 | 0-1 | 0-1 |
| 8372 | 1-2 | 1-2 | 1-2 | 1-2 | 1-2 | 0-1 | 0-1 | 0-1 |

[a]Reprinted from MS thesis submitted by M. Smaron as partial fulfillment of the requirements for the MS degree from the Department of Microbiology, Loyola University of Chicago, on February, 1974.
[b]Goat anti-rabbit conjugate used at a 1:20 dilution
[c]Di = organism grown diphasically
[d]Bld = organism grown on blood
[e]NRS = normal rabbit serum
[f]Fluorescent staining intensity rated in degrees from 0 to 4+, with 4+ being maximum intensity

that anti-1408 Bld reacted with 5 of the 6 reference strains and only 1 of the clinical isolates. The antisera were tested against possible related bacteria; i.e., L. acidophilus, C. diptheriae, C. xerosis, C. hofmanii, H. influenzae, N. asteroides, A. bovis, S. mutans.

Certain of the antisera did react with certain heterologous bacteria but after these antisera were adsorbed with the apparent cross-reacting bacteria, no significant decrease in the homologous cross-reacting titer was observed (117).

As stated previously, anti-T94 Di, anti-594 Di, anti-14018 Di, anti-14018 Bld, anti-8226 Di, anti6488D Di and anti 6488W Di antisera did differ in their reactivity with the isolated antigens when tested by the Ouchterlony technique. All the antisera formed precipitin bands with antigens (i) and (o) but only anti-T94 Di, anti-594 Di, anti-14018 Bld and anti-14018 Di antisera reacted with antigen (m). Only those antisera which reacted with antigen (m) formed precipitin bands with all the C. vaginale isolates tested. This occurred when the source of antigen was either sonicated (Table 14) or broth extracts (Table 15). Anti-8226 Di antiserum reacted with 11 of the 15 organisms tested whether the source of antigen was sonicated or broth extracts (Tables 14 and 15). Anti-8226-Di antiserum reacted with 8 of the 15 isolates tested when the source of antigen was the sonicated extracts (Table 14) and 13 of the 15 isolates tested when the source of antigen was the broth extracts (Table 15). Anti-6488W Di antiserum, reacted only with 6488W Di when the source

TABLE 14

Immunodiffusion Reactions of Sonicated Extracts of *C. vaginale* Isolates with Antisera Prepared Against Reference Strains of *C. vaginale*

| Organism | Antisera | | | | | | |
|---|---|---|---|---|---|---|---|
| | 14018 Di | 14018 Bld | 594 Di | T94 Di | 8226 Di | 6488D Di | 6488W Di |
| Reference strains | +[1] | + | + | + | + | + | -[2] |
| 14018 | + | + | + | + | + | + | - |
| T94 | + | + | + | + | + | + | - |
| 594 | + | + | + | + | + | + | - |
| 8226 | + | + | + | + | + | + | - |
| 6488D | + | + | + | + | + | + | - |
| 6488W | + | + | + | + | + | - | + |
| Clinical isolates | | | | | | | |
| 1637 | + | + | + | + | + | + | - |
| 6234 | + | + | + | + | + | + | - |
| 144 | + | + | + | + | + | + | - |
| V28 | + | + | + | + | + | - | - |
| V44 | + | + | + | + | + | - | - |
| 359 | + | + | + | + | - | - | - |
| 1544 | + | + | + | + | - | - | - |
| 1575 | + | + | + | + | - | - | - |
| 8315 | + | + | + | + | - | - | - |

[1] + = at least one precipitin band formed

[2] - = no precipitin band formed

TABLE 15

Immunodiffusion Reactions of C. vaginale Isolates with Antisera

Prepared Against Reference Strains of C. vaginale

| Organism | Antisera | | | | | | |
|---|---|---|---|---|---|---|---|
| | 14018 Di | 14018 Bld | 594 Di | T94 Di | 8226 Di | 6488D Di | 6488W Di |
| Reference strains | +[1] | + | + | + | + | + | + |
| 14018 | + | + | + | + | + | + | + |
| T94 | + | + | + | + | + | + | + |
| 594 | + | + | + | + | + | + | + |
| 8226 | + | + | + | + | + | + | + |
| 6488D | + | + | + | + | + | + | + |
| 6488W | + | + | + | + | + | + | + |
| Clinical isolates | | | | | | | |
| 1637 | + | + | + | + | -[2] | + | - |
| 6234 | + | + | + | + | + | + | - |
| 144 | + | + | + | + | + | + | + |
| V28 | + | + | + | + | + | - | - |
| V44 | + | + | + | + | + | - | - |
| 359 | + | + | + | + | - | - | - |
| 1544 | + | + | + | + | - | + | - |
| 1575 | + | + | + | + | + | - | + |
| 8315 | + | + | + | + | - | + | + |

[1] + = at least one precipitin band formed
[2] - = no precipitin band formed

of antigen was the sonicated extracts (Table 14) and 9 of the 15 isolates tested when the source of antigen was the broth extracts (Table 15). Anti-14018 Di, anti-14018 Bld, anti-594 Di, anti-T94 Di, anti-8226 Di, anti-6488D Di, anti-6488W Di antisera did not react with the media controls.

## E. CRUDE CHEMICAL AND PHYSICAL CHARACTERIZATION OF ANTIGENIC DETERMINANTS

### 1. Polyacrylamide Gel Electrophoresis

Fig. 14 indicates the pattern of precipitin bands which developed against the gels containing the C. vaginale broth or saline extracts. Three precipitin bands developed against the broth extract (Fig. 14a) and these were designated according to their rate of migration; that is, the fastest moving band, which migrated with the tracking dye, was designated (f); the slowest moving band was designated (s); and the intermediate band was designated (im). In other experiments, when the column fractions 1, 3 and 4 were electrophoresed, it was found that antigenic component (i), present in fraction F1, migrated approximately the same distance as (f), antigenic component (o), present in fraction F3, migrated approximately the same distance as (s); and fraction F4, containing antigenic components (m) and (o), formed 2 precipitin bands corresponding to (s) and (im).

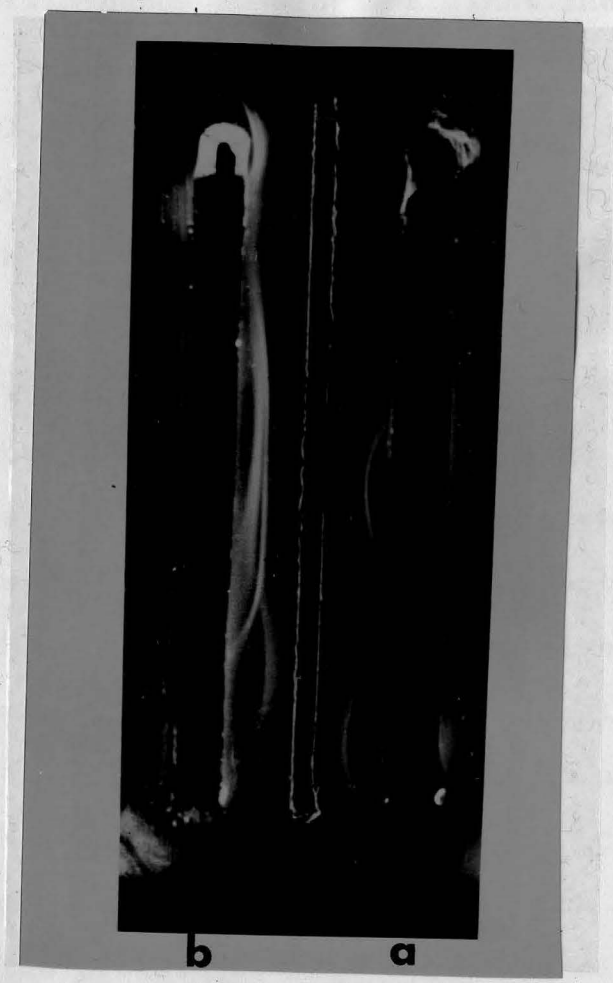



Figure 14. Polyacrylamide gel-immunodiffusion patterns of C. vaginale broth extract and cell washings. The broth extract (a) and washings (b) of C. vaginale were electrophoresed on gradient gels, and immediately after electrophoresis, the gels were placed on a slide and agar was added and allowed to solidify, as described in Materials and Methods sections C1 and C3. Anti-14018 Di antiserum was placed in the trough cut adjacent to the gels.

Five precipitin bands developed against the saline extract (Fig. 14b). Two of the bands developed against material present in the sample gel. The location of the three remaining precipitin bands was similar to those produced with the gel containing the broth extract but some minor variations were noted, that is: (a) the fastest migrating antigen did not travol with the tracking dye, and (b) the (s) and (im) bands in the saline extracts corresponding to antigens (m) and (o) in the immunodiffusion studies were found over a greater length of the gel.

A preliminary study was performed to select carbohydrate and protein stains which would be the most sensitive in detecting the material being examined. The Periodate-Schiff stain was selected as the carbohydrate stain, because it appeared to be more sensitive than the alpha naphthol stain. Coomassie brilliant blue appeared more sensitive than Buffalo Black or nigrosin and was selected as the protein stain. Fig. 15 indicates the staining patterns produced in the gels in which the broth and saline extracts were electrophoresed and stained with Periodate-Schiff, Coomassie brilliant blue, and Alcian blue. The areas in which antigens (i), (m) and (o) are located were stained by Coomassie brilliant blue, Periodate-Schiff and Alcian blue stains. However, differences in the staining patterns of the broth and saline extracts were noted; that is, (1) a greater number of bands were observed with the saline extract, and (2) a broad band, which migrated with the tracking dye and was stained intensely

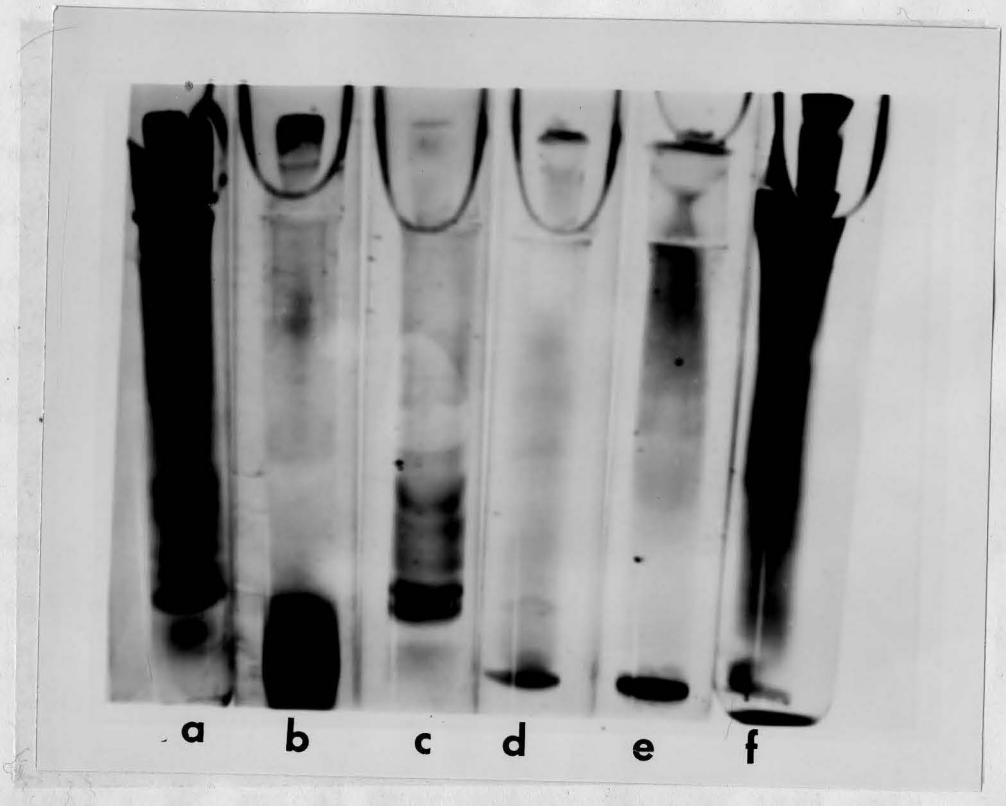



Figure 15. Polyacrylamide gel staining patterns of C. vaginale broth extract and cell washings. The broth extract (a, b, and c) and cell washings (d, e, and f) of C. vaginale were electrophoresed on gradient gels, and the gels were subsequently stained with Periodate-Schiff stain (a and f), Alcian blue, (b and e) and Coomassie brilliant blue (c and d) as described in Materials and Methods sections C1 and C2.

by the Alcian blue stain, was seen in the saline extract. Since the staining patterns of gels in which column fractions were electrophoresed were not as distinct as those produced by the saline and broth extracts, these are not presented in this dissertation. Sudan black B stained the bottom of gels on which either the saline or broth extracts were electrophoresed and also stained material in the sample gel of the latter, indicating the presence of lipid in these areas. No lipid staining material was found in the areas in which antigens (m) and (o) were located. Although antigen (i) did migrate to the same area that was stained by Sudan black B, subsequent experiments demonstrated that antigen (i) does not contain lipid material.

## 2. Physical and Chemical Characterization of Antigenic Determinants

### a. Heat stability

Heating the broth extract at 60° C or 100° C for 30 min did not alter the antigenic activity. Antisera reacted with the same dilution of either the heated or unheated broth extract; that is, anti-14018 Di and anti-P4 antisera reacted with a dilution of 1:16, anti-F3 antiserum reacted with a dilution of 1:6, and anti-F1 antiserum reacted with a dilution of 1:2 of either treated or untreated antigen.

b. Extractability with chloroform-methanol

The C. vaginale antigens present in the broth extract were not extracted by chloroform-methanol. Antisera reacted with the same dilution of the material which remained after extraction and with the unextracted material; that is, anti-14018 Di and anti-P4 antisera reacted with a dilution of 1:16, anti-F3 antiserum reacted with a dilution of 1:6, and anti-F1 antiserum reacted with a dilution of 1:2 of either extracted or unextracted broth material. Of a total of 2 g of material tested, approximately 100 mg of the material was extracted by the chloroform-methanol procedure. The material was subsequently tested by thin layer chromatography and was shown to migrate in a lipid solvent. (This was performed courtesy of Dr. S. Shelley of the Department of Pathology, Loyola University).

c. Effect of treatment with protease

Broth extract was treated with protease for 24 h to determine the effect of this enzyme on antigenic activity. The control consisted of broth extract dissolved in distilled water and left at the same conditions of temperature, etc. Of 100 mg of the control material, 43 mg was recovered following dialysis and lyophilization (Table 16). Of the 100 mg applied to the protease column, 25 mg was recovered following dialysis and lyophilization. The control was shown to contain

TABLE 16

Effect of Protease Treatment on

*C. vaginale* Broth Extract

| | Treated[1] | Untreated[2] |
|---|---|---|
| Total OD units | 5.2 | 7.8 |
| Weight (mg) | 25 | 43 |

[1]Broth extract (100 mg) was subjected to treatment by an enzite agarose protease column (3 ml) for 24 h at 23° C. The column eluate was adjusted to 5 ml and an aliquot read at 280 nm. The eluate was dialyzed, lyophilized, weighed, and tested for antigenic activity by the Ouchterlony technique.

[2]The control consisted of broth extract (100 mg) not treated with protease but left at 23° C for 24 h and subsequently tested as stated above.

7.8 OD units compared to 5.2 OD units for the protease treated material. Antisera reacted with the same dilution of either the treated or untreated broth extract; that is, anti-14018 Di and anti-P4 antisera reacted with a dilution of 1:15, anti-F3 antiserum reacted with a dilution of 1:5 and anti-F1 antiserum reacted with a dilution of 1:2 of either protease-treated or untreated antigen.

d. Effect of treatment with urea

C. vaginale antigens were not affected by the presence of urea. Both the urea-treated and control (untreated) material reacted at the same dilutions when tested against anti-14018 Di antiserum. The urea treated extract was applied to a DEAE cellulose column and antigens (i), (m) and (o) were recovered (further discussion in Results section B2).

e. Effect of periodate oxidation

When activity of the control and periodate treated materials were compared (Table 17), no significant decrease in OD units or weight was observed. The OD of the control was 2.7 while the OD of the periodate treated material was 2.2. Of the original 200 mg sample, 160 mg of the control and 140 mg of the periodate-treated extract was recovered following dialysis and lyophilization. When the periodate-treated extract was tested against anti-14018 Di antiserum,

TABLE 17

Effect of Periodate Treatment on

C. vaginale Broth Extract

| | Treated[1] | Untreated[2] |
|---|---|---|
| Total OD units | 2.2 | 2.7 |
| Weight | 140 | 160 |

[1] Broth extract (200 mg) dissolved in 0.1 M lithium periodate buffer, pH 7.2, was left at 23° C for 72 h and glucose added to decompose the excess periodate. The extract was dialyzed, adjusted to 5 ml and an aliquot read at 280 nm. Subsequently, the extract was lyophilized and tested for antigenic activity by the Ouchterlony technique.

[2] The control consisted of broth extract (200 mg) not treated with periodate but left at 23° C for 24 h and subsequently tested as stated above.

no precipitin bands formed, while the control material reacted at a 1:15 dilution with anti-14018 Di antiserum.

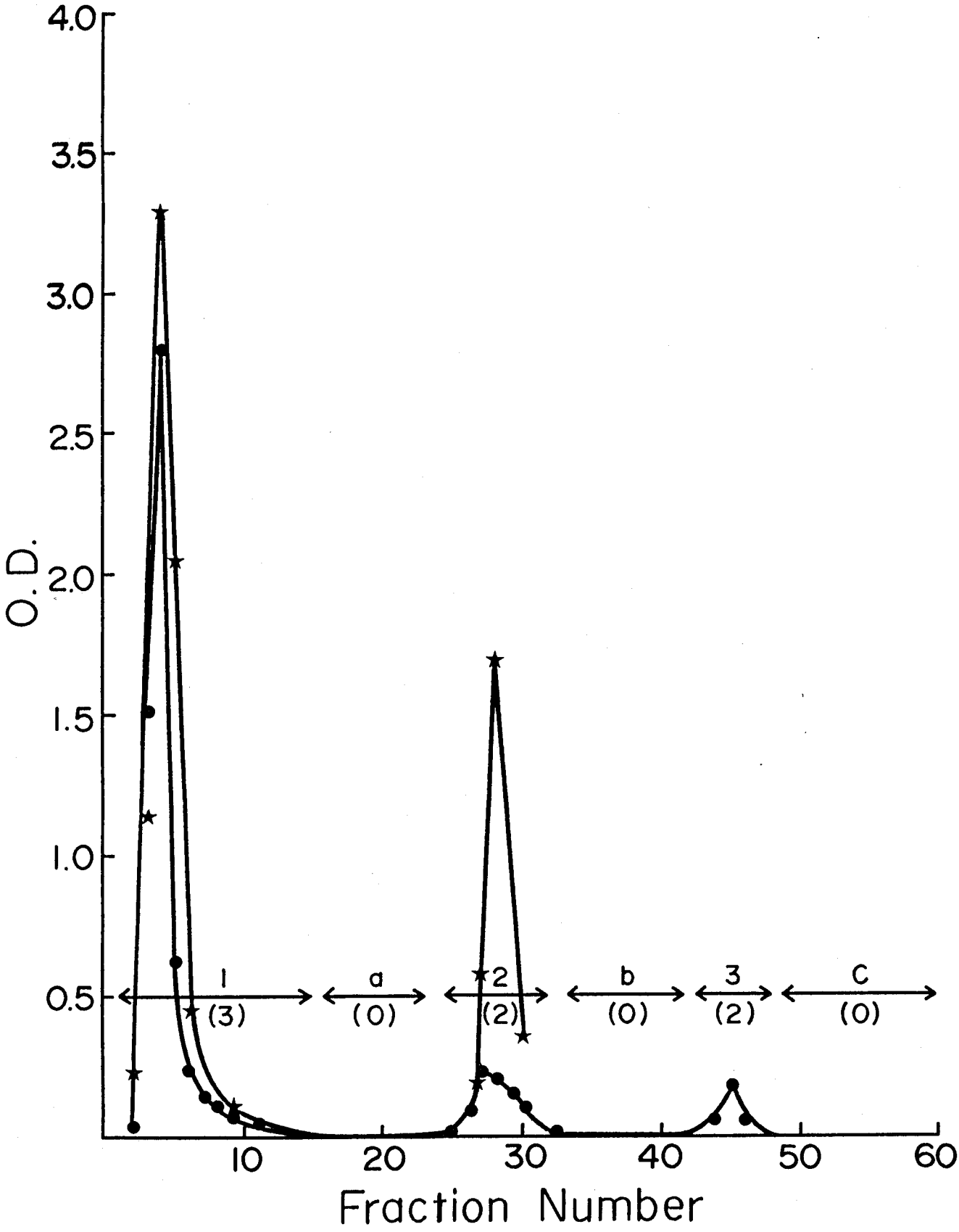
f. Ability to bind to Con A-Sepharose

Fraction 1 was washed from the Con A-Sepharose column by the equilibrating buffer, fraction 2 was eluted from the Con A-Sepharose column with the equilibrating buffer containing 1 M NaCl, and fraction 3 was eluted from the Con A-Sepharose column with the equilibrating buffer containing 0.2 M α-methyl glucose (Fig. 16). The weights of the fractions were as follows: fraction 1 -- 177 mg, fraction 2 -- 63 mg, fraction 3 -- 23 mg, fraction a -- 11 mg, fraction b -- 17 mg, fraction c -- >1 mg. Fractions 1 and 2 contained material that absorbed at 280 nm, suggesting the presence of protein, and also contained carbohydrate as detected by the Dubois procedure (25). Fraction 3 contained material that absorbed at 280 nm but could not be tested for carbohydrate because the eluting buffer contained α-methyl glucose. Fractions a, b, and c did not contain material which absorbed at 280 nm. Fractions a and b did not contain detectable carbohydrate and fraction c could not be tested for carbohydrate because the buffer in this fraction contained alpha-methyl glucose.

Anti-14018 Di and anti-BE-i, m, o antisera each produced precipitin bands with fractions 1, 2, and 3, but not with fractions a, b or c. The precipitin bands formed by each

Legend Con A-Sepharose Column

Figure 16. Chromatography of the broth extract of C. vaginale on Con A-Sepharose. A 2.0 ml sample of the C. vaginale broth extract was applied to a 1 x 30 cm column of Con A-Sepharose, and unbound material was washed off the column with the equilibrating buffer (0.01 M acetate buffer, pH 6.8, containing $10^{-3}$ M $CaCl_2$, $10^{-3}$ M $MgCl_2$, and $10^{-3}$ M $MnCl_2$) at a flow rate of 0.3 ml/min. A stepwise elution was performed using the equilibrating buffer containing 1 M NaCl followed in 12 h by the equilibrating buffer containing 0.2 M α-methyl-glucose. The optical density at 280 nm (o) and carbohydrate content (x) were determined as described in Material and Methods section. The bar and letter or number designation indicate the manner in which fractions were pooled for further analyses. The parentheses indicate the number of antigens detected in the pooled fractions.


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
O.D.
1
(3)
a
(0)
2
(2)
b
(0)
3
(2)
C
(0)
10
20
30
40
50
60
Fraction Number

antiserum with fractions 2 and 3 were weak. Anti-F3-o antiserum produced a precipitin band with fractions 1, 2 and 3, although the bands which formed with fractions 2 and 3 were weak. This indicates that antigen (o) was present in fractions 1, 2 and 3. Anti-P4-m antiserum produced precipitin bands with fractions 1, 2 and 3, although the precipitin bands formed with fractions 2 and 3 were weak. This indicates that antigen (m) was present in fractions 1, 2 and 3. Anti F1-i antiserum did produce a precipitin band with fraction 1, but did not react with fraction 2 or 3. This indicates that antigen (i) was present in fraction 1 but could not be detected in fractions 2 or 3.

g. Amino acid analyses

Amino acid analyses were performed courtesy of Dr. C. F. Lange of the Department of Microbiology, Loyola University of Chicago on a Spinco model 120 C amino acid analyzer according to the method of Spackman (110a).

# IV. DISCUSSION

In this study, C. vaginale antigens were isolated and immunologically characterized. A primary objective, therefore, was to devise an extraction procedure by which soluble C. vaginale antigens could be obtained in relatively large quantities and in the least degraded form. Various extraction procedures previously utilized for other organisms were tested to determine whether they could be used to isolate soluble C. vaginale antigens. The extracts were examined by the Ouchterlony technique employing polyvalent antiserum (anti-14018 Di) previously described by Vice and Smaron (110, 117) to estimate the number and amount of antigen(s) released. When washed whole cells were used as the source of antigen (Table 5), only a few of the extraction procedures yielded detectable quantities of antigen, i.e., ether, lysozyme, and lysozyme with EDTA. Extraction of C. vaginale cells with alkali, acid, phenol or by autoclaving did not consistently release antigen. However, these were only pilot studies and several factors can be postulated to account for the fact that these extraction procedures did not release antigen: (a) the cells or reagents may have been tested at improper concentrations, (b) the antigens may be labile under the conditions used for extraction, and/or (c) the reagents may be unable to disrupt the bonds necessary for the release of antigens.

Examination of the broth supernatant and the cell washings revealed that they consistently contained greater quantities of C. vaginale antigens than did the extracts obtained by chemical extraction of the whole cells. Ouchterlony analyses (Fig. 2) revealed the presence of three C. vaginale antigens in the broth extract which appeared to form bands of identity with the three C. vaginale antigens detected in the washings. The cell washings contained significantly more non-antigenic compounds as revealed by PAGE-immunodiffusion experiments (Fig. 14) than the broth extract. Most importantly, the broth extract from all the C. vaginale isolates tested reacted with antiserum (anti-14018 Di) prepared against the cells of the ATCC strain (110) suggesting that the extracts contained a common antigen. Therefore, it was decided that the broth extract would be employed as the source of antigen in the remaining studies. Although the supernatants from cultures of both the diphasic medium and the PSD broth contained significant quantities of antigen, the PSD broth was chosen as the growth medium since it is more simple in composition. Attempts to further simplify the PSD growth medium by substituting maltose, a dialyzable carbohydrate, yielded decreased quantities of antigen and starch was therefore retained as the carbohydrate source.

Once the PSD culture supernatant was established as the source of antigen, a number of reagents including ammonium sulfate, hydrochloric acid and ethanol (Table 5) were tested for their ability to partially purify them, i.e., to separate the antigens

from the PSD broth components. It was also felt that these same methods could possibly serve also to isolate the antigens, i.e., to separate the antigens from each other. It was established that precipitation by HCl did not give consistently high yields of antigen, and alkali extraction resulted in partial loss of the antigenic activity. Alkali frequently has been used to deproteinize (6a), and thereby purify extracts containing polysaccharide antigens; however, alkali is known to disrupt O-acetyl groups and these may be an important antigenic determinant (121c, 123f). The experimental results (Table 6) established that both ammonium sulfate and ethanol effectively precipitated C. vaginale antigens present in the broth extract, in good yield, but no conditions could be found which selectively isolated any one of these antigens in large quantity. The scheme which was finally utilized to obtain the antigens involved a combination of ammonium sulfate precipitation followed by alcoholic precipitation which resulted in a 32 fold purification of the C. vaginale broth extract antigens. The resulting precipitate was next used in experiments designed to isolate the antigens.

Column chromatography, based on the principle of molecular sieving, revealed that the antigens appear to range in size since they were detected at many points along the elution curves. Also based on the fact that the antigens were eluted near the carbohydrate exclusion limits of the G75 Sephadex columns, the molecular weight of the antigens was crudely estimated to be approximately $5 \times 10^4$ daltons or less.

Although G75 Sephadex columns did not isolate the antigens from each other, a further partial purification was apparently achieved (Fig. 3). The broth extract was eluted from G75 Sephadex columns in 2 peaks, both of which contained protein and carbohydrate. All of the antigenic activity was detected in peak 1; in contrast, peak 2, which constituted approximately 50% of the material, did not contain C. vaginale antigens. From this it can be concluded that G75 Sephadex columns could be used to obtain purer C. vaginale antigen preparations, but further experiments would be necessary to clarify this possibility.

Immunoelectrophoretic studies of the antigen extract showed that the three C. vaginale antigens present in the broth extract differed in charge. One of the antigens, later identified as antigen (i) , was shown to migrate at the same rate as albumin, which has a pI of 4.8. Two antigens, later identified as antigens (o) and (m) , remained near the antigen well and appear to migrate at the same rate as gamma globulins which have a pI range of 6.8 to 7.3. The differences in the pI's indicated that ion exchange cellulose could prove to be a suitable separation media and experiments showed that DEAE cellulose isolated two of the three antigens antigens (i) and (o) . These antigens were isolated when the following conditions were employed (Fig. 6). (a) DEAE columns were initially equilibrated to pH 7.7 with a 0.01 M Tris-HCl buffer, (b) the broth extract was dialyzed for 12 h against a 0.01 M citric acid-$Na_2HPO_4$ buffer prior to its

application on the column and (c) the bound antigens were eluted by a 24 h pH gradient ranging from pH 7.2 employing a 0.01 M citric acid-$Na_2HPO_4$ buffer to a pH 2.8 using the same type of buffer. As shown in Fig. 7, antigen (i) was detected in the void volume of the column which was designated fraction F1. Antigen (o) was eluted in the third peak which was designated fraction F3. Both antigen (m) and antigen (o) were detected in the fourth peak which was designated fraction F4. These experiments also gave critical information since it was shown that the initial pH gradient (Fig. 6) which resulted was an important condition critical for isolation of the antigens. Although it was not investigated, an ionic strength and/or anion binding phenomenon may be an important point here. This effect of pH gradient was demonstrated when urea was added in an attempt to improve antigen isolation. Urea prevented the initial pH rise (Fig. 6) and the antigens were no longer isolated. Regardless of the many methods employed (Table 1) none proved effective for the isolation of antigen (m). Antigen (m), which was not separated by any physical method attempted, was subsequently isolated from fraction F4 by reaction with a monospecific anti-m antiserum which was obtained by absorbing anti-14018 Di with purified antigens (i) and (o) (Fig. 1). Thus, all three antigens were isolated one from another.

To further characterize the antigens, rabbits were immunized with DEAE fraction F2 containing antigen (i) , with DEAE fraction F2 containing antigen (o) or with immune antigen (m)-anti-(m)

precipitates. The resulting antisera were absorbed to monospecificity and were designated anti-F1-i, anti-F3-O, and anti-P4-m, respectively. Studies utilizing anti-P4-m antiserum indicated that antigen (m) is a common and unique C. vaginale antigen. The evidence to support this conclusion is: (a) antigen (m) was detected in the broth extracts and sonicated extracts of all the C. vaginale isolates tested (Tables 11 and 12); (b) antigen (m) was not detected on any of the possible related bacteria tested including L. acidophilus, C. diptheriae, C. cervicis, and vaginal diptheroid (8372); and (c) no antigenic relationship of antigen (m) with antigens (i) or (o) was demonstrable. Additional evidence which suggests that antigen (m) is a common antigen was obtained by demonstrating that only those C. vaginale reference strain antisera (anti-14018 Di, anti-14018 Bld, anti-594D, and anti-T94) which contained anti-m antibodies were also capable of reacting with all of the C. vaginale isolates (Tables 14 and 15). Also anti-BE antiserum, which produced a precipitin band with antigen (m), was shown to produce precipitin bands with all of the C. vaginale isolates.

While antigens (i) and (o) also appear to be unique to C. vaginale, they were not detectable in all the species tested (Tables 10, 11 and 12). Anti-F1-i antiserum, monospecific for antigen (i), anti-F3-o antiserum, monospecific for antigen (o), and anti-P4-m, monospecific for antigen (m), do not react with other possible related bacteria including L. acidophilus, C. diptheriae,

C. cervicis and vaginal diptheroid (8372) establishing the uniqueness of the antigens. However, antigen (i) and antigen (o) appear to share an antigenic determinant (Fig. 7, 10 and 12). While neither antigen (i) nor antigen (o) could be demonstrated to be "common" antigens there may be an explanation for this. It may be that both antisera have a relatively low antibody concentration and these may be low avidity antibodies. Attempts to prepare higher titered antisera by injecting immune precipitates were unsuccessful. This suggests yet another possibility; i.e., that the purified antigens may be poor immunogens, a common finding (6a) with may bacterial antigens.

Anti-14018 Di antiserum, prepared against the whole cells of C. vaginale, had been shown in previous studies (110, 117) to react specifically with C. vaginale and this antiserum was used in these studies to determine the best source of antigen. However, since the broth extract was selected as the source of C. vaginale antigen, it was necessary to demonstrate that the antigens detected by anti-14018 Di antiserum in the broth extract were identical to those present on the C. vaginale cells. Antisera prepared against antigens in the broth extract (anti-BE) and against whole C. vaginale cells (anti-14018 Di) did react with the same organism to the same degree, suggesting that the antigens are identical. Moreover, adsorption of anti-BE antiserum and anti-14018 Di antiserum with washed cells completely removed antibody activity directed against both C. vaginale cells and the broth extract.

The adsorption experiments support the idea that the three C. vaginale antigens detected in the broth extract and on the whole cells are identical and also indicate that these are cell surface antigens.

Although the above data suggests that the three C. vaginale antigens are located on the cell surface, only anti-F1 antiserum of the four antisera prepared against the broth extract antigens reacted positively when tested for antibody activity by the indirect fluorescent antibody technique. These results could be due either to (a) the location of the antigens or (b) the avidity of the antisera. If the avidity of an antiserum is low, the bound antibodies are more readily dissociable during washing (112a). Generally, an antiserum which gives a good precipitin response is applicable for fluorescent studies (121e) because even a 50 percent loss of bound antibody will not alter the observation (112b). Anti-F1-i antiserum, however, has the lowest precipitating activity of the four antisera prepared since it produced a precipitin band with a 1 to 2 dilution of the broth extract. Anti-F1 antiserum reacted fluorescently at a titer of 20 against the type strain and also fluoresced with four of the reference strains (14018, 594, T94 and 8226) and four of the clinical isolates (359, 1544, 6234 and 8315). In contrast, anti-F1-i produced a precipitin band only with the type strain (14018). These variations in reactivity could be due to the different sensitivities of the two methods employed and to the low concentration of antibody in anti-F1 antiserum.

Anti-BE-i, m, o antiserum reacts with fraction F1 by Ouchterlony analyses (Fig. 9) and has the greatest reactivity of all the antisera tested. Anti-BE-i, m, o antiserum produces a precipitin band with a 1:22 dilution of the broth extract (Table 9). However, anti-BE antiserum, in contrast to anti-F1 antiserum, does not react well by the indirect fluorescent technique. A possible explanation for the difference in fluorescent activity between anti-F1 and anti-BE antisera is that they detect different determinants on antigen (i). Absorption experiments were attempted to answer this question but were unsuccessful due to the problems with increased viscosity of the antiserum caused by the starch present in the column fractions which were used for the absorption.

The data obtained using anti-F3 antiserum favors the location hypothesis as a probable explanation for the negative fluorescent reactions. Although anti-F3 antiserum does not react in the indirect fluorescent antibody technique, peptone-absorbed anti-F3 antiserum (anti-F3-o antiserum) does produce a better precipitin reaction than peptone-absorbed anti-F1 antiserum (anti-F1-i antiserum) (Table 9). Anti-F3-o antiserum reacts with a 1 to 6 dilution of the broth extract while anti-F1-i antiserum reacts with a 1 to 2 dilution of the broth extract. Furthermore, anti-F3-o antiserum reacts better with the broth extracts than with the sonicated extracts of the C. vaginale isolates tested (Tables 11 and 12). When the broth extracts were used, anti-F3-o antiserum reacted with all six of the reference strains (14018, 594, T94,

8226, 6488D and 6488W) and three clinical isolates (V28, V44, and 144) (Table 12); however, when the sonicated extracts were used, only three reference strains (14018, 594 and T94) reacted with anti-F3-o antiserum (Table 11). On the basis of the above data, it appears that either (a) antigen (i) is located closer to the cell surface than antigen (o), or (b) the determinant of antigen (o) is blocked (e.g., by configuration) from recognition while on the cell surface, but as antigen (o), is released into the broth, the antigenic determinant is exposed and capable of reacting with anti-F3-o antiserum.

Antigen (m) also appears to be blocked from recognition while on the cell surface. Anti-P4 antiserum does not fluoresce when reacted against whole cells, but does give a good precipitin response with the broth extract at a 1 to 16 dilution (Table 9). Further evidence for the subsurface location of antigen (m) is indicated by experiments with anti-T94 antiserum. Anti-T94 antiserum reacts (Tables 14 and 15) with antigen (m) and all the C. vaginale isolates when the Ouchterlony technique is used, but only fluoresces (Table 13) with 8 of the 15 C. vaginale isolates (14018, 6488D, 594, T94, 1575, V28, 8315 and V44). Therefore, it appears that antigen (m) has a subsurface location on the cell.

Attempts were made to "crudely" characterize the three C. vaginale antigens. First, the antigens were migrated on polyacrylamide gels and the gels were subsequently reacted against anti-14018 Di antiserum by immunodiffusion. The patterns obtained by combining

these techniques (PAGE-immunodiffusion patterns) (Fig. 14) suggest that the antigens differ in charge. Antigen (i), present in fraction F1, appears to be the most positively charged of the three antigens since it migrated the furthest in the gels. This data correlates with that obtained when the broth extract was tested by immunoelectrophoresis. The PAGE-immunodiffusion patterns (Fig. 14b) produced by the cell washings are comparable to the patterns produced by the broth extracts (Fig. 14a) except for minor variations. When the cell washings were examined, antigens (m) and (o) appear to migrate over a greater length of the gel. Also, precipitin bands were formed opposite the sample gel with the cell washings but not with the broth extract.

This data may indicate that the antigens in the cell washings exhibit a greater range of charge and/or size. The stained gels suggested (Fig. 15) that the antigens are glycoproteins or polysaccharides, since the portions of the gels containing the antigens were stained by Coomassie blue and the periodate-Schiff stain.

Further attempts to chemically categorize the antigens were made by employing procedures generally considered to be useful for the simple characterization of a compound as a carbohydrate, lipid or protein (5, 14, 54, 57, 86, 128). The antigens were shown to be carbohydrate or, possibly, glycoprotein since they are: (a) destroyed by periodate, (b) able to bind to Con A-Sepharose, (c) not destroyed by protease, (d) not disrupted by urea, and (e) not extracted nor destroyed by chloroform-methanol.

Since chloroform-methanol did not extract the antigens nor degrade them, it does not appear that lipid plays a major role in the determinant site (14, 128). It does appear that all three antigens contain carbohydrate since they are not detectable after periodate oxidation (Table 17) (5,6). Although proteins, i.e., albumin, are affected by periodate, their antigenic activity generally is not destroyed (46). The presence of carbohydrate in antigens (m) and (o) is further confirmed by their ability to bind to Con A-Sepharose (Fig. 16). Although antigen (i) could not be detected in the material eluted from the Con A-Sepharose column, this may be due to the low concentration of antibody in anti-F1 antiserum and/or the low concentration of antigen (i). Con A has been shown to bind compounds containing free glucose and mannose units (24). The antigenic determinants do not appear to contain significant amounts of protein since they are not disrupted by urea and are not broken down by protease (Table 16) (5, 54, 57, 86).

It is possible, however, that the antigens are glycoproteins or glycopeptides but either (a) the carbohydrate moiety of the compound is protecting the protein component from destruction and/or (b) the protein moiety is not part of the antigenic determinant. Amino acid analyses of fraction F3 indicated that it contains approximately 55 percent protein. However, Ouchterlony analyses indicated that, beside antigen (o), fraction F3 also contained two compounds which are derived from the proteose peptone in the

medium and they may contribute a significant part of the amino acids detected. The amino acid analyses also indicated that fraction F3 contained a significant amount of glucosamine, which may be the binding sight for the Con A attachment. It is interesting to note that lysozyme alone released antigens from C. vaginale which suggests that it is truly a gram positive organism as reported by Zinnerman and Turner (127), Dunkelberg et al. (31), Edwards (32) and Reyn et al. (99). Gram negative organisms, in contrast, usually require the presence of EDTA in order for lysozyme to be effective in the release of antigens. Also, because antigen (m) is unique to C. vaginale and is detectable on all the isolates, this suggests that C. vaginale is not related to other vaginal organisms and probably should be placed in a genus distinct from the Corynebacterium.

A striking similarity is apparent between C. vaginale antigens (i) and (o) and the C-polysaccharide and C-capsular polysaccharide isolated from Streptococcus pneumoniae and alpha Streptococci (58, 59, 60, 104). The streptococcal C-polysaccharide and C-capsular polysaccharide antigens and the C. vaginale antigens (i) and (o): (a) are detected on the surface of the respective cells and in the growth media, (b) cross-react but also appear to contain unique determinants, (c) exist as a heterogeneous population of molecular sizes, (d) are detected on some, but not on all, strains of the respective species, and (e) are not good immunogens. Generally, in order to produce a high titered antiserum against the

capsular antigens of S. pneumoniae the whole cells rather than the isolated capsular material are used. Similarly, only immunization with the whole cells of C. vaginale (anti-14018 Di) gave rise to a high titer fluorescent antiserum. Capsular polysaccharide and C-polysaccharide-like antigens have also been found in other genera including Neisseria (47).

The apparent toxicity observed when antigen (o) was used to immunize rabbits is similar to that observed with the C polysaccharide-mucopeptide complex isolated from group A Streptococci (102). The rabbit injected with precipitates containing antigen (o) died and no antibody activity was detected in the sera of the animal. The two animals injected with fraction F3, which also contains antigen (o), became ill. Amino acid analyses indicated the presence of glucosamine in fraction F3. N-acetyl glucosamine has been reported by Schwab to be associated with the toxicity in the C-polysaccharide-mucopeptide complex isolated from group A Streptococci (105).

Preliminary studies have, in fact, revealed that antiserum prepared against the whole cells of C. vaginale (anti-14018 Di) formed a precipitin band against an alcohol-extract of cell washings of S. pneumoniae but did not react with alcohol-extract of cell washings of Klebsiella pneumoniae. Further studies, utilizing more purified antigen preparations, would be necessary to clarify the apparent similarities between C. vaginale antigens and the C-polysaccharide and C-capsular polysaccharide of S. pneumoniae.

In conclusion, this study has demonstrated that C. vaginale possesses three diffusable antigens which appear to be polysaccharide or glycoprotein in nature. One of the antigens, (m), is unique and characteristic for C. vaginale. There is a striking similarity between the properties of these C. vaginale and the C-polysaccharide antigens of S. pneumoniae. The antiserum used in this study (anti-14018) which was prepared against the type strain of C. vaginale reacted with extracts prepared from S. pneumoniae but not with other bacteria including L. acidophilus, C. diptheriae, C. xerosis, C. cervicis (110) and K. pneumoniae. The following similarities between C. vaginale and the genus Streptococcus can also be noted: (a) acetic acid as major volatile acid (31, 106), (b) guanidine to cytosine ratio of approximately 40 (10a, 10b), (c) microaerophilic, gram variable coccobacillary organisms, (d) colony morphology, and (e) catalase negative (10a, 10b). Based on these observations, this author concludes that C. vaginale is more closely related to the genus Streptococcus than Corynebacterium and might more appropriately be redesignated Streptococcus vaginale. Further studies would be needed to clarify this point.

## V. SUMMARY

Three C. vaginale antigens were isolated and immunologically characterized. The source of the C. vaginale antigens was the supernatant fluid from 72 h peptone-starch-dextrose broth cultures. The antigens were partially purified by ammonium sulfate precipitation followed by ethanol precipitation. DEAE cellulose columns were used to isolate two of the three antigens. Antigen (i) was detected in the void volume of the column, designated fraction F1. Antigen (o) was eluted by a pH gradient in the third 280 nm absorbing peak emerging from the column, designated fraction F3. The fourth 280 nm absorbing peak eluted from the column, designated fraction F4, contained antigens (m) and (o).

Antigen (m) was subsequently separated from fraction F4 by immune precipitation. Anti-14018 Di antiserum prepared against the whole cells of the ATCC strain 14018 contained antibodies directed against all three antigens. After anti-14018 Di antiserum was absorbed with fraction F3 to remove anti-(o) antibodies, fraction F4 was added to obtain a precipitate which contained isolated antigen (m).

The isolated antigens were used to prepare antisera and absorptions were done to make the antisera monospecific. Thus, adsorbed anti-F1 antiserum reacted specifically with antigen (i),

absorbed anti-F3 antiserum reacted specifically with antigen (o), and absorbed anti-P4 antiserum reacted specifically with antigen (m). These monospecific antisera were subsequently utilized in Ouchterlony analyses, which revealed that antigen (m) is common to all C. vaginale isolates tested. Antigen (m) does not appear to be present on other possible related bacteria including L. acidophilus, C. diptheriae, C. cervicis or vaginal diptheroids. Antigens (i) and (o) appear to be unique to C. vaginale, but were not detected on all C. vaginale isolates.

All three C. vaginale antigens appear to be large molecular weight polysaccharide or glycoprotein antigens which are located in the surface layers of C. vaginale cells. The antigens were destroyed by periodate oxidation and were bound, although somewhat weakly, by Con A-Sepharose. The antigens were not affected by urea, or protease and could not be extracted by chloroform-methanol. Immunoelectrophoresis and polyacrylamide gel-immunodiffusion experiments, performed at a pH of 8.6 and 8.9, respectively, suggest that antigen (i) is an acidic polymer and antigens (m) and (o) are relatively neutral compounds.

The apparent similarities between C. vaginale antigens (o) and (i) and Streptococcal C-polysaccharide and C-capsular polysaccharide antigens as reported by Schiffman et al. (104) and Schwab (102, 105) were discussed. Thus, the data presented in this study indicate that C. vaginale is an unique organism which does not appear to be immunologically related to the genera Corynebacterium or Lactobacillus.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

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