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## Observations on the Adherence of Bacteroides Bivius to Female Genital Tract and Oral Epithelial Cells

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OBSERVATIONS ON THE ADHERENCE OF  
BACTEROIDES BIVIUS TO FEMALE GENITAL TRACT  
AND ORAL EPITHELIAL CELLS

by

Susan V. Meade

**Library - Loyola University Medical Center**

A Thesis Submitted to the Faculty of the Graduate  
School of Loyola University of Chicago in Partial  
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Master of Science

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## VITA

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## TABLE OF CONTENTS

|  | Page |
|--|------|
| ACKNOWLEDGEMENTS.....  | ii   |
| VITA.....  | iii  |
| LIST OF TABLES.....  | vi   |
| LIST OF FIGURES.....   | vii  |
| LIST OF ABBREVIATIONS.....   | viii |
| INTRODUCTION.....  | 1    |
| MATERIALS AND METHODS.....   | 16   |
| Organisms.....   | 16   |
| Media and growth conditions.....   | 18   |
| Buffers.....   | 18   |
| Examination for capsules.....  | 19   |
| Growth and viability determinations.....   | 20   |
| (i) Growth curves.....   | 20   |
| (ii) Viability under aerobic conditions.....   | 21   |
| Preparation of organisms for adherence assay.....  | 22   |
| Preparation of epithelial cell suspensions.....  | 23   |
| Adherence assay.....   | 24   |
| Trypsin treatment of epithelial cells.....   | 27   |
| Analysis of data.....  | 28   |
| RESULTS.....   | 29   |
| Characterization of growth of organisms used<br>in this investigation.....                 | 29   |
| 1. Growth of <u>B. bivius</u> ATCC 29303 and<br><u>B. fragilis</u> ATCC 23745 in BHIS..... | 29   |
| 2. Determination of working concentra-<br>tions of organisms.....                          | 29   |
| 3. Oxygen tolerance of organisms.....  | 32   |
| Encapsulation of <u>Bacteroides</u> spp.....   | 35   |
| Evaluation of epithelial cell suspensions prior<br>to assay.....                           | 37   |

|  |     |
|--|-----|
| 1. Effectiveness of washing procedure on epithelial cell suspensions.....                        | 37  |
| 2. Epithelial cell viability.....  | 41  |
| Development of adherence assay procedure.....  | 41  |
| Adherence of oral streptococci to buccal epithelial cells using assay protocol as developed..... | 45  |
| Adherence of <u>B. bivius</u> to genital tract epithelial cells.....                             | 48  |
| 1. Effect of time of incubation on adherence.....  | 48  |
| 2. Effect of growth medium on adherence....  | 50  |
| 3. Effect of divalent cations on adherence.....  | 51  |
| 4. Effect of gram stain treatment on adherence of <u>Bacteroides</u> spp. to BEC....             | 55  |
| 5. Adherence of clinical isolates of <u>B. bivius</u> .....                                      | 56  |
| 6. Adherence of <u>B. bivius</u> to VEC of patients at risk for infection.....                   | 58  |
| Adherence of <u>B. bivius</u> to buccal epithelial cells.  | 59  |
| 1. Inhibition of adherence by sugars.....  | 59  |
| 2. Effect of pH on adherence of <u>B. bivius</u> to BEC.....                                     | 62  |
| Investigation of possible role of fibronectin on adherence.....                                  | 77  |
| DISCUSSION.....  | 85  |
| SUMMARY.....   | 120 |
| LITERATURE CITED.....  | 122 |

LIST OF TABLES

| Table |  | Page |
|-------|--|------|
| 1.    | Survival of <u>B. fragilis</u> ATCC 23745 and <u>B. bivius</u> ATCC 29303 when exposed to atmospheric oxygen under assay conditions..... | 36   |
| 2.    | Effect of multiple washings on resident flora counts.....  | 40   |
| 3.    | Adherence of oral streptococci to buccal epithelial cells.....   | 46   |
| 4.    | Adherence of <u>B. fragilis</u> and <u>B. bivius</u> to CEC, VEC and BEC at 30 min and 1 hr incubation.....                              | 49   |
| 5.    | Adherence of <u>B. bivius</u> to buccal epithelial cells under standard assay conditions.....  | 54   |
| 6.    | Comparison of bacterial counts by phase microscopy and gram stain of adherence assay....   | 57   |
| 7.    | Adherence of <u>Bacteroides</u> species to VEC from women in the post-partum period.....   | 60   |
| 8.    | Viability of genital tract epithelial cells as determined by exclusion of trypan blue dye...   | 61   |
| 9.    | Effect of carbohydrates and concanavalin A on adherence of <u>B. bivius</u> to BEC.....  | 63   |

## LIST OF FIGURES

| Figure  | Page |
|---|------|
| 1. Representative growth curve of <u>B. fragilis</u> ATCC 23745 and <u>B. bivius</u> ATCC 29303 at 37°C in BHIS.....            | 31   |
| 2. Survival of <u>B. fragilis</u> ATCC 23745 and <u>B. bivius</u> ATCC 29303 when exposed to atmospheric oxygen on BHIAS.....   | 34   |
| 3. Photomicrograph of dry film india ink smears of <u>B. fragilis</u> ATCC 23745 and <u>B. bivius</u> ATCC 29303.....           | 39   |
| 4. Photomicrograph of adherence assay to buccal epithelial cells.....   | 53   |
| 5. Adherence of <u>B. bivius</u> to BEC in sodium phosphate buffer.....   | 66   |
| 6. Adherence of <u>B. bivius</u> to BEC in sodium acetate buffer.....   | 68   |
| 7. Adherence of <u>B. bivius</u> to BEC in Tris-maleate buffer.....   | 71   |
| 8. Adherence of <u>B. bivius</u> to BEC in Tris-HCl buffer.....   | 73   |
| 9. Adherence of <u>B. bivius</u> to BEC in Tris-maleate buffer supplemented with varying concentrations of sodium chloride..... | 76   |



## ABBREVIATIONS

|                 |  |
|-----------------|--|
| BEC .....       | buccal epithelial cells                    |
| BHIS .....      | supplemented brain heart infusion<br>broth |
| BHIAS .....     | supplemented brain heart infusion<br>agar  |
| BSA .....       | bovine serum albumin                       |
| °C .....        | degrees centigrade                         |
| C-section ..... | Caesarean section delivery                 |
| CEC .....       | cervical epithelial cells                  |
| cfu/ml .....    | colony forming units per milliliter        |
| DNA .....       | deoxyribonucleic acid                      |
| Fig. ....       | figure                                     |
| x g .....       | times force of gravity                     |
| hr .....        | hour                                       |
| LUMC .....      | Loyola University Medical Center           |
| <u>M</u> .....  | molar                                      |
| ug .....        | microgram                                  |
| um .....        | micrometer                                 |
| min .....       | minute                                     |
| ml .....        | milliliter                                 |
| <u>mM</u> ..... | millimolar                                 |
| % .....         | percent                                    |
| sp., spp. ....  | species                                    |

TSA ..... trypticase soy agar  
TSB ..... trypticase soy broth  
VEC ..... vaginal epithelial cells

## INTRODUCTION

It is the purpose of this thesis to examine the possible role of adherence in the pathogenesis of obstetric and gynecologic infections due to Bacteroides bivius. In order to address this problem, the following studies will be undertaken: (1) a description of the basic growth characteristics of B. bivius; (2) development of a suitable adherence assay system; and (3) a survey of adherence of B. bivius to epithelial cells of the female genital tract in populations considered to be at risk for infection with this organism.

Members of the genus Bacteroides are widely recognized as significant human pathogens. They have been implicated in a variety of clinical infections including intra-abdominal sepsis, bacteremia and female genital tract infections [57]. Members of the Bacteroides fragilis group and the Bacteroides melaninogenicus - Bacteroides asaccharolyticus group are the most frequently isolated members of the genus in clinical specimens [39, 83].

Bacteroides bivius was first described by Holdeman and Johnson in 1977 [66]. Previously known as Bacteroides group PS, it is phenotypically similar to B. melaninogenicus ss. intermedius. It has been shown by

Holdeman and Johnson to be distinctly different from both B. melaninogenicus and from another phenotypically similar species, B. disiens by DNA homology studies.

B. bivius is a saccharolytic, proteolytic, anaerobic gram negative pleomorphic bacillus, frequently more coccoid than bacillary in appearance [66]. Like other members of the genus Bacteroides, it is non-motile, and major acid end products from carbohydrate fermentation include succinic and acetic acids, with isovaleric acid produced in smaller quantities and some isolates may produce small amounts of isobutyric acid as well [66]. It differs from the 'fragilis' group of Bacteroides species in its inability to grow in the presence of 20% bile and its inability to ferment sucrose [65]. It differs phenotypically from B. melaninogenicus in its lack of pigment production [66]. The ability to ferment lactose distinguishes it from the other proteolytic, saccharolytic species, B. disiens [66]. B. bivius has been shown by Fraser and Brown to produce neuraminidase while B. disiens does not [42].

Frequent resistance to penicillin has been reported by a number of different investigators. In the original species description, Holdeman and Johnson found 71% of the strains tested were resistant to penicillin [66]. Other reports of penicillin resistance have run from 52% to 97% of isolates examined [49, 86, 141, Snyderman et al., Abstr.

Int. Conf. Antimicrob. Agents Chemother., 1978, No. 359]. Timewell et al., and Lacroix et al., have isolated and characterized beta-lactamases from a group of penicillin resistant B. bivius isolates and found them to be of a molecular weight similar to those of B. fragilis beta-lactamases (30,000 to 40,000) but possessing isoelectric points different from those of both B. fragilis and those of the rest of the 'melaninogenicus' group [86, 173, 174]. Susceptibility to clindamycin, metronidazole, chloramphenicol, erythromycin, cefoxitin, cefotaxime, cefoperazone and piperacillin has been frequently reported [30, 41, 49, 66, 77, 152, and Snyderman et al., Abstr. Int. Conf. Antimicrob. Agents Chemother., 1978, No. 359].

In B. fragilis, drug resistance has been reported to be transferable by plasmids. Tally et al., among others, has found clindamycin and erythromycin resistance to be associated with the transfer of a plasmid between B. fragilis strains [167]. Welch, et al., have reported that a similar plasmid can transfer resistance between species, from B. fragilis to B. uniformis [185]. And, Martinez-Suarez et al., have demonstrated the plasmid-mediated transfer of chloramphenicol resistance from B. uniformis to B. fragilis [96]. Transfer of clindamycin or tetracycline resistance in vivo in an experimental abscess model was observed by Butler et al., although in this study evidence of concurrent plasmid transfer was not

sought [12]. The evidence for plasmid transfer of beta-lactamase production is somewhat scanty. Butler et al., have reported some success in transferring ampicillin resistance between strains of B. fragilis [165, Butler et al., Clin. Res. Abstr., 1980, 28:365A]. While in these experiments they showed transfer of two types of plasmids along with resistance, the two plasmids were shown not to bear the ampicillin resistance gene. The transfer of beta-lactamase resistance due to the acquisition of a unique substrate profile enzyme has been reported between B. fragilis strains and from B. fragilis to B. vulgatis by Sato et al., although this was not specifically reported as plasmid mediated [123]. It is unlikely that the penicillin resistance of B. bivius has been acquired by transfer of beta-lactamase production from B. fragilis due to the differences of the nature of the respective enzymes [86, 173, 174]. The location of the beta-lactamase gene in B. bivius has not been determined.

B. bivius is a common component of the vaginal flora of normal healthy females [32, 90, 125]. In one study by Hamman, B. bivius was the most frequently isolated anaerobic species in a total of 212 vaginal and cervical swabs from normal healthy women [61]. It was also found to be the most common anaerobic isolate in another study by Hill involving 65 premenopausal women, accounting for 22 isolates in 34% of patients [64]. An earlier study by the

same group found B. bivius in 33% of vaginal swab specimens from healthy females [Hill et al., Abstr. 78th Ann Meet. Am. Soc. Microbiol., 1978, p. 282]. It has been isolated from the oropharynx and from gingival plaque of normal healthy adults [33, 83] and has recently been reported by one group to be the most frequently isolated anaerobic organism in semen [67].

B. bivius has received increased attention in the clinical literature over the last few years for its possible association with infections of the female genital tract. Early clinical reports of involvement of this organism in a variety of gynecologic, obstetric and post-partum infections included vaginitis [91, 148, 170], bartholin cyst infection [83], acute salpingitis [83], acute pelvic inflammatory disease [172], post-hysterectomy wound infections [4], breast abscess [88], intra-amniotic infections [56, 100, 101, 59], chorioamnionitis following cervical cerclage [21] , and puerperal and post-abortal uterine infections [54, 83, 102, 141]. It continues to be reported in a number of infectious processes of the female reproductive tract [7, 8, 9, 46, 47, 55, 69, 78, 164, and Wood, S.P., Abst. 84th Ann. Meet. Am. Soc. Microbiol., 1984, p. 20]. With rare exception, B. bivius has been recovered as part of a mixed flora from the infected site. As in other mixed infections, the pathogenic significance of a single species is not easily

established, and one must search for other evidence, such as production of bacteremia. There has been one reported case of isolation of B. bivius in the blood of a patient with post-partum endometritis and one report of bacteremia developing in a patient with post-abortal complications [48, 83].

Improvements in anaerobic culture techniques over the last decade or so, coupled with an increased awareness by physicians of the substantial role of anaerobic organisms in infectious processes, have enabled diagnostic laboratories to provide increased documentation of the presence of anaerobic organisms in a variety of disease states [39]. Thus, many infections previously catagorized as 'multi-agent', 'non-specific', or 'sterile abscess' may instead represent the inability to isolate and define the etiologic agent. A possible explanation for the reported increase of B. bivius associated obstetric and gynecologic infections may accordingly be due either to an increase in the number and types of specimens cultured by physicians for anaerobic organisms or to an increased ability of diagnostic laboratories to isolate and identify this particular organism. Since it does appear to be frequently involved in significant female and possibly neonatal morbidity [100, 101], we feel that an analysis of possible virulence factors which might contribute to the pathogenesis of infection with this organism is warranted.



Host specificity and tissue tropisms have been shown for a number of organisms. Early studies by Ellen and Gibbons have shown that adherence of Streptococcus pyogenes to rat tongue and Escherichia coli to rat urinary bladder was affected by specificity of both bacterial and epithelial surfaces [35]. Subsequent studies by Gibbons et al., demonstrated the specificity of the adherence of human and rat strains of streptococci to their respective mammalian tissues [51]. Alkan et al., have observed that group A streptococci isolated from the throat adhere better to buccal epithelial cells of healthy donors in vitro , while those isolates from skin sites adhere preferentially to epithelial cells from the skin [2]. Additional studies by Gibbons and others have shown the selectivity of the attachment of bacteria to the various ecological niches in which the indigenous flora are found [35, 50].

An infecting organism must colonize the host as the initial step in the development of an infectious disease [106]. A variety of different organisms have been demonstrated to attach to the mucosal surfaces of the host during natural or experimentally induced infections. Pneumococci were found by Selinger and Reed to adhere to pharyngeal cells of carriers [Selinger and Reed, Clin. Res. Abstr., 1978, 26:405A]. The presence of colonization factor antigens (CFA/I and CFA/II) has been shown to be

requisite in the development of diarrheal disease in enterotoxigenic strains of E. coli [36, 37]. Strains that did not possess CFA antigens were unable to adhere to and colonize the mucosal surface of the small intestine. Regardless of their ability to produce diarrheogenic enterotoxins, CFA negative mutants failed to produce diarrhea [124]. A similar relationship has been found in diarrheal disease in newborn pigs produced by E. coli possessing K88 antigen [72]. Organisms lacking the K88 antigen failed to attach to the intestinal mucosa and thus failed to reach high numbers in the small intestine. Vibrio cholerae strains that are less virulent have been shown to adhere less well to intestinal mucosal surfaces [71]. Preliminary work with Campylobacter spp. indicates a similar prerequisite for intestinal adherence may be important in development of disease with this organism [23]. The virulence of gonococci has been shown to be associated with the presence of pili and the ability of these pili to mediate attachment to epithelial cells [116, 181].

Non-bacterial pathogens have also been shown to have a specificity of adherence to mammalian tissues. Candida albicans, the yeast most frequently isolated from clinical specimens, has been shown to adhere to epithelial cells from the mucosal surfaces of sites frequently involved in candidal colonization and infection [80, 130].

In general, studies have shown that organisms involved in infections at specific sites adhere well to the respective target tissues in vitro. Gould et al., found that those organisms most frequently implicated in endocarditis were those that demonstrated an increased ability to adhere to heart valve tissue in vitro [58]. Those species of Candida that adhere to buccal and vaginal epithelial cells are those which are found to colonize those mucosal surfaces most frequently, suggesting a correlation between adherence and the ability to colonize tissues [81].

Host factors may also be a determinant in tissue tropisms and the ability of an organism to cause disease in a given individual. Selinger et al., have shown that strains of streptococci capable of producing rheumatic fever adhere readily to pharyngeal cells of patients with rheumatic heart disease as compared to cells from controls [132]. Candy et al., have reported that E. coli isolated from infants with protracted diarrhea and bacterial overgrowth of the small intestine adhered significantly more to buccal cells obtained from these children than it did to buccal cells from patients with acute diarrhea, healthy adults and human fetal intestinal epithelium [15]. In addition, the susceptibility of pigs to diarrhea produced by E. coli K88 has been shown by Sellwood et al., to be associated with a phenotypic expression of recep-

tors that allow the organisms to adhere to intestinal brush border cells of the piglets [133].

Periurethral epithelial cells from females with recurrent urinary tract infections have been shown by several investigators to bind E. coli in higher numbers than epithelial cells from patients who are not prone to such infections [41, 73, 157]. Stamey et al., have observed that women with recurrent urinary tract infections not only carry Enterobacteriaceae at an increased rate; in addition, the cervico-vaginal fluid lacks specific antibody to the organisms that colonize the vaginal introitus of these women [149]. They suggest that women not prone to recurrent infection have detectable levels of specific antibody that prevents introital colonization by indigenous fecal flora. Kallenius et al., have found that pyelonephritogenic strains of E. coli interact with glycolipids of the P blood group system [74]. These glycolipids have been isolated from human uroepithelial cells by Leffler and Svanborg-Eden, among others [89]. Kallenius et al., suggest that the greater capacity of uroepithelial cells from women prone to infection to bind these E. coli may be due to differences in density and/or accessibility of these glycolipids to the fimbrial receptors [74].

Some organisms have been shown to adhere in an opportunistic fashion to target tissues and thus increase the

likelihood of colonization and possible subsequent infection in certain patient populations. Pregnant and diabetic women are at increased risk of developing candidal vaginitis [104]. Candida albicans was shown by Segal et al., to adhere in higher numbers to vaginal epithelial cells from women in these two populations than it did to vaginal epithelial cells from non-fecund, nondiabetic women. Several studies from the laboratory of D.E. Woods have shown that gram negative organisms such as Klebsiella pneumoniae and Pseudomonas aeruginosa adhere to oropharyngeal cells of patients who are colonized with these or other gram negative bacilli [68, 187, 190]. Patients predisposed to gram negative respiratory infections such as cystic fibrosis patients or those suffering the stress of acute illness exhibit specific in vivo alterations on the epithelial cell surface that allow these organisms to adhere to and colonize the respiratory tract [188, 189]. These patients exhibit increased salivary protease activity and a concurrent decrease in cell surface fibronectin, associated with an increased adherence of gram negative organisms.

The adherence of anaerobic organisms to tissues has received relatively little attention to date. In one study, Onderdonk et al., looked at the ability of B. fragilis and other Bacteroides species to adhere to rat mesothelial cells in vivo [110]. Okuda et al., have also

recently evaluated the adherence of B.gingivalis, B. asaccharolyticus and B. melaninogenicus to human buccal epithelial cells [109]. Recently, Pruzzo et al., evaluated the relationship between adherence of B. fragilis to epithelial cells and subsequent sensitivity to phagocytosis and killing by human neutrophils [115]. A study by Mardh and Westrom examined the ability of B. fragilis, B. melaninogenicus and Fusobacterium glutinosum to adhere to exfoliated vaginal epithelial cells [95].

Two primary techniques have been employed to study the phenomenon of adherence. Early studies on the adherence of oral streptococci to buccal epithelial cells by Gibbons and van Houte [52] used a membrane filtration system. In this system, the cell suspension is poured onto a membrane filter (mounted in an appropriate apparatus), a weak vacuum is applied to the filter, and the cells are washed free of bacteria by allowing buffer and/or media to flow over the cells. This procedure has been used both as a way of initially preparing epithelial cells for assay, as well as for removal of non-adherent test organisms after incubation of the assay suspensions. Membrane filtration techniques have been widely used in one form or another by many investigators [31, 68, 79, 80, 103, 109, 144, 187, 191]. In most instances, the resultant washed cell preparations were examined for adherent bacteria by preparing a stained smear, although

at least one investigator reports examination of the cell suspension by direct microscopy using a filled counting chamber [157].

The other technique used is that of differential centrifugation [41, 73, 85, 99, 107, 108, 132, 136, 145]. In these assays, bacteria and epithelial cells were combined in desired ratios as is done in the filtration techniques, incubated, and the cells washed free of nonadherent bacteria by centrifugation at relatively low gravitational force (200 - 350 x g) for short periods of time, which sediments the larger epithelial cells while leaving the smaller bacterial cells in suspension in the supernatant. In some instances, differential centrifugation was followed by membrane filtration before staining for visual examination [73, 111, 146].

As an alternative to tedious visual examination for adherent bacteria, some investigators have used radiolabeled organisms [19, 127, 187]. Labeled bacteria and epithelial cells are combined and incubated, nonadherent bacteria eliminated by filtration or differential centrifugation, the cells resuspended in scintillation cocktail and radioactivity measured. With the specific activity of the bacterial suspension known (counts per minute per bacterium) and the number of epithelial cells in the aliquot determined, the number of bacteria adherent to the cells can be calculated from the radioactivity measured.

There have also been a few studies using epithelial cells in tissue culture rather than freshly harvested epithelial cells [18, 23, 62, 126, 160]. Most studies using tissue culture cells evaluate adherence by visual means after staining. Still other studies have used fresh tissue slices or organ cultures of mouse trachea [118] and human fallopian tube [182]. In the mouse trachea model, in some instances, animals are sacrificed and tracheas infused with bacterial suspensions in situ before dissection of the trachea and subsequent evaluation of adherence [119], or alternatively, fresh slices of trachea are exposed to a bacterial suspension in a petri dish, washed and then fixed and prepared for electron microscopic examination [117]. In the human fallopian tube model, pieces of fallopian tube removed during the course of hysterectomy were placed into tissue culture dishes, mucosal side up, and incubated in suitable tissue culture medium for 24 hr prior to exposure of tissues to a gonococcal suspension. Adherence was monitored by electron microscopy in these experiments [169].

Quantitative subculture techniques have been used occasionally in evaluation of adherence to tissue culture cell lines. In one report, the bacterial suspension was added to cell monolayers, non-adherent bacteria subsequently washed off, and the cells then treated with a proteolytic enzyme to release them from the slide sur-



face. Cells in the resultant suspension were enumerated by hemocytometer exam and serial dilutions and subcultures to agar medium made to determine bacterial concentration on (in) the cell suspension. After incubation, the bacterial counts were then correlated with the cell counts to determine degree of adherence [93]. In another variation, a suspension of test organism (of known quantity) is added to tissue culture cells. After appropriate incubation, aliquots of suspension were serially diluted, subcultured, and the degree of adherence calculated by the difference in bacterial counts between the original inoculum and the supernatant counts after exposure to the test cells [160].

There are many factors to be considered in evaluating the significance of B. bivius in pelvic infections. Tissue trauma, vascular supply, the presence of foreign bodies such as intra-uterine contraceptive devices, estrogen and/or progesterone levels, and the presence of other microbiological flora or potentially pathogenic species may all be contributory in development of disease due to B. bivius. It is our intent to initiate this evaluation by examining what role, if any, adherence may play in this complex process.

## MATERIALS AND METHODS

Organisms. Bacteroides fragilis ATCC 23745 and B. bivius ATCC 29303 (American Type Culture Collection, Rockville, MD) were used throughout this study. In some experiments, isolates of B. bivius obtained from clinical sources as well as from another culture collection were used for comparison. DUMC OB13B is an isolate recovered from the blood of a neonate and was kindly supplied by the laboratory of Dr. Gale B. Hill at Duke University Medical Center. LUMC 525059 was obtained from the Clinical Microbiology Laboratory of Loyola University Medical Center and was originally isolated from the uterus of a patient with post-partum endometritis. VPI 8587 represents another blood isolate and was supplied by Ms. Elizabeth Cato at Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg, VA. In addition, the following organisms were used to study various aspects of the adherence test system: Streptococcus salivarius SS2, S. salivarius G9S2, and S. salivarius CM6, all kindly provided by Dr. Ronald Gibbons of Forsyth Dental Center, Boston, MA; S. mutans GS5, kindly provided by Dr. William Yotis of Loyola University Department of Microbiology, and a strain of S. salivarius obtained from the Loyola University Medical Center Clinical Microbiology Laboratory (LUMC

strain).

In one series of experiments, fresh clinical isolates of Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae from respiratory specimens at LUMC were used in evaluation of trypsinization of BEC. Pseudomonas aeruginosa DG 1 was kindly supplied by Dr. D.E. Woods for use in these experiments as well.

Organisms were stored at  $-70^{\circ}\text{C}$  in sterile defibrinated sheep blood (Scott Laboratories, Inc., Fiskeville, RI). When needed for adherence studies, organisms were thawed and subcultured from frozen storage. The number of passages prior to freezing and prior to inclusion in the various culture collections is unknown. In the case of the clinical isolates from LUMC, the number of passages is believed to be minimal (on the average, approximately 3 times).

Confirmation of identification of all anaerobic isolates was performed using the API 20A identification system (Analytab Products, Plainview, NY) and gas chromatographic analysis of end products as recommended by the Anaerobe Laboratory of Virginia Polytechnic Institute and State University [65], using a Dohrmann gas chromatograph (Envirotech Corp., Mountain View, CA). Identification of streptococci was confirmed using the API 20S identification system. Identification of gram negative bacilli was confirmed using the API 20E system.

Media and growth conditions. Streptococci were grown in trypticase soy broth (BBL, Cockeysville, MD) for 24 to 48 hr at 37°C under anaerobic conditions as described by Gibbons and van Houte [52]. Bacteroides spp. were grown in brain heart infusion broth or agar (BBL), supplemented with 0.5% yeast extract (BBL), 5 µg/ml hemin (Gibco, Grand Island, NY) and 10 µg/ml vitamin K<sub>3</sub> (Sigma Chemical Co., St. Louis, MO) (referred to as BHIS and BHIAS, respectively). Organisms were incubated at 37°C in an anaerobic chamber containing 85% N<sub>2</sub>, 10% H<sub>2</sub> and 5% CO<sub>2</sub> (Forma Scientific, Marian, OH) for 24 to 48 hr. All media were pre-reduced before use by allowing to equilibrate in the anaerobic chamber for a minimum of 24 hr prior to use. In those experiments using Enterobacteraceae or Pseudomonas sp., organisms were grown aerobically either overnight at 37°C on trypticase soy agar with 5% sheep blood (BBL) or for 12 hours at 37°C in trypticase soy broth.

Buffers. For washing and suspension of streptococci, 0.067 M phosphate buffer containing 0.15 M NaCl (PBS), pH 6.0 was used. For anaerobic organisms, 0.01 M PBS, pH 7.0 was used. In some experiments, the following buffers were used at 0.01 M concentrations: Tris-hydroxymethyl-amino-methane-maleate (Tris-maleate), pH 5.5 - 8.5; sodium acetate, pH 4.5 - 5.5; sodium phosphate, pH 6.5 - 7.5; PBS, pH 6.5 - 7.5; Tris-hydrochloride (Mallinckrodt,

Inc., Paris, KY). All buffers were verified for correct pH  $\pm$  0.02 pH units using a pH meter (Model 12-B, Corning Medical Instruments, Medfield, MA, fitted with a Corning #476022 electrode). The pH was adjusted using NaOH, HCl, or in one instance  $H_2SO_4$ . In one experiment, Hank's Balanced Salt Solution (HBSS), pH 7.0 (Gibco) was used as the buffer system. The following chemicals were used in preparation of the buffers: sodium acetate, acetic acid, sulfuric acid, sodium hydroxide and magnesium chloride (J.T. Baker Chemical Co., Phillipsburg, NJ); monobasic sodium phosphate and dibasic sodium phosphate (Fisher Scientific Co., Fair Lawn, NJ); Tris-hydroxymethyl-amino-methane (TRIZMA Base) (Sigma Chemical Co.); maleic acid and Tris-hydrochloride (Mallinckrodt, Inc., Paris, KY).

Examination for capsules. The Bacteroides species used in these studies were examined for the presence or absence of capsules using the dry india ink film technique of Butt et al., [13] as modified and described by Cruickshank et al., [28]. A loopful of 6% glucose (Mallinckrodt, Inc.) is put on a clean glass slide and either a loopful of broth culture or a small amount of plate culture is added and mixed. A small loopful of india ink (Rapidograph, Bloomsbury, NJ) is added and mixed. The mixture is spread over the slide in a thin film with the edge of a second glass slide in the manner used for blood films. The slides are

dried thoroughly, fixed by flooding with methyl alcohol (Mallinckrodt, Inc.) for 1 minute, drained and allowed to dry. The organisms are stained with an aqueous crystal violet solution such as that used in the gram stain for one minute. The slide is rinsed with running water and allowed to air dry before examination with the oil immersion objective. A clear zone around the majority of stained bacterial cells is considered a positive test for the presence of capsular material.

#### Growth and viability determinations.

(i) Growth curves. Organisms were removed from frozen storage, thawed, subcultured to fresh BHIS and incubated anaerobically at 37°C for 18 hr (B. fragilis) or 32 hr (B. bivius) until turbid (approximately  $10^8$  to  $10^9$  cfu/ml). This turbid culture was diluted 1:100 in prewarmed BHIS and 0.5 ml of this dilution was inoculated into tubes containing 50 ml of prewarmed media. The tubes were placed on an aliquot mixer (Model 4651, Ames Co., Elkhart, IN) and incubated anaerobically at 37°C. At regular intervals, aliquots were removed, serially diluted in 0.01 M PBS, pH 7.0 and subcultured onto BHIAS. Colony count plates were done in duplicate and were incubated anaerobically for 48 hours. All media and buffers were prereduced in the anaerobic chamber prior to use. All manipulations were performed in the anaerobic glove box

under anaerobic conditions. In some experiments, organisms were grown to stationary phase, serial dilutions made in pre-reduced PBS, and colony counts and absorbance at 660 nm determined on the dilutions. This procedure was performed to determine the absorbance corresponding to  $1 \times 10^8$  cfu/ml to be used in the adherence assay.

(ii) Viability under aerobic conditions. Organisms were grown under anaerobic conditions for 24 hr at 37°C in BHIS. Cells were washed once in prereduced PBS and resuspended to a concentration of  $1.0 \times 10^8$  c.f.u./ml as determined by spectrophotometric measurement. This suspension was then serially diluted in PBS and subcultured onto prereduced BHIAS. After streaking for isolation, plates were removed from the chamber and left at room temperature on the bench top for specified intervals. After the given oxygen exposure, plates were returned to the chamber for incubation at 37°C for 48 hr before determining viable cell counts. In one experiment, viability was determined under the conditions of the adherence assay procedure; that is, bacterial suspensions were prepared at working concentration, added to assay tubes containing PBS instead of epithelial cell suspension, and incubated aerobically at 37°C for 30 min or 1 hr. Aliquots of this "mock" assay were then serially diluted and subcultured to BHIAS to determine viable cell counts.

All manipulations except centrifugation were per-

formed in the anaerobic chamber using materials that had been allowed to equilibrate in the chamber for 24 - 48 hr prior to use. All determinations were performed in duplicate.

#### Preparation of organism suspensions for adherence assay.

After approximately 48 hr incubation, broth grown organisms were harvested by centrifugation at 1500 x g (Model UV, International Equipment Co., Needham Heights, MA) for 20 min, and washed once in appropriate buffer. Organisms grown on agar were harvested from the plate using a buffer moistened swab, suspended in appropriate buffer, and washed once in a similar manner. In one experiment, organisms were grown to late log phase, approximately 10 hr for B. fragilis and 17 hr for B. bivius prior to harvesting for the adherence assay. Streptococci were resuspended in PBS to yield a cell suspension having an absorbance at 550 nm of 0.6, containing a cell concentration of approximately  $1 \times 10^8$  cfu per ml as described by Gibbons and van Houte [52]. Anaerobic organisms were resuspended to an absorbance at 660 nm of 0.370 for B. fragilis and 0.180 for B. bivius to yield a cell suspension containing approximately  $1 \times 10^8$  cfu per ml. These standardized suspensions were diluted 1:1 for use in the assay system to yield a working concentration of  $5 \times 10^7$  cfu per ml. Cell suspensions were gram stained prior to



use to confirm microscopic morphology and purity.

Preparation of epithelial cell suspensions. Vaginal and cervical epithelial cells were obtained from normal healthy women presenting with no medical complaints and receiving no medication at the time of routine gynecologic examination at the Loyola University Outpatient Center Obstetrics and Gynecology Clinic. After obtaining the informed consent of the patient, a clean, non-lubricated speculum was inserted into the vagina and the cervix and vaginal walls visualized. An Ayers spatula used for obtaining a Pap smear was inserted and the lateral vaginal walls gently scraped for 10 to 20 sec with the broad end of the spatula. The spatula was placed into a 50 ml polypropylene tube (Falcon Division of Becton-Dickinson & Co., Oxnard, CA) containing 30 ml PBS, pH 7.0 and agitated to dislodge the cells. In some instances, a cervical sample was obtained with the curved end of the spatula in a similar manner and the cells suspended in a separate tube of PBS. Gram stains were routinely performed on the initial cell suspensions to insure that there were no "clue cells" present. Patients with bacterial vaginosis typically have vaginal epithelial cells with many small gram negative and gram variable rods and coccobacilli covering their surfaces [113]. The presence of such cells would: (1) make examination of cells for adherent Bacter-

oides species impossible, and, (2) indicate an asymptomatic infection, rendering such samples unacceptable for our study.

Initially, cells were washed a minimum of 4 times by centrifugation at 300 x g for 5 min to determine the effectiveness of the cell washing procedure. A drop of cell suspension was transferred to a glass slide after each wash, allowed to dry, and then gram stained to evaluate the effectiveness of the washing procedure in removing resident bacterial flora. After the final wash, cells were resuspended in PBS to a concentration of  $5 \times 10^4$  as determined by direct microscopic count using a Neubauer counting chamber. Cells were used in the adherence assay within 4 hours of collection. Viability of cells in the epithelial cell suspensions was determined by exclusion of 0.4% trypan blue dye [99].

Human buccal epithelial cells were used as control cells and were obtained by vigorous scraping of the oral mucosa of laboratory volunteers with a wooden tongue blade for 30 sec per side. The blade was then agitated in 30 ml PBS to dislodge the cells. The cells were washed and evaluated in a manner similar to that of the vaginal epithelial cells.

Adherence assay. Polypropylene Eppendorf microfuge tubes (Brinkman Instruments, Inc., Westbury, NY) were pre-

treated overnight with 0.1% bovine serum albumin (BSA) (U.S. Biochemical Corp., Cleveland, OH) in PBS. BSA was aspirated before performing adherence test. To these tubes is added 0.5 ml each of epithelial cell suspension ( $5 \times 10^4$  cells/ml) and bacterial suspension ( $5 \times 10^7$  cfu/ml). The tube containing the adherence reaction mixture was incubated at  $37^\circ\text{C}$  on an aliquot mixer (Ames) for specified time periods. After incubation, the contents of each tube were filtered using a  $10 \mu\text{m}$  pore diameter polycarbonate membrane filter (Nuclepore Corp., Pleasanton, CA) in a syringe adaptor (Millipore Corp., Bedford, MA) fitted to a 10 ml plastic syringe (B.D. #5604, Becton-Dickinson Division, Rutherford, NJ). This pore size retains epithelial cells and allows free bacteria to pass on through. The cells were washed 4 times with 10 ml buffer, the membrane removed and pressed to a glass slide, and a clean pipette rolled across the back of the membrane to transfer the cells to the slide as described in the product literature. The membrane was peeled off the slide, the slide allowed to dry and subsequently gram stained for microscopic detection of adherent bacteria. Slides were examined at 1000 X magnification using a Bausch & Lomb DynaZoom microscope. The average number of bacteria per cell was determined by counting the bacteria adherent to 50 epithelial cells. Only cells appearing intact were evaluated. Cells that

appeared disintegrated or torn, cells folded over on themselves or large cellular clumps were not evaluated. In some instances, we determined the ratio of the number of cells with adherent test organisms to the total number of cells counted.

In order to evaluate the effect of the gram stain procedure on bacteria adherent to the cells, a series of experiments was performed in which the cells were transferred to the glass slide, a drop of PBS was added to the cells and a glass coverslip mounted over them and sealed with Vaspar (I.-M., Inc., Lexington, KY). The cells were then examined by phase microscopy (Zeiss Binocular Microscope, Model 47-34-15, Oberkochen, West Germany). The number of bacteria per epithelial cell was determined by counting the bacteria adherent to 50 epithelial cells.

In one experiment the assay was modified to include a variety of carbohydrate solutions as inhibitors, and their effect on adherence was observed. The following compounds were tested at 25 mg/ml, to determine possible inhibition of adherence due to carbohydrate specific adhesin - receptor interactions between bacteria and epithelial cells: D-glucose (Mallinckrodt, Inc.), L-fucose (Mann Research Labs, Inc., New York, NY), D-galactose, D-maltose, D-mannose (Nutritional Biochemical Corp., Cleveland, OH), L-rhamnose (Pfanstiehl Labs, Inc., Waukegan, IL), N-acetyl-D-glucosamine,  $\alpha$ -methyl-D-

glucopyranoside,  $\alpha$ -methyl-D-mannopyranoside (Sigma Chemical Co.) [108]. Concanavalin A (Sigma), a lectin that binds specifically to mannose and glucose residues, was also tested at a concentration of 0.5 mg/ml to evaluate the possible role of these carbohydrate residues in bacterial adherence to epithelial cells [108]. The assay was performed in the standard manner, with the B. bivius suspended in the various carbohydrate solutions and then added to the epithelial cell suspension.

Trypsin treatment of epithelial cells. Trypsinization of buccal epithelial cells to remove surface fibronectin was carried out using several modifications of the procedure described by Woods et al., [190]. After the final wash in PBS, the specimen was divided into equal aliquots and dispersed into 15 ml polycarbonate tubes (Corning, Corning, NY) containing 0.01 M PBS, pH 7.6. Trypsin (Sigma Chemical Co.) was added to these cell suspensions at varying concentrations and the cell suspensions incubated at 37°C for 15 min. Trypsin inhibitor (Sigma) or phenylmethylsulfonyl fluoride, PMSF (Sigma) was then added in appropriate concentrations to inactivate the trypsin. Cells were centrifuged at 300 x g for 5 min, the supernatant removed by aspiration and the cells then resuspended to working concentration. Control cells in this procedure consisted of cells suspended in PBS,

incubated along with trypsinized cells, and to which trypsin inhibitor was added after incubation.

Analysis of data. Counts of adherent bacteria per cell, and of percentage of cells with adherent bacteria, are expressed as mean values  $\pm$  standard error of the mean. Statistical significance of differences was determined using the Student's t test. Statistical significance was set at P values less than 0.05. In some cases, analysis of variance (ANOVA) techniques were employed to determine differences in populations under study.

## RESULTS

### Characterization of growth of organisms used in this investigation.

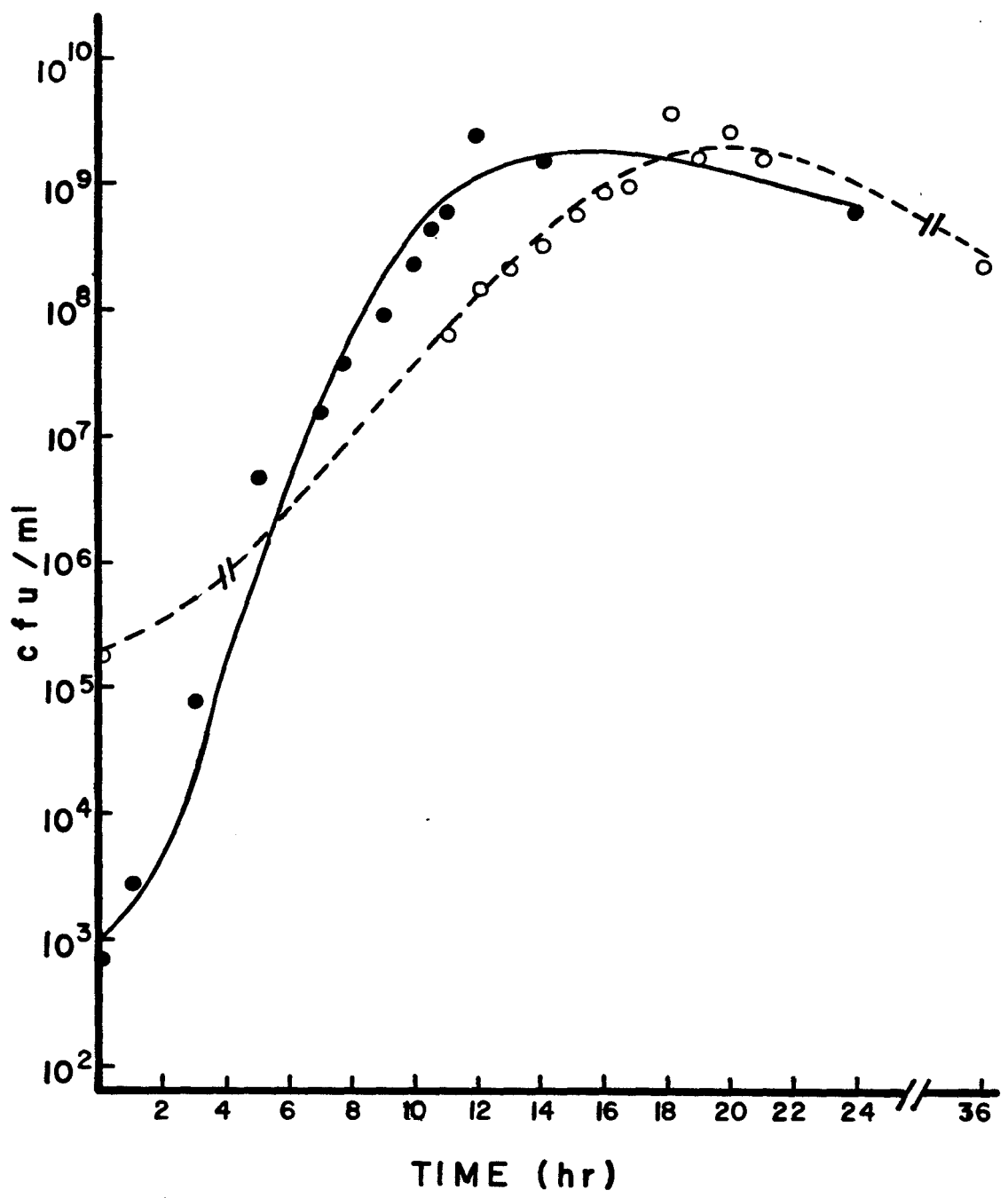
Except for the original species description [66], little has been reported in the literature concerning the growth characteristics of B. bivius. We first set about to characterize the growth of our organisms in the medium used in this study.

1. Growth of B. bivius ATCC 29303 and B. fragilis ATCC 23745 in BHIS. A representative growth curve for B. fragilis and B. bivius obtained by the method described in Materials and Methods is shown in Fig. 1. B. fragilis was found to be a more rapidly growing organism, with stationary phase being reached in 12 to 14 hrs and an average generation time of approximately 30 min. Stationary phase was attained by the B. bivius culture in 18 to 20 hrs and in this medium, B. bivius had a generation time of approximately 80 min.

2. Determination of working concentration of organisms. So that we could standardize the inoculum used in the assay procedure, the correlation between absorbance measurements and organism concentration for these bacteria had to be determined. Serial dilutions of a stationary phase culture were made in PBS in the anaerobic chamber,

Fig. 1. Representative growth curve of B. fragilis ATCC 23745 (—●—●—) and B. bivius ATCC 29303 (--○---○--) at 37°C in BHIS. Each point represents the mean viable count of duplicate samples. At regular intervals, aliquots of culture were removed, serially diluted in PBS, and plated onto BHIAS. Colony count plates were incubated anaerobically for 48 hr before counts were determined.

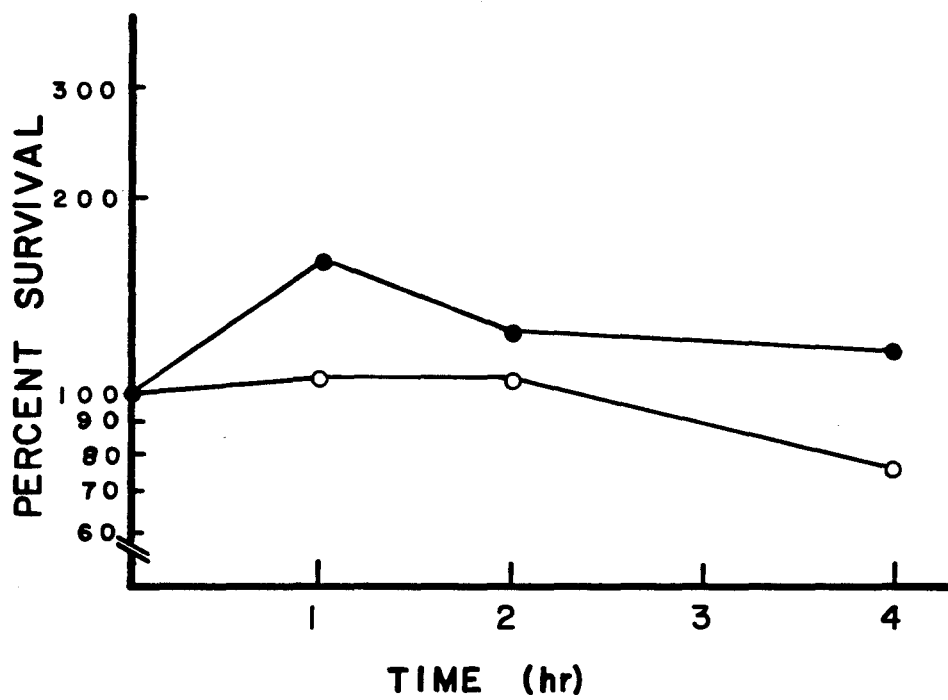




colony count determinations made on the resultant suspensions, and the absorbance of these suspensions determined at 660 nm. The relationship between absorbance and viable cell numbers for both organisms was found to be linear between  $10^7$  and  $10^9$  cfu/ml and the absorbance corresponding to  $10^8$  cfu/ml was established at 0.370 for B. fragilis and 0.180 for B. bivius (data not shown). Confirmation of these values was made by intermittent checks on the organism suspensions prepared for use in the assay.

3. Oxygen tolerance of organisms. Hagen et al., [60] have reported that viability of B. fragilis exposed to aerobic conditions on solid media at 25°C was essentially unchanged over a 72 hr period. Since our preliminary observations had suggested that our B. bivius was relatively sensitive to oxygen exposure for even brief periods of time, we determined the relative viability of the two stock strains over a 4 hr period as described in Materials and Methods. Fig. 2 illustrates the viability upon exposure to atmospheric oxygen while on BHIAS. In the case of B. fragilis, viability was essentially unchanged over 4 hours. With B. bivius, survival after exposure to atmospheric oxygen for 4 hours was 76% of the original inoculum. While apparently more oxygen sensitive than B. fragilis, repeated experiments showed that B. bivius was not as oxygen intolerant as was originally

Fig. 2. Survival of B. fragilis ATCC 23745 (—●—●—) and B. bivius ATCC 29303 (—○—○—) when exposed to atmospheric oxygen on BHIS. Organisms were grown anaerobically to stationary phase in BHIS, harvested and serially diluted in PBS, plated in duplicate and exposed to aerobic conditions for specified time periods.



suspected.

Organisms were also assessed for their survival under the conditions of the assay procedure. Organism suspensions were diluted to working concentrations, added to the microfuge tubes (without epithelial cell suspensions), and subjected to the incubation conditions of the assay procedure. As can be seen in Table 1, in an environment that is totally aerobic, the viability of these organisms is somewhat decreased under these conditions. B. fragilis survival decreased to only 37% of the original count after 1 hr exposure to non-prereduced PBS and the increased temperature of incubation. Survival of B. bivius was even more adversely affected, with the viability decreasing by more than one log after 30 min and more than 3 logs after 1 hr exposure.

#### Encapsulation of Bacteroides spp.

B. fragilis ATCC 23745, B. bivius ATCC 29303 and the three clinical isolates used in the adherence assays were examined for the presence of a capsule by the dry india ink flim technique of Butt et al., [13]. Kaspar et al., [75] and Babb and Cummins [5] have used this india ink technique to demonstrate the presence of a capsule on B. fragilis, although the latter also report the observation of a capsule on B. vulgatis, B. thetaiotaomicron and B. ovatus that Kaspar's group did not find. Figure 3

TABLE 1

Survival of B. fragilis ATCC 23745 and B. bivius ATCC 29303  
when exposed to atmospheric oxygen under assay conditions. <sup>a</sup>

| Organism           | mean cfu/ml               |                                |  |
|--------------------|---------------------------|--------------------------------|--|
|                    | time 0                    | 30 min                         | 1 hr   |
| <u>B. fragilis</u> | $2.7 \pm 0.9 \times 10^7$ | nd <sup>b</sup>                | $1.0 \pm 0.1 \times 10^7$ (37%) <sup>c</sup> |
| <u>B. bivius</u>   | $4.5 \pm 0.8 \times 10^7$ | $1.9 \pm 1.0 \times 10^6$ (4%) | $1.5 \pm 0.5 \times 10^4$ (0.1%)             |

<sup>a</sup> Organisms were suspended to working concentration ( $5 \times 10^7$  cfu/ml), added to microfuge tubes containing an equal amount of PBS, and incubated aerobically for 30 min or 1 hr at 37°C before subculturing to determine survival.

<sup>b</sup> not determined

<sup>c</sup> Numbers in parentheses refer to percent survival of original inoculum.

shows a definite capsule seen on B. fragilis while none is seen on any of the B. bivius strains tested.

Evaluation of epithelial cell suspensions prior to assay.

1. Effectiveness of washing procedure on epithelial cell suspensions. The effectiveness of multiple washings for the removal of resident bacterial flora was initially evaluated using buccal epithelial cells, and was then determined using clinical specimens during evaluation of the assay procedure. A gram stain was performed on the epithelial cell suspensions after each stage of the washing procedure. Table 2 shows the average number of bacteria per cell after each wash for all 3 types of epithelial cells examined. Analysis of counts by the paired Student's 't' test showed that there was no significant change in bacterial counts between successive washes. Only Mardh and Westrom [95] have addressed the question of how many washings are necessary for removal of resident bacterial flora and they reported that resident bacterial flora counts were significantly reduced after 3 washes using a 14  $\mu$ m pore diameter filter. In our evaluation, although the difference between the first and second washes was not highly significant ( $P_{BEC} > 0.2$ ,  $P_{VEC} = 0.07$ ,  $P_{CEC} = 0.12$ ), the differences were slightly greater than in subsequent washes. Differences in the resident flora counts obtained after the first versus the

Fig. 3. Photomicrograph of dry film india ink smears of B. fragilis ATCC 23745 (A) and B. bivius ATCC 29303 (B), prepared as described in Materials and Methods. Organisms were grown on BHIAS for 48 hr prior to examination for capsular material. Bar represents 10  $\mu\text{m}$ .



A



B

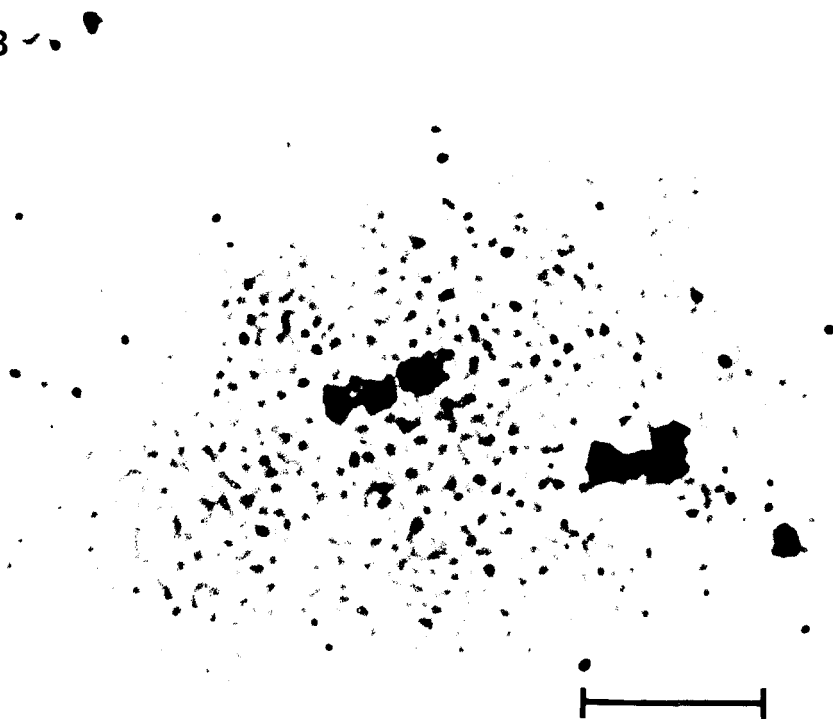


TABLE 2

Effect of multiple washings on resident  
bacterial flora counts. <sup>a</sup>

| Number of<br>washings | bacteria/epithelial cell |                      |                       |
|-----------------------|--------------------------|----------------------|-----------------------|
|                       | buccal <sup>b</sup>      | vaginal <sup>c</sup> | cervical <sup>d</sup> |
| 1                     | 8.9 ± 5.0                | 14.3 ± 5.0           | 10.4 ± 4.3            |
| 2                     | 6.6 ± 2.8                | 11.5 ± 4.0           | 7.3 ± 2.6             |
| 3                     | 8.1 ± 4.4                | 10.5 ± 4.4           | 5.9 ± 2.5             |
| 4                     | 6.6 ± 3.6                | 8.1 ± 3.1            | 5.4 ± 2.1             |

<sup>a</sup> Epithelial cells were collected in PBS as described in Materials and Methods, centrifuged at 300 x g for 5 min and gram stains made on resultant cell sediment after aspiration of supernatant.

<sup>b</sup> n = 6

<sup>c</sup> n = 23

<sup>d</sup> n = 20

third or fourth washes were also not significant ( $P > 0.2$ ) for all three cell populations. As a result, we chose 2 washes for the preparation of epithelial cells for the assay, as repeated washing of cells served no purpose in reducing the resident flora, and each successive wash reduced the total cell yield.

2. Epithelial cell viability. Mean cell viability was determined on epithelial cells prior to their use in the assay. For BEC, the viability was found to be  $5.1 \pm 1.1\%$  by exclusion of trypan blue. VEC were found to have a mean cell viability of  $29.3 \pm 5.9\%$ , and that of CEC was  $38.6 \pm 5.3\%$ .

#### Development of adherence assay procedure.

Initial attempts to reproduce the streptococcal assays of Gibbons and van Houte [52] met with repeated disappointment. As originally described, they used  $14 \mu\text{m}$  pore size polypropylene filters, which are no longer available. We chose instead to use  $10 \mu\text{m}$  polypropylene filters (Gelman, Ann Arbor, MI) and compare that to differential centrifugation techniques for epithelial cell preparation. A sample of buccal epithelial cells was divided into two aliquots. One aliquot was centrifuged at  $250 \times g$  for 5 min with the resultant epithelial cell concentration at  $3.9 \times 10^4$  cells per ml. Membrane filtration with a vacuum was difficult to control, and

resulted in the loss of many cells in the process. The resultant cell suspension ( $2.5 \times 10^3$  cells/ml) was far below that needed for the assay ( $5 \times 10^4$  cells/ml).

We therefore decided to model our system after that of Kallenius and Winberg [73] and Svanborg-Eden [157] in which differential centrifugation for isolation of epithelial cells was utilized. Using these techniques, we attempted to test adherence of oral strains of streptococci to BEC. Washing of non-adherent bacteria from the epithelial cells was to be done as in the initial washing of resident bacterial flora; that is, at low gravitational force for a short time period. When smears of the reaction assay mixture were examined after four washings, the bacterial suspension in the background was so great as to make determination of adherence impossible. Distribution of bacteria between cells appeared identical to that over the surface of the cells. In order to more effectively eliminate non-adherent bacteria, it was decided that we would again filter the cell suspension with the 10  $\mu$ m membrane filter, allowing bacteria to pass through, and retaining the epithelial cells for examination. After incubation, the reaction mixture was poured into the barrel of a 10 ml syringe that was fitted with a filter unit. The cell suspensions were washed four times with 10 ml PBS, and the filter was removed and pressed to a clean glass slide to transfer epithelial cells. When the smears

were examined, only a rare epithelial cell was found on the slide. Pre-coating the slide with albumin as had been reported by some investigators [95, 144] did not seem to increase the yield of epithelial cells for microscopic examination and frequently made evaluation more difficult due to excess protein on the slide.

Examination of the reaction tubes that were being used at that time (12 x 75 mm polystyrene, Falcon #2054, Becton-Dickinson & Co., Oxnard, CA) showed that the epithelial cells were clinging to the tube walls, even though polystyrene tubes are widely used because of their non-adherent tendencies. Similar adherence was noted with polypropylene tubes (Falcon #2063), as well as glass tubes proved no better. The epithelial cells tended to adhere tenaciously to the walls of any vessel into which they were placed, thus making cell recovery for microscopic exam exceedingly difficult.

We tried several techniques that would allow us to use this tenacity to our advantage, but ran into unexpected obstacles. Attempts were made to allow the cells to associate with the wells of several different types and sizes of tissue culture plates, the wells of which could also serve as a convenient container for the assay reaction mixture. Although the epithelial cells did adhere to the bottom of the wells, microscopic exam of the cells by either transillumination or by phase-contrast

could not be performed at more than 100 X magnification due to the thickness of the plastic. .It was therefore impossible to evaluate the presence of bacteria on the cell surfaces.

Allowing BEC to adhere to glass or plastic coverslips which could subsequently be stained and mounted for examination was also attempted. Although cells adhered better to plastic than to glass coverslips, in both instances, the cells tended to clump at the edges of the coverslips, causing much distortion and making microscopic exam difficult. The same difficulty was noted with round coverslips.

Speziale et al., reported in a recent paper the use of a 0.1% solution of BSA in PBS for preincubation of their plastic tubes to minimize nonspecific binding of proteins and bacteria to their tubes [147]. Adaptation of this technique allowed us recovery of a high percentage of the original cell suspension for microscopic examination. At the suggestion of Dr. A.W. Simpson in the laboratory of Dr. E.H. Beachey at the University of Tennessee [personal communication], we chose to use 1.5 ml polypropylene microfuge tubes for the assay. The tubes were pre-treated with 0.1% BSA in PBS for 24 hr prior to use.

Recovery of BEC using these BSA treated tubes was evaluated in triplicate for 2 different BEC samples. After 15 min, cell recovery was  $63 \pm 7.8\%$  of the original

suspension, and after 2 hr was found to be  $71 \pm 4.6\%$  of the original suspension (a difference that was not statistically significant). Originally, only 4 - 26% of the cell suspensions were recovered from the untreated tubes.

The tendency of the epithelial cells to stick to surfaces also created a problem when we again attempted to reproduce the original studies of Gibbons and van Houte [52], as we were unable to get the epithelial cells to transfer from membrane filters to the slide for microscopic examination. It was discovered rather fortuitously that we could readily transfer the cells to a glass slide after filtration on the smooth surfaced Nuclepore polycarbonate filters instead of polypropylene filters of the fiber-matrix type.

Adherence of oral streptococci to buccal epithelial cells using assay protocol as developed.

Having resolved the major obstacles encountered in the assay procedure, we sought to confirm its suitability by using it in assessment of adherence of oral streptococci as was reported by Gibbons [52]. The results are shown in Table 3. Two of the strains supplied by Dr. R.J. Gibbons, were used in his original studies (S. salivarius G9S2 and SS2). The results obtained with these two strains in his studies showed a 15-fold increase and a

TABLE 3

Adherence of oral streptococci to  
buccal epithelial cells. a

| Organism                  | bacteria/cell |
|---------------------------|---------------|
| PBS control               | 0.3           |
| <u>S. salivarius</u> SS2  | 8.7           |
| <u>S. salivarius</u> G9S2 | 60.6          |
| <u>S. salivarius</u> CM6  | 109.1         |
| <u>S. mutans</u> GS5      | 1.7           |
| <u>S. salivarius</u> LUMC | 0.5           |

a Assay was performed in 0.067M PBS,  
pH 6.0 with bacteria : BEC ratio  
approximately 1000:1



5-fold increase, respectively, over the control bacterial counts. Our results showed a 202-fold increase and a 29-fold increase, respectively, over the control counts. The S. mutans GS5 is the same strain used in his studies, but was not obtained from his laboratory. In our hands, this strain has shown a slight but consistent adherence to BEC (6-fold in this experiment, and ranging from 2 to 15 fold in previous experiments (data not shown). Although our results differed quantitatively from those of Gibbons, the general trend seems to be the same; that is, adherence of strain G9S2 was greater than that of strain SS2 and S. salivarius adheres better than S. mutans. The slight adherent capacity of our strain may be due either to strain differences or to individual variations in BEC used. Virtually no adherence was detected in the LUMC strain of S. salivarius in this experiment and in previous experiments throughout the assay development (data not shown). The system used for speciation of this isolate identified it as S. salivarius with an atypical biochemical profile. It may thus be either an atypical variant of S. salivarius that does not adhere, or may, in fact, not be S. salivarius. In any case, the consistent non-adherence of this isolate would indicate that this test system does not allow for random deposition of bacteria onto the epithelial cell surface during the filtration process that could erroneously be interpreted as adherence.

### Adherence of *B. bivius* to genital tract epithelial cells.

Using the assay system that we have devised, we initiated our investigation into the adherence characteristics of *B. bivius*.

1. Effect of time of incubation on adherence. The first series of assays were performed to determine the effect of time of incubation on the adherence of *B. bivius* and of the control organism, *B. fragilis*, to vaginal, cervical and buccal epithelial cells. For these experiments, organisms were grown in liquid medium (BHIS) and adherence was evaluated at 30 min and 1 hr of incubation. For these initial evaluations, no distinction was made to separate the counts of normal, resident flora from those of adherent test organisms. This may at first seem an unenlightened decision, but it was initially not a problem, since only organisms morphologically consistent with the resident flora seen on the PBS control slide were usually seen in the assay smears. In this first series of six clinical specimens, there was no significant difference found among any of the cell types, organisms, or time of incubation, as is shown in Table 4. Analysis by the paired Student's 't' test was performed to evaluate differences in mean bacterial counts for the different cell types, for different incubation periods, or for differences between adherence of the two organisms, and was found to be not significant. In fact, the only

TABLE 4

Adherence of B. fragilis and B. bivius to CEC, VEC and  
BEC at 30 min and 1 hr incubation. <sup>a,b</sup>

| Organism           | number of bacteria/cell <sup>c</sup> |           |            |           |           |            |
|--------------------|--------------------------------------|-----------|------------|-----------|-----------|------------|
|                    | 30 min                               |           |            | 1 hr      |           |            |
|                    | CEC                                  | VEC       | BEC        | CEC       | VEC       | BEC        |
| PBS control        | 1.0 ± 0.9                            | 1.2 ± 0.9 | 8.5 ± 4.6  | 0.5 ± 0.5 | 0.8 ± 0.5 | 8.4 ± 4.6  |
| <u>B. fragilis</u> | 0.7 ± 0.6                            | 0.6 ± 0.3 | 9.2 ± 5.0  | 0.7 ± 0.6 | 0.7 ± 0.6 | 8.2 ± 4.5  |
| <u>B. bivius</u>   | 0.5 ± 0.5                            | 0.8 ± 0.5 | 12.1 ± 5.5 | 0.5 ± 0.4 | 0.7 ± 0.4 | 10.3 ± 5.3 |

<sup>a</sup> Organisms and epithelial cells were collected and prepared as described in Materials and Methods. Organisms were grown in BHIS. Adherence assay was performed in PBS, pH 7.0 and assay was incubated for 30 min or 1 hr before cells were washed free of non-adherent bacteria and gram stained for microscopic exam.

<sup>b</sup> Results shown are from 6 patient samples.

<sup>c</sup> Results are expressed as total bacteria per cell.

experiment that showed any adherence of the test organisms was with a sample of buccal epithelial cells that showed gram negative bacilli that were morphologically consistent with the B. bivius suspension. In addition, moderate adherence of B. bivius to 2 other BEC samples was subjectively noted on tests not included in these data, performed on other days. A 30 min incubation period was used in all subsequent assays, as increased incubation had no effect on adherence.

2. Effect of growth medium on adherence. Growth conditions affect the formation of bacterial surface structures such as pili which are known to participate in bacterial adhesion. Vibrio sp. [176], Neisseria sp. [98, 186]. and Pseudomonas sp. [183] produce surface fimbriae or pili maximally in liquid culture, while maximal CFA fimbria production in enterotoxigenic E. coli occurs on agar medium [36]. In addition, Onderdonk et al., have shown that the presence of capsular polysaccharide in B. fragilis is required for in vivo adherence to rat mesothelial cells [110], and, although not shown specifically for Bacteroides sp., it is known that media composition can affect bacterial surface components [29].

In view of these facts, we examined adherence of BHIS and BHIAS grown organisms to vaginal and cervical epithelial cells. Five patient specimens were observed for differences in adherence, and again, there was no signif-

icant adherence to cells of either vaginal or cervical origin by either species, irrespective of growth medium.

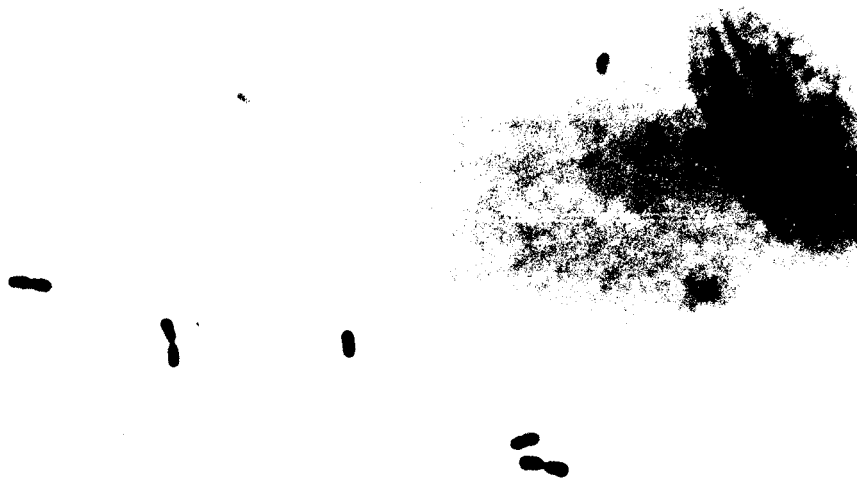
However, there was again an observed adherence of B. bivius to BEC. In four out of five of the control BEC run in this series of experiments, B. bivius was obviously adherent to a certain percentage of the cells (Fig. 4). Table 5 shows mean counts of B. bivius to these buccal epithelial cells, as well as the percentage of cells involved in this adherence phenomenon. In these particular experiments, all BEC were obtained from the same volunteer. Although both the percentage of cells with B. bivius on them and the average number of organisms adherent to the cells was greater with agar grown organisms, in neither instance was the difference statistically significant ( $P > 0.1$ ). All subsequent tests were performed using organisms grown on the agar medium.

In one experiment, we grew B. fragilis for 10 hr and B. bivius for 17 hr in BHIS. When compared to organisms grown for approximately 48 hr in the same medium, these organisms as the end of the log phase of growth did not exhibit any different adherence to BEC.

3. Effect of divalent cations on adherence. Both bacterial and epithelial cells have a net negative surface charge and must therefore overcome the resultant repulsive force between like-charged surfaces if adhesion is to take place [106]. The negative charges on the opposing

Fig. 4. Photomicrograph of adherence assay to buccal epithelial cells. PBS control (resident flora) (A), and BEC with adherent B. bivius (B). Inset: B. bivius grown at 37°C for 48 hr on BHIAS. Bar represents 10  $\mu$ m.

A



B

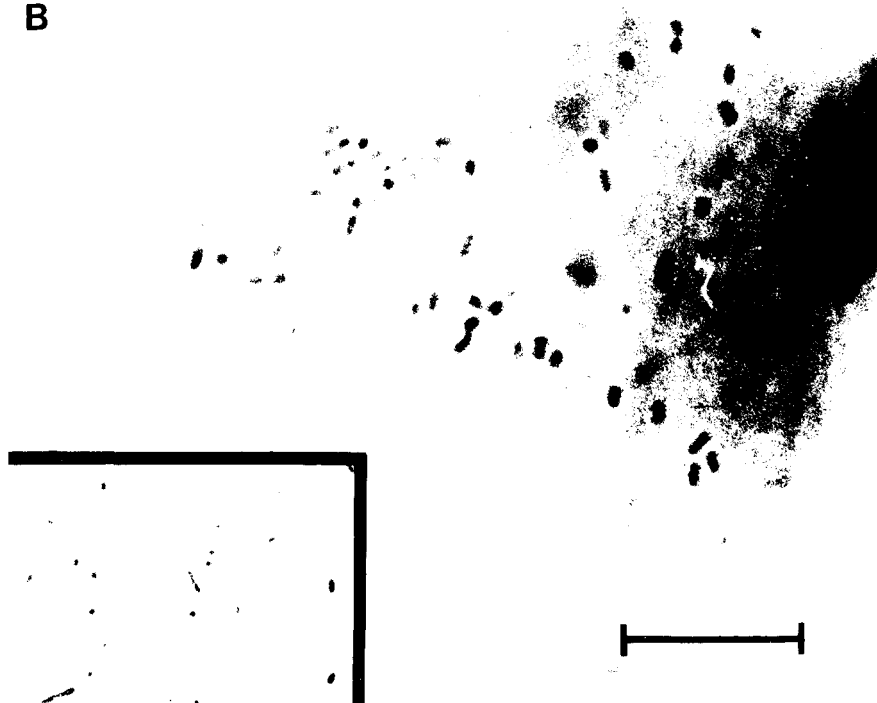


TABLE 5

Adherence of B. bivius to buccal epithelial cells  
under standard assay conditions. <sup>a</sup>

| Growth<br>medium <sup>b</sup> | number of bacteria/cell |                           |                                       | % cells involved |
|-------------------------------|-------------------------|---------------------------|---------------------------------------|------------------|
|                               | PBS control             | total<br>bacterial counts | gram negative <sup>c</sup><br>bacilli |                  |
| BHIS                          | 4.4 ± 2.9               | 8.3 ± 5.0                 | 5.1 ± 2.6                             | 32 ± 13.6        |
| BHIAS                         | 4.4 ± 2.9               | 14.4 ± 6.2                | 12.6 ± 4.5                            | 52 ± 15.2        |

<sup>a</sup> Results of 5 experiments.

<sup>b</sup> Computed by subtracting bacterial counts in PBS control (resident background flora) from total bacterial counts in assay samples.

<sup>c</sup> Organisms were grown on specified medium for 48 hr prior to harvesting as described in Materials and Methods.



surfaces may be reduced by the addition of cations to the system which act to decrease the gap between them by binding to anionic groups on both surfaces and thus decreasing the repulsive forces, permitting apposition of bacteria and epithelial cells; or, the ions may be forming a bridge between cell surfaces [17, 53].

Hank's Balanced Salt Solution was chosen as a source of divalent cations in the adherence system, as the  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  concentrations are within physiological range (0.001M). HBSS was used as the buffer/suspension medium instead of PBS in the evaluation of two vaginal and cervical specimens. Subjective evaluation of the resultant smears showed no increase in the occurrence of either broth or agar grown Bacteroides sp. present on either vaginal or cervical epithelial cells, with mean bacterial counts similar to those obtained with PBS (data not shown). Again, slight adherence of B. bivius to buccal control cells was noted with no enhancement observed in HBSS.

4. Effect of gram stain treatment on adherence of Bacteroides spp. to BEC. In order to determine if the combination of solvents and possible fluid force used in gram staining of smears might wash off loosely adherent bacteria, the assay reaction mixtures were examined by phase microscopy without drying and staining of the cell suspensions. The assay was performed in duplicate and

examined by both methods. We chose to evaluate BEC in this experiment as it was becoming apparent that consistent adherence was observed only with the buccal cells. The results are shown in Table 6. Higher counts were obtained by gram stain examination, both for resident flora and for the Bacteroides sp. in all but one instance, indicating that the staining procedure is not removing adherent organisms and that they may be more visible after staining.

5. Adherence of clinical isolates of B. bivius.

Since it is impossible to know how many times ATCC and other culture collection strains have been subcultured and thus how surface components or other pathogenic factors may have been affected in passage, we chose to compare the adherence of fresh clinical isolates of B. bivius in order to decrease the possibility that these virulence factors had been lost. The ATCC strain and 3 other strains as described in Materials and Methods were examined for their adherence to vaginal epithelial cells in 3 normal females. These isolates also exhibited virtually no adherence to VEC. One strain, VPI 8587, another culture collection strain, exhibited significant adherence to the cells of one of the duplicate assays in patient #081. In the smear resulting from this assay, there were approximately 10 cells on the entire slide that were virtually covered with gram negative bacilli morphologically

TABLE 6

Comparison of bacterial counts by phase microscopy  
and gram stain of adherence assay. <sup>a</sup>

| Organism            | mean number<br>bacteria/cell | mean number<br>bacilli/cell | % cells with<br>adherent bacilli |
|---------------------|------------------------------|-----------------------------|----------------------------------|
| <u>PBS control:</u> |                              |                             |                                  |
| phase exam          | 4.9                          | 0                           | 0                                |
| gram stain          | 9.8                          | 0                           | 0                                |
| <u>B. fragilis:</u> |                              |                             |                                  |
| phase exam          | 3.6                          | 0.01                        | 2                                |
| gram stain          | 12.0                         | 0.14                        | 4                                |
| <u>B. bivius:</u>   |                              |                             |                                  |
| phase exam          | 3.6                          | 2.0                         | 32                               |
| gram stain          | 9.7                          | 1.6                         | 35                               |

<sup>a</sup> Adherence assay performed in duplicate as described in Materials and Methods. For phase microscopy, cells were transferred to slide as is standard, a drop of PBS was added to cells on slide, and a coverslip applied to and sealed over the cells with Vaspar.

consistent with B. bivius. The duplicate sample was devoid of such cells when examined at length.

These strains also demonstrated a slight, but consistent adherence to the control BEC. When compared to all the other BEC evaluations using the ATCC strain of B. bivius, the degree of adherence was not significantly different. The ATCC strain shows a mean of  $4.3 \pm 1.0$  bacilli per epithelial cell ( $n = 15$  BEC evaluations), while for the four additional isolates, the counts in 4 experiments was as follows: OBl3B,  $2.7 \pm 2.1$  bacilli/cell ( $P = 0.5$ ); VPI 8587,  $0.4 \pm 0.2$  bacilli/cell ( $P = 0.06$ ); and LUMC 525059,  $1.1 \pm 0.6$  bacilli/cell ( $P = 0.12$ ). Although values differed from those found for the ATCC strain, none of them were statistically significant.

6. Adherence of B. bivius to VEC of patients at risk for infection. With no apparent adherence observed in normal, healthy, non-pregnant females, we then elected to look at epithelial cells from women undergoing Caesarean section delivery, a population at increased risk of developing endometritis in the post-partum period, and from whom this organism is most frequently isolated.

Vaginal epithelial cells were collected from six women at the time of C-section, usually after the surgeon had closed the incision and before the patient left the operating room. In addition, VEC were collected 24 hr after surgery from three patients who were convalescing

with no complications. We also had the opportunity to collect VEC from 3 women who developed symptoms of post-partum endometritis. Cells were processed and evaluated for adherence by our standard protocol, and, in addition, cell viability was determined on these populations. The results are shown in Table 7. Again, there was no adherence demonstrated to VEC in any of these populations. There was, however, a marked difference in the cell viability in these populations. Viability of VEC from women at the time of surgery was  $4.5 \pm 1.6\%$ , a figure much different from that of the normal non-pregnant population, and from women sampled 24 hr after surgery ( $P = 0.02$  and  $P = 0.006$ , respectively) (Table 8). When analyzed by the non-paired 't' test, the differences between the other populations was found to be non-significant. Additional statistical analysis by ANOVA methods indicates that these cells are different from the other populations sampled ( $P < 0.01$ ), and thus VEC from women at time of delivery are less viable than those collected at other times.

#### Adherence of *B. bivius* to buccal epithelial cells.

In view of the fact that we consistently observed adherence of *B. bivius* to BEC, we decided to investigate this phenomenon at greater depth.

1. Inhibition of adherence by sugars. Adherence of some *E. coli* strains to buccal mucosa can be blocked by D-

TABLE 7

Adherence of Bacteroides species to VEC from  
women in the post-partum period. <sup>a</sup>

| Organism           | gram negative bacilli/cell             |   |   |
|--------------------|--|---|---|
|                    | VEC collected<br>at surgery<br>(n = 6) | VEC collected<br>24 hr post<br>surgery<br>(n = 3) | VEC collected<br>endometritis<br>work-up<br>(n = 3) |
| <u>B. fragilis</u> | 0.05 ± 0.03                            | 0.003 ± 0.003                                     | 0   |
| <u>B. bivius</u>   | 0.1 ± 0.09                             | 0.07 ± 0.07                                       | 0.006 ± 0.006                                       |

<sup>a</sup> VEC were collected from women at the time of Caesarean section, 24 hours post Caesarean section, or at the time of evaluation for symptoms of endometritis by the attending physician.

TABLE 8

Viability of genital tract epithelial cells  
as determined by exclusion of trypan blue dye. <sup>a</sup>

| Cell type (n)                              | percent viable cells |
|--|----------------------|
| CEC (13)                                   | 38.6 $\pm$ 5.3       |
| VEC (14)                                   | 29.3 $\pm$ 5.9       |
| VEC collected<br>at surgery (6)            | 4.5 $\pm$ 1.6        |
| VEC collected<br>24 hr post<br>surgery (3) | 40.0 $\pm$ 13.8      |
| VEC endometritis<br>work-up (3)            | 34.7 $\pm$ 2.7       |

<sup>a</sup> Cells were diluted 5:1 in 0.4% trypan blue, allowed to stand 5 min, and examined at 400 X to determine percentage of cells unstained by dye.

mannose or derivatives such as  $\alpha$ -methyl-D-mannopyranoside [105, 108] and adherence of V. cholerae to intestinal brush border cells can be inhibited by L-fucose and L-fucosides [71]. We chose to examine whether a variety of different carbohydrates might affect the observed adherence of B. bivius to BEC. The results are shown in Table 9. Although individual counts showed marked variation, with several of the cell suspensions having a relatively higher than usual background distribution of unattached bacteria, none of the tested compounds were capable of inhibiting the association of B. bivius with BEC, suggesting that this association is not mediated through a carbohydrate - receptor interaction. Although this does not rule out the possibility that other carbohydrates may be involved, these sugars represent those most frequently tested and reported to be involved in adherence phenomena [108, 182].

## 2. Effect of pH on adherence of B. bivius to BEC.

In light of these data, it was thought that carbohydrates were probably not involved in the association of B. bivius to BEC, and perhaps this might be a non-specific physico-chemical phenomenon related to surface charge distributions. As such, this association should be affected by changes in pH, as increases in hydrogen ion concentrations may act to redistribute and/or counteract the net negative repulsive forces between the two cells. We examined



TABLE 9

Effect of carbohydrates and concanavalin A  
on adherence of B. bivius to BEC. <sup>a</sup>

| Supplement                       | <u>B. bivius</u> /cell | % cells with<br>adherent organisms |
|----------------------------------|------------------------|------------------------------------|
| PBS control                      | 9.5                    | 72                                 |
| L-fucose                         | 9.5                    | 84                                 |
| D-galactose                      | 10.1                   | 82                                 |
| D-glucose                        | 6.1                    | 72                                 |
| D-maltose                        | 7.8                    | 77                                 |
| D-mannose                        | 12.7                   | 88                                 |
| L-rhamnose                       | 7.0                    | 81                                 |
| N-acetyl-D-<br>glucosamine       | 11.2                   | 62                                 |
| $\alpha$ -methyl-D-<br>glucoside | 8.5                    | 69                                 |
| $\alpha$ -methyl-D-<br>mannoside | 8.9                    | 83                                 |
| concanavalin A                   | 7.0                    | 70                                 |

<sup>a</sup> Carbohydrates were used at 25 mg/ml and concanavalin A was used at 0.5 mg/ml as described in Materials and Methods. Counts shown are representative of at least 2 determinations.

several different buffer systems, with and without supplementation with salts to observe the ionic effects on this phenomenon.

The assay was performed in four different buffer systems over a wide pH range (pH 4.5 - 8.5) with PBS acting as the control system (pH range 6.5 - 7.5). The results from the first experiment (data not shown) were inconsistent and did not correlate with a change in pH. As a result, we decided to examine the individual buffers supplemented with various amounts of different salts.

Sodium phosphate buffer was prepared with and without 0.15M NaCl (PBS). In this buffer system, addition of NaCl appeared to have a depressive effect on the number of B. bivius adherent to BEC (Fig. 5). At the same time, sodium acetate buffer was used unsupplemented and supplemented with 0.018M NaCl (a sodium concentration equivalent to that in sodium phosphate buffer) in order to observe and compare results to the phosphate buffer system (Fig. 6). While adherence at low pH in unsupplemented buffer is low, addition of small amounts of NaCl (0.018M) increased adherence of B. bivius to BEC, most markedly at pH 5.5, a result that we did not anticipate.

We then chose to determine if this was a sodium specific phenomenon; that is, we asked if NaCl in amounts equivalent to that of saline (0.15 M) consistently had a depressive effect on adherence, or would other ions have a

Fig. 5. Adherence of B. bivius to BEC in 0.01 M sodium phosphate buffer (—●—●—) and in 0.01 M sodium phosphate buffer supplemented with 0.15 M NaCl (PBS) (—○—○—). Assay was performed as described in Materials and Methods using pooled BEC from 2 volunteers. Results shown are representative of at least 2 determinations.

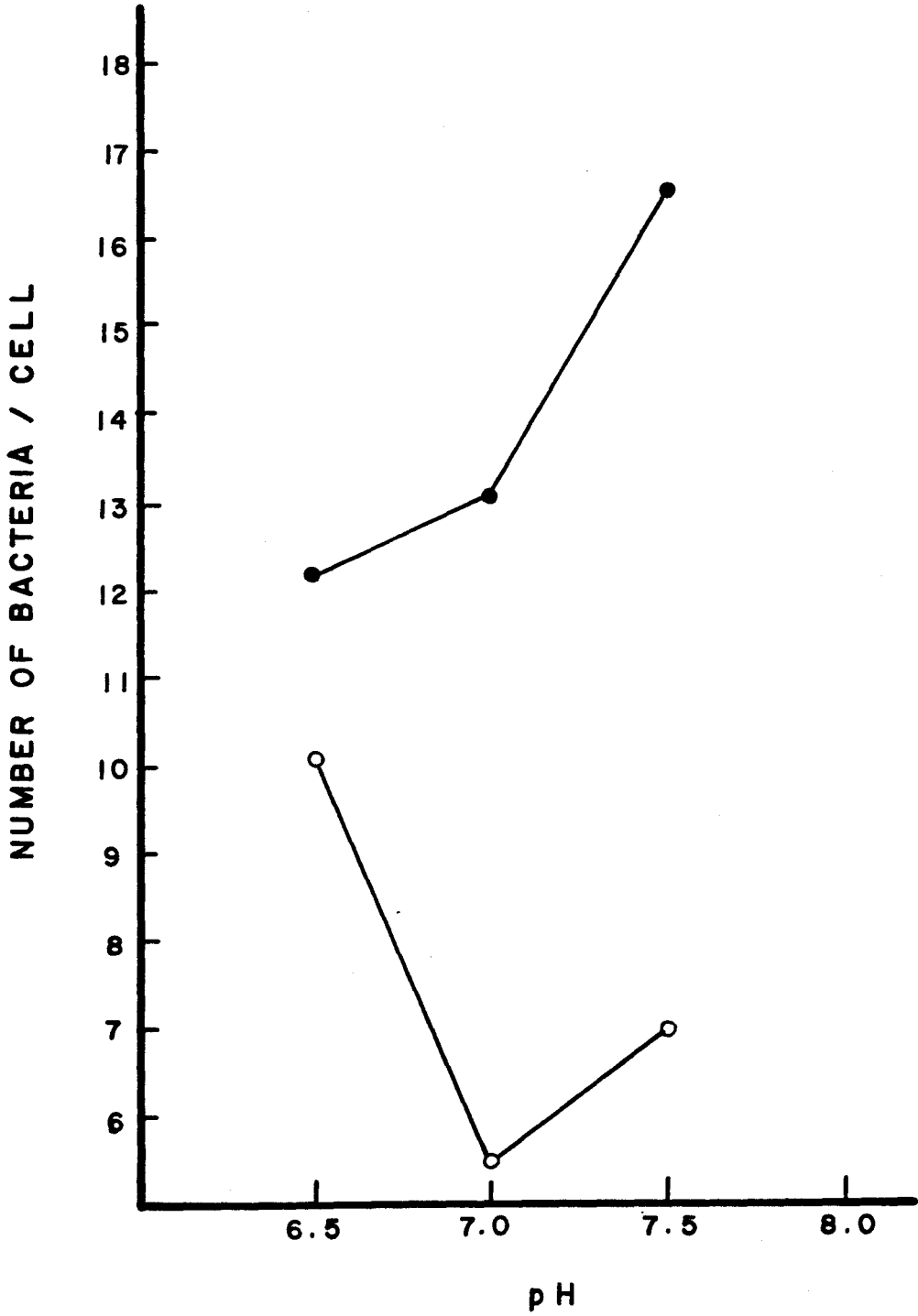
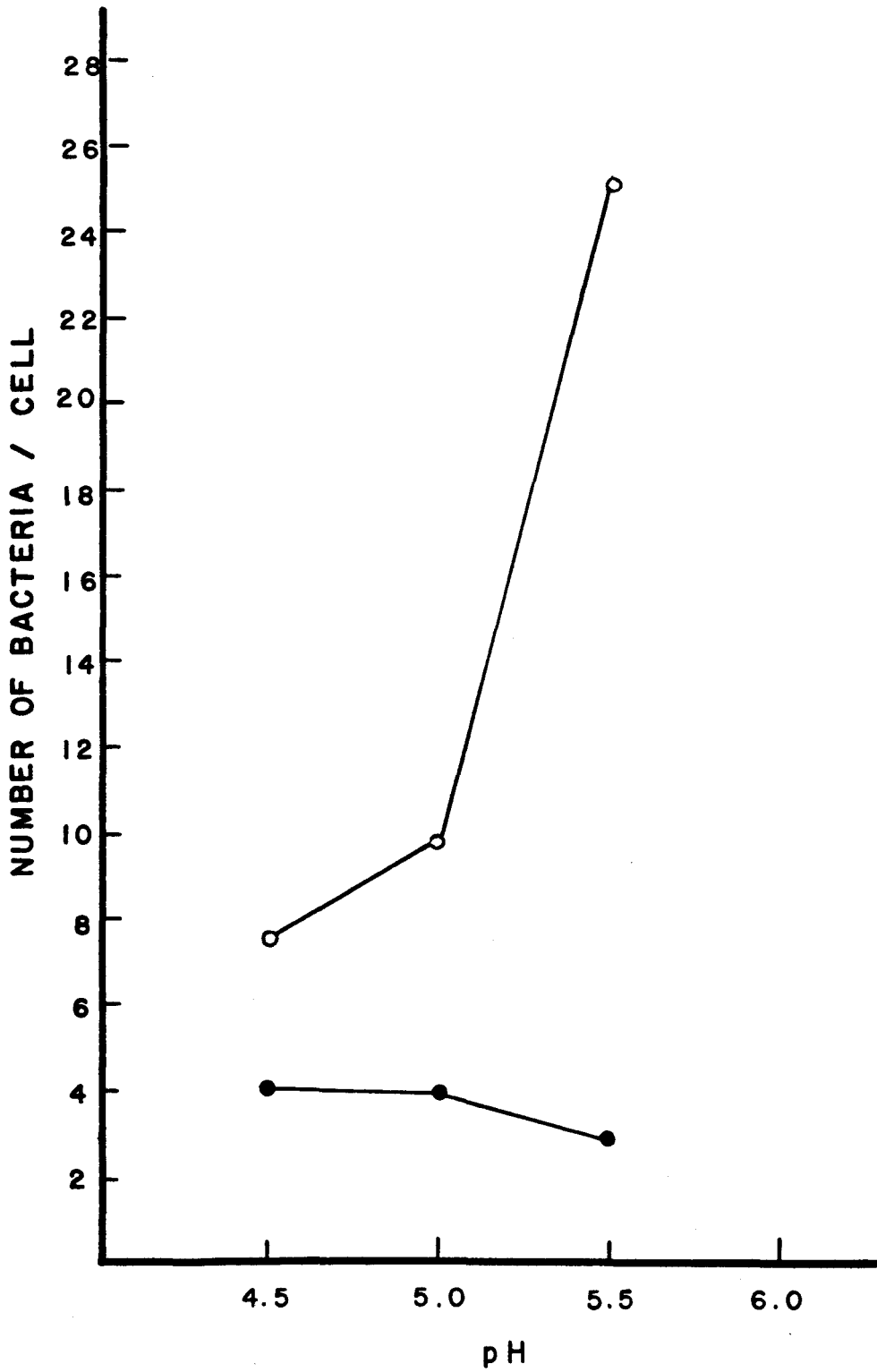


Fig. 6. Adherence of B. bivius to BEC in 0.01 M sodium acetate buffer (—●—●—) and in 0.01 M sodium acetate buffer supplemented with 0.018 M NaCl (—○—○—). Assay was performed as described in Materials and Methods using pooled BEC from 2 volunteers. Results shown are representative of at least 2 determinations.



similar effect? When the original buffer systems were evaluated, Tris-maleate showed what appeared to be a pH dependent effect on adherence (Fig. 7). Evaluation of a Tris-maleate buffer system would eliminate the contribution of phosphate ion, although there is a small amount of sodium ion present (0.004M to 0.006M, depending on the pH in question), resulting from the NaOH used in its preparation. For comparison, we also evaluated a Tris-HCl buffer system, which has neither sodium nor phosphate ions (Fig. 8).

In the Tris-maleate system, addition of NaCl, sodium citrate or  $MgCl_2$  resulted in a decrease in the number of B. bivius adherent to the cells. We therefore could not specifically implicate either sodium or chloride ions. In the Tris-HCl system, NaCl, KCl and LiCl (at 0.15M) were evaluated and found to have just the opposite effect; that is, with either of these salts present, adherence was at least equal to or increased over that observed in unsupplemented buffer. In this system,  $MgCl_2$  at equivalent concentration markedly inhibited adherence of B. bivius to BEC.

At this point, the exact effect of salts on the observed adherence of B. bivius to BEC was still obscure. In sodium phosphate buffer and in Tris-maleate buffer, NaCl in near physiologic concentrations (0.15M) decreased adherence, while the same salt in Tris-HCl buffer markedly

Fig. 7. Adherence of B. bivius to BEC in 0.01 M TRIS-maleate buffer ( —●—●— ) supplemented with 0.16 M NaCl ( —○—○— ), 0.16 M sodium citrate ( —■—■— ) or 0.15 M magnesium chloride ( —△—△— ). Assay was performed as described in Materials and Methods using pooled BEC from 2 volunteers. Results shown are representative of at least 2 determinations.



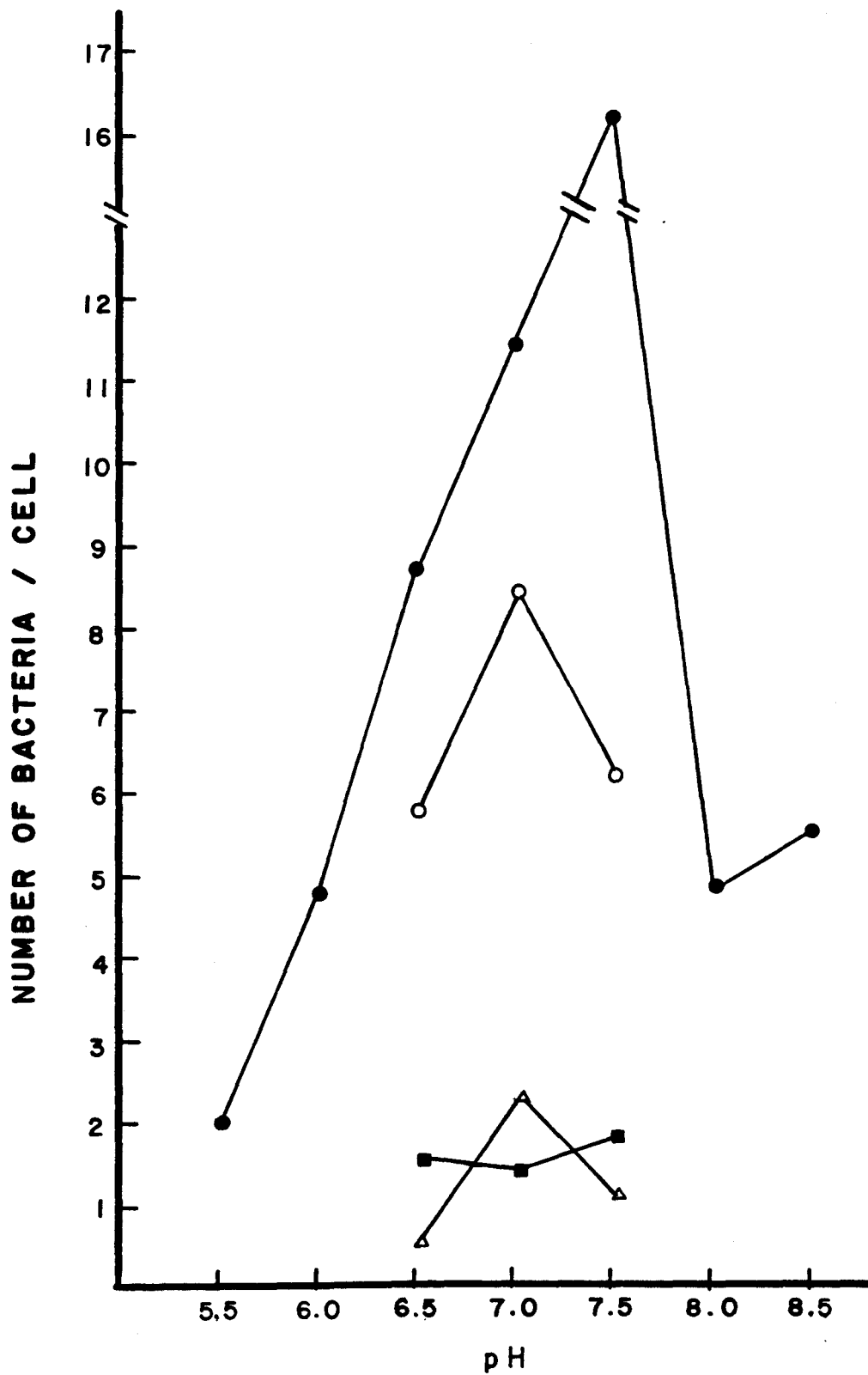
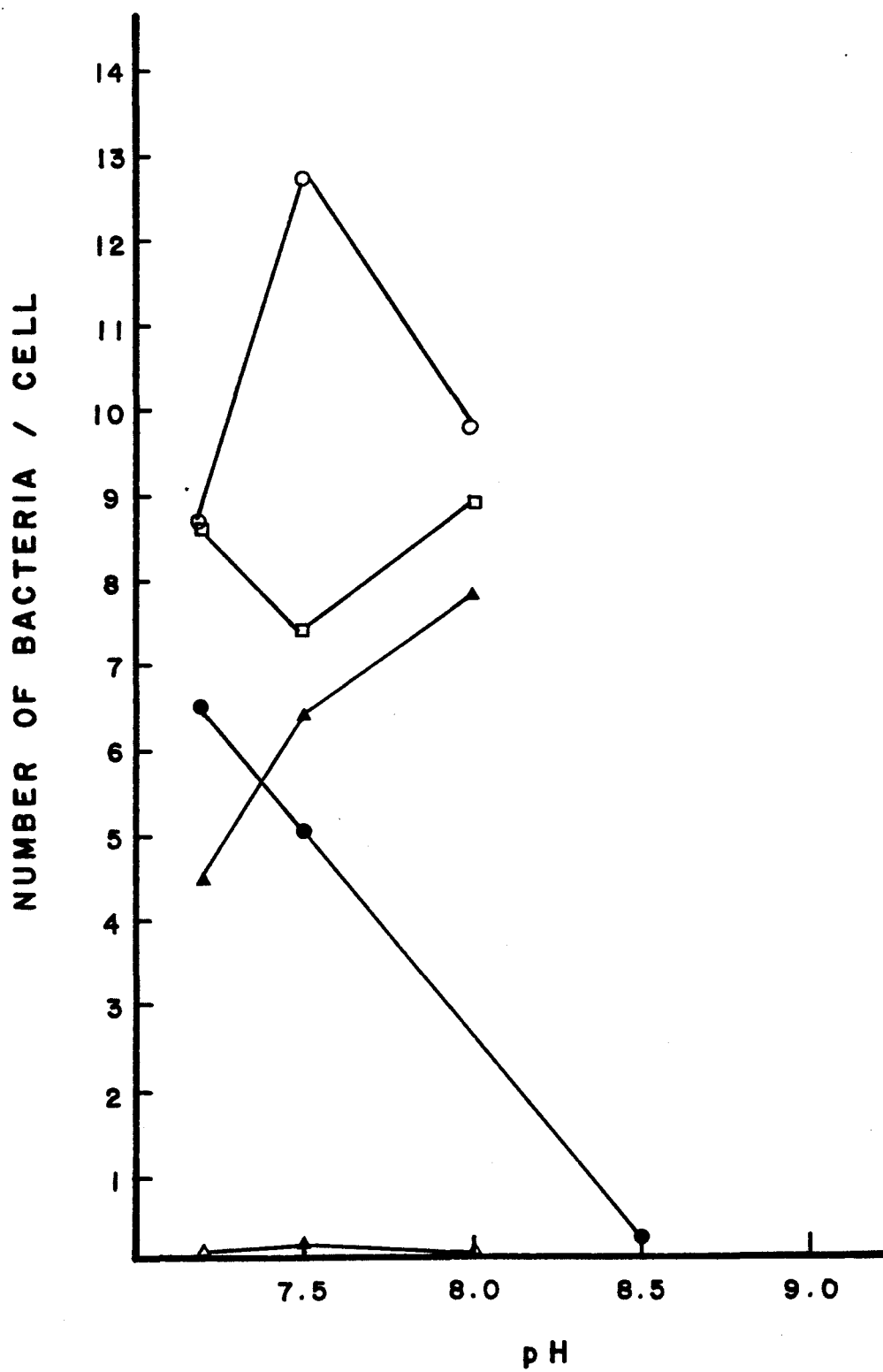


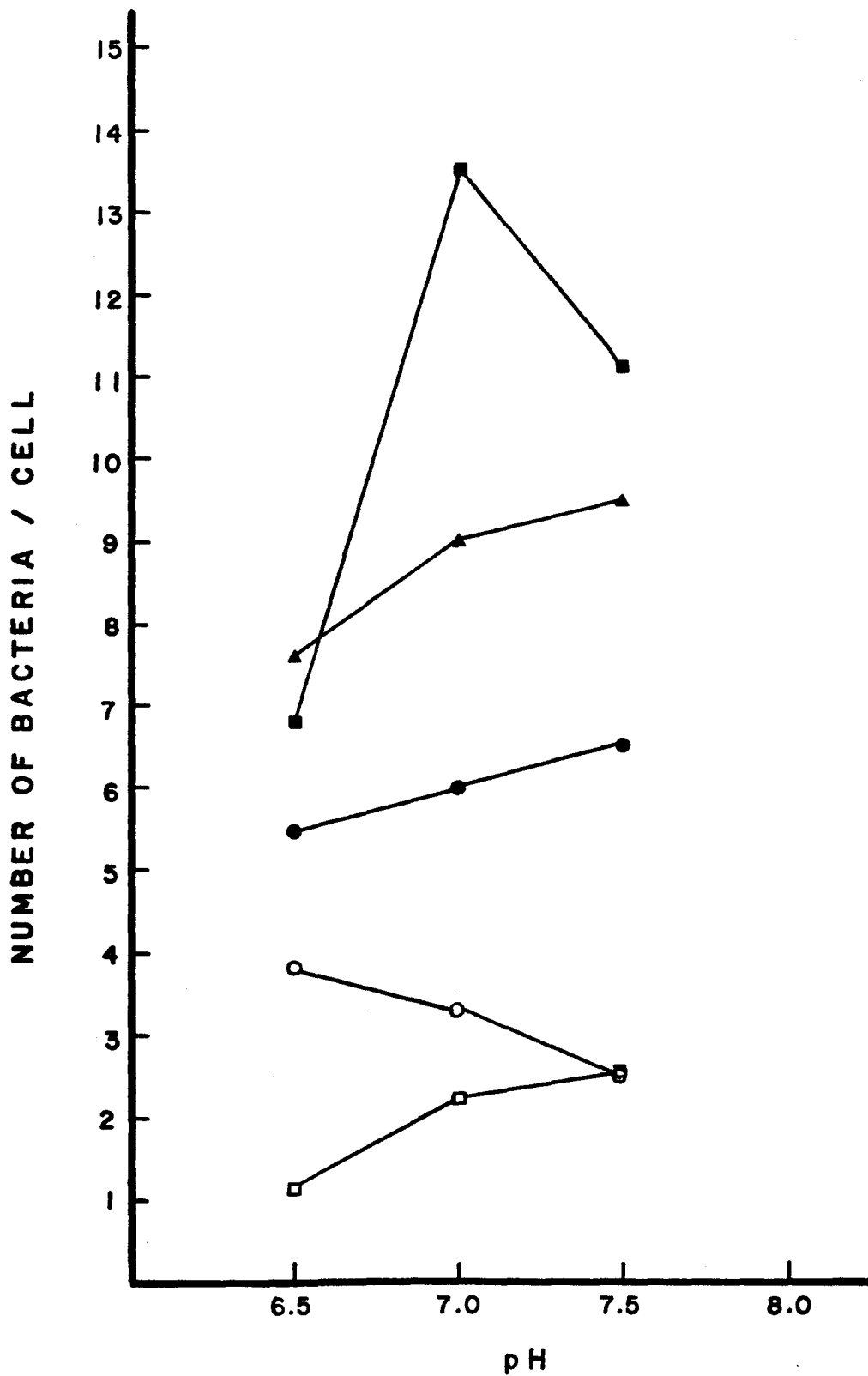
Fig. 8. Adherence of B. bivius to BEC in 0.01 M TRIS-HCl buffer ( —●—●— ), supplemented with 0.15 M NaCl ( —○—○— ), 0.15 M potassium chloride ( —□—□—□— ), 0.15 M lithium chloride ( —▲—▲—▲— ) or 0.15 M magnesium chloride ( —△—△—△— ). Assay was performed as described in Materials and Methods using pooled BEC from 2 volunteers. Results shown are representative of at least 2 determinations.



enhanced adherence. Magnesium chloride at low levels (1 mM as found in HBSS) had no effect, while when present at high concentration (0.15M), markedly inhibited adherence in both Tris-HCl and Tris-maleate buffer.

Since it appeared to be not specifically a chloride ion effect, nor an effect attributable to sodium ion, it was thought that the alteration of adherence may be a concentration phenomenon. In pursuit of the answer, we tested varying concentrations of NaCl in Tris-maleate buffer (a buffer that exhibited relatively high adherence values) (Fig. 9). At low concentrations (0.05M and 0.1M), adherence was increased over that observed in unsupplemented buffer, while at higher concentrations (0.15M and 0.2M), the observed adherence was diminished over the baseline. It should be noted here that in this particular experiment there was no dramatic peak of adherence observed at pH 7.5 in unsupplemented buffer. As a rule, it has been characteristic of this assay that while general trends are maintained from experiment to experiment, absolute values for adherence may vary greatly from day to day. The inhibitory effect of physiologic concentrations of NaCl seen in Figure 9 has been a consistently observed phenomenon in both the Tris-maleate and the sodium phosphate buffer systems. Conversely, relatively low concentrations of NaCl (18 mM to 50 mM) have shown a reproducible enhancement of adherence in the sodium acetate and

Fig. 9. Adherence of B. bivius to BEC in 0.01 M TRIS-maleate buffer ( —●—●— ) supplemented with 0.05 M NaCl ( —■—■— ), 0.1 M NaCl ( —▲—▲— ) 0.15 M NaCl ( —○—○— ) or 0.2 M NaCl ( —□—□— ). Assay was performed as described in Materials and Methods using pooled BEC from 2 volunteers. Results shown are representative of at least 2 determinations.



Tris-HCl systems, as well as in the Tris-maleate buffer system.

Investigation of possible role of fibronectin in adherence.

Several recent reports [1, 68, 147, 177, 187, 189, 190] have suggested that the presence or absence of the surface glycoprotein fibronectin may be a determining factor in the colonization of epithelial cells by a variety of organisms. Several of these investigators have utilized treatment of respiratory tract epithelial cells with proteases (which removes surface fibronectin) to evaluate the role of fibronectin in bacterial adherence. Accordingly, we set upon a series of experiments to evaluate the possible role of fibronectin in adherence (or non-adherence) of B. bivius.

Initially, the trypsinization procedure used was similar to that as described by Woods, et al., [190] using cold PBS and two centrifugations to eliminate enzyme before performing the adherence assay. It was found that approximately 50% of the original cell suspension was lost during these centrifugations. We elected to stop enzyme action by addition of trypsin inhibitor and then only one centrifugation was needed to concentrate the cells for assay, thus circumventing the problem and increasing the cell yield.

Several studies have shown that gram positive organ-

isms will bind to fibronectin [1, 114, 147, 177]. Also, studies have shown that cells that are poor in surface fibronectin content bind fewer gram positive organisms [1, 177]. Although none of the aforementioned reports have documented the loss of gram positive organisms from trypsin treated cells, it would seem logical to assume that such would be the case. Trypsin was used at a concentration of 2.5  $\mu\text{g/ml}$  in the original studies by Woods et al., [68, 190]. When we used this concentration, not only did the gram negative organisms tested not adhere in increased numbers, but the counts of resident bacterial flora (streptococci in the case of BEC and lactobacilli in the case of VEC) did not decrease significantly. On the basis of this observation, it was decided to increase the concentration of trypsin used in epithelial cell digestion. When trypsin was used at a concentration of 2.5 mg/ml (admittedly a high concentration, but equivalent to that used in studies by Sobel et al., in trypsinization of VEC [144]), the resultant cell suspension contained much debris and very few intact cells. Epithelial cell counts were reduced by 1 to 2 logs.

To determine whether the enzyme was digesting the intact cells or if they were being rendered more fragile and thus more susceptible to any subsequent manipulations such as the stress of centrifugation, the trypsinization process was observed microscopically at intervals during



the incubation period. Cells were suspended in 60 x 15 mm tissue culture plates (Falcon, Division of Becton - Dickinson, Oxnard, CA), trypsin was added to a final concentration of 2.5 mg/ml and the plates were incubated at 37°C for 20 min. Cells were set up in duplicate and one plate of each pair was examined microscopically at 5 min intervals at 440 X to observe for cell lysis, the pairs being alternated so that sub-optimal enzyme action upon exposure to cooler temperature was minimized. After incubation, trypsin inhibitor was added and the suspensions swirled gently to mix. Cell counts were determined to compare trypsinized and PBS control cells. Upon observation, the cells appeared intact at all intervals. No gross cell lysis was observed. After centrifugation, there was a 34% loss in control cells during the entire procedure and a 66% loss of trypsin treated cells. Examination of stained smears of trypsinized cells showed markedly decreased numbers of cells and much cellular debris present. The cellular debris appeared to contain large amounts of resident flora trapped within. It is uncertain whether this represents resident flora trapped on what is left of the cell membrane surface, or whether these are bacteria released during trypsinization and subsequently caught in the resultant debris.

We altered the assay procedure at this point to obtain enough cells to count. When trypsinized cells were

used, after filtration and washing of cell suspensions, the membrane filter was removed from the adaptor and the filter pressed to and allowed to dry on the slide without removal, thus maximizing the number of cells available for evaluation. The membranes were cleared prior to gram staining by submersion in chloroform for 8 to 10 min, followed by a few rinses in 95% ethanol as recommended by the manufacturer [Nuclepore Corp., Bulletin of "Diagnostic Cytology"]. Bacterial counts obtained by clearing of membranes in this manner were comparable to those obtained in the usual "transfer" procedure.

VEC from five normal females were subjected to trypsinization and the adherence assay, as were BEC. Statistical analysis of the resultant data showed that adherence of the B. fragilis and B. bivius to VEC was not significant. P values were never less than 0.4 whether cells were trypsinized or not. If one compares trypsinized versus non-trypsinized cells, the counts of resident bacteria per cell were significantly different. Mean bacterial counts for VEC were  $0.6 \pm 0.1$  organisms per cell prior to trypsin treatment and were  $0.1 \pm 0.03$  after trypsinization ( $P = 0.005$ ). For BEC, counts were  $25.7 \pm 7.5$  before trypsin and  $3.8 \pm 1.8$  after trypsin treatment ( $P = 0.02$ ). Resident gram positive flora were reduced by trypsinization of the cells. Again, B. bivius consistently adhered to trypsinized BEC, but no more so than to

nontrypsinized cells.

These data, however, provide only indirect evidence of the negligible role of fibronectin in adherence of B. bivius to BEC or VEC. The trypsin procedure we used was developed somewhat empirically and we could not at this point be sure that fibronectin was being removed by our trypsin treatment. The trypsin concentration used in these evaluations was quite high and was quite destructive to the cells. The question arose as to whether the procedure could be used to duplicate the results with BEC observed by Woods et al., in their studies. His original description of the phenomenon utilized "recent clinical isolates" of P. aeruginosa and K. pneumoniae [68], not readily obtainable reference strains. In order to attempt to reproduce his trypsinization data, we tested fresh clinical isolates of K. pneumoniae and P. aeruginosa from respiratory specimens at LUMC, a mucoid and a non-mucoid strain of P. aeruginosa isolated from a cystic fibrosis patient, and a P. aeruginosa isolate from a patient in the intensive care unit known to be colonized with the organism.

Since the working concentration of trypsin appeared to be unduly harsh on the integrity of the epithelial cells, it was also decided that we should re-evaluate the concentration of trypsin being used, evaluating a range from 25  $\mu\text{g/ml}$  to 1.0  $\mu\text{g/ml}$ . Although cations were deemed

unimportant in our B. bivius assay procedure, we supplemented PBS used in this particular experiment with 0.001 M  $MgCl_2$  to more closely reproduce those conditions reported in the literature. PBS was used at pH 7.2 for the assay, and the incubation time was increased to 1 hr. The pH of the trypsinization procedure was not reported in the literature, but PBS supplemented with  $MgCl_2$  was made up at pH 7.6 (optimal pH for trypsin activity) and used for the trypsinization step.

We had chosen to use trypsin inhibitor to terminate trypsin activity rather than several washes in ice cold PBS as was done by Woods as too many cells were being lost through the washings. It is possible that the various protein components of the trypsin inhibitor (a partially purified ovomucoid preparation) may coat or otherwise "mask" and interfere with various receptors and/or adhesions. Adherence of group A streptococci to BEC has been reported by Simpson et al., to be inhibited by serum [137]. To eliminate the possibility of non-specific interference by an ill-defined protein preparation, we chose to use the chemical serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) to terminate trypsin activity [45].

In no case examined did we observe a marked increase in adherence to trypsinized cells over that seen in untreated control cells. Woods et al., have shown that P.

aeruginosa and K. pneumoniae adhere to BEC with reduced surface fibronectin at a rate of from 5 to 12 times greater than they adhere to BEC with normal, intact fibronectin [187, 189, 190]. Although we would see slight adherence of these organisms (approximately 0.5 bacilli per cell), the difference between the number of organisms adherent to untreated vs. trypsinized cells was never significantly increased. It was in the range of 0.4 - 0.5 bacilli per cell (data not shown), and in no case was there ever a marked difference between treated and untreated cells, regardless of the trypsin concentration used, the organism used, the growth medium used, or the method of trypsin inactivation.

We obtained P. aeruginosa DGI from Dr. Woods. This strain was used in some of his early studies and has been reported to adhere well to BEC that have lost surface fibronectin. We followed his published procedure, using buffers supplemented with 0.001 M  $MgCl_2$  at pH 7.2. The organism was grown to log phase in TSB for 12 hr at 37°C. Epithelial cells were trypsinized at 37°C for 15 min and the adherence assay incubated for 2 hours at 37°C as stated in his previous reports. Deviations from his methods were what we considered to be minor. Trypsin treatment was carried out at pH 7.6, an optimum for trypsin activity and to which he makes no reference in his reports. We trypsinized cells with 5.0  $\mu g/ml$ , 2.5  $\mu g/ml$

and 1.0  $\mu\text{g/ml}$  trypsin, whereas his results have been based on treatment with 2.5  $\mu\text{g/ml}$  trypsin. The major deviation would appear to be our termination of protease activity with 1  $\text{mM}$  PMSF instead of with multiple washes in PBS, a technique we found unsuitable due to excessive cell loss. Controls in this experiment consisted of BEC suspended in PBS to which was added: 1) nothing, 2) 0.1 ml PMSF in 95% ethanol, or 3) 0.1 ml 95% ethanol. Although this particular experiment was performed only once, the results showed essentially no difference in adherence of P. aeruginosa DGI to trypsin or non-trypsin treated cells. In all instances, counts of bacilli per cell were minimal, ranging from 0.02 to 0.3 bacilli per BEC (data not shown). We therefore were unable to duplicate the trypsinization experiments of Woods et al.

## DISCUSSION

It seems to be generally accepted that anaerobic organisms grow at a slower rate than do most facultative or strictly aerobic organisms. While most guidelines for anaerobic bacteriology recommend a 48 hr incubation rather than 18-24 hr before examination for growth of anaerobic organisms [3, 40, 65], most experienced anaerobic bacteriologists agree that organisms in the Bacteroides fragilis group grow quite well after overnight incubation. Our growth curves (Fig. 1) would tend to substantiate that opinion. B. fragilis ATCC 23745 reached maximal stationary phase approximately 10 hr after exponential growth was initiated, while B. bivius ATCC 29303 achieved stationary phase after approximately 16 hr of exponential growth. While there is no data in the literature concerning the rapidity with which B. bivius may be isolated and identified from clinical material, our data and our practical experience with this organism would suggest that it would require 24 to 36 hr initial incubation.

Because of initial difficulties encountered in establishing reproducible absorbance/concentration correlations, we suspected that perhaps B. bivius was relatively intolerant to oxygen exposure. While B. fragilis has been shown to be tolerant to oxygen exposure

[60, 92, 168], to our knowledge there have been no specific reports concerning some of the more recently described Bacteroides sp. such as our B. bivius. When we observed the effects of exposure to room air over a four hour period, we found that while approximately 25% of the original inoculum decreased over the four hour period (Fig. 2), this did not reflect our initial random observations where it appeared to decrease by almost 1 full log over the same period. These initial observations were made during our growth curve studies and in many instances probably involved organisms in the exponential phase of growth, that phase in which organisms are most sensitive to oxygen exposure [65]. While Tally et al., have shown that oxygen tolerance is related to the levels of superoxide dismutase (SOD) produced by a variety of anaerobic organisms (including 7 Bacteroides isolates) [166], there are no direct reports of SOD production by B. bivius. SOD catalyzes the dismutation of superoxide free radicals generated in the reduction of molecular oxygen as follows:



with the resultant hydrogen peroxide produced also capable of being a destructive oxidizing compound to microorganisms. Production of catalase by members of the B. fragilis group [65] may afford them extra protection against



exposure to oxygen and might explain the relative differences in aerotolerance that we observed in our studies between B. fragilis, a producer of catalase and B. bivius, a non-catalase producer.

The deleterious effects of oxygen exposure were found to be exaggerated under the conditions of the adherence assay (Table 1). One possible explanation for this phenomenon is that at the increased temperature of incubation, the enzyme SOD may have increased activity and is producing hydrogen peroxide at an increased rate from superoxide radicals present. Once formed, the hydrogen peroxide may have a more deleterious effect on an organism such as B. bivius that does not have catalase present to further protect itself from oxidation by these compounds.

It is desirable to remove resident bacterial flora from epithelial cell surfaces prior to assaying for bacterial adherence in order to facilitate visual examination. Virtually all studies of bacterial adherence to epithelial cells include a preliminary washing of the epithelial cells [10, 11, 14, 19, 20, 25, 31, 41, 52, 73, 79, 80, 81, 85, 95, 107, 109, 112, 130, 131, 132, 144, 155, 156, 190] to remove mucus and unattached bacteria. Only one investigative group has reported on the relative effectiveness of the washing procedure. Mardh and Westrom found that washing VEC 3 times (by membrane filtration) significantly reduced resident flora prior to addition of

the test bacteria [95]. Our examination of preliminary washing of epithelial cells by differential centrifugation showed there to be a quantitative but statistically insignificant reduction in bacterial flora on repeated washings (Table 2). This would imply a rather stable association between bacterial flora and the cell surface. We elected to prepare our epithelial cells for assay by using two washes by differential centrifugation. At that point, resident bacterial counts had been reduced, and subjectively, there were relatively few unattached bacteria in the background when the smears were read.

While removing free, unattached bacteria, repeated cell washings may also be removing cell surface mucus, the importance of which has not been demonstrated in most adherence systems. V. cholerae has been reported by Freter to associate with the intestinal mucus gel prior to epithelial cell adherence [43], and conversely, Slots and Gibbons have shown that saliva could inhibit the attachment of B. melaninogenicus to BEC [140]. Most investigators have not addressed the role of native secretions in epithelial adherence evaluations, and it appears to be standard protocol to remove surface secretions and debris prior to adherence testing. With no significant differences in bacterial counts noted after each washing in our system, and with very little data available on the effects of removal of mucus secretions on bacterial adherence,

perhaps the sagacity of such a widespread technique should be evaluated. While it may be relatively easy to collect saliva, clarify it by high speed centrifugation, and then re-introduce it into an assay system for evaluation, collection and assessment of vaginal, bronchial or intestinal secretions might present more of a technical problem.

Mean cell viability was also determined on the epithelial cell preparations. For BEC, it was found to be quite low. It seems to be generally accepted that buccal epithelial cells are mostly dead when stained by trypan blue [16]. Beachey has found that no matter how long buccal surfaces are scraped, the resultant cells have no more than 5% viability [6] and our findings would agree with this figure. Svanborg-Eden has also reported less than 10% viability for buccal epithelial cells and approximately 80% viability for the uroepithelial cells used in her studies [153]. Vaginal cell viability was not reported by Mardh and Westrom in their studies [95], nor in any other studies to date.

Our results would suggest that exfoliated vaginal epithelial cells represent a different population in terms of cell viability, having a mean viability of from 29.3% up to 40.0%, depending on the population in question (Table 8). The exception would be VEC obtained from women at the time of Caesarean section delivery, whose cells were markedly less viable. In our normal, non-pregnant

women, cells were collected at all stages of the menstrual cycle, although the majority of women were seen in the gynecology clinic during weeks 3 and 4. During the follicular phase, individual cells are exfoliated in the course of normal, rapid cell regeneration [180]. In the luteal phase, as the onset of the menses approaches, vaginal epithelial cells begin to desquamate and an associated cytolysis occurs, which one would expect to result in less viable cells when evaluated by trypan blue exclusion. There appeared to be no obvious correlation between stage of the menstrual cycle and cell viability (data not shown).

During pregnancy, hyperemia and venous stasis cause an extension of the whole vaginal surface and the epithelial layer becomes greatly thickened [82]. The initial stages of labor are accompanied by chemical changes in vaginal wall colloids that make them more hydrophilic and more capable of undergoing structural changes associated with delivery. While resultant vaginal delivery may have a direct effect on the sloughing off of vaginal epithelium, when the fetus is delivered surgically, there would be little direct effect on vaginal epithelium. It is possible that the cellular changes induced by labor may result in rapid cell death during prolonged labor, which could be reflected as decreased cell viability in our surgical VEC specimens. The reasons for increased viability of cells 24 hr post delivery is less clear. It

is possible that within 24 hours after labor-induced cellular changes, there may be an increased desquamation of superficial vaginal epithelium, although such has not been reported in the literature. When our samples were obtained at 24 hours after delivery, the vaginal epithelium may have begun regeneration to compensate for loss of epithelium associated with delivery. While cellular viability was not significantly increased at this time, the viability was slightly above that in the non-pregnant population, and it is possible that the mean epithelial cell viability may increase even more during the days following labor as the superficial vaginal epithelium regenerates. In the case of this particular group of samples, the majority of them were collected by obstetrical residents at night, the specimens being refrigerated until the next morning when they would be processed. While random checks showed that cell viability did not change much over 6 to 8 hr when refrigerated, the possibility exists that these cells exhibited a decreased viability due to the time period involved in their processing.

The initial development of a reproducible adherence assay involved far more problems than we could ever have anticipated. Although a variety of different techniques were being used by the groups studying adherence, the literature gives no indication of the extraordinary nature

of epithelial cell allegiance to any and all surfaces with which it comes into contact. A colleague of E.H. Beachey, Andrew Simpson, has found that no matter how one manipulates or pretreats tubes used in epithelial adherence assays, the cell yield is poor, and the assay results seem to be unaffected [personal communication]. We have found, however, that pretreatment of polypropylene microfuge tubes with 0.1% BSA gave us a much better cell yield for adherence evaluation.

While most adherence evaluations use freshly collected epithelial cells, usually obtained by a superficial scraping of the mucosal or epidermal surface, some investigators have used tissue culture cell lines in their studies. A human intestinal cell line (Intestine 407) has been used in evaluation of adherence of E. coli [18, 62, 178] and Campylobacter sp. [23], amniotic cells (WISH) for adherence of N. gonorrhoeae [63], and HeLa cells for the study of E. coli [84, 126], E. coli and C. albicans [94] and N. gonorrhoeae [160]. In addition, primary monkey kidney cells were used in a comparative study by Mackowiak and Marling-Carlson in evaluation of Staphylococcus aureus, N. meningitidis and Streptococcus viridans adherence [93]. In these systems, known pathogenic strains of organisms have been shown to adhere to various cell lines, but few studies have addressed the correlation between cell culture adherence and adherence to exfoliated cells.

Clausen and Christie showed that enteropathogenic strains of E. coli adhered both to cells from intestinal biopsies of patients from whom they were isolated as well as to the human epithelial cell line, Hep II [24]. Pruzzo et al., found that strains of B. fragilis that adhered well to BEC adhered well to Intestine 407 cells but very poorly to HeLa cells [115].

It is a generally held opinion that the surface of a derived tissue culture cell shows little more than a superficial resemblance to cells of living tissues of an animal. The immunology literature is replete with reports of cultures of lymphoid and other cell lines that have either lost some antigenic determinants or have additional antigens not present on the cells prior to their transformation [38]. While tissue culture systems provide a well-defined, controlled system within which to work, such a system may have no relevance to in vivo relationships and tissue colonization by a given organism. Svanborg-Eden reports that only 50% of strains that attach in the HeLa cell system of Vosbeck will adhere in her exfoliated uroepithelial cell system [134]. These systems could, however, provide a controlled system in which to study adherence kinetics or cellular interactions at the molecular level. Primary short term cell cultures could also provide a means of studying the adhesiveness of different bacterial strains to biopsy material, particularly when

epithelium cannot be sampled by superficial scraping, such as the intestine or fallopian tube. Sobel et al., did find that several vaginal organisms (group B streptococci, Lactobacillus sp., Gardnerella vaginalis, C. albicans and E. coli) adhered in a similar fashion to exfoliated epithelial cells and to vaginal tissue explants, but not to fibroblast cultures [142]. Their data also suggests that at least in a vaginal cell system, the topmost, more mature cells that are sampled in exfoliation may be the best cells for evaluation. They found that bacteria adhered in particular to those cells in the process of desquamating and did not adhere to newly dividing young cells in the culture.

Evaluation of adherence can be done by several methods, the two most popular being a visual microscopic exam for bacteria adherent to epithelial cells, and a radiolabeling technique for the quantitation of cell-associated bacteria. Mackowiak and Marling-Carlson did a recent comparative evaluation of adherence techniques [93]. Their evaluation utilized a cultered cell line rather than exfoliated epithelial cells, so some of the problems we encountered in establishing our protocol were not evident in their appraisals. Quantitatively, each technique gave different results, with light microscopy giving higher bacterial counts per cell. Their opinion was that each technique had inherent advantages and



limitations and overall preferred the radiolabeling method. They felt that visual exam might pose a problem in differentiating bacteria with similar cellular characteristics or organisms that may stain poorly such as Haemophilus sp. The main advantage to visual evaluation was the ability to estimate the percentage of cells involved. In comparing methods, they found that light microscopy demonstrated more variability, with results of assays of the same slides by a single observer on different days varying not more than 20%. The vast majority of visual adherence evaluations use bacterial counts obtained from examination of 50 epithelial cells [1, 35, 41, 53, 73, 79, 95, 107, 108, 111, 132, 142, 143, 144, 145, 146, 191], although reports range from 30 cells evaluated [105] up to 100 [14, 15, 31, 80] or as high as 200 [10, 11, 22]. Among the major investigators in this field, relatively few have addressed the number of cells to be evaluated in their studies. In those reports that did [52, 111, 153], experimental error never increased significantly when 2 to 3 times as many cells were evaluated. In general, most investigators have chosen 40 to 50 cells for evaluation as a reasonable concession to substantial variations inherent in this method.

Parsons and Schmidt report large variances in the number of bacteria bound per vaginal cell, with the standard error of the mean being  $\pm$  the entire mean for

most data, regardless of the investigator evaluating the smear [111]. They also found significant differences in counts made on the same smear by different examiners, although the general trend of adherence (low, medium or high) was maintained among examiners. Moi et al., also found large variations in bacterial adherence to VEC within a given assay sample as well as between individual samples [103]. While this seems to be a problem inherent to these evaluations, they suggest this may be due in part to different stages in the keratinization of the VEC in a sample. An increased degree of keratinization has been shown by Sklavounou and Germaine to be associated with an increased adherence of S. salivarius and S. mitis to oral epithelial cells [139]. However, vaginal squamous epithelium is not as highly keratinized, so it is not possible to determine this association without concurrent staining for and evaluation of the epithelial cells for keratinization.

During the course of our studies, we did repeat counts on 67 different assays, usually done because counts obtained in the duplicate tests were in extreme disparity. Smears that were re-counted were found to differ from the original counts by  $29.4 \pm 2.5\%$ . When these duplicate counts were compared using the paired Student 't' test, they were found not to be significantly different ( $P = 0.82$ ). Thus, the visual evaluation technique in our hands

provided similar reproducibility when compared to other investigators. It is, in general, a very tedious and time consuming technique, and if large numbers of specimens are to be done frequently, it is definitely worth considering the use of a radiolabelling technique. The original decision to use a visual assay technique was based on what was thought to be ease of use. Cost was a secondary, but not insignificant consideration. The use of radioactive materials carries with it certain constraints in terms of labware and equipment that we felt could be avoided by use of simple supplies required for the microscopic evaluations. If cost of both the isotope and extra labware can be justified, and if scintillation equipment is readily available, the time saved by using a radiolabelling method would be of much relief to the investigator.

Our attempts to use our protocol to reproduce the studies done by Gibbons in evaluating adherence of oral streptococci were acceptable (Table 3). While quantitatively different from the original reports [52], the same general trends were evident in the adherence we observed. There was some initial concern about the possible interference of BSA used to pretreat the tubes, since serum albumin has been shown by Simpson et. al., to inhibit the binding of streptococci to epithelial cells through the interaction of streptococcal lipoteichoic acid and the free fatty acid binding sites of albumin [137]. No

interaction was observed with E. coli and albumin. BSA was shown by Johanson et al., to have no effect on the binding of P. aeruginosa, Klebsiella pneumoniae or E. coli to BEC [68]. We were able to essentially duplicate the data of Gibbons using BSA treated tubes in our method. We thus felt that it was a suitable system for the evaluation of adherence and that the residual BSA that was present in the tubes did not interfere with adherence.

We examined a number of different parameters reported to have an effect on adherence phenomena. Mardh and Westrom compared 30 min and 3 hr incubation periods in their study and found no significant increase in adherence with increased incubation [95]. They subsequently chose 30 min as their routine incubation period. Other standard assays have used 30 min incubations periods [2, 15, 25, 41, 51, 52, 73, 103, 105, 107, 108, 109, 111, 112, 127, 128, 132, 146, 191], with occasional variations of 40 min [10, 11], 1 hr [35, 121, 156], and 2 hr [14, 144]. Other than the studies by Mardh and Westrom, there are relatively few studies that have compared adherence over different incubation times. Kubin et al., in evaluation of group B streptococcal adherence to VEC and BEC found that maximal adherence was obtained by 50 min incubation [85]. Svanborg-Eden et al., have reported that most strains of E. coli adhere to uroepithelial cells maximally during the first 60 min of incubation [155], and Cheney et al., found

that association of enteropathic E. coli with rabbit intestinal brush border cells was maximal after 15 min incubation [22]. We chose to evaluate adherence at 30 min and 1 hr (Table 4), and found that there was no significant adherence of either B. bivius or B. fragilis at either time period. Therefore we chose 30 min for our standard assay protocol, as increased incubation times did not increase adherence. These figures represent total bacterial counts. At this point we were not distinguishing between resident flora and test organisms. We were, however, beginning to note a slight adherence of B. bivius to BEC.

The next variable that was evaluated was adherence of organisms grown on solid versus liquid medium. Jones and Freter have reported that V. cholerae will adhere to rabbit intestinal brush border cells when grown in trypticase soy broth, but not when grown on trypticase soy agar [71]. Bacterial surface architecture is known to be reflective of the medium in which cells are grown, with static broth culture encouraging pilus production in many species [98, 176, 183, 186]. Slots and Gibbons [140] and Okuda et al., [109] have demonstrated the presence of pili on the black pigmented Bacteroides species, B. gingivalis, B. asaccharolyticus and B. melaninogenicus. When Onderdonk et al., studied 14 strains of Bacteroides, most of them members of the B. fragilis group, none were found

to have pili by electronmicroscopic examination [110]. Pruzzo et al., have reported the presence of pili in unencapsulated strains of B. fragilis that also exhibited hemagglutination of human and guinea pig erythrocytes and adherence to an intestinal cell line [115]. Strains that demonstrated neither hemagglutinating nor adherent abilities were devoid of pili. We are unaware of any reports on the presence of pili on other members of the genus Bacteroides. In our studies, neither B. fragilis nor B. bivius exhibited adherence to genital tract epithelial cells, whether grown in broth or on agar media. B. bivius grown on BHIAS showed increased adherence to BEC compared to organisms grown in BHIS, although this was not a statistically significant preferential adherence (Table 5). Therefore, organisms grown on BHIAS were used in all our experiments to maximize adherence. It is interesting to note that the piliated strains of B. fragilis noted by Pruzzo et al., showed adherence to BEC [115], while our B. fragilis strain, a known encapsulated organism, has consistently failed to demonstrate any adherence.

Divalent cations have been reported to be required for adherence of V. cholerae to rabbit brush borders [71], for the attachment of V. parahemolyticus to a human fetal intestinal cell line [17] and for the attachment of E. coli to intestinal cells [Carl Deneke, New England Medical

Center, personal communication]; additionally, Woods et al., use PBS supplemented with 0.001 M magnesium chloride in all of their studies of adherence of gram negative organisms to BEC [187, 188, 189, 190]. Our use of Hank's balanced salt solution as a source of divalent cations had no effect on the adherence of either B. fragilis or B. bivius to VEC, CEC or BEC. Results were comparable to those obtained with unsupplemented PBS.

In view of the fact that numerous descriptions exist of adherence phenomena in the absence of divalent cations [2, 10, 11, 14, 16, 18, 19, 20, 22, 25, 31, 35, 41, 51, 52, 79, 81, 85, 105, 107, 108, 109, 111, 112, 121, 130, 131, 132, 140, 153, 155, 156], one must assume that it is not a critical component of all adherence interactions. Sugarman et al., evaluated the role of the divalent cation of zinc in adherence of a wide variety of gram-negative and gram-positive organisms to HeLa cells [151]. They found that zinc enhanced the adherence of piliated gram-negative and gram-positive organisms, but not that of non-piliated organisms, in a dose related fashion. They suggest that this is a pilus specific phenomenon as addition of mercury, magnesium, manganese or sodium cations did not result in a similar increase in adherence. While divalent cations are requisite in some adherence interactions, our data suggests that it is unimportant in the development of adherence of B. bivius

and B. fragilis to VEC, CEC or BEC. Therefore, we did not include divalent cations in our routine studies.

The effect of the gram stain procedure on adherence was examined by comparing counts from gram stained smears with those of wet mounts from the same assay mixture examined by phase contrast microscopy. As seen in Table 6, the differences in counts with the two methods are within ranges seen previously when duplicate gram stains from the same assay mixture are compared. The comparative study by Mackowiak and Marling-Carlson [93] found that counts were consistent for any one technique, but that counts may differ from one procedure to another. When examined by phase microscopy, we noted that vacuoles within the epithelial cells were frequently difficult to distinguish from organisms on the surface. It was also sometimes difficult to determine if an organism was a coccus or a bacillus (some of the Bacteroides organisms are cocco-bacillary and streptococci may be somewhat elongated). The higher counts in the gram stained evaluations may thus be the result of a conservative tendency to not call a "vacuole/bacterium" a bacterium by phase exam, while in a gram stained specimen, the bacterial nature is unequivocal. These differences were limited to counts of resident cocci (represented by total mean number of bacteria per cell), as counts of bacilli were fairly consistent.



All of our evaluations of adherence demonstrated no preferential adherence of either B. fragilis or B. bivius to epithelial cells of the female genital tract. One of the parameters that we evaluated was a possible differential adherence to vaginal versus cervical epithelium. In obtaining a cervical sample, examining physicians were instructed to use the curved end of an Ayers spatula , the objective being that the narrower curved end would sample cells at the squamo-columnar junction, close to the endocervical canal. With columnar rather than squamous epithelium lining the endocervical canal, and extending onto the ecto-cervix [138], we should have been able to detect cervical cells in our samples. Tall columnar epithelial cells never accounted for more than 8% of the total cells present, and the majority of samples contained no columnar cells at all. Therefore, our inability to distinguish between adherence to vaginal and cervical epithelium may result from comparing squamous populations indistinguishable morphologically as much as it could be due to a non-adherent tendency on the part of the organisms tested. While manipulation and sampling of the vaginal mucosal surface is a relatively innocuous procedure, additional measures to procure the endocervical columnar cells would cause the patient discomfort and other risks such as possible infection of the uterine cavity. We did not feel that the study warranted placing

normal volunteers at such risk.

When we examined adherence of clinical isolates of Bacteroides sp., we also observed no adherence, except to BEC. Kaspar et al., have shown that B. fragilis has less capsular material as it is serially passaged in vitro on blood agar [76]. His group has subsequently shown that this capsule enhanced the adherence of these organisms to rat mesothelium [110]. It has also been shown by Swanson et al., that the pili of N. gonorrhoeae are involved in their attachment to HeLa cells, and that these pili are lost on in vitro passage of the organisms [159, 160]. Although presence of capsular material or pili on B. bivius has not been reported, our examination for capsular material was negative in all of the B. bivius strains used in this study. We examined the adherence of clinical isolates that had not been passaged to any great extent, in the hopes of finding some adherence to genital tract epithelial cells. These isolates did not demonstrate adherence to VEC, but did adhere to BEC in numbers comparable to those seen with the ATCC strain of B. bivius.

One spurious incidence of adherence to VEC was noted in these experiments. There were approximately 10 cells on one of the duplicate assays that were virtually covered with organisms resembling B. bivius, while no such cells were discovered on the other sample, as well as in the control or the B. fragilis assay smears. A detailed

review of this patient's medical record revealed that she had a history of Weber-Christian disease. This disease is a disease of cutaneous and visceral fat and is characterized by subcutaneous nodules with inflammatory cells within fat lobules and lipophagic granulomas [87]. Acute attacks are treated with steroids, otherwise no other treatment is recommended. It is unclear from her record whether she was taking prednisone at the time she was seen in the gynecology clinic. How this disease or its therapy might have affected her epithelial cells so that they might show a differential adherence of B. bivius is unclear. There is no other explanation for this one and only incidence of B. bivius adherence to VEC.

We chose to look at additional patient populations for 2 specific reasons. Women who have undergone Caesarean section delivery are at increased risk of developing post-partum endometritis, an infection from which B. bivius is frequently isolated [9, 46, 47, 48, 49, 55, 141]. We thought that genital tract epithelium may be altered in some way during pregnancy to make it more susceptible to colonization by and perhaps subsequent infection with this organism.

Epithelial cells from patients prone to certain infections have been shown to preferentially bind bacteria associated with those infections. Fowler and Stamey [41], Kallenius and Winberg [73] and Svanborg-Eden [156, 157]

have shown that uroepithelial cells from women with a history of recurrent urinary tract infection will bind a greater number of E. coli than do cells from women not prone to such infections. Secondly, studies by Woods et al., have demonstrated a change in respiratory tract epithelium in patients suffering acute stress such as acute respiratory distress or surgery [189]. In these cases, secretory protease levels increase with a subsequent decrease in the surface glycoprotein, fibronectin, and a resultant increase in colonization by gram negative bacilli.

We felt that the stress of labor or Caesarean delivery might be capable of causing a similar alteration in genital tract epithelial cells that might endow them with a different adherence characteristic or profile. As can be seen in Table 7, there was no significant adherence of either B. fragilis or B. bivius to VEC collected at the time of surgery, 24 hr after Caesarean section delivery, or in those patients who show clinical symptoms of post-partum endometritis. This does not necessarily suggest that surface fibronectin is not lost in these patients, rather that the physiological stress of labor and delivery does not alter adherence characteristics of epithelial cells to our test organisms. This does suggest that vaginal adherence is not important in the development of post-partum infections with B. bivius in high risk

populations.

Mardh and Westrom have reported that B. fragilis adhered to VEC at a rate not significantly different from that of Lactobacillus acidophilus [95]. The fact that they observed detectable levels of adherence of B. fragilis (mean number of bacteria per cell = 10.8) while we consistently observed little if any adherence could be explained by their use of a recent clinical isolate for their experiments. Although we did not evaluate a recent isolate of B. fragilis in our studies, we did study recent isolates of B. bivius and found that there was no preferential adherence to VEC with clinical strains.

Our data is somewhat difficult to reconcile with a recent report by Moi et al., evaluating the adherence of a number of newly described organisms associated with non-specific vaginitis, including B. bivius [103]. They reported low, but detectable adherence of B. bivius to VEC that was pH dependent. They found B. bivius attached better with decreasing acidity, the highest values of  $15.7 \pm 13.4$  adherent B. bivius per cell obtained at pH 7.5. At pH 4.0, only  $4.0 \pm 3.9$  B. bivius were found to adhere, and at pH 5.5,  $6.5 \pm 4.6$  bacteria per cell were observed. Several aspects of their report should be addressed.

First, examination of their figures illustrates the high degree of variance within the counts obtained, as evidenced by the extremely high standard deviations

reported, a phenomenon confirmed in most all of our evaluations. When variation is so extreme, one could expect counts between studies to disagree, especially when the quantitative value is low, as in this instance. Other investigators have noted the relatively large variations inherent in this technique. Parsons and Schmidt report that standard deviations observed in their counts were frequently  $\pm$  the value of the entire mean counts [111], and the data of Svanborg-Eden also suggest an inherent variation in these determinations [153].

Second, they used a higher bacteria to epithelial cell ratio (5000:1 as compared to 1000:1 as we chose), which one could argue might contribute to their higher counts. Reports of bacteria to epithelial cell ratios in adherence studies range from a low of 100:1 in the studies of Cheney et al., [22], to a high of 10,000:1 in the studies of Candy et al., [15], Clegg et al., [25], Fowler and Stamey [41] and Parsons and Schmidt [111], with the vast majority using values in the 1000:1 to 5000:1 range [19, 20, 35, 51, 52, 105, 108, 109, 112, 121, 128, 132, 143, 156, 191].

Svanborg-Eden found saturation of the binding capacity of E. coli to uroepithelial cells occurred at a ratio of 1000:1 bacteria to epithelial cell [153, 155], and Mardh and Westrom reported that adherence of N. gonorrhoeae increased proportionately to the bacterial density up

to the maximum tested of a 10,000:1 bacteria:epithelial cell ratio [95]. Reid et al., have also observed that adherence of E. coli to uroepithelial cells increases as the ratio of bacteria to epithelial cells increases up to 1000:1, after which there is no significant increase in adherence beyond that point. Gibbons has reported that in general, 10% or less of available bacterial cells become associated with buccal epithelial cells [52] and others have reported that only a fraction of available cells of S. faecalis, V. cholerae or N. gonorrhoeae associate with epithelial cells [70, 158].

Having sufficient numbers of bacteria present to saturate potential receptors on the epithelial cell would appear to be the primary consideration. The majority of reports suggest that in most of the evaluations, critical concentrations should be obtained using a 1000:1 to 5000:1 range. It is unlikely, therefore, that this would account for discrepancies between the data of Moi et al., and our own.

A more likely explanation would be their evaluation procedure. Their counts were obtained by what is potentially a more sensitive technique, the use of fluorescein tagged specific antibody to B. bivius. This technique would increase the detectability of an organism which stains somewhat pale against the amorphous background of the epithelial cell surface. Fluorescent antibody

techniques may also give false-positive results due to cross-reacting antigens between species. Weissfield and Sonnenwirth have previously shown that commercial fluorescent antibody kits for detection of B. melaninogenicus cross-reacted with B. bivius organisms [184]. While the antibody used by Moi et al., was prepared by them, the possibility also exists that their positive fluorescent results may have resulted from cross-reaction with other organisms present as resident flora.

Of some interest is their evaluation at different pH values. The early studies on vaginal adherence by Mardh and Westrom [95] used a pH of 7.2 for the majority of their evaluations. With the exception of N. gonorrhoeae, organisms adhered in higher numbers, but not significantly so, to VEC when evaluated in minimal essential medium at pH 7.2 than they did in PBS at pH 4.5. The results reported by Moi also show increased adherence at higher pH values. Studies by Botta on adherence of group B streptococci to VEC have found adherence greater at pH 7.2 than at pH 5.4 [10, 11]. Zawaneh et al., found maximal adherence of group B streptococci occurred at pH 5.5. This is intriguing, since normal pH values in the vagina are quite low, ranging from 4.0 to 5.0 in healthy women of child-bearing age [179]. One might expect adherence of organisms found in such an environment as normal flora to adhere better at a pH near that in which they routinely



exist.

The above data, in conjunction with the report of Mardh and Westrom showing increased adherence of N. gonorrhoeae at acidic pH, suggests that adherence to VEC may be important at least with organisms considered potentially pathogenic in the female genital tract. Although neither group B streptococcus nor N. gonorrhoeae causes vaginitis, and group B streptococcus may be considered as normal flora in a non-pregnant female, both of them can cause subsequent disease as they gain access to the upper genital tract. The report by Moi et al., on adherence of other genital tract pathogens also lends credence to adherence as a potential pathogenic factor in the development of genital tract infections.

The incidence of post-partum endometritis is reported to be up to seven times greater in patients who undergo Caesarean section delivery than in similar patients having vaginal deliveries [26]. Premature rupture of membranes (rupture at any time before onset of contractions) also shows a strong correlation with development of maternal infections [161]. Even with normal rupture of membranes, bacteria from the lower genital tract ascend into the amniotic cavity at the onset of labor [162]. Operative intervention then may produce conditions appropriate for selective survival and proliferation of anaerobic organisms. The blood supply at the site of the incision is

impaired, and when this tissue is inoculated with organisms introduced from the lower genital tract, a nidus for infection is established. The suture material present as a foreign body further acts as a matrix within which infection may flourish, encouraged by collection of serum and fibrin around the suture. The multiplication and growth of anaerobic bacteria is encouraged in such an avascular, traumatized and possibly necrotic area which has a lower redox potential than well perfused tissues [40].

Studies continue to report a high incidence of isolation of this organism [163, McNeeley et al., Abstr. 25th Int. Conf. Antimicrob. Agents Chemother., 1985, No. 32; Landers et al., Abstr. 25th Int. Conf. Antimicrob. Agents Chemother., 1985, No. 34; Phillips et al., Abstr. 25th Int. Conf. Antimicrob. Agents Chemother., 1985, No. 35]. While our studies have not ruled out any other of a number of factors that may be involved in development of infection with B. bivius, a more likely explanation for the increased attention given to this organism is an increased awareness on the part of both physicians and microbiologists of its occurrence and significance. Modern diagnostic equipment and methods that were not widely available at the time of the initial reports of this organism are now commonly used in many laboratories. Recovery and identification of this organism, with a

subsequent increase of reports in the clinical literature may have resulted from this advancement in technology. The literature, as has been noted, rarely has documented B. bivius as the sole agent in gynecologic and obstetric infection, and reports of central nervous system or hematologic involvement are rare. Its consistent occurrence in conjunction with other genital tract flora in the majority of reports suggest that it is either being isolated from inappropriately collected, vaginally contaminated specimens, or that it is interacting synergistically with other microbial flora to produce a polymicrobial infection and is unable to produce disease of its own accord.

The lack of adherence of B. bivius to VEC suggests that its presence in the vagina and detectable adherence to vaginal epithelial cells may not play a critical role in the development of infections with this organism. Our original premise was to evaluate adherence of this organism in the hopes of finding a situation analogous to that of the respiratory tract or urinary tract; that is, that there might be a differential adherence of B. bivius to genital tract epithelial cells in different patient populations. Either a certain percentage of the normal population might exhibit adherence with this organism, thus predisposing them to subsequent infection, or perhaps patients with predisposing conditions such as Caesarean

section delivery might show a change in epithelial cell interaction with this organism. Although our population sample is not extensive, it would appear that other more important virulence factors promote the development of obstetrical and gynecologic infections with B. bivius.

Further laboratory evaluations of adherence and infection with B. bivius is warranted to better elucidate factors involved in susceptibility to infection. In the same way that endometrial aspirates or swabs are more appropriate for culture, endometrial tissue may be a more appropriate specimen for adherence testing, although enthusiastic agreement on the part of patients or physicians to provide such material is less likely than in this study. However, a correlative study on endometrial adherence characteristics and subsequent development of post-partum endometritis involving B. bivius would be of interest.

A more detailed evaluation of the possible role of fibronectin might also be considered. The source of fibronectin on buccal cells has not been established, but is likely to originate from saliva as it is detected only on the superficial epithelial cells (somewhat distal from the underlying connective tissue) and furthermore, no fibronectin has been detected in intercellular spaces or within the cytoplasm of epithelial cells [135, 192]. While fibronectin has been reported to occur in amniotic

fluid and to be synthesized by amniotic epithelioid and fibroblastic cells in culture [27], we are unaware of reports of its presence on the surface of genital tract epithelial cells.

It has been proposed by Abraham et al., that fibronectin, in adsorbing to epithelial cells, may either bind to the gram negative receptor on the cell or may sterically hinder the interaction of organisms with these receptors [1]. Svanborg-Eden has shown that the receptor on the epithelial cell that binds to pylonephritogenic E. coli is a glycolipid [154], but the possible role of fibronectin in binding to or blocking this receptor has not been investigated. Indeed, there is no evidence suggesting the presence of fibronectin on uroepithelial cells.

An assay to detect the presence of fibronectin on genital tract epithelial cells should be developed, followed by comparative studies in patients delivering vaginally, by Caesarean section, and those with obstetrical complications. Thus, the presence or absence of fibronectin on genital tract epithelium and its possible role in predisposition to colonization and infection in the post-partum period could be determined.

It should be noted that the role of fibronectin in blocking adherence of gram-negative organisms has been shown only with aerobic and facultative organisms such as

E. coli, K. pneumoniae and P. aeruginosa [1, 45, 136, 150, 187, 189, 190]. While it appears to be directly involved in the binding of gram-positive organisms such as S. pyogenes and S. aureus [1, 177] to epithelial cells, it has recently been shown to be important in the adherence of virulent strains of Treponema pallidum (a gram negative organism) to chinese hamster ovary and human tumor 1080 cell culture monolayers [171]. Thus, its occurrence on the surface of genital tract epithelium could play a critical role in the development of infections with organisms considered pathogenic to the genital tract. Although we cannot be certain that our trypsinization procedure effectively removed fibronectin (or other surface proteins) from BEC, adherence of B. bivius to BEC was unaffected by our trypsin treatment.

The only significant adherence noted with B. bivius in our studies was with buccal epithelial cells. Further studies on the nature of the observed adherence of B. bivius to buccal epithelial cells started with attempts to inhibit this adherence with carbohydrates. As far back as 1957, Duguid and Gilles reported that E. coli adherence to intestinal epithelial cells could be blocked by D-mannose. Subsequently, work by Sharon et al., has shown that E. coli binds specifically and reversibly to D-mannose residues on the epithelial cell surface [108]. While binding to surface sugars may not be the exclusive

mechanism of attachment to host cells, several investigators have evaluated inhibition by sugars in examination of adherence phenomena [71, 105, 182]. Adherence of B. bivius to BEC was not inhibited by any of the carbohydrates tested (Table 9). As can also be seen in Table 9, the percentage of cells with B. bivius adherent varied from 62 to 88%. Similar variation was also seen in all other assays in which adherence of B. bivius to BEC was shown (ranging from as low at 1% to as high as 80%). These observations lead us to believe that this is not a specific adhesin - receptor interaction, but perhaps was a non-specific physico-chemical association between the cells.

The data obtained from our pH and salt supplementation evaluations is not clear cut. In general, adherence in unsupplemented buffers was found to be highest at pH 7.0 to 7.5 and declined with pH change in either direction. Supplementation of buffers with a variety of salts gave no definitive correlation between ionic species present and inhibition or enhancement of adherence. Neither did a change in ionic strength. Indeed, results varied, depending on which buffer system was being evaluated. We were able to show an effect on adherence in supplementing Tris-maleate buffer with different concentrations of NaCl (Fig. 9). At lower concentrations (0.05 M and 0.1 M) there was an increase in adherence, while at

0.15 M and 0.2 M NaCl, adherence was decreased when compared to unsupplemented buffer.

Although adherence of B. bivius to BEC is of little, if any, clinical significance, even attempted speculation does not explain the observed adherence. Maximal adherence to BEC in unsupplemented buffer was observed at moderate pH values (7.0 - 7.5) (Figs. 5, 7, 8, 9), but the normal range of salivary pH is 5.6 - 7.0 [97]. The sodium concentration in saliva is 5.7 - 6.7 mM [97], at least half that at which we supplemented any of our buffers. It would thus appear that the maximal adherence we observed occurred under conditions quite unlike that which would be encountered in vivo.

A variety of ionic species were shown to affect adherence, but in a non-predictable manner that varied with the buffer system being employed, suggesting that ionic interactions are important, but probably not the sole explanation for the observations. The general non-correlative nature of this adherence suggests the occurrence of a non-specific phenomenon. While it may well be that this cellular association is due to electrostatic interactions and charge distributions, the reproducibility suggests a more specific but as yet unexplained mechanism.

Our ability to demonstrate adherence to which even our remote speculation cannot attach clinical importance,



should remind us and others that adherence data must be reviewed critically before the in vitro data is applied clinically. Any given adherence phenomenon should be completely and specifically characterized. This is not to say that previous reports suggesting the importance of adherence to the development of disease is to be doubted. However, we do suggest that the specificity of the association should be investigated. Differences in pH and ionic strength reflecting physiological reality, and most importantly, blocking by known inhibitors of adherence, should be carefully defined before attaching clinical significance to adherence data.

## SUMMARY

A reproducible assay for the evaluation of adherence to female genital tract epithelial cells was developed. The assay was used to assess the adherence of Bacteroides bivius and B. fragilis to epithelial cells from different patient populations. In this study, no significant adherence of either stock strains or recent clinical isolates of B. bivius to cells from normal, healthy females, or from patients predisposed to infections with this organism was observed. Factors previously reported to influence in vitro adherence such as growth conditions of the organisms, time of incubation and presence of divalent cations were found to have no effect on the results.

A coincidental finding in these studies was a consistent adherence of B. bivius to the buccal epithelial cells. Investigations were pursued to evaluate this adherence. Sugars frequently implicated in bacterial - epithelial cell interactions were found to have no inhibitory effect on the association of B. bivius with BEC. This adherence was found to be influenced by changes in hydrogen ion concentration and by the presence of a variety of ionic species when introduced into the assay system. The observed effects were found to be inconsistent and varied with the buffer system being evaluated. The

data suggest that the observed adherence may be a non-specific physico-chemical association rather than a specific adhesin - receptor interaction.

The need to critically evaluate any observed in vitro adherence phenomena is discussed in light of our observations.

The data suggest that bacterial adherence to VEC or CEC is unlikely to be a critical pathogenic factor for infection with B. bivius in the female genital tract.

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## APPROVAL SHEET

The thesis submitted by Susan V. Meade has been read and approved by the following committee:

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The final copies have been examined by the co-directors of the thesis and the signatures which appear below verify the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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