1978

In Vivo and In Vitro Interactions between Bacteroides fragilis and Escherichia coli: Their Role in Intra-Abdominal Abscess Formation

James Carl Hagen

Loyola University Chicago

Recommended Citation
Hagen, James Carl, "In Vivo and In Vitro Interactions between Bacteroides fragilis and Escherichia coli: Their Role in Intra-Abdominal Abscess Formation" (1978). Dissertations. Paper 1743.
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IN VIVO AND IN VITRO INTERACTIONS BETWEEN

BACTEROIDES FRAGILIS AND ESCHERICHIA COLI:

THEIR ROLE IN INTRA-ABDOMINAL ABSCESS FORMATION

by

James C. Hagen

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy
1978
ACKNOWLEDGEMENTS

TO: Dr. T. Hashimoto, not only for the long hours and effort he has spent to train me as a scientist and teacher, but for the kindness, understanding and extreme patience he has shown as a friend.

TO: Drs. H. J. Blumenthal, W. S. Wood, and J. Balis for the valuable suggestions, support, and laboratory facilities so helpful for my studies.

TO: Mrs. T. Hashimoto, for the great generosity and skill in preparing figures for this dissertation as well as manuscripts and presentations in the past.

TO: Dr. Ruth Emyanitoff, for her kind assistance in the preparation of this dissertation, and for her many years of friendship during my studies.

TO: Diana Contreraz, for her typing skills and many kindnesses as a friend.

TO: The faculty and students of the Department of Microbiology for their continued support and friendship.

TO: Eli Lilly Company and Loyola (GRSC) for their financial support.

TO: Elwin Fosedahl (Upjohn Co.) for the information and literature he has given us.

Most importantly, to my wife and companion, Colleen, without whose support and understanding this dissertation could never have been completed. Her rare ability to combine the talents of a physician while retaining the insight of a wife have made my life during this investigation not only bearable but truly enjoyable.
LIFE

James Carl Hagen was born to Carl and Beverly Hagen in Grand Rapids, Michigan, on March 30, 1950. He graduated from Lowell High School in June of 1968 and received his Bachelor of Science degree from the Department of Microbiology at Michigan State University in June of 1972. He began his studies in the Department of Microbiology at the University of Montana under the guidance of Dr. J. A. Rudbach. In December of 1973 he received his Master of Science degree with a thesis title of "Study of the Inactivation of Endotoxin by Dogfish Shark Serum Components," and began his Ph.D. studies at Loyola University of Chicago in January, 1974. He is a member of Sigma Xi, the American Society for Microbiology, and the American Association for Dental Research, and is currently an Instructor in the Department of Microbiology at Loyola University School of Dentistry.
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ABBREVIATIONS

°C    degree centigrade
EDTA  ethylenediamine tetraacetic acid
LD_{50} fifty percent lethal dose
Fig.  figure
g     gram
h, hr hour
μg    microgram
μl    microliter
μm    micrometer
ml    milliliter
mm    millimeter
mM    millimolar
min   minute
M     molar
nm    nanometer
NBT   nitroblue tetrazolium
N     normal
PYG   Peptone yeast glucose
RBC   red blood cell
subsp. subspecies
SOD   superoxide dismutase
TSA   Trypticase soy agar
TSB   Trypticase soy broth
INTRODUCTION

In recent years, there has been a renewed interest in anaerobic bacteriology and diseases caused by anaerobic organisms. A review of recent literature demonstrates that there has been an increase in the isolation of such bacteria from clinical specimens. In 1964, in a 5 hospital combined study, anaerobic bacteria were reportedly isolated from only 2% of 1388 surgical wounds (Committee on Trauma, National Research Council, 1964). Bornstein et al. (1964) found that only 3% of specimens in the clinical laboratory contained anaerobic bacteria. In 1973, Davis et al. reported a 15% isolation rate from 7500 specimens. Leigh (1974) studied the Bacteroides isolates during a 4 year time period and reported a continuous increase in rate from 13% in 1970 to 83% in the first 6 months of 1974. The increase in rate of isolation of anaerobic bacteria, especially of *B. fragilis*, has been noted by other investigators (Gorbach and Bartlett, 1974; Altemeier et al., 1967; Thadepalli et al., 1973; Martin, 1974; Polk and Kasper, 1977; Saksena et al., 1968).

The reported increase in isolation of anaerobic bacteria might be due to a) an actual increase in the number of infections caused by anaerobic bacteria, b) an apparent increase in infections because of increased awareness of physicians as to possible presence of anaerobic bacteria in specimens and c) more importantly, the greatly improved techniques of anaerobic bacteriology.

Certain underlying diseases are known to predispose individuals
to infection by anaerobic bacteria. These include malignancies and diabetes. With increased longevity, the incidence of these diseases has increased considerably in recent years. Undoubtedly, this has contributed to an actual increase in the number of anaerobic infections and concomitant number of anaerobic species isolated. Other factors which might predispose to such infections include prolonged use of corticosteroids, antimicrobial agents or antineoplastic agents.

Many investigators (Marcoux et al., 1970; Felner and Dowell, 1971; Clark et al., 1977; Saksena et al., 1968; Finegold et al., 1970) noted a striking number of malignancies associated with infection by anaerobic bacteria. Marcoux et al. (1970) found that of 123 patients with Bacteroides bacteremia, 35 had carcinoma (21 with colon carcinoma). In a study (Felner and Dowell, 1971) of 250 patients with Bacteroides bacteremia, 57 had malignant neoplasms. Finegold et al. (1970) reported that 16% of 1736 patients with anaerobic infections had malignancies. In other studies, 25 of 174 patients (Clark et al., 1977) and 26 of 112 patients (Saksena et al., 1968) were shown to have underlying malignancies. Other investigators (Chow and Guze, 1974; Bodner et al., 1970) have also noted the prevalence of malignant neoplasms in patients with anaerobic infection indicating that malignancies might be a predisposing factor for anaerobic infections.

A second important predisposing factor is previous treatment by antibiotics, specifically, neomycin and the other aminoglycosides. In one study (Marcoux et al., 1970), 75 of 123 patients with anaerobic infection had prior antibiotic treatment. Finegold et al., (1970) noted that antibiotic therapy preceded a disproportionately large
number of anaerobic infections. These findings are supported by the reports of others (Bodner et al., 1970; Bornstein et al., 1964; Saksena et al., 1968).

Diabetes (Bornstein et al., 1964; Bodner et al., 1970; Saksena et al., 1968; Finegold et al., 1970; Clark et al., 1977), corticosteroid therapy (Bodner et al., 1970; Saksena et al., 1968; Finegold et al., 1970), organ transplants (Finegold et al., 1970), and treatment with antineoplastic agents (Clark et al., 1977; Bornstein et al., 1964) were also noted as being closely associated with infection by anaerobic bacteria. It is worth noting that in the study of Marcoux et al. (1970) 62% of the patients had disease of the gastrointestinal tract, and all patients were shown to have some form of underlying disease.

Another possible reason for increased incidence of anaerobic infection is the increased longevity of the human population. Studies by Ellner and Wasilauskas (1971), Sinkovics and Smith (1970) and Felner and Dowell (1971) have also demonstrated an increased incidence of anaerobic infection with increase in age.

The reported rise in number of anaerobic infections might also be due to an increased awareness by the physician as to the possible involvement of anaerobic bacteria in the disease process (Martin, 1974; DeHaan et al., 1974; Clark et al., 1977).

Infinitely more important in accounting for the apparent rise in number of anaerobic infections are the improved techniques of anaerobic bacteriology (Leigh, 1974; Appelbaum et al., 1976; Moore et al., 1969; Holland et al., 1977). The improved methods for sample collec-
tion, transportation and storage, isolation and identification, as well as development of suitable culture media are jointly responsible for higher rates of isolation of anaerobes from clinical specimens.

A major difficulty in the collection, transportation, and storage of specimens suspected of containing anaerobic bacteria has been a loss in viability of the organisms due to their exposure to oxygen and low temperature. The effect of these parameters on clinically significant anaerobic bacteria is important in determining appropriate handling of clinical specimens. Contrary to earlier beliefs, recent investigations have demonstrated that clinically significant anaerobic bacteria are quite tolerant to oxygen exposure (Bartlett et al., 1976; Loesche, 1969; Tally et al., 1975). In 1969, Loesche studied the effect of differing oxygen concentrations and time of oxygen exposure on a variety of anaerobic bacteria from stock cultures. From the results of this study, he classified the bacteria into two groups: strict and aerotolerant anaerobes. Although strict, or extremely oxygen sensitive (EOS), anaerobes can be routinely isolated from the normal flora by rigid anaerobic techniques, Attebery (1971) and Rosenblatt et al. (1974) failed to isolate any EOS organisms from clinical material. Onderdonk et al. (1974) did not isolate EOS from experimental abscesses caused by rat cecal contents. It is now relatively well established that most organisms commonly isolated from anaerobic infections are found in the moderate, or aerotolerant group of anaerobes (Bartlett et al., 1976). Ueno (1968) found that he could isolate B. fragilis from pus after as long as one week of storage.

Since the oxygen tolerance of stock cultures may differ signifi-
cantly from that of fresh clinical isolates, Tally et al. (1975) compared oxygen tolerance of fresh and stored cultures of *B. fragilis* and other organisms commonly associated with anaerobic infections. They found that most clinically significant anaerobic bacteria could tolerate exposure to oxygen for at least 8 hours and frequently for longer periods of time. They suggested that primary isolation and many manipulations of anaerobic bacteria could be done on the laboratory bench in room air.

In addition to the problem of oxygen exposure, clinical specimens that cannot be processed immediately are often subjected to storage at refrigerator temperatures. This prevents the overgrowth of facultative forms that occurs either aerobically or anaerobically at room temperature. Finegold et al. (1974) recommended that specimens that could not be processed within 2 hours after collection should be refrigerated in order to suppress the undesirable proliferation of coexistent facultative anaerobes. However, authors of other clinical laboratory manuals (Dowell and Hawkins, 1974; Holdeman and Moore, 1972) cautioned not to store specimens at low temperature because of possible detrimental effects of chilling on anaerobic bacteria. Other investigators have reported similar findings when certain facultative and obligate anaerobic bacteria were suddenly exposed to low temperature (Gorrill and McNeil, 1960; Hegarty and Weeks, 1940; Strange and Dark, 1962; Traci and Duncan, 1974). This unresolved problem as to the effects of low temperature needs to be further investigated.

In the past, techniques for the isolation of anaerobic bacteria have been cumbersome, time consuming, and too expensive for routine
clinical use. However, one of the major improvements in anaerobic bacteriology has been the development of devices that make routine anaerobic bacteriology possible. Three devices currently in use are: a) the anaerobe jar; b) the roll tube; c) the anaerobic chamber.

In early models of the anaerobe jar (McIntosh and Fildes, 1916; Stokes, 1958), atmospheric oxygen was removed by catalyzing its union with hydrogen gas. However, the external source of explosive hydrogen gas posed a serious hazard. Recently a self contained gas generating packet has been developed (Brewer and Allgier, 1966) for production of a hydrogen-carbon dioxide atmosphere. The union of oxygen and hydrogen can be safely catalyzed, leaving a carbon dioxide atmosphere. Finegold (1977) demonstrated that oxygen was reduced to less than 0.2% of atmospheric levels by 1 hour using a commercially available gas generating system (GasPak, BBL). Since moderately oxygen sensitive anaerobes can survive and grow under these conditions (Loesche, 1969) and extremely oxygen sensitive anaerobes have not been recovered from clinical specimens (Rosenblatt et al., 1974), this system is quite effective in the isolation of anaerobic pathogens. Due to its ease of use and compact nature, this technique is now in use in most anaerobic bacteriology laboratories.

The roll tube, a system in which each sample tube serves as its own miniature anaerobic chamber, was developed and modified by Hungate (1950), Bryant (1963) and Moore (1966). A much higher isolation rate of strict anaerobes can be obtained with this technique. However, the need for bulky tanks of gas and special media, and inherent mechanical complexity make this technique more valuable to the research laboratory
than to the clinical labs. Lastly, anaerobic chambers have been developed (Drasar, 1967; Aranki et al., 1969; Leach et al., 1971) in which to perform bacteriology on clinical specimens or study the growth and characteristics of the anaerobic bacteria (Drasar, 1967). This technique has the advantages of providing continuous anaerobic conditions and continuous viewing without the need of hydrogen or catalysts, but requires a great deal of room and a large initial expenditure. Studies by Killgore et al., (1972) and Rosenblatt et al., (1974) demonstrated that all three of these methods were comparable in their ability to recover anaerobes from clinical specimens.

Many anaerobic bacteria, including clostridia, Actinomyces, Peptostreptococcus, Peptostreptococcus, Fusobacterium and Bacteroides have been implicated as, or shown to be, pathogenic in man. However, Bacteroides, especially B. fragilis, was by far the most frequently isolated anaerobic bacterium from clinical infections (Holland et al., 1977; Lorber and Swenson, 1975; Moore et al., 1969). B. fragilis is an obligately anaerobic, gram negative rod which was first isolated by Veillon and Zuber (1897). As part of the normal flora, it makes up 95% of the colonic bacteria, outnumbering the aerobic microorganisms by 100-1000 to 1. Feces contains approximately $10^{11}$ Bacteroides per gram of material, the predominant species being B. fragilis (Finegold et al., 1974). The species B. fragilis has been divided into the 5 subspecies of fragilis, vulgatus, ovatus, thetaiotaomicron, and dis- tasonis. Subspecies thetaiotaomicron and vulgatus have been shown to be isolated from 90-100% of fecal specimens from healthy volunteers (Ueno et al., 1974). In contrast, B. fragilis subsp. fragilis was
only isolated in 30-40% of the samples, and was usually 1-2 logs lower in concentration than the others.

As a member of the normal flora of the body, B. fragilis plays a number of roles. B. fragilis in the intestinal tract is known to be a prime producer of vitamin K for the human body (Gibbons and Engle, 1964). It also plays a major role in the degradation of bile acids in the gut and is vital to the proper recycling of these acids (Hill and Drasar, 1968). Treatment which reduces counts of Bacteroides in the mouse intestine has been shown to render mice more susceptible to Salmonella infections (Bohnhoff et al., 1964).

B. fragilis has been associated with infections in virtually every part of the human body, the most commonly encountered clinical presentation being the abscess (Gorbach and Bartlett, 1972). Such infections almost always arise from an endogenous source, occurring only when the primary defense mechanisms have been breached, as in trauma resulting from intestinal surgery, wounds (Nichols et al., 1972; Thadepalli et al., 1973) or the other predisposing diseases or deficiency states mentioned earlier. These opportunistic infections are most commonly associated with pleuropulmonary, intra-abdominal, and female genital tract regions, as well as the bloodstream. In an extensive study of anaerobic isolates, DeHaan et al. (1974) reported that whenever anaerobes were isolated in the following cases, it was Bacteroides 100% of the time from osteomyelitis cases, 89% from septicemias, 81% from intra-abdominal infections, 70% from infections of the pelvis and genital tract, 64% from lower respiratory tract infections, and 62% from soft tissue infections. Of 81 clinical specimens
collected from infections of various areas of the body, 17.4% were found to contain *B. fragilis* (Moore et al., 1969). In this study, the next most frequently isolated bacterial species was *Clostridium perfringens*, at 9%. Holland et al. (1977) found that a far greater percentage of *B. fragilis* (52%) was isolated from specimens collected over a 14 year time period. Martin (1974) reported a 23% isolation rate for *B. fragilis*. Other investigators have also demonstrated *B. fragilis* to be the most frequently isolated bacteria from anaerobic infections (Nobles, 1973; DeHaan et al., 1974).

A study of the *B. fragilis* subspecies isolated from clinical samples revealed that subsp. *fragilis* was the most frequently isolated. Holland et al. (1977) reported that 44% of the *B. fragilis* isolates were subsp. *fragilis*, and the subsp. *thetaiotaomicron* was next with a 27% isolation rate. However, as mentioned earlier, *B. fragilis* subsp. *fragilis* is isolated much less frequently than subsp. *thetaiotaomicron* and *vulgatus* from normal individuals. The prevalence of infections by subsp. *fragilis* when compared to the infrequency of infections by subsp. *thetaiotaomicron* and subsp. *vulgatus* may indicate an active role for *B. fragilis* subsp. *fragilis* in the pathogenic process.

Although the frequent involvement of *B. fragilis* in anaerobic infections is well documented, the exact mechanisms whereby this organism produces infection have never been completely elucidated. There are at least three major unsolved problems concerning the infections caused by *B. fragilis*. a) The ability of *B. fragilis* alone to cause infections has never been established. Interaction with other microorganisms may be necessary in the pathogenesis of such infections.
b) Pathogenic factors in *B. fragilis* have never been identified.

c) Although many antibiotics and types of therapy have been used for treatment of *B. fragilis* infections, results show only moderate success. A more effective method for the prophylaxis and chemotherapy of *B. fragilis* infections needs to be developed.

It has been speculated for some time that synergistic interactions between facultative and obligate anaerobes might play an important role in the development of intra-abdominal infections (Meleney et al., 1932; Altemeier, 1942; Hite et al., 1949; Finegold and Rosenblatt, 1973; Hill et al., 1974; Lorber and Swenson, 1975; Stone et al., 1975; Onderdonk et al., 1976). However, there have only been a few demonstrations of actual synergistic interactions between these two types of microorganisms. In 1932, Meleney et al. showed that gangrenous lesions could be produced in guinea pigs by mixtures of microaerophilic, or anaerobic, streptococci, and staphylococci, but not by either organism alone.

Altemeier (1942) studied the bacterial isolates from 100 cases of perforated appendix in humans, of which 96 cases contained both aerobes and anaerobes. He found that while pure cultures of organisms from infections were nonpathogenic, combinations were often able to cause progressive infections. Hite et al. (1949) demonstrated similar results using *B. melaninogenicus*, streptococci and other organisms found as normal flora of the oral cavity. More recently, Onderdonk et al. (1976) reported that intra-abdominal abscesses failed to form after implantation of *Escherichia coli* or *B. fragilis* alone into the peritoneal cavity of rats. However, abscesses were produced in 100% of
the animals that received the combination of these organisms, suggesting that intra-abdominal abscess formation was indeed related to synergy between anaerobes and facultative bacteria. However, the nature of these interactions and the mechanisms involved are not known.

Despite the many reports suggesting that synergistic interactions of *B. fragilis* and facultative anaerobes are essential for the development of these anaerobic infections, several investigators (Hill et al., 1974; Renz et al., 1975; Onderdonk et al., 1976; McConville et al., 1976; Walker and Wilkins, 1976) reported that *B. fragilis* alone could cause minute intra-abdominal abscesses in experimental animals when bacteria were inoculated along with certain materials.

The mechanism(s) of *B. fragilis* pathogenicity is essentially unknown. *B. fragilis* is not known to produce any exotoxins which participate in the pathogenic process, although various exoenzymes, endotoxin, and a capsule have been identified for this organism. *B. fragilis* has been found to produce a wide variety of extracellular enzymes. Various strains produced extracellular DNAase (Porschen and Sonntag, 1974), phosphatase (Porschen and Spaulding, 1974), penicillinase, hyaluronidase, chondroitin sulfatase, protease, and lipase (Rudek and Hague, 1976). Although these enzymes are thought to allow the spread of infections caused by some facultative organisms, their role in the pathogenic process of *B. fragilis* remains to be elucidated. Another potentially damaging enzyme shown to be produced by *B. fragilis* is an extracellular heparinase (Gesner and Jenkin, 1961; Bjornson et al., 1970). A heparinase has been hypothesized as being responsible for phlebitis and dissemination of anaerobic infections.
(Bjornson and Hill, 1973; Felner and Dowell, 1971; LeFrock et al., 1976; Meyer and Finegold, 1976). This type of thrombophlebitis has been associated with *B. fragilis* septicemia (Bjornson and Hill, 1973). However, at present, the in vivo production of a heparinase by *B. fragilis* has never been demonstrated and the role of *B. fragilis* heparinase in abscess formation still remains to be elucidated.

Endotoxin has been identified in *B. fragilis*. However, Kasper (1976) has shown the endotoxin of *B. fragilis* to be much less toxic than the lipopolysaccharides of *Salmonella* or *E. coli*, possibly due to its novel structure. *B. fragilis* lipopolysaccharides were shown to lack both 2-keto-3 deoxyoctonate and heptose, 2 essential sugars found in classical endotoxins (Heath et al., 1966; Osborne, 1966). A very loose association between polysaccharide and lipid moieties of the lipopolysaccharide was also demonstrated. Possibly due to these structural characteristics, *B. fragilis* endotoxin could not induce two classic examples of endotoxin toxicity, chick embryo lethality and the localized Shwartzman reaction. This might also account for the low incidence of disseminated intravascular coagulation in patients with *B. fragilis* sepsis, as reported by Yoshikawa et al. (1974).

Once *B. fragilis* bypasses the primary defense mechanisms, it must somehow protect itself against the phagocytic defenses. Recent work by Kasper (1976) demonstrated that a capsule was produced by *B. fragilis* subsp. *fragilis* but was not produced by the other subspecies. Subsequently, Onderdonk et al. (1977) showed that capsular material alone was able to induce abscess formation, and that encapsulated strains of *B. fragilis* adhered to rat peritoneal epithelium better
than unencapsulated strains (Onderdonk et al., 1978). This might explain the increased frequency of isolation of subspecies *fragilis* when compared to the other subspecies. However, contrary to these findings, Babb and Cummins (1978) demonstrated that three of the other *B. fragilis* subspecies, *vulgatus*, *thetaiotaomicron*, and *ovatus*, also possessed capsules and cautioned that capsules may not be a unique pathogenic factor of subsp. *fragilis*.

The third main question concerns the selection of proper and effective antibiotics for chemotherapy of anaerobic infections. The confusion arises from the question as to which organisms antibiotic therapy should be directed in mixed bacterial infections. Polk (1977) infers that only the facultative component of such infection needs to be treated. He found that a failure to treat surgical infections of the large intestine with cephaloridine resulted in a fourfold rise in infection rate. Since cephaloridine is ineffective against certain anaerobic bacteria, Polk inferred that anaerobic organisms were not the prime source of infection. It was suggested that only the facultative component of such infections needs to be treated. On the other hand, Onderdonk et al. (1976) has suggested that *B. fragilis*, in an experimental mixed infection of *B. fragilis* and *E. coli*, was a necessary component for abscess formation. If this hypothesis is true, treatment of *B. fragilis* alone would prevent abscesses from forming, but would not prevent a potentially lethal peritonitis caused by facultative gram negative rods.

Treatment of *B. fragilis* infections poses a very serious problem because of the extreme resistance of this organism to many chemothera-
apeutic agents, including aminoglycosides (Finegold and Sutter, 1971), β-lactam antibiotics and others (Kislak, 1972; Martin et al., 1972; Zabransky et al., 1973). Previously, tetracycline was the drug of choice for anaerobic infections. However, due to a rise in resistance of B. fragilis to this antibiotic (Nastro and Finegold, 1972) it is no longer recommended. Chloramphenicol is effective against B. fragilis, but is reserved for seriously ill patients due to its association with aplastic anemia (Finegold, 1975). Clindamycin, the 7-chloro derivative of lincomycin, has been shown to be extremely effective against B. fragilis (Haldane and van Rooyen, 1972). However, because of its association with pseudomembranous enterocolitis, now known to be caused by a toxin produced by Clostridium difficile (George et al., 1977), routine therapy with this antibiotic has often been avoided (Cohen et al., 1973; Scott et al., 1973; Viteri et al., 1974).

Recently, good results have been obtained with carbenicillin against anaerobic infections involving B. fragilis by in vitro testing (Sutter and Finegold, 1975) and in vivo clinical studies (Meny et al., 1975; Swenson and Lorber, 1976). There have also been promising results with metronidazole in the treatment of anaerobic infections (Staneck and Washington, 1974; Whelan and Hale, 1973; Willis et al., 1976; Tally et al., 1975), although this agent has not yet been approved by the Federal Drug Administration for use in the United States.

Another problem with the treatment of anaerobic infections concerns the ability of antibiotics to reach the extravascular site of infection (Gerding et al., 1976). A number of investigators (Alexander
et al., 1973; Tight et al., 1975; Gerding et al., 1976) have studied penetration of antibiotics into subcutaneously or intraperitoneally implanted capsules. However, there has been great variability in results due to bleeding, infection, differing routes of antibiotic inoculation, and inconsistent degree of vascularity.

The most prevalent thought is that until a time when pathogenesis of these mixed infections is understood, therapy should be directed against all significant components of the infection, including both aerobic and anaerobic bacteria (Finegold, 1977; Chow et al., 1976; Thadepalli et al., 1973; Weinstein et al., 1974). Chow and Guze (1974) performed a prospective study in humans on the effectiveness of various antibiotic combinations for control of both aerobic and anaerobic components, and indicated the need for both types of antibiotics. Other investigators (Bach et al., 1977; Griffith et al., 1977) have also studied the effectiveness of various combinations of antibiotic therapy in humans.

It is the purpose of this dissertation to critically investigate the in vivo and in vitro interactions between B. fragilis and E. coli. In order to approach this problem, the following studies will be undertaken: (1) An animal model of intra-abdominal abscess formation will be developed in which the ability of B. fragilis, E. coli, or the combination of both organisms, to cause abscess infections in rats will be studied; (2) The effect of type of bacteria, inoculum size and suspending material on abscess formation will be studied; (3) The nature of the interactions between B. fragilis and E. coli will be studied in vitro. Finally, (4) factors produced by E. coli that
stimulate the growth of *B. fragilis* will be partially characterized.
MATERIALS AND METHODS

I. Isolation and maintenance of cultures.

_Bacteroides fragilis_ subsp. _fragilis_ (strain 8044) and _Escherichia coli_ (strain 8077) were used throughout this study. Other organisms used were _B. fragilis_ strains 2701, 8368, 7728, and 6880, _Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa_, and _Staphylococcus aureus_. All cultures were initially isolated from clinical specimens by the clinical microbiology laboratories and kindly provided by Dr. J. Vice, Foster G. McGaw Hospital.

The identity of all _B. fragilis_ strains was confirmed using techniques described by Holdeman and Moore (1972). These included gas chromatographic analysis and sugar fermentation testing. Samples were prepared for gas chromatographic analysis as follows. Organisms were grown at 37°C for 48 h under anaerobic conditions in peptone yeast glucose medium (PYG, Difco Labs). Volatile acids were removed by acidifying the culture and then extracting with ethyl ether (Mallin- crodt, Inc.). Non-volatile fatty acids were removed from the acidified culture by adding methanol and sulfuric acid, incubating at 55°C for 30 min, and finally extracting with chloroform (Mallincrodt, Inc.). Approximately 14 μl of chloroform or ether was injected into a Beckman GC-5 gas chromatograph equipped with a hydrogen flame detector and a Beckman recorder. Chromosorb W 80-100 mesh in a 6' x 1/8 inch stainless steel column was used with LAC-1-R-296 (diethylene glycol + adipate) as the partition liquid (Beckman Instruments, Inc.).
Hydrogen gas (Liquid Carbonic Corp.) was used as the carrier, and a combination of hydrogen and air (Liquid Carbonic Corp.) was used to fuel the hydrogen flame detector.

Stock cultures of each strain were stored in 1.0 ml defibrinated sheep blood (BBL) at -60°C (Model SZB-659, Revco Co.). For routine work, cultures were maintained at 37°C on blood agar (BBL) or enriched media as described below.

II. Media

A. Liquid media. Several different complex media were used in these studies. Enriched trypticase soy broth (enriched TSB) consisted of trypticase soy broth (TSB, BBL), supplemented with 0.5% yeast extract (BBL), 0.05% hemin (Eastman Organic Chemical Div., Eastman Kodak Co.), and 0.0005% menadione (Sigma Chem. Co.), and was routinely used for growth and maintenance of cultures. It was also used for studying oxygen and cold tolerance of anaerobic bacteria. Bacto-peptone (5%, Difco Labs), 5% tryptone peptone (Difco Labs), and 5% thiopeptone peptone (BBL) were tested for their ability to support growth of B. fragilis alone, E. coli alone, or the combination of both. On the basis of these findings, Bacto-peptone was chosen for the study of mixed culture interactions of B. fragilis and E. coli.

A modification of the semi-synthetic medium of Roberts et al. (1955) was also used to study mixed culture interactions. The semi-synthetic medium contained 1% Casamino acids (Difco Labs), 0.0026% sodium sulfate, 0.3% potassium phosphate (monobasic), 0.3% sodium chloride, and 0.0001% magnesium chloride. The pH of this medium after autoclaving was 7.0. For certain experiments, Bacto-peptone or semi-
synthetic medium was conditioned (spent) by aerobic growth of *E. coli* for 24 h at 37°C. The cultures were then centrifuged at low speed (setting 6, International Clinical Centrifuge, International Equipment Co.) to remove the bulk of bacteria and then filtered using a membrane filter (0.45 μm pore size, Millipore Co.), for sterilization.

**B. Solid media.** Enriched Trypticase soy agar (enriched TSA) was prepared by the addition of 1.5% agar (Difco Labs) to enriched TSB described above. Blood agar (5% defibrinated sheep blood) was obtained from BBL Laboratories. To examine the survival of *B. fragilis* at 4°C, Mueller-Hinton agar (BBL) and Schaedlers agar (BBL) supplemented with 0.05% hemin were used. In some cases, when a minimal medium for *B. fragilis* was required, TSA was used without supplementation.

**C. Selective media.** To selectively isolate *B. fragilis* from *E. coli* in cultures containing both organisms, samples were plated on enriched TSA containing kanamycin (Sigma Chem. Co.) or streptomycin (Eli Lilly) to a final concentration of 100 μg/ml. Aminoglycosides at this concentration were found to inhibit growth of *E. coli*, while allowing growth of *B. fragilis*. To selectively isolate *E. coli* from mixed cultures containing *B. fragilis*, samples were plated on enriched TSA and incubated aerobically.

**D. Vitamins, cofactors and supplements.** Bacto-peptone was supplemented with various compounds, alone or in combination, and the ability of *B. fragilis* to grow was then determined. Supplements added were: 0.05% hemin, 0.0005% menadione, vitamin *B₁₂* (Sigma Chem. Co.), ammonium sulfate, or a vitamin mixture (MEM-vitamin solution, 100x,
Lot Cl233C, Grand Island Biological Co.). Several compounds were tested to determine their effect on the survival of *B. fragilis* at 4°C in enriched TSB. For these experiments, various concentrations of magnesium chloride, sucrose (Merck and Co., Inc.), calf serum (Flow Labs, Inc.), or combinations of these materials were used. Unless the source of chemicals was specifically mentioned, reagent grade chemicals were used.

III. **Anaerobic system.**

Routinely, cultures were placed in anaerobe jars using the BBL Gas Pak system and incubated at 37°C. In some experiments, individual tubes or bottles were placed under anaerobic conditions by flushing with 10% CO₂ and 90% N₂ (anaerobe grade, Benster Specialty Gas Co.) and tightly stoppered. Each bottle could then serve as an individual sample. Once the microenvironment of the bottle was disturbed by sampling, it was discarded.

For experiments utilizing pus containing anaerobic bacteria, samples were placed in a chamber maintained under reduced conditions by flowing nitrogen gas (anaerobe grade, Benster Specialty Gas Co.).

IV. **Growth determinations.**

A. **Turbidity.** Turbidity of cultures was measured in a Klett-Summerson colorimeter using a red number 54 filter. A standard curve of Klett units vs. viable cell number of *B. fragilis* or *E. coli* was prepared. Using this information, an inoculum of known concentration could then be prepared for in vivo or in vitro experiments.

B. **Viable counts.** Bacterial suspensions were appropriately
diluted in enriched TSB or sterile distilled water and 0.1 ml was spread with a sterile bent glass rod on the surface of the appropriate agar medium. Cultures containing *B. fragilis* alone, or *B. fragilis* in mixed culture with *E. coli*, were plated on enriched TSA containing 100 μg kanamycin or streptomycin/ml, and incubated at 37°C for 24-48 h under anaerobic conditions. Cultures containing *E. coli* were plated in duplicate on enriched TSA and incubated at 37°C for 24 h under aerobic conditions. The number of colony forming units/ml of culture were then determined.

V. Susceptibility of *B. fragilis* to oxygen and low temperature.

A. Oxygen tolerance. To determine the ability of *B. fragilis* to survive exposure to atmospheric oxygen, approximately 100 cells were plated on enriched TSA and exposed to atmospheric oxygen at room temperature. After various time periods of oxygen exposure, plates were incubated under anaerobic conditions at 37°C for 48 h and viable cell counts determined.

B. Cold tolerance. For determination on solid media, *B. fragilis* was inoculated on enriched TSA plates to approximately 150 cells/plate. Sets of inoculated plates in separate jars were placed at a specified temperature both under aerobic and anaerobic conditions. The anaerobic cultures were first incubated at 37°C for 1 h to obtain complete anaerobiosis. At selected intervals, duplicate plates were removed from each set and incubated at 37°C anaerobically to determine the number of viable cells remaining. To test the effect of chilling on *B. fragilis* suspended in liquid media, 50 ml portions of enriched
TSB were dispensed into 60 ml dropping bottles. A sufficient number of bottles were prepared so that each bottle would only be sampled once before discarding. Bottles were stored overnight in an incubator at 37°C and then allowed to come to room temperature before use. Bottles to be used for anaerobic cultures were preincubated overnight at 37°C in a GasPak (BBL) anaerobe jar. Each bottle was inoculated with 0.5 ml of a diluted culture of B. fragilis to obtain a final concentration of approximately $10^5$ cells/ml. The anaerobic cultures were immediately flushed with 10% CO$_2$ + 90% N$_2$ and tightly stoppered, while the aerobic cultures were covered with sterile aluminum foil. All bottles were then placed at the appropriate temperatures for various lengths of time and viability counts determined.

The cold tolerance of microorganisms in pus collected from experimentally produced abscesses was determined as follows: Pus was pooled from a number of abscesses and thoroughly mixed while kept under anaerobic conditions. A series of tubes was prepared, each containing 0.1 g of pus. Tubes were flushed with anaerobic gas and then divided into three sets. One set was placed at room temperature and the second set at 4°C and the third set at 15°C. Another three sets of tubes were flushed with air. Again, one set was placed at room temperature and the others at 4°C and 15°C. All tubes were tightly sealed to avoid desiccation. Duplicate sample tubes were removed from each set at designated time periods and again weighed. Five ml of saline (0.85% NaCl) were added to each tube, the tubes mixed thoroughly, and viable counts were determined as a number of organisms/g of pus.
VI. Interaction of *B. fragilis* and *E. coli* in vitro.

A. Effect of *E. coli* growth on the growth of *B. fragilis*. The kinetics of growth of *B. fragilis* and *E. coli* in a mixed culture were first determined. *E. coli* and *B. fragilis* each at a concentration of $10^5$ cells/ml were inoculated to enriched TSB. At various times during incubation, samples were removed, appropriately diluted in sterile saline, and 0.1 ml was plated on solid media. Number of *E. coli* was determined by plating samples on enriched TSA and incubating aerobically. The number of *B. fragilis* was determined by plating samples on enriched TSA containing 100 µg kanamycin/ml and incubated anaerobically. Colony counts were performed 24 h later after incubation at 37°C.

To test the effect of the initial concentration of *E. coli* on *B. fragilis* growth, the inoculum size of *E. coli* was varied from $10^1$ cells/ml to $10^3$ cells/ml. Viable counts of each were determined at designated intervals.

To determine the effect of selective inhibition of *E. coli* on the growth of *B. fragilis* in mixed culture, kanamycin was added to the mixed cultures at designated time intervals. Kanamycin was added to a final concentration of 100 µg/ml. Samples were taken at designated times and viable counts were then determined as previously described.

Since our preliminary experiments showed that growth of *B. fragilis* was enhanced when it was incubated with *E. coli*, an experiment was devised to determine if cell to cell contact was necessary for this interaction. *B. fragilis* and *E. coli* were physically separated
by a dialysis membrane. Dialysis tubing containing 50 ml of peptone was placed into a flask containing 100 ml of the same medium. Using a bent glass tube, the medium inside of the dialysis tubing was inoculated with *E. coli*. Medium outside the dialysis tubing was inoculated with *B. fragilis*. The flask was then placed aerobically at 37°C and growth of *B. fragilis* observed after 24 h and 48 h.

B. **Effect of other organisms on *B. fragilis* growth.** *E. coli* was replaced by $10^5$ cells/ml of *Klebsiella pneumoniae* or *Proteus vulgaris* in mixed culture growth. Ability to stimulate *B. fragilis* growth was then determined at 24 h and 48 h as previously described.

C. **Effect of conditioned medium on growth of *B. fragilis*.** The ability of *E. coli* conditioned peptone and semi-synthetic medium to stimulate growth of *B. fragilis* was determined by two methods and stimulatory factors then further characterized in conditioned semi-synthetic medium.

1. **Test in liquid medium.** Various amounts of *E. coli* spent medium, ranging from 0 to 50 ml, were added to 50 ml of stored medium. The volume of medium in each tube was brought to 10 ml with sterile saline. All tubes were inoculated with $10^5$ cells *B. fragilis*/ml, and incubated anaerobically at 37°C for 24 h.

2. **Test on solid medium.** The growth stimulatory activity of spent media for *B. fragilis* was determined on solid media as follows. The surface of TSA or enriched TSA was spread with approximately $10^6$ cells of *B. fragilis*. Wells, 6.5 mm in diameter, were cut with a metal #5 cork borer and then filled with 0.25 ml of conditioned peptone or semi-synthetic media. Plates were incubated at
37°C under anaerobic conditions for 48 h. Stimulation was defined as an enhanced growth around wells when compared to control wells containing saline or distilled water.

3. Characterization of *B. fragilis* stimulatory factors produced by *E. coli*. The stability of the stimulatory factors under various conditions was determined by treating separate samples of *E. coli* spent semi-synthetic medium in a variety of ways. Spent medium was placed in a boiling water bath for 30 min and then cooled to room temperature. Other samples were either (a) adjusted to pH 2.0 with HCl (Baker Chem. Co.), placed at room temperature for 1 h, and then neutralized with NaOH, or (b) adjusted to pH 12.0 with NaOH, placed at room temperature for 1 h, and then neutralized with HCl.

After determining stability of the stimulatory materials to heat, acid, and alkali, their physical and chemical properties were further characterized. Spent medium was dialyzed extensively against distilled water to determine if the stimulatory materials were macromolecular, or relatively small molecules. Cation exchange resin (Cl⁻ charged, Dowex 50 W, 100-200 mesh, Sigma Chem. Co.), or anion exchange resin (H⁺ charged, Dowex 1-X8, 100-200 mesh, Sigma Chem. Co.) was used to remove negatively charged or positively charged materials respectively. Activated charcoal (Norit A, Baker Chem. Co.) was used nonspecifically to remove certain substances. To determine if the stimulatory materials were lipid in composition, spent medium was extracted with ether (Mallincrodt, Inc.). All treated spent media was then qualitatively assayed for stimulatory activity using the solid medium assay as described above.
VII. Antibiotic susceptibility.

Susceptibility of *B. fragilis* and *E. coli* to cephalothin (Keflin, Eli Lilly), cephalaxin (Eli Lilly), cefazolin (Eli Lilly), cephaloridine (Eli Lilly), cefamandole (Eli Lilly), penicillin (Sigm Chem. Co.), kanamycin (Sigma Chem. Co.), gentamicin (Schering), streptomycin (Eli Lilly), and erythromycin (Sigma Chem. Co.) was determined and expressed as minimal inhibitory concentration (MIC).

Organisms grown anaerobically in enriched TSB for 24 h at 37°C were diluted 1 to 100 into enriched TSB, and used as the inoculum. This inoculum contained an average of 100 organisms/ml as determined by viable cell counts. The antibiotics were diluted in sterile distilled water and 0.5 ml of each antibiotic dilution plus 0.5 ml of inoculum were added to 9.0 ml of enriched TSB to obtain final concentrations ranging from 0.39 to 1,000 μg of antibiotic/ml. The tubes were incubated at 37°C for 24 h in a GasPak system. The first tube showing no turbidity was taken as the end point of the minimal inhibitory concentration (MIC).

VIII. Measurement of dissolved oxygen.

The dissolved oxygen level in broth media was measured using a model 53 oxygen monitor equipped with a Clark oxygen electrode (Yellow Springs Instrument Co.). Measurements were obtained as percent of total saturation of the medium with oxygen (saturation produced by extensive vortexing). In accord with the studies of Morris (1975), the oxygen content of broth media for bacterial growth was accepted as 250 μmoles oxygen/1 at saturation. Oxygen concentration of broth
media in this study was calculated as follows:

\[
\text{\textmu moles oxygen/l in} = \% \text{ saturation} \times 250 \text{ \textmu moles/l test medium}
\]

IX. Preparation of superoxide dismutase (SOD) and its effect on \textit{B. fragilis}.

Superoxide dismutase was prepared from bovine blood (Lincoln Slaughter House, Chicago, Ill.). Red blood cells (RBC's) were separated from 2 liters of citrated whole blood by centrifugation for 12 min at 4,000 g (Model B-20, International Equipment Co.) and washed twice in saline.

The red cells were lysed by diluting them 1:1 in distilled deionized water. Hemoglobin was then precipitated and the superoxide dismutase was prepared by the procedure of McCord and Fridovich (1969).

To determine superoxide dismutase activity, a nitro blue tetrazolium (NBT) assay was used (Beauchamp & Fridovich, 1971). This assay is based on the use of xanthine oxidase to catalyze the oxidation of xanthine, resulting in the generation of superoxide radicals. NBT intercepts the radicals and becomes oxidized, resulting in an increase in absorbance at 560 nm. The reaction mixture consisted of 0.3 ml 2.2 x 10^{-8} \text{ N xanthine oxidase (Sigma)}, 1 \times 10^{-3} \text{ N xanthine}, 0.3 ml 2.5 \times 10^{-4} \text{ N NBT (Sigma)}, 0.3 ml 1 \times 10^{-3} \text{ N ethylene diamine tetraacetic acid (EDTA)}, 0.3 ml 0.5 \text{ N sodium carbonate, 0.3 ml of enzyme and 1.2 distilled water. One unit of superoxide dismutase is defined as the amount necessary to produce 50\% inhibition of the rate of reduction of NBT by xanthine oxidase. The extracted SOD was found}
to have an activity of 6.5 units/ml. After determining the SOD activity, the crude extract was added in varying concentrations to Bactopeptone medium to test the effect of its presence on the growth of *B. fragilis*.

X. **Experimental intra-abdominal abscess formation in rats.**

A. **Animals.** Female white rats (outbred Sprague-Dawley albino, Scientific Small Animal Laboratory and Farm, Inc., Arlington Heights, Ill.) ranging from 250 to 300 g in weight were used in the present investigation. Animals were placed 5 per cage before and following the surgical procedures and were maintained on Purina Rat Chow (Ralston Purina, St. Louis, Mo.) and water *ad libidum*.

B. **Preparation of implants.** Two types of implants were used to introduce microorganisms into the abdominal cavity while minimizing their diffusion. These were: a) an artificial fecal material impregnated with a bacterial inoculum, or b) a plasma or blood clot impregnated with a bacterial inoculum. Artificial fecal material was prepared by adding equal quantities of minced beef (80% lean hamburger, Jewel Food Stores) to cellulose fiber (Whatman filter paper, #1, W. & R. Balston, Limited). Filter paper had been broken into cellulose fiber by mixing extensively in an Omni-Mixer (Sorvall, Inc.). A small piece (0.35-0.4 g wet weight) of autoclaved artificial fecal material was placed inside a #0 gelatin capsule which was then inserted into a #1 gelatin capsule. Gelatin capsules (Eli Lilly & Co.) had been sterilized by exposure to UV light (20 cm from a Westinghouse Sterilamp 782L-20) for 48 h prior to use. Cultures were grown for 18 to
24 h in enriched TSB and diluted to the desired concentration. Immediately before implantation into animals, a predetermined number of organisms was impregnated into the artificial fecal material.

Plasma or blood clots were prepared as follows: male and female rats (Locke-Erickson Co.), 400-500 g in weight, were bled by cardiac puncture using 10 ml syringes that had been flushed with heparin solution (Lipo-Hepin, 5000 units/ml, Upjohn Co.). After collection, the blood was placed into 0.7 ml red top Vacutainer tubes containing Lipo-Hepin (Upjohn Co.). The heparinized blood was centrifuged to remove red blood cells, and the plasma then sterilized by filtration through membrane filters (0.45 μm pore size, Millipore Corp.). Immediately before surgery 1.0 ml of plasma was distributed to each of the several tubes. To prevent fibrinolysis 0.05 ml of aminocaproic acid (Amicar, Lederle) was added to each tube. The plasma was inoculated with 0.1 ml of a bacterial suspension after which 0.3 ml of thrombin (Parke-Davis, 5000 units/25 ml) was added to initiate clot formation. A firm clot formed within 30 seconds that exhibited little, if any, fibrinolysis and clot retraction. If a blood clot was desired rather than a plasma clot, red cells were not removed.

C. Rat abdominal surgery. Rats were anesthetized by intramuscular injection of 0.07 ml Innovar (fentanyl plus droperidol, Pitman-Moore, Inc.). The abdomen of each rat was then shaved and the skin disinfected with 70% ethanol. A 1.5-2.0 cm incision was made on the posterior midline through the abdominal wall and into the peritoneum so as to form an artificial capsule around the inoculum with
mesentery and intestine. The peritoneum was closed with 4 or 5 continuous sutures of 3-0 monofilament (Ethilon, Ethicon Co.).

D. Evaluation of abscess formation. An abscess was defined as a circumscribed collection of pus. Carefully isolated abscesses were removed on day 42 and weighed. Smears of pus were prepared for light microscopic examination. Viable counts of *B. fragilis* and/or *E. coli* from washings of the peritoneal cavity and of pus from the abscesses were determined as previously described.

Animals that had been implanted with plasma clots or blood clots were necropsied at 3 h, 6 h, 12 h, 24 h, 3 days, 7 days or 14 days. Presence or absence of abscesses, as well as general appearance of the animals, was noted.

E. Histologic examination of intraperitoneal abscesses. Animals were necropsied at 1 day, 3 days, 7 days and 12 days after implantation of the artificial fecal material. Histologic examination was performed both on the abscesses and on tissues immediately surrounding the site of implantation.

Immediately after necropsy, tissues for histology were fixed in buffered formalin (pH 7.2) for 48 h, embedded in paraffin, sectioned, and stained with either hematoxylin-eosin (H and E) or phosphotungstic acid hematoxylin (PTAH).

XI. Light microscopy and photomicrography.

All light microscopy was performed with a Nikon light microscope fitted with both phase contrast and plain objectives. Photomicrographs were made on panchromatic film (Plus-X, Kodak) with a Nikon
camera equipped with an automatic exposure system attached to the microscope. Film was developed with Microdol developer (Kodak).

XII. Analysis of data.

Determination of the statistical significance of differences was made by using a Student's t test. For comparing percent survival of *B. fragilis* and *E. coli* in pus after exposure to oxygen or low temperature, and for analyzing differences in weight of intra-abdominal abscesses produced in rats, data were expressed as mean ± standard error of the designated number of values. For comparing percent survival of *B. fragilis* and *E. coli* after in vitro exposure to oxygen or low temperature, data was expressed as mean ± standard deviation of the designated number of values. A difference was considered statistically significant if the p value was less than 0.05.
RESULTS

I. Characterization of B. fragilis used in this investigation.

Prior to actual experimentation with B. fragilis, its growth characteristics, antibiotic sensitivity patterns, and susceptibility to oxygen and low temperature were examined. This was necessary because the strain of B. fragilis used (strain 8044) was a clinical isolate which had not been previously characterized.

A. Growth of B. fragilis in enriched TSB. In order to standardize inoculum for in vitro and in vivo experiments, the time course of growth of B. fragilis in enriched TSB and the relationship of culture turbidity to viable cell number were determined.

A typical growth curve of B. fragilis, obtained by the method described in Materials and Methods, is shown in Fig. 1. Since the relationship between Klett unit reading and viable cell number (24 h cultures) held a linear correlation up to $10^9$ cells/ml (200 Klett units), an exact number of B. fragilis cells could be readily determined for inoculation purposes.

B. Antibiotic susceptibility of B. fragilis. The susceptibility of B. fragilis strain 8044 to various antibiotics is summarized in Table 1. This antibiogram was used for preparation of a medium that would be selective for B. fragilis when mixed cultures of B. fragilis and E. coli were studied.

Of all the cephalosporin derivatives tested, B. fragilis was most susceptible to cephazolin, with a minimal inhibitory concentration
Fig. 1. Representative growth curve of *B. fragilis* in enriched TSB at 37°C. Each point represents the mean viable count of duplicate samples as described in Materials and Methods.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/ml) for B. fragilis subsp. fragilis</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalothin</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>3.12</td>
<td>3.12</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>6.25</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>6.25</td>
<td>3.12</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>&gt; 1000</td>
<td>3.12</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&gt; 1000</td>
<td>12.5-25</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&lt; 1.56</td>
<td>3.12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Minimal inhibitory concentration (MIC) determined by the broth dilution technique described in Materials and Methods.

<sup>b</sup>ND = not done.
(MIC) of 3.12 μg/ml (Table 1). *B. fragilis* was also susceptible to quite low levels of erythromycin with an MIC of < 1.56 μg/ml. *B. fragilis* was not susceptible to any aminoglycoside tested, even at levels of > 1000 μg/ml, confirming earlier reports (Finegold and Sutter, 1971; Martin et al., 1972).

C. **Survival of *B. fragilis* after storage in atmospheric oxygen.** During the collection, transportation, storage and handling of clinical specimens containing anaerobic bacteria, some exposure to atmospheric oxygen is unavoidable. In order to determine whether *B. fragilis* might be inactivated under our experimental conditions, sensitivity of *B. fragilis* to atmospheric oxygen was determined.

During 24 h of oxygen exposure, essentially no *B. fragilis* viability was lost (Fig. 2). Even after 72 h, only 2-3% viability was lost. This information indicates that handling and manipulation of our *B. fragilis* strain can be undertaken on the bench under aerobic conditions.

D. **Survival of *B. fragilis* after storage at low temperature.** In some cases during our investigation, mixed cultures containing *B. fragilis* might be stored at low temperature to avoid the overgrowth of coexistent facultative bacteria. Finegold et al. (1974) recommended that if specimens had to be stored for longer than 2 h, they should be refrigerated to avoid this type of overgrowth. However, other investigators (Dowell and Hawkins, 1974; Finegold et al., 1974), reported that low temperature was lethal for anaerobic bacteria and that these organisms should not be stored in the cold. To resolve this controversy and to determine whether our experimental conditions
Fig. 2. Survival of *B. fragilis* after exposure to atmospheric oxygen and low temperature. Predetermined numbers of 18 h old *B. fragilis* cells were plated in duplicate on enriched TSA (○-○), or suspended in enriched TSB (Δ-Δ), and then exposed to chilling (4°C) for specified periods under aerobic conditions. The number of surviving cells after exposure to 4°C was determined as described in Materials and Methods. Duplicate sets of inoculated plates (enriched TSA) were left at 25°C (●●) under aerobic conditions.
might cause a loss in viability of *B. fragilis*, sensitivity of *B. fragilis* to low temperature was determined. Results of this experiment can be seen in Fig. 2 and 3. *B. fragilis* was killed rapidly at 4°C, but there was essentially no loss of viability at 25°C. A similar loss in viability at 4°C was observed when *B. fragilis* was plated on enriched Mueller-Hinton Agar, enriched Schaedler's agar, or blood agar (Fig. 3). Although data are not shown here, other strains (8268, 7728, 6880 and 2701) of *B. fragilis* exhibited a similar sensitivity to low temperature.

The effect of chilling on *B. fragilis* in liquid medium was also tested. Over 50% viability was lost within the first 12 h after which the rate of loss slowed until a loss of 80% viability was demonstrated at 72 h (Fig. 2).

In order to clarify whether this inactivation of *B. fragilis* at 4°C was due to the lethal effect of a higher oxygen concentration dissolved in the cold media, or due to injuries induced by cold, we determined the loss of viability of *B. fragilis* exposed to low temperature (4°C) under both aerobic and anaerobic conditions. It appears that the inactivation of *B. fragilis* was a result of cold injury since loss of viability occurred at 4°C either in the presence or absence of oxygen (Fig. 4). Under aerobic conditions, cells suspended in liquid media (Fig. 4B) appeared to be more susceptible (p < 0.005) to cold than those plated on solid media (Fig. 4A). However, under anaerobic conditions there seemed to be no significant difference in the extent of killing of *B. fragilis* between solid and liquid media.

To determine if the ability to survive prolonged exposure to low
Fig. 3. Survival of *B. fragilis* after exposure to low temperature. Predetermined numbers of 18 h old cells were plated in duplicate on various solid media and exposed to 4°C and 25°C for a specified time under aerobic conditions, and the numbers of surviving cells were determined as described in Materials and Methods. Symbols: △-△, enriched Mueller-Hinton Agar; x-x, enriched Schaedler's Agar; o-o, enriched TSA; Δ-Δ, commercial blood agar. Solid lines represent the exposure at 4°C and the dotted line represents the exposure at 25°C.
Fig. 4. Survival of _B. fragilis_ after storage of fresh isolates at low temperature. Predetermined numbers of cells were either suspended in enriched TSA (A), or plated on enriched TSB (B) and exposed to 4°C both under aerobic and anaerobic conditions for 12 h and the numbers of surviving cells were determined as described in Materials and Methods. The controls refer to the number of _B. fragilis_ cells inoculated at time 0. Mean and standard deviation of at least 3 values are indicated in each bar.
temperature differed between cells in exponential phase (6 h) and stationary phase (18 h), cultures of both ages were exposed to low temperature (Fig. 5). Under all conditions tested, older cultures (18 h) were more susceptible to chilling than younger cultures (6 h). Tally et al. (1975) noted that repeated subculturing of B. fragilis might reduce its susceptibility to oxygen. To examine this possibility, the decrease in susceptibility of B. fragilis (18 h culture) to chilling after repeated transfers on blood agar medium was determined (Fig. 6). B. fragilis cells transferred on blood agar more than 15 times became significantly (p < 0.05) more tolerant to cold than the fresh isolate.

In attempting to minimize the lethal effect of low temperature on B. fragilis, various materials were added to enriched TSB before storage of B. fragilis (Table 2). The detrimental effect of chilling on B. fragilis was minimized when 5 mM magnesium chloride and 1.0 M sucrose were added. Calf serum or divalent cations alone added to the enriched TSB did not protect B. fragilis from the lethal effect of low temperature.

From the above data, it is obvious that long periods of exposure to low temperature are lethal to B. fragilis under most conditions tested, and that storage of this organism at low temperature should be avoided.

E. Survival of B. fragilis in experimentally produced pus. To determine whether B. fragilis cells in pus were similarly affected by low temperature, pus containing B. fragilis, E. coli, or mixed cultures of both organisms, was collected from experimental animals and tested.
Fig. 5. Survival of exponentially growing and stationary phase *B. fragilis* after storage at low temperature. Predetermined numbers of 6 h and 18 h old *B. fragilis* (freshly isolated) were either plated on enriched TSA (A and B) or suspended in enriched TSB (C and D) and exposed at 4°C for 12 h, either under aerobic conditions (A and C), or anaerobic conditions (B and D). The numbers of surviving cells were determined as described in Materials and Methods. The controls represent the number of *B. fragilis* cells inoculated at time 0. Mean and standard deviation of at least 3 values are indicated on each bar.
Fig. 6. Survival of subcultured *B. fragilis* after storage at low temperature. Predetermined numbers of either freshly isolated or repeatedly subcultured *B. fragilis* (both were 18 h cultures) were plated on enriched TSA (A and B), or inoculated in enriched TSB (C and D), and exposed at 4°C both under aerobic (A and C) and anaerobic (B and D) conditions. The number of surviving cells after 12 h of exposure was determined as described in Materials and Methods. The controls represent the number of cells inoculated at time 0 (approximately $1 \times 10^5$ cell/ml final concentration). Mean and standard deviation of at least 3 values are shown on each bar.
TABLE 2.

Effect of selected compounds on the survival of
*B. fragilis* at 4°C in enriched TSB<sup>a</sup>

<table>
<thead>
<tr>
<th>Stored in enriched TSB plus:</th>
<th>% Survival after 12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
</tr>
<tr>
<td>1 mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>4</td>
</tr>
<tr>
<td>5 mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>13</td>
</tr>
<tr>
<td>10 mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6</td>
</tr>
<tr>
<td>5 mM CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>9</td>
</tr>
<tr>
<td>5 mM MgCl&lt;sub&gt;2&lt;/sub&gt; + 0.1 M Sucrose</td>
<td>11</td>
</tr>
<tr>
<td>5 mM MgCl&lt;sub&gt;2&lt;/sub&gt; + 0.5 M Sucrose</td>
<td>48</td>
</tr>
<tr>
<td>5 mM CaCl&lt;sub&gt;2&lt;/sub&gt; + 1.0 M Sucrose</td>
<td>97</td>
</tr>
<tr>
<td>5 mM CaCl&lt;sub&gt;2&lt;/sub&gt; + 0.5 M Sucrose</td>
<td>30</td>
</tr>
<tr>
<td>10% Calf serum</td>
<td>8</td>
</tr>
<tr>
<td>20% Calf serum</td>
<td>9</td>
</tr>
<tr>
<td>50% Calf serum</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Trypticase soy broth containing yeast extract (0.5%), hemin (0.05%), and menadione (0.0005%).
Percent of survival of *B. fragilis* in pus, in which *B. fragilis* was the only infectious agent, is shown in Fig. 7A and B. *B. fragilis* in the pus lost viability slowly but progressively at 4°C whether it was stored under aerobic or anaerobic conditions. However, when it was stored at 25°C, no significant loss of viability occurred within 24 h under both aerobic and anaerobic conditions. Since most anaerobic infections are mixed infections containing both strict and facultative anaerobes, we investigated the survival of *B. fragilis* and *E. coli* in pus in which both microorganisms coexisted. The results of these experiments are summarized in Fig. 8. While the survival pattern of *B. fragilis* in such pus (Fig. 8A and 8C) was similar to that seen above (Fig. 7A and 7B), there was a significant increase in number of *E. coli* when pus was stored at 35°C (Fig. 8B and 8D). Apparently, chilling was also detrimental to *E. coli* whether pus was stored under aerobic or anaerobic conditions. In an effort to find an alternative condition for storage of pus that allows the maximum number of *B. fragilis* to survive while suppressing the overgrowth of coexistent *E. coli*, we tested the survival of both organisms in pus stored at 15°C. The results of such an experiment are summarized in Table 3. It is apparent that, at this temperature, most *B. fragilis* remained viable for at least 24 h while the overgrowth of coexistent *E. coli* was minimized.

F. Preliminary animal experiments. In an attempt to establish an intra-abdominal abscess model suitable for studying the interaction between *B. fragilis* and *E. coli* in vivo, several preliminary experiments were performed.
Fig. 7. Survival of *B. fragilis* in pus after storage at 25°C or 4°C.
Pus containing *B. fragilis* was collected from experimentally produced intraperitoneal abscesses in rats. Samples of pus were placed under aerobic (A) or anaerobic (B) conditions at 25°C (•••) or 4°C (o-o) for varying lengths of time. Number of surviving cells was determined as described in Materials and Methods. Points and bars represent mean ± standard error of at least three values. Absence of a bar indicates that the standard error is smaller than the area covered by the point itself.
Fig. 8. Survival of *B. fragilis* and *E. coli* in pus after storage at 25°C or 4°C. Pus containing *B. fragilis* and *E. coli* was collected from experimentally produced intraperitoneal abscesses in rats. Samples of pus were placed under aerobic (A and B) or anaerobic (C and D) conditions at 25°C (●-●) or 4°C (○-○) for varying lengths of time. Number of surviving cells was determined as described in Materials and Methods. Points and bars represent mean ± standard error of at least three values. Absence of a bar indicates that the standard error is smaller than the area covered by the point itself.
TABLE 3.
Change in viable cell number of *B. fragilis* and *E. coli* in pus at 15°C.

<table>
<thead>
<tr>
<th>Time (h) of storage</th>
<th><em>B. fragilis</em> (monoinfection)</th>
<th><em>B. fragilis</em> (mixed infection)</th>
<th><em>E. coli</em> (mixed infection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aerobic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>anaerobic&lt;sup&gt;c&lt;/sup&gt;</td>
<td>aerobic&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100 ± 3</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>84 ± 6</td>
<td>116 ± 8</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>24</td>
<td>76 ± 13</td>
<td>87 ± 5</td>
<td>94 ± 11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard error  
<sup>b</sup>Stored aerobically  
<sup>c</sup>Stored anaerobically  
<sup>d</sup>Each figure represents the mean of at least three samples
1. Inoculation of bacterial suspensions. Rats (5 per group) were implanted with gelatin capsules containing a suspension of $10^8$ cells *B. fragilis* or $10^8$ cells *B. fragilis* plus $10^8$ cells *E. coli*. By 24 h, a lethal peritonitis had resulted in 100% mortality of animals implanted with *B. fragilis* and *E. coli*. On necropsy, intraperitoneal abscesses could not be detected (Fig. 9C). No mortality was observed by 15 d in animals implanted with *B. fragilis* alone (Fig. 9A). Representative animals from this group were necropsied at 4 d, 8 d, or 15 d. Peritonitis could not be detected at any time, and intraperitoneal abscesses did not form.

This model is not suitable for our studies because of the lack of formation of intraperitoneal abscesses. Abscesses probably did not form because of the rapid diffusion of microorganisms from the area of implantation.

2. Implantation of rat fecal material. Intraperitoneal abscesses often result from some type of trauma or surgery that causes spillage of colonic contents. Rats (5 animals) were implanted with gelatin capsules containing rat fecal material as described previously by Deysine et al. (1967). As seen with the previous model, all animals formed well defined abscesses (Fig. 9B), and lived until necropsy at 15 d.

Although this model is effective in causing anaerobic intra-abdominal abscesses, several problems make it unsuitable for a critical study of such infections. These include the undefined nature of feces, the multiplicity of bacterial species, and also the bacterial end products that lend to the complexity of this system.
Fig. 9. Rats infected with \textit{B. fragilis}, \textit{B. fragilis} in combination with \textit{E. coli}, or with rat fecal material. Animals (5 rats per group) were implanted with a single gelatin capsule containing (A) $10^8$ cells of \textit{B. fragilis}, (B) fresh rat fecal material, or (C) $10^8$ cells of \textit{B. fragilis} and $10^8$ cells of \textit{E. coli}. All animals in group C were dead by 24 h. Animals infected with \textit{B. fragilis} alone or rat fecal material were necropsied on day 15. As designated by the arrow in B, abscesses were reproducibly formed in animals infected with the fecal material.
3. **Implantation of bacteria impregnated in "artificial feces."**

To avoid the problems of using actual fecal material to cause abscesses, an artificial feces was developed. This was defined as a mixture of sterile ground meat and cellulose fiber, into which a known bacterial inoculum had been impregnated. The artificial feces was then placed into a gelatin capsule and implanted in the peritoneal cavities of rats.

Intra-abdominal abscesses were formed when the artificial fecal material contained certain bacteria or bacterial mixtures. The ultimate size of these abscesses depended on the types of bacteria which had been impregnated into the sterile meat and cellulose.

It is obvious from these results that it is possible to utilize the basic model of Deysine et al. (1967) to cause intraperitoneal abscesses while retaining the ability to carefully define the inoculum size and composition implanted into rats. For this reason, the artificial feces model of intra-abdominal abscess formation was adopted for our studies.

II. **Interaction between B. fragilis and E. coli in vivo.**

A. **Enhancement of B. fragilis pathogenicity by E. coli.** The rat intra-abdominal abscess model developed by Deysine et al. (1967) and used by Weinstein et al. (1974) was modified (see Materials and Methods) to determine whether the pathogenicity of *B. fragilis* could be enhanced by *E. coli*. The results of the experiments using the modified animal model are summarized in Table 4.

When gelatin capsules, containing artificial fecal material impregnated with *B. fragilis* alone, *E. coli* alone, or the combination
TABLE 4.

Average weight and bacteriological data of abscesses experimentally produced in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Organisms impregnated in artificial fecal material</th>
<th>Weight of encapsulated material (g)</th>
<th>Frequency of abscess formation (%)</th>
<th>Organisms recovered from peritoneal cavity pus (cells/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No bacteria</td>
<td>0.69 ± 0.22</td>
<td>0</td>
<td>Sterile</td>
</tr>
<tr>
<td>2</td>
<td>B. fragilis</td>
<td>0.73 ± 0.40</td>
<td>100</td>
<td>Sterile B. fragilis 3.2 x 10^9</td>
</tr>
<tr>
<td>3</td>
<td>E. coli</td>
<td>2.80 ± 1.22</td>
<td>100</td>
<td>Sterile E. coli 3.85 x 10^8</td>
</tr>
<tr>
<td>4</td>
<td>B. fragilis and E. coli</td>
<td>4.04 ± 1.69</td>
<td>100</td>
<td>Sterile B. fragilis 6.5 x 10^9 and E. coli 1.7 x 10^9</td>
</tr>
</tbody>
</table>

a 10 animals were used for each group.

b Mean ± standard deviation.

c Since only sterile artificial fecal material was found within the encapsulation, we did not consider these as abscesses.

d The LD$_{50}$ of B. fragilis for rats could not be determined with an inoculum of as high as 10$^{10}$ cells. None of the animals had died at 42 days and abscesses were minute in size with all doses of B. fragilis used.

e The LD$_{50}$ of E. coli for rats was 1.15 x 10$^8$ cells. All surviving animals from groups given 10$^5$ to 10$^9$ E. coli exhibited large intraperitoneal abscesses at 42 days. None of the animals given 10$^6$ E. coli died within the 42 days of observation.
of both organisms, were implanted into the peritoneal cavities, various sizes of abscesses were formed intra-abdominally in 42 days (Table 4). Implantation of capsules containing artificial feces alone resulted in encapsulation of the inoculum without the marked accumulation of pus. Representative photographs of rats from each experimental group are shown in Fig. 10. When $10^8$ cells of *B. fragilis* were added to the artificial fecal base and implanted into the peritoneal cavity, only small intra-abdominal abscesses were formed (Fig. 10B). The average weight of *B. fragilis* induced abscesses was $0.73 \pm 0.40$, not significantly larger than the encapsulated granule produced by the sterile artificial fecal inoculum ($0.69 \pm 0.22$). However, *B. fragilis* in pure culture was isolated at $3.2 \times 10^9$ cells per gram of pus. When $10^6$ cells of *E. coli* were added to the artificial fecal base and implanted into animals, large abscesses ($2.80 \pm 1.22$) were formed (Fig. 10C) which were significantly larger than the control group ($p < 0.001$). The abscesses produced by *E. coli* contained larger quantities of pus than abscesses caused by *B. fragilis* and were shown to contain $3.85 \times 10^8$ cells of viable *E. coli* per gram of pus (Table 4). When $10^8$ cells of *B. fragilis* and $10^6$ cells of *E. coli* were combined and implanted into animals (Fig. 10D), the resultant abscesses ($4.04 \pm 1.69$) were significantly larger than the control ($p < 0.001$) or those formed by *B. fragilis* alone ($p < 0.001$), although they were not significantly different from those produced by *E. coli* alone ($p < 0.10$). Abscesses produced by the combination of both organisms were filled with pus that contained both viable *E. coli* and *B. fragilis* ($1.7 \times 10^9$ and $6.5 \times 10^9$ cells/g pus, respectively). In all cases,
Fig. 10. Intra-abdominal abscess formed in rats by *E. coli* or *B. fragilis* or by the combination of both organisms. Animals (10 rats per group) were implanted with a double gelatin capsule containing artificial feces impregnated with (A) no bacteria (control), (B) $10^9$ *B. fragilis* cells, (C) $10^6$ *E. coli* cells, or (D) $10^9$ *B. fragilis* cells combined with $10^6$ *E. coli* cells. Arrows indicate the encapsulated sterile feces in Fig. 1A, and the abscesses (Fig. 1B to 1D) formed in rats as specified above.
the peritoneal cavity of the rats was sterile at the time of necropsy.

Both _B. fragilis_ and _E. coli_ alone caused the formation of intra-peritoneal abscesses in rats. The combination of these organisms caused abscesses that appeared larger than those caused by either alone, although they were not statistically different in weight from those induced by _E. coli_. These results are significantly different from those reported by Onderdonk et al. (1976) in that _B. fragilis_ and _E. coli_ could independently cause intraperitoneal abscesses to form. To our knowledge, this is the first demonstration of intraperitoneal abscesses caused by a facultative anaerobe alone. Onderdonk et al. (1976) reported that neither organism could cause abscesses unless the other was present.

B. Lethality of _B. fragilis_ and _E. coli_ for the rat. In order to determine the maximum number of _E. coli_ or _B. fragilis_ that could be implanted in the rat to cause abscess formation without causing lethality, the LD$_{50}$ of each organism was determined.

_B. fragilis_ in concentrations ranging from $10^5$ to $10^{10}$ cells/ml was impregnated in artificial fecal material and implanted into the peritoneal cavity of rats. The LD$_{50}$ of _B. fragilis_ in this model system could not be determined with an inoculum as high as $10^{10}$ cells. None of the animals had died by 42 days postoperatively.

_E. coli_ in concentrations ranging from $10^5$ to $10^{10}$ cells/ml was also impregnated into artificial fecal material and implanted in rats. The LD$_{50}$ of _E. coli_ for rats in this model system was found to be $1.15 \times 10^8$ cells.

On the basis of these findings, an inoculum of $10^8$ cells _B. fra-
gilis, $10^6$ cells E. coli, or the combination of both organisms was used in our animal model of anaerobic infection.

C. Histology of experimentally produced abscess. The histologic examination of abscesses caused by B. fragilis, E. coli or the combination of both, was performed. A typical cross section through an abscess formed by implantation of gelatin capsules containing artificial fecal material impregnated by B. fragilis and E. coli is shown in Fig. 11.

The outside of the abscess is seen to the right of the micrograph. In the older, outermost area of the abscess wall, granulation tissue (GT) and proliferation were evident with fibrosis extending to the surrounding adipose tissue (A). Closer to the lumen of the abscess, (L) there were increasing numbers of phagocytic cells (P). Plasma cells and macrophages were more prominent in this area. In the lumen of the abscess was a large amount of exudate, primarily debris, bacteria and polymorphonuclear leukocytes. This histopathologic description is also typical for abscesses sectioned from human tissues. There was no difference in histologic appearance of this abscess and those caused by B. fragilis alone or E. coli alone when animals were necropsied at 42 d.

Since differences in response to these organisms might be more visible at an earlier time after implantation, tissue surrounding the gelatin capsule and artificial fecal material was observed histologically at 1 d, 3 d, and 7 d postoperatively. At 24 h after implantation, tissue surrounding the abscess caused by B. fragilis or the combination of B. fragilis and E. coli was found to have microthrombi
Fig. 11. Cross section of an intraperitoneal abscess formed in rats by implantation of $10^8$ cells *B. fragilis* cells and $10^6$ cells of *E. coli*. Animals were implanted with a double gelatin capsule containing artificial fecal material as described in Materials and Methods and were necropsied at 42 days. Abscess tissue was fixed in buffered formalin, embedded in paraffin, sectioned, and stained with H and E. Letters: (L) lumen, (P) phagocytic cells, (GT) granulation tissue, (A) adipose tissue.
present. However, microthrombi seen at this time could be explained by the severe vasculitis observed and not necessarily to any specific response of \textit{B. fragilis}. It is interesting that at 7 d, tissues infected with \textit{B. fragilis} appeared to have an arteriolization of the capillaries in the granulation tissue. However, the meaning of this finding is, as yet, obscure.

It is clear from this brief examination of tissues that the response seen in animals infected with \textit{B. fragilis} (or combinations with \textit{E. coli}) is different from that seen in animals infected with \textit{E. coli} alone. Extensive studies need to be performed to determine what is causing these pathogenic effects and to study them more critically \textit{in vitro}.

D. Factors affecting intraperitoneal abscess formation caused by \textit{B. fragilis}. To study the actual role of each bacterial component pathogenesis, the effect of inoculum size of \textit{B. fragilis} and/or \textit{E. coli}, on abscess formation were determined.

1. Effect of inoculum size on abscess formation. When the size of \textit{B. fragilis} inoculum was varied from $10^7$ to $10^{10}$ cells, all abscesses formed were small and not significantly different in weight from the encapsulated mass seen in the control animals (Table 5). Variation in the inoculum size of \textit{E. coli} from $10^6$ cells to $10^8$ cells did not result in any difference in abscess size (Table 6). In fact, abscesses caused by an inoculum of $10^7$ or $10^8$ cells of \textit{E. coli} were slightly smaller than those caused by $10^6$ cells of \textit{E. coli}. An inoculum of higher than $10^8$ cells resulted in lethal peritonitis.

When animals were implanted with both \textit{B. fragilis} and \textit{E. coli}
TABLE 5.
Average weight of abscesses experimentally produced in rats by *B. fragilis*

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Number of <em>B. fragilis</em> impregnated in artificial fecal material</th>
<th>Weight of encapsulated material&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>None</td>
<td>0.69 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>$10^{10}$ cells</td>
<td>0.85 ± 0.26</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>$10^9$ cells</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>$10^8$ cells</td>
<td>0.73 ± 0.13</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>$10^7$ cells</td>
<td>0.67 ± 0.33</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± standard error

<sup>b</sup> When no bacteria were impregnated, only sterile artificial fecal material was found within the encapsulation and no pus was formed.
TABLE 6.

Average weight of abscesses experimentally produced in rats by *E. coli*

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Number of <em>E. coli</em> impregnated in artificial fecal material</th>
<th>Weight of encapsulated materiala</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>None</td>
<td>0.60 ± 0.09b</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>$10^8$ cells</td>
<td>2.18 ± 0.41</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>$10^7$ cells</td>
<td>2.94 ± 0.31</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>$10^6$ cells</td>
<td>4.04 ± 0.56</td>
</tr>
</tbody>
</table>

a Mean ± standard error.

b When no bacteria were impregnated, only sterile artificial fecal material was found within the encapsulation and no pus was formed.
(Table 7) even small numbers of bacteria were sufficient to cause the 
formation of large abscesses. Representative abscesses caused by 
B. fragilis, E. coli or both in combination are seen in Fig. 10.

2. Effect of suspending material on abscess formation.
Intraperitoneal abscesses are most often associated with trauma or 
spillage of intestinal contents into the abdomen. As shown above, an 
artificial fecal material in conjunction with bacteria was necessary 
to cause infection. This closely mimics colonic spillage without 
introducing the complexities of actual fecal material.

Another factor associated with ability of these organisms to 
initiate infection might be the presence of clots formed during trauma 
or abdominal surgery. Although the clots resorb internally after a 
short time interval, they might still serve as an initial focus of 
infection.

Animals implanted with plasma clots that had been impregnated 
with bacteria did not cause the formation of intraperitoneal abscesses 
by day 7. Clots began to dissolve immediately on implantation and had 
disappeared by day 7. In contrast to this when blood clots were im-
pregnated with bacteria, small well defined abscesses formed and per-
sisted to necropsy on day 14 (Fig. 12). It appears that localization 
of bacteria was necessary initially. However, if blood is present and 
a focus of infection forms, suspending material might not be necessary, 
and pathogenicity of the organisms could be determined without the 
further complication of a prolonged foreign body response.

It appears that for intra-abdominal abscesses to form, localiza-
tion of bacteria was initially necessary. However, after initiation
TABLE 7.

Average weight of abscesses caused by low numbers of *B. fragilis* and *E. coli*

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Organisms impregnated in artificial fecal material</th>
<th>Weight of encapsulated material$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>No bacteria</td>
<td>0.69 ± 0.09$^b$</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td><em>B. fragilis</em>$_i$(10$^4$ cells) <em>E. coli</em>$_i$(10$^4$ cells)</td>
<td>3.26 ± 0.41</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td><em>B. fragilis</em>$_i$(10$^3$ cells) <em>E. coli</em>$_i$(10$^3$ cells)</td>
<td>3.28 ± 0.49</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td><em>B. fragilis</em>$_i$(10$^2$ cells) <em>E. coli</em>$_i$</td>
<td>3.20 ± 0.21</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td><em>B. fragilis</em>$_i$(10$^1$ cells) <em>E. coli</em>$_i$(10$^1$ cells)</td>
<td>2.48 ± 0.49</td>
</tr>
</tbody>
</table>

$^a$ Mean ± standard error.

$^b$ When no bacteria were impregnated, only sterile artificial fecal material was found within the encapsulation and no pus was formed.
Fig. 12. Intra-abdominal abscess in rats caused by implantation of blood clots impregnated with $10^8$ cells of B. fragilis. The arrow indicates a representative abscess formed in rats as specified above.
of the infection, solid material was no longer necessary. It should also be noted that the presence of blood was necessary, possibly due to its content of nutrients, vitamins, and/or iron.

III. Effect of E. coli on the growth of B. fragilis in vitro.

Although data from other investigators (Onderdonk et al., 1976) suggest that a synergistic interaction between aerobic and facultative organisms is involved in abscess formation, it is still not clear from our data (Table 4, Fig. 10) whether B. fragilis and E. coli actually interact to enhance the formation of intra-abdominal abscesses in rats. In order to investigate the nature of interactions between these two organisms more critically, their growth in a mixed culture was examined in vitro.

A. Growth of B. fragilis and E. coli in peptone media.

1. Enhancement of B. fragilis growth in the presence of E. coli. B. fragilis rapidly lost viability when incubated alone in stored Bacto-peptone broth (lot #540620) at 37°C. In the same medium, however, E. coli grew optimally with a short lag period and a doubling time of approximately 20 min (Fig. 13). When B. fragilis was inoculated in the same medium together with E. coli, the B. fragilis showed an initial decline in viability as it did in pure culture. However, after 9 h to 12 h of incubation, growth of B. fragilis was initiated and eventually reached near maximal levels of growth in 24 h. This phenomenon was repeatedly observed whether the mixed cultures were incubated under aerobic or anaerobic conditions. In one particular lot of Bacto-peptone broth (Difco, peptone, lot #630023) the viability of
Fig. 13. Enhancement of *B. fragilis* growth by *E. coli* and change in dissolved oxygen level during incubation. Cells were grown in Bacto-peptone (lot #540620) under aerobic conditions at 37°C for 24 h. Dissolved oxygen (o-o) was measured using an oxygen monitor equipped with a Clark oxygen electrode. Each point represents the mean of at least two samples. Symbols: •-•, viable *E. coli* when grown alone or in combination with *B. fragilis*; Δ-Δ, viable *B. fragilis* alone; ●-●, viable *B. fragilis* when grown with *E. coli* in a mixed culture.
B. fragilis in a mixed culture dropped so rapidly that essentially no viable B. fragilis cells could be recovered from the culture by 3 h (Fig. 14). However, B. fragilis growth initiated at 12 h and progressed normally, reaching the maximal level by 24 h. These observations clearly indicate that under this condition, the initial growth of E. coli is required for the subsequent growth of B. fragilis.

2. Suitability of various peptone media for studying the in vitro interaction between B. fragilis and E. coli. Because it was experienced in the early phase of this in vitro study that reproducible results could be obtained only when the same batch of peptone was used, various peptone media were examined to see whether only certain types of peptone were suitable for the present study.

Various lots of Bacto-peptone and other commercially available peptone media were tested under the same experimental conditions as employed for testing the lot #540620. The results of the test are summarized in Tables 8 and 9. As evident in the tables, good to fair growth of B. fragilis occurred in the absence of E. coli in most of the peptone media tested. Although data are not shown, all peptone media supported good growth of E. coli. Since it was desirable for our purpose to use a medium in which B. fragilis could grow only in the presence of E. coli, it was decided to use Bacto-peptone lot #540620 throughout the subsequent study.

3. Enhancement of B. fragilis growth by E. coli while separated by a dialysis membrane. To determine if physical contact between the two organisms is necessary for B. fragilis growth to occur, the organisms suspended in peptone broth were separated by a dialysis
Fig. 14. Enhancement of *B. fragilis* growth by *E. coli* in Bactopeptone (lot #630023). Cultures were grown under aerobic conditions at 37°C for 24 h. Each point represents the mean of at least two samples. Viable cell numbers of less than 10 cells/ml could not be detected. In some cases, cell number dropped below this limit and were again countable at a later time. Symbols: •-•, *E. coli* alone or in combination with *B. fragilis*; ▲-▲, *B. fragilis* alone; ○-○, *B. fragilis* growing with *E. coli*. 
<table>
<thead>
<tr>
<th>Medium</th>
<th>Time stored&lt;sup&gt;c&lt;/sup&gt; (h)</th>
<th>Growth&lt;sup&gt;d&lt;/sup&gt; (Klett units at 540)</th>
<th>Dissolved oxygen&lt;sup&gt;e&lt;/sup&gt; (μmoles/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>0</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>192.5</td>
</tr>
<tr>
<td>Bacto-peptone</td>
<td>0</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tryptone</td>
<td>0</td>
<td>46</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td>Thiotone</td>
<td>0</td>
<td>75</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>75</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>21</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Growth and oxygen levels are representative of at least 2 determinations.

<sup>b</sup> Supplemented with 0.05% hemin.

<sup>c</sup> Stored at room temperature for 24 h under aerobic conditions.

<sup>d</sup> The medium was inoculated with $2.5 \times 10^5$ *B. fragilis* cell/ml, and incubated under anaerobic conditions at 37°C.

<sup>e</sup> Measured by a Clark type electrode immediately before inoculation.
<table>
<thead>
<tr>
<th>Lot number</th>
<th>Growth(^b^)  (viable cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>540620</td>
<td>10</td>
</tr>
<tr>
<td>561172</td>
<td>7.8 x 10(^4^)</td>
</tr>
<tr>
<td>564852</td>
<td>1.7 x 10(^2^)</td>
</tr>
<tr>
<td>604607</td>
<td>1.19 x 10(^9^)</td>
</tr>
<tr>
<td>630023</td>
<td>1.41 x 10(^9^)</td>
</tr>
</tbody>
</table>

\(^a^\) Stored at room temperature for 24 h under aerobic conditions and supplemented with 0.05% hemin.

\(^b^\) The media was inoculated with 2.5 x 10\(^7^\) B. \textit{fragilis} cells/ml and incubated under anaerobic conditions at 37\(^\circ^C\) for 48 h. Growth is representative of at least 2 determinations.
membrane and then incubated aerobically at 37°C (data not shown). After 24 h of incubation, \textit{E. coli} grew from an original inoculum of approximately $10^5$ cells/ml to an optimal level of $10^9$ cells/ml, whereas no growth of \textit{B. fragilis} occurred. In fact, viable number of \textit{B. fragilis} dropped from $10^5$ to $10^3$ cells/ml within that period. However, by 48 h, \textit{B. fragilis} grew to a level of $2 \times 10^7$ cells/ml.

A similar result was obtained when the same culture system was incubated anaerobically. These results indicate that direct cell to cell contact is not necessary for enhancement of \textit{B. fragilis} growth by \textit{E. coli} and that a possible factor(s) involved is dialyzable.

4. Effect of selective inhibition of \textit{E. coli} growth on the survival and growth of \textit{B. fragilis} in mixed culture. Kanamycin is inhibitory to the growth of \textit{E. coli} but not \textit{B. fragilis}. To determine the extent of \textit{E. coli} growth necessary for survival and subsequent growth of coexistent \textit{B. fragilis}, kanamycin (100 µg/ml, final concentration) was added to separate sets of mixed cultures at various times of incubation. The results of these experiments are illustrated in Fig. 15.

When kanamycin was added to the mixed culture at 0 time, \textit{E. coli} rapidly lost viability and dropped to a level of 20 cells/ml at 4 h and no growth of \textit{B. fragilis} was observed (Fig. 15A). When the antibiotic was added at 2 h to the mixed culture in which \textit{E. coli} had grown from $10^5$ cells/ml to $2 \times 10^6$ cells/ml, most of the \textit{E. coli} were rapidly killed. However, \textit{B. fragilis} began growing at 10 h, reaching $2 \times 10^6$ cells/ml at 24 h. In case kanamycin was added at 4 h (Fig. 15C), when \textit{E. coli} had grown to $10^8$ cells/ml, initiation of \textit{B. fragilis}
Fig. 15. Effect of *E. coli* on the growth of *B. fragilis* in Bacto-peptone medium. Cultures were grown under aerobic conditions at 37°C for 24 h. Kanamycin (100 µg/ml) was added at 0 time (A), 2 h (B) or 4 h (C) to selectively inhibit *E. coli* growth. Viable counts were determined as described in Materials and Methods. Each point represents the mean of at least two samples. Symbols: •••, *E. coli* viable counts •••••••, *B. fragilis* viable counts.
growth occurred slightly earlier, reaching approximately $10^8$ cells/ml by 24 h (Fig. 15).

5. **Effect of inoculum size of *E. coli* on growth of *B. fragilis* in mixed culture.** In order to determine the effect of the initial inoculum size of *E. coli* on *B. fragilis* growth in the mixed culture system, two different inoculum sizes, $2 \times 10^3$ cells/ml, and $10^1$ cells/ml, were tested. As shown in Fig. 16, *B. fragilis* growth in the mixed culture was not significantly altered when the inoculum size was reduced from $10^5$ (Fig. 16) to $2 \times 10^3$ or $10^1$ cells/ml. However, when initial levels of *E. coli* were reduced to $10^1$ cells/ml, viable numbers of *B. fragilis* obtained at 24 h were significantly lower than that observed with the $10^3$ or $10^5$ cell inoculum of *E. coli*.

It is apparent from these observations that, under certain conditions, growth of *B. fragilis* is markedly enhanced by the initial coexistence of *E. coli*.

6. **Mechanism of the enhancement of *B. fragilis* growth by *E. coli* in Bacto-peptone medium.** A variety of mechanisms might be responsible for the enhancement of *B. fragilis* growth by *E. coli* in a mixed culture. These include the following: a) Dissolved oxygen level in media used was too high to allow *B. fragilis* growth. The utilization of oxygen by *E. coli* during its growth reduced oxygen to a very low level, allowing growth of *B. fragilis*; b) Preformed toxic substances might be present, or exposure of media to storage might cause the production of toxic peroxides or superoxides. *E. coli* could utilize or alter toxic substances on its growth, and is capable of ridding the media of toxic oxygen products by its catalase and superoxide dis-
Fig. 16. Effect of \( E. \) coli inoculum size on the enhancement of \( B. \) fragilis growth in Bacto-peptone medium. \( 2 \times 10^3 \) cells (A) or \( 10^1 \) cells (B) of \( E. \) coli were inoculated with \( 10^5 \) cells of \( B. \) fragilis to Bacto-peptone medium and incubated under aerobic conditions at 37°C for 24 h. Viable cell number was determined as described in Materials and Methods. Each point represents the mean of at least two samples. Symbols: \( \times-\times \), growth of \( B. \) fragilis alone, \( \circ-\circ \), growth of \( B. \) fragilis when in combination with an inoculum of \( 10^1 \) cells of \( E. \) coli/ml; Growth of \( E. \) coli after inoculation of \( 5 \times 10^3 \) cells/ml (\( \bullet-\bullet \)), or \( 10^1 \) cells/ml (\( \mathbf{A-\mathbf{A}} \)) with \( B. \) fragilis was also.
mutase enzymes. *B. fragilis* could then initiate growth; c) *E. coli* might produce and excrete vitamins or nutrients stimulatory to *E. coli*. This (these) substance(s) might be some type of material already defined as a growth factor for *B. fragilis*, or may be an as yet unidentified stimulatory material(s).

To determine which of these mechanisms were actually involved in *E. coli* induced enhancement of *B. fragilis* growth in mixed culture, each of the possibilities was analyzed individually.

a. **Dissolved oxygen.** The dissolved oxygen level of variously treated Bacto-peptone media and growth of *B. fragilis* in each are shown in Table 10. The level of dissolved oxygen in media immediately after autoclaving was approximately $30 \times 10^{-6}$ moles/l. *B. fragilis* grew in this medium or in freshly autoclaved media that had been placed immediately under anaerobic conditions and stored there before use. However, if media were stored aerobically, the dissolved oxygen level increased to $67 \times 10^{-6}$ moles/l by 24 h and increased further to $190 \times 10^{-6}$ moles/l after 48 h. *B. fragilis* never grew either aerobically or anaerobically in peptone stored for 24 h or longer after autoclaving.

The growth of *E. coli* in stored peptone medium reduced dissolved oxygen to a level below that observed in freshly autoclaved medium. After oxygen level was lowered by *E. coli*, initiation of *B. fragilis* growth occurred. It has been demonstrated (data not shown) that in a mixed culture shaking under aerobic conditions, *B. fragilis* will not grow. In the same respect, vortexing freshly autoclaved peptone diminished the amount of *B. fragilis* growth (Table 10). The reduction
TABLE 10.
Growth of *B. fragilis* and oxygen levels\(^a\) in Bacto-peptone medium (5%) stored under various conditions

<table>
<thead>
<tr>
<th>Conditions of storage</th>
<th>Growth(^c) (cells/ml)</th>
<th>Dissolved oxygen(^d) at time of inoculation ((\mu)moles/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Freshly autoclaved)</td>
<td>(5.5 \times 10^8)</td>
<td>30</td>
</tr>
<tr>
<td>Vortexed (2 min)</td>
<td>(8.0 \times 10^6)</td>
<td>187.5</td>
</tr>
<tr>
<td>24 h under anaerobic conditions (24°C)</td>
<td>(4.9 \times 10^9)</td>
<td>10.2</td>
</tr>
<tr>
<td>24 h under aerobic conditions</td>
<td>(2.4 \times 10^3)</td>
<td>67.5</td>
</tr>
<tr>
<td>48 h under aerobic conditions</td>
<td>10</td>
<td>192.5</td>
</tr>
<tr>
<td>24 h aerobically, then 24 h anaerobically</td>
<td>(8.3 \times 10^2)</td>
<td>10</td>
</tr>
<tr>
<td>24 h aerobically, then boiled 30 min.</td>
<td>(3.6 \times 10^6)</td>
<td>47.5</td>
</tr>
</tbody>
</table>

\(^{a}\) Growth and oxygen levels are representative of at least 2 determinations.

\(^{b}\) Supplemented with 0.05% hemin and kept at 25°C during storage.

\(^{c}\) Media were inoculated immediately after the designated treatment to a final concentration of \(2.5 \times 10^2\) cells/ml of *B. fragilis* and incubated at 37°C for 24 h anaerobically.

\(^{d}\) Measured by a Clark type electrode immediately before inoculation.
of dissolved oxygen, then, is vital to the growth of B. fragilis. However, this may not be the only action of E. coli that enhances B. fragilis growth.

If oxygen alone were responsible for inability of B. fragilis to grow, stored peptone media, which was treated by procedures to lower oxygen level without altering other parameters, ought to subsequently allow growth of B. fragilis. Media which was initially stored for 24 h under aerobic conditions, was subsequently placed under anaerobic conditions for 24 h or boiled for 30 min before inoculation with B. fragilis. B. fragilis grew only slightly in stored media that was boiled before inoculation, and actually lost viability in stored peptone placed under anaerobic conditions for 24 h before inoculation (Table 10). This would indicate that reduction of oxygen cannot completely account for enhancement of B. fragilis growth. In confirmation of these findings, other facultative microorganisms, P. vulgaris, K. pneumonia and S. aureus, all known to rapidly reduce oxygen tension in media, were grown in mixed culture with B. fragilis (Table 11). S. aureus did not stimulate growth of the anaerobe. Although P. vulgaris and K. pneumoniae did enhance B. fragilis growth, enhancement was not observed until 48 h, whereas enhancement by E. coli was observed at 24 h.

E. coli is apparently responsible for reducing oxygen level in media, a requirement for growth of the B. fragilis. However, this mechanism is not alone sufficient to account for the optimal B. fragilis growth seen in mixed culture. Other mechanisms must also be participating in this phenomenon.
TABLE 11.

Enhancement of *B. fragilis* growth by other organisms in 5% Bacto-peptone

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth of <em>B. fragilis</em> viable cell/ml at 24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>1.72 x 10^7</td>
<td>6.0 x 10^8</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>7.1 x 10^4</td>
<td>4.9 x 10^8</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>10^3</td>
<td>1.9 x 10^9</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>ND^c</td>
<td>3 x 10^4</td>
</tr>
</tbody>
</table>

^a^ Supplemented with 0.05% hemin

^b^ Media were inoculated with 2.5 x 10^5 *B. fragilis* cells/ml, 3 x 10^5 cells/ml of *P. vulgaris* or *K. pneumoniae*, or 3 x 10^4 cells/ml of *S. aureus*, and incubated aerobically at 37° C. Growth is representative of at least 2 determinations.

^c^ ND = not done.
b. Removal of toxic superoxide radicals or other growth inhibitors. To determine if removal of superoxide radicals would allow growth of *B. fragilis* in peptone broth, superoxide dismutase (65 units activity/ml), prepared as described in Materials and Methods, was added to stored peptone (Table 12). When superoxide dismutase was not added, all *B. fragilis* lost viability by 24 h. Addition of superoxide dismutase to the medium slowed the rate of viability loss, although by 48 h complete loss in viability of *B. fragilis* was still observed.

It is possible that *E. coli* is utilizing or removing toxic substances in the stored peptone that are inhibitory to the growth of *B. fragilis*. To test this possibility, the stored peptone was treated with activated charcoal, filter sterilized and then inoculated with *B. fragilis*. The result of this study is shown in Table 13. *B. fragilis* grew in such a peptone medium to a level of $10^9$ cells/ml indicating that there are some toxic substances in stored peptone that can be removed or altered by *E. coli* when in mixed culture.

c. *E. coli* production of nutrients or vitamins that stimulate the growth of *B. fragilis*. To determine if preformed structures or internal components of *E. coli* would stimulate growth of *B. fragilis*, heat killed *E. coli* were inoculated along with *B. fragilis* to stored peptone under anaerobic conditions. As many as $10^8$ cells of *E. coli* were heat killed by boiling for 30 minutes. Addition of the heat killed *E. coli* to stored peptone did not stimulate the growth of *B. fragilis* under any experimental condition tested (data not shown).

Stored peptone was supplemented with hemin, menadione, ammonium
TABLE 12

Effect of superoxide dismutase on the growth of *B. fragilis* in 5% Bacto-peptone medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>viable cells/ml at</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td></td>
</tr>
<tr>
<td>Stored peptone</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Stored peptone + 65 units/ml SOD</td>
<td>3.0 x 10^2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Control (freshly autoclaved peptone)</td>
<td>1.6 x 10^9</td>
<td>1.7 x 10^9</td>
<td></td>
</tr>
</tbody>
</table>

*a* Supplemented with 0.5% hemin.

*b* Media were inoculated with 2.5 x 10^5 *B. fragilis* cells/ml and incubated anaerobically at 37° C. Growth is representative of at least two determinations.

*c* Stored aerobically for 24 h at 25° C.
TABLE 13.

Growth of *B. fragilis* in Bacto-peptone medium<sup>a</sup> (5%) treated with activated charcoal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth&lt;sup&gt;b&lt;/sup&gt; viable cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
</tr>
<tr>
<td>Stored medium adsorbed with activated charcoal</td>
<td>$3.28 \times 10^9$</td>
</tr>
<tr>
<td>Control (freshly autoclaved)</td>
<td>$1.45 \times 10^9$</td>
</tr>
</tbody>
</table>

<sup>a</sup> Supplemented with 0.05% hemin.

<sup>b</sup> Media were inoculated with *B. fragilis* to a final concentration of $2.5 \times 10^5$ cells/ml and incubated anaerobically at 37° C for 24 h. Growth is representative of at least 2 determinations.
sulfate, ferrous sulfate, and/or a vitamin mixture (Table 14). All of these substances are known to be stimulatory to *B. fragilis* under other circumstances (Varel and Bryant, 1974). In no case did these materials enhance the growth of *B. fragilis*.

An experiment was devised to determine if *E. coli* produced substances that were stimulatory to *B. fragilis*. Stored medium (5.0 ml) was supplemented with 0-5.0 ml *E. coli* spent medium, and the volumes equilibrated to 10 ml with sterile saline. Tubes were then inoculated with *B. fragilis* to $10^5$ cells/ml. Addition of 2 ml or more of spent medium allow optimal growth of *B. fragilis* to $10^9$ cells/ml by 24 h (Table 15). This suggests that the spent medium contained material(s) produced by *E. coli* capable of stimulating growth of *B. fragilis*. A dilution of toxins was not responsible for *B. fragilis* growth since stored medium to which various amounts of sterile saline had been added did not support growth of *B. fragilis*.

From the data presented above, it is likely that at least three different mechanisms are jointly responsible for growth of *B. fragilis* in mixed culture in stored peptone. These are: 1) removal of dissolved oxygen, 2) removal or inactivation of toxic substances and 3) production of a factor(s) stimulatory to the growth of *B. fragilis*.

B. Interaction of *B. fragilis* and *E. coli* when grown in mixed culture in semi-synthetic medium. The data presented in the preceding section strongly indicate that the stimulation of *B. fragilis* growth by *E. coli* in mixed cultures is mediated, at least in part, by a substance or substances accumulating in the medium as a result of *E. coli* growth. To further characterize such substances it was thought
TABLE 14

Growth of *B. fragilis* in Bacto-peptone medium (Lot #540620) supplemented with various additives

<table>
<thead>
<tr>
<th>Supplements</th>
<th>Growth(^a) viable cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemin (0.05%)</td>
<td>10</td>
</tr>
<tr>
<td>Hemin + Menadione (0.0005%)</td>
<td>10</td>
</tr>
<tr>
<td>Hemin + Cyanocobalamin (ng/ml)</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>500</td>
<td>10</td>
</tr>
<tr>
<td>Hemin + Ammonium sulfate (mM)</td>
<td>10</td>
</tr>
<tr>
<td>0.03</td>
<td>10</td>
</tr>
<tr>
<td>0.15</td>
<td>10</td>
</tr>
<tr>
<td>0.30</td>
<td>10</td>
</tr>
<tr>
<td>0.60</td>
<td>10</td>
</tr>
<tr>
<td>3.00</td>
<td>10</td>
</tr>
<tr>
<td>Hemin + Menadione (0.0005%) + Cyanocobalamin (50ng) + Ammonium sulfate (0.3 mM) + Vitamin mix</td>
<td>(3.5 \times 10^2)</td>
</tr>
</tbody>
</table>

\(^a\)The media were inoculated with \(2.5 \times 10^5\) *B. fragilis* cells/ml and incubated at 37° C for 24 h anaerobically. Growth is representative of at least two determinations.
TABLE 15.

Growth of B. fragilis in stored Bacto-peptone medium supplemented with various amounts of spent medium

<table>
<thead>
<tr>
<th>Stored peptone^a</th>
<th>Spent peptone^b</th>
<th>Diluent (H₂O)</th>
<th>B. fragilis growth^c (viable cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>3.0 x 10³</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>5</td>
<td>1.1 x 10³</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>4</td>
<td>5.7 x 10⁸</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>3</td>
<td>2.3 x 10⁹</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2</td>
<td>2.5 x 10⁹</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1</td>
<td>2.3 x 10⁹</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0</td>
<td>2.6 x 10⁹</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>0</td>
<td>2.9 x 10⁹</td>
</tr>
</tbody>
</table>

^a Stored at room temperature for 24 h under aerobic conditions.

^b Spent by 24 h of E. coli growth and filter sterilized before use.

^c The media was inoculated with a 2.5 x 10⁵ B. fragilis cells/ml and incubated under anaerobic conditions for 48 h at 37°C. Growth is representative of at least 2 determinations.
that a synthetic or semi-synthetic medium was more preferred to complex peptone media. On the basis of several preliminary experiments, a modification of the semi-synthetic medium of Roberts et al. (1955) was selected and used throughout this investigation.

1. Kinetic studies of enhancement of B. fragilis growth by E. coli. B. fragilis slowly lost viability when incubated alone in the semi-synthetic medium, either aerobically or anaerobically (Fig. 17). No initial rapid drop in B. fragilis viability, as that seen in certain peptone media (Fig. 13, 14), was observed in this medium. This observation indicates that toxic substances that were shown to be present in specific lots of peptone media are not present in the semi-synthetic medium. Absence of toxic substances in this medium was further confirmed by demonstrating that semi-synthetic medium would not inhibit B. fragilis growth using the agar diffusion method described below (Fig. 18). In contrast to the loss in viability of B. fragilis in pure culture, B. fragilis, in mixed culture with E. coli, increased in number from approximately $10^5$ cells/ml to $10^7$ cells/ml in 48 h.

2. Enhancement of B. fragilis growth in mixed culture after inhibition of E. coli by kanamycin. To determine how much E. coli growth was necessary to initiate the growth of B. fragilis, E. coli was inhibited by the addition of kanamycin (final concentration, 100 µg/ml) at various times after inoculation of mixed cultures. When kanamycin was added at 0 time or 2 h, growth of B. fragilis did not occur (Table 16). In fact, a slight loss in viability was noted. However, kanamycin added at 4 h did not prevent the subsequent enhancement of B. fragilis growth.
Fig. 17. Stimulation of *B. fragilis* growth by the growth of *E. coli*

in semi-synthetic medium. Approximately $10^5$ cells of *B. fragilis*

*E. coli*, or the combination of both were inoculated to semi-

synthetic medium, and incubated at $37^\circ$C under aerobic conditions.

Viable cell number was determined as described in Materials and

Methods. Each point represents the mean of at least two samples.

Symbols: ■■, *E. coli* alone or mixed with *B. fragilis*; △△,

*B. fragilis* alone; ○○, *B. fragilis* mixed with *E. coli*.
Fig. 18. Lack of inhibition of *B. fragilis* growth on enriched 
TSA by stored peptone or semi-synthetic medium. The 
surface of enriched TSA was spread with approximately 
$10^6$ cells of *B. fragilis* and wells cut. The wells 
were filled with 0.25 ml of (A and B) stored peptone, 
(C and D) semi-synthetic medium, or (E) hemin (control).
TABLE 16.

Effect of *E. coli* growth<sup>a</sup> on the growth of *B. fragilis* in semi-synthetic medium

<table>
<thead>
<tr>
<th>Time of supplementation with kanamycin&lt;sup&gt;b&lt;/sup&gt; (h)</th>
<th><em>B. fragilis</em> growth&lt;sup&gt;c&lt;/sup&gt; (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None added</td>
<td>1.6 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>1.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>8.0 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>1.7 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Broth was inoculated with 2.5 x 10<sup>5</sup> cells each of *B. fragilis* and *E. coli*.

<sup>b</sup> *E. coli* growth was selectively halted at the designated times by addition of kanamycin to a final concentration of 100 ug/ml.

<sup>c</sup> Viable cell number of *B. fragilis* was determined after anaerobic incubation for 48 h at 37° C.
3. Demonstration of the B. fragilis growth stimulatory activity of E. coli spent media by the plate agar diffusion method.

As described above, B. fragilis growth was stimulated by E. coli in broth media. To confirm this finding, and to establish a rapid method for qualitative determination of the presence of B. fragilis growth stimulatory materials, a plate agar diffusion technique was developed. Wells were cut in TSA plates, B. fragilis seeded onto the plate surfaces and the wells filled with E. coli spent media. After seeded plates containing stored peptone or semi-synthetic medium were incubated for 24 h at 37°C under anaerobic conditions, visual inspection of the plates revealed no stimulation of B. fragilis growth (Fig. 19). However, it was clearly demonstrated, as seen in Fig. 19, that spent peptone and spent semi-synthetic medium did stimulate the growth of B. fragilis, confirming results observed in the broth method. This technique will be used to determine the ability of spent media treated in various manners to retain the stimulatory ability.

C. Some properties of the B. fragilis growth stimulatory factor(s) produced by E. coli. In order to further define some of the properties of the stimulatory factors produced by E. coli, spent semi-synthetic medium was treated in a variety of manners and then assayed for the capacity to stimulate B. fragilis growth in the plate assay system. Some general properties of the stimulatory materials can be seen in Table 17 and effect of spent medium on B. fragilis stimulation after various treatments observed in Fig. 20. The stimulatory material was found to be quite stable to heat, acid or alkali. Treatment by boiling for 30 min, or exposure to pH 2.0 or pH 12.0 for 30 min, did
Fig. 19. Stimulation of *B. fragilis* growth by *E. coli* spent peptone and spent semi-synthetic medium. The surface of TSB was spread with approximately $10^6$ cells of *B. fragilis* and wells cut. The wells were filled with 0.25 ml of (A) hemin alone (control), (B) stored peptone medium, (C) spent peptone medium, (D) spent semi-synthetic medium, or (E) semi-synthetic medium.

**TABLE 17**

<table>
<thead>
<tr>
<th>Treatment of spent peptone</th>
<th>Stimulation of <em>B. fragilis</em> growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>Boiling (30 min)</td>
<td>+</td>
</tr>
<tr>
<td>Dialysis (against distilled H$_2$O)</td>
<td>-</td>
</tr>
<tr>
<td>Acid (pH 2.0, 1 h)</td>
<td>+</td>
</tr>
<tr>
<td>Alkali (pH 12.0, 1 h)</td>
<td>+</td>
</tr>
<tr>
<td>Cationic resin (Cl$^-$ charged)</td>
<td>-</td>
</tr>
<tr>
<td>Anionic resin (H$^+$ charged)</td>
<td>-</td>
</tr>
<tr>
<td>Ether extraction</td>
<td>+</td>
</tr>
</tbody>
</table>

*Stimulation determined on solid medium. Approximately $10^6$ cells of *B. fragilis* were spread on the surface of either TSA or enriched TSA. Wells were cut, and treated spent medium placed in the wells. Growth stimulation was defined as enhanced growth around the wells.
Fig. 20. Stimulation of *B. fragilis* growth by spent semi-synthetic medium subjected to various treatments. The surface of TSA was spread with approximately $10^6$ cells of *B. fragilis*. Wells were cut and filled with 0.25 ml of test fluids. Wells A and F contain hemin (control), well E contains semi-synthetic medium (concentrated 20-fold), and well G contains 20-fold concentrated spent semi-synthetic medium. The other wells contain spent semi-synthetic medium concentrated 20-fold and treated by (B) boiling for 30 min., (C) acid (pH 2.0), 1 h (D) alkali (pH 12.0), 1 h, (H) dialyzed extensively against distilled water, (I) exposure to cationic (Cl$^-$ charged) resin, or (J) exposure to anionic (H$^+$ charged) resin.
not destroy capacity to stimulate _B. fragilis_ growth. The molecular weight of the material appeared to be less than 10,000 since dialyzed spent medium no longer stimulated _B. fragilis_. Ether did not extract the stimulatory materials, although it was removed by both cationic and anionic exchange resins.
DISCUSSION

This investigation has provided some new and significant information as to the role and the nature of microbial interactions in the pathogenesis of intraperitoneal abscess formation. First, it was shown that the combination of *B. fragilis* and *E. coli* caused the formation of large intra-abdominal abscesses in rats, although abscess weight was not statistically different from that observed in infections induced by *E. coli* alone. Both *B. fragilis* and *E. coli* were independently able to cause intraperitoneal abscess formation.

Second, an interaction between *E. coli* and *B. fragilis* was demonstrated for the first time in vitro; the growth of *B. fragilis* was greatly enhanced by *E. coli* growth in certain peptone media and a semi-synthetic medium. In addition, the present study has clarified two important questions pertaining to the susceptibility of *B. fragilis* and *E. coli* to oxygen and low temperature.

I. Susceptibility of *B. fragilis* and *E. coli* to oxygen and low temperature.

*B. fragilis* is a mesophilic (optimal growth temperature: 37°C) and obligately anaerobic organism. Since it was often necessary to handle this organism in open air, or to store bacterial specimens for later processing, we initially investigated the effects of oxygen and other storage conditions on the survival of this organism. Although it has been relatively well established that clinically important anaerobic bacteria are tolerant to exposure to atmospheric
there is still controversy as to whether anaerobic bacteria can be safely exposed to low temperature (Finegold et al., 1974; Dowell and Hawkins, 1974; Holdeman and Moore, 1972). We believe that preliminary experiments investigating the effects of these parameters are essential for drawing valid conclusions from our experiments.

Unlike some of the strict anaerobes, _B. fragilis_ was found to be quite oxygen tolerant, although no growth took place under aerobic conditions (Fig. 2, 4, 5, 6). This high degree of resistance to oxygen in our strain of _B. fragilis_ does not seem to be unique to this particular isolate. The data published from several other laboratories also indicate that _B. fragilis_ is in general quite tolerant to oxygen (Bartlett et al., 1976; Loesche, 1969; Tally et al., 1975). The biochemical basis of this unique oxygen tolerance of the anaerobe, _B. fragilis_, has been recently elucidated. Tally et al. (1977) and others (Gregory et al., 1978) have demonstrated that low levels of superoxide dismutase, the enzyme that converts toxic superoxide radicals to a nontoxic form, can be found in _B. fragilis_. More detailed biochemical reactions involved in this detoxifying mechanism are found in reviews by Fridovich (1975) and Halliwell (1978). Most strict anaerobes do not contain this enzyme and are rapidly rendered nonviable by the action of superoxide radicals generated in the aerobic environment.

It is generally accepted practice in microbiology that cultures stored for later processing are kept at low temperatures, most commonly at 4°C. However, whether one may store cultures containing
strict anaerobes in a similar manner has been disputed for some time among clinical microbiologists. Some (Dowell and Hawkins, 1974; Holdeman and Moore, 1972) warned that specimens should not be exposed to chilling since anaerobes are highly susceptible to low temperatures. On the other hand, Finegold et al. (1974) recommended that these specimens should be refrigerated to slow down the growth of coexistent facultative bacteria if the specimens could not be cultured within 2 h.

Our data (Fig. 2-8) show that B. fragilis was rapidly killed at low temperature, both in vitro and in experimentally produced pus, under most experimental conditions tested. Other investigators (Gorrill and McNeil, 1960; Hegarty and Weeks, 1940; Strange and Dark, 1962) have also demonstrated the lethal effect of low temperature on gram negative organisms. Strange and Dark (1962) demonstrated that chilling was lethal to Enterobacter aerogenes to varying degrees, depending on the phase of growth, the diluent, and the concentration of bacteria. In 1972, Leder demonstrated that glycerol would protect against lethality from cold shock by increasing osmotic pressure. In agreement with this finding, we have shown that increasing osmotic pressure by the addition of CaCl₂ and sucrose (Table 2) could greatly reduce the lethality normally observed after exposure to low temperature. A leakage of endogenous constituents (Strange and Dark, 1962) due to a precipitation (Ring, 1972) or crystallization (Leder, 1972) of certain membrane lipids was thought to be responsible for the lethality to microorganisms. Whether a similar mechanism may be responsible for the killing of B. fragilis remains to be determined.
On the basis of the data obtained in our preliminary experiments, (a) all cultures were processed immediately after collection, or freshly grown cultures were routinely used so that no prolonged storage periods were required, and (b) experiments involving B. fragilis were performed on the bench under aerobic conditions except that all anaerobes were incubated under anaerobic conditions.

II. In vivo and in vitro interaction of B. fragilis and E. coli.

Because the animal model devised in this study not only enabled us to accurately control the type and number of bacteria to be implanted into the peritoneal cavity, but also allowed the implanted organisms to remain at the site of inoculation, we were able to evaluate the abscess forming ability of B. fragilis and E. coli more precisely. In addition, the foreign material in which the microorganisms were impregnated roughly simulated fecal material, both in consistency and in the fact that it contained a ground meat mixed with cellulose. Since our model employed a defined artificial fecal material free from unidentified enteric bacteria and their products, we believe that it was better suited for this type of study than any other models previously reported.

Several investigators (Meleney, 1932; Hite et al., 1949; Lorber and Swenson, 1975; Stone et al., 1975; DeHaan et al., 1974; Finegold and Rosenblatt, 1973) have suggested that a "synergistic" mechanism between aerobic and anaerobic microorganisms might be involved in the development of anaerobic infections. However, in most instances, evidence presented in support of their conclusion is far from con-
vincing. Furthermore, the term "microbial synergy" has been used indiscriminantly, and often incorrectly. Synergism, in the strict sense, refers to a bilateral relationship in which both populations benefit from each other (Frederickson, 1977). In most cases, (Lorber and Swenson, 1975; Stone et al., 1975; DeHaan et al., 1974; Finegold and Rosenblatt, 1973), the involvement of microbial synergy in the formation of anaerobic abscess was suggested only on the basis of the fact that high percentages of specimens collected from anaerobic infections contain a mixed flora of aerobic and anaerobic bacteria. Considering the complex nature of microbial flora contaminating wounds or the abdominal cavity, it is not too surprising to find a variety of microorganisms, both aerobic and anaerobic, in the infected lesions. There has been no convincing evidence presented in prior studies indicating that both aerobic and anaerobic bacteria synergistically participate in the development of such infections.

There have been, however, a few investigations that have provided some experimental evidence to support this theory of synergism. Altemeier (1942) performed some rather preliminary studies on the pathogenicity of mixed cultures in guinea pigs. He reported that a mixed flora of facultative aerobes and anaerobic bacteria was isolated from 96/100 patients with perforated appendix. Subcutaneous implantation of pure cultures of the organisms into guinea pigs failed to produce gangrenous lesions. However, when certain combinations of these organisms were implanted, animals developed extensive cellulitis accompanied by gangrene. Somewhat similar findings were reported by other investigators (Meleney, 1932; Hite et al., 1949). More recently,
an apparently true synergism between *E. coli* and *B. fragilis* in the formation of intraperitoneal abscess in rats was reported by Onderdonk et al. (1975). In their study, no animals implanted with *E. coli*, enterococcus, *B. fragilis* or *F. varium* alone developed intra-abdominal abscesses. Likewise, the combination of either anaerobes alone (*B. fragilis* and *F. varium*), or aerobes alone (*E. coli* and enterococcus), did not cause abscesses. However, 89-100% of animals receiving combinations of one aerobe and one anaerobe developed abscesses by 7 days. The specific combination of *E. coli* (1.3 x 10^7 cells/ml) and *B. fragilis* (2.5 x 10^7 cells/ml) produced intra-abdominal abscesses in 100% of the animals.

Our results (Tables 4, 5, Fig. 10) agree with the conclusion of Onderdonk et al. (1975) in that the combination of *B. fragilis* and *E. coli* resulted in the formation of large intra-abdominal abscesses. However, in contrast to their findings, we have found both *E. coli* and *B. fragilis* to be independently capable of causing intraperitoneal abscesses to form in rats (Table 4, 5, Fig. 10). Our results are consistent with the observations of others that *B. fragilis* alone can cause intra-abdominal abscesses in experimental animals (Hill et al., 1974; McConville et al., 1976; Renz et al., 1975; Walker and Wilkins, 1976). In our animal model, it was possible to produce intra-abdominal abscesses with *E. coli* alone (Table 4, 6, Fig. 10). To our knowledge this is the first study demonstrating intraperitoneal abscess formation by a gram negative facultative rod. Our finding is difficult to reconcile with the data reported by Onderdonk et al. (1975), who showed that presence of both *E. coli* and *B. fragilis* was necessary for
the abscess formation. However, Onderdonk et al. (1975) and others (Nichols et al., 1978), have used sterile cecal contents (which contain a multitude of bacterial endproducts, lysed bacteria, and bacterial components), as well as barium sulfate, to help initiate and potentiate abscess formation. The problems associated with this use of a complex inoculum and unnatural additives was avoided by our use of an artificial fecal material. In addition, Onderdonk et al. (1975) used rat strains of *B. fragilis* and *E. coli*, whereas organisms used in this investigation were human isolates from actual intraperitoneal abscesses. The strain of rats used in each study also differed.

The elucidation of types of microorganisms actually involved in pathogenesis is a very significant consideration when determining appropriate antibiotic therapy for these infections. Currently, there are two major approaches for the treatment of anaerobic abscesses. First, if truly synergistic interaction is involved in abscess formation as shown by Onderdonk et al. (1975), treatment of either component alone should prevent abscess formation. In fact, some investigators (Polk, 1977; Stone et al., 1975) suggested that if only the aerobic component were treated, anaerobic infections could be prevented. Polk (1977) observed that failure to treat mixed aerobic-anaerobic infections with cephaloridine resulted in a fourfold rise in infection rate. Stone et al. (1975) performed a study on the effectiveness of clindamycin or cephaloridine on patients with infections resulting from abdominal disease, surgery or trauma. Use of cephalothin, which is relatively ineffective against *B. fragilis* resulted in an infection rate of 3%. These data suggest that treat-
ment of the aerobic component of the mixed flora will be sufficient to reduce rate of infection. In contrast to this, several investigators (Busch et al., 1976; Griffith et al., 1977; Klastersky and Husson, 1977; Weinstein et al., 1974) have emphasized the need for treatment of both aerobic and anaerobic flora or a mixed infection. Using a model of abdominal sepsis developed earlier, Weinstein et al. (1974) studied the efficacy of gentamicin alone, clindamycin alone, or the combination of both antibiotics in the treatment of anaerobic infections. He demonstrated that gentamicin, effective against the aerobic flora, greatly reduced peritonitis, but not the rate of abscess formation, whereas clindamycin, affecting *B. fragilis*, greatly reduced the rate of abscess formation, but not peritonitis. Both antibiotics were necessary for optimal therapy of a mixed infection. Fass et al. (1978) demonstrated the efficacy and safety of this combination in treatment of clinical anaerobic infections. Other investigators (Busch et al., 1976; Griffith et al., 1977; Klastersky and Husson, 1977) studied various combinations of therapy for the treatment of anaerobic infections and suggested that both aerobic and anaerobic organisms should be treated since too little is known about the pathogenesis of such mixed infections to do otherwise. The results of our animal studies demonstrate experimentally that both aerobic and anaerobic flora should be treated in a mixed infection. Since both *B. fragilis* and *E. coli* were individually able to cause intraperitoneal abscesses, it would be necessary to treat both organisms to completely prevent abscesses from forming.

Bowel spillage, resulting from disease, trauma, or abdominal
surgery, often results in intra-abdominal abscess formation. However, depending on the conditions of spillage, infection may not occur, a peritonitis may develop, only an abscess might form, or a peritonitis followed by abscess might be seen. Until now, there has been little experimental data to explain the factors which determine the outcome of spillage of intestinal contents. Using our animal model, the effect of various factors on such infections was examined in an attempt to more clearly understand the actual role of *B. fragilis* and *E. coli* in pathogenesis of intra-abdominal sepsis.

It is possible that no infection will result from spillage of colonic contents into the peritoneal cavity. If the material is quite fluid, and number of organisms small, the contaminating material will rapidly diffuse throughout the peritoneal cavity and be effectively phagocytized by the body's defense mechanisms. We have shown that if microorganisms are not held within a localized area by the some type of solid material or semi-solid material, abscess formation does not occur (Fig. 9). In no case did direct inoculation of *B. fragilis* alone, or the combination of *B. fragilis* and *E. coli*, into the peritoneal cavity result in intraperitoneal abscesses in rats. *E. coli* was rapidly phagocytized and could not be isolated from the abdominal cavity by 3 days, whereas *B. fragilis* could still be isolated on day 15 from the abdomen (data not shown). Although *B. fragilis* has been shown to be readily engulfed and killed by rabbit polymorphonuclear leukocytes in vitro (Casciato et al., 1975; Bjornson and Hill, 1973), the chemotactic activity of *B. fragilis* endotoxin for phagocytic cells appeared to be greatly reduced when compared to classical gram nega-
tive endotoxins (Sveen, 1977). *B. fragilis* lipopolysaccharide did generate a leukocyte chemotactic factor (Sveen, 1978) even though phagocytic cells did not appear to be effectively mobilized in the *B. fragilis* infection.

Recently, a polysaccharide capsule has been discovered surrounding *B. fragilis* subsp. *fragilis* (Kasper and Seiler, 1975) and has been implicated in the pathogenesis of this organism. Kasper et al. (1977) demonstrated that all strains of subsp. *fragilis* possessed this polysaccharide, whereas other subspecies of *B. fragilis* did not have the capsule. Rowley (1971) has suggested that the thickness of a bacterial capsule is a major factor in determining virulence. Onderdonk et al. (1977) showed that encapsulated strains of *B. fragilis* usually caused intraperitoneal abscess in rats whereas unencapsulated strains usually did not induce abscess. In their model, capsular material alone actually induced abscess formation. It was also shown (Onderdonk et al., 1978) that encapsulated strains of *B. fragilis* adhered to rat peritoneal epithelium better than unencapsulated strains, suggesting a role in virulence of the organism. Contrary to this finding that only subspecies *fragilis* possesses a capsule, Babb and Cummins (1978) has shown that three of the other four subspecies, *vulgatus*, *thetaiotaomicron* and *ovatus*, also possess capsular material. Consequently, the role of this capsule in the pathogenesis of *B. fragilis* still remains unclear.

If the number of certain bacteria, especially *E. coli*, spilled during trauma is high, the risk of severe peritonitis exists. The problem arises from the fact that *E. coli* contains a classical endo-
toxin and is normally found in levels of $10^8$ to $10^{10}$ bacteria per g of colonic contents (Attebery et al., 1971). In fact, only *B. fragilis* outnumbers *E. coli* as normal flora of the human colon. Peritonitis is usually induced by the classical endotoxin of facultative gram negative bacteria. It is known to elicit fever, the Shwartzman reaction, disseminated intravascular coagulation (DIC), and vascular collapse in humans. This does not appear to be the case for *B. fragilis*. This may be because the lipopolysaccharide structure of *B. fragilis* is atypical when compared to that of facultative forms in that heptose and 3-keto-2-deoxyoctonate are absent (Hofstad and Kristofferson, 1970). Possibly due to this difference, DIC has not been observed in most clinical *B. fragilis* infections (Yoshikawa et al., 1974). In our animal model of abscess infection, peritonitis has been notably absent when as many as $10^{10}$ *B. fragilis* cells were placed into the peritoneal cavity of rats. In contrast to this, $10^8$ cells of *E. coli*, with its classical endotoxin, resulted in a 50% lethality in rats. Various in vivo and in vitro assays, such as mouse lethality, chick embryo lethality, rabbit biphasic fever response, Shwartzman reaction and reactivity in the Limulus assay have all been used to demonstrate the low toxicity of *B. fragilis* endotoxin (Kasper, 1976; Sveen and Milner, 1977). No matter how the endotoxin was extracted (the phenol-water, trichloroacetic ethylenediamine tetraacetic acid, or liquid phenol-chloroform petroleum ether method), endotoxic activity as measured by skin inflammation in rabbits, was low (Hofstad et al., 1977). This lack of toxicity may explain, in part, the inability of *B. fragilis* to induce the peritonitis that is
so commonly associated with infections caused by other gram negative rods.

Whether or not peritonitis results from spillage, abscess formation will most likely occur if organisms have been localized by solid or semi-solid components of intestinal material. In our animal model, the addition of artificial fecal material or blood clots to the bacterial inoculum resulted in abscess formation (Table 4, 5, 6, 7, Fig. 10, 12). It appears that these materials prevent rapid dissemination of bacteria through the peritoneal cavity, while allowing inoculated bacteria to grow in situ. Other investigators have also demonstrated the necessity or potentiating effect of various substances on ability of the organisms to cause abscesses. These substances included feces, (Deysine et al., 1967) cecal contents and barium sulfate, (Onderdonk et al., 1975), mucin (Hill et al., 1974) or semi-solid agar (Walker and Wilkins, 1976).

The body responds to such a localized contamination by surrounding and walling off the foreign material with sticky omentum. One such factor which might contribute to the walling off process is the enzyme heparinase. In a report in 1961, Gesner and Jenkin described a heparinase produced by certain strains of Bacteroides. Many reports (Bodner et al., 1970; Felner and Dowell, 1971; LeFrock et al., 1976; Nastro and Finegold, 1972) have described thrombophlebitis associated with human B. fragilis infections. A heparinase has been suggested as responsible for this occurrence. Such an enzyme would interfere with normal homeostatic mechanisms involved in preventing clot formation. It would tend to allow clots to form, resulting in a
dissemination of disease and possibly phlebitis. However, to this point, production of a heparinase by B. fragilis in vivo has never been demonstrated. We have found that when B. fragilis was implanted into the peritoneal cavity of rats, microthrombi formed by 24 h around the area of implantation (data not shown). Control animals implanted with E. coli did not form microthrombi. These preliminary data suggest that heparinase may indeed be formed. Carrizosa et al. (1977) has demonstrated that in a rabbit model of experimental endocarditis produced by a nonheparinase producing B. fragilis subsp. vulgatus, phlebitis did not occur. Although vegetations on the heart appeared fragile and likely to disseminate, emboli were not found.

The severity of the abscess, and abscess size, are undoubtedly determined by a variety of factors such as inoculum size, inherent pathogenic potential of each organism and enhanced pathogenicity due to microbial interactions. Surprisingly, size of abscess formed was not necessarily due to number of bacteria present in the contaminating material. Infection with B. fragilis alone in our animal model uniformly resulted in small abscesses, whether the inoculum size was \(10^6\) organisms or 10,000 time larger (Table 5). Likewise, the size of abscess caused by E. coli remained larger whether the inoculum size was \(10^5\) or \(10^7\) cells, at which point animals began to die from peritonitis. A most important observation was that extremely large intraperitoneal abscesses were formed even when as low as 10 cells of B. fragilis and 10 cells of E. coli were impregnated into artificial fecal material and implanted into the peritoneal cavities of rats (Table 7). This would, in part, explain the relative ineffectiveness
of prophylactic antibiotics in preventing the development of abscess infections following colonic surgery. It has been shown (Clark et al., 1977) that preoperative antibiotics only reduce the level of intestinal bacteria to $10^3$ organisms/g of colonic contents. Undoubtedly, reducing the number of bacteria in spillage material will reduce chances of infection, especially in terms of peritonitis. However, the number of bacteria remaining are more than sufficient to initiate abscess formation, especially if both aerobic and anaerobic flora are allowed to interact.

We have described above some of the factors which probably determine the outcome of spillage of colonic contents containing \textit{B. fragilis} and \textit{E. coli}. However, the actual mechanisms of interaction between aerobic and anaerobic bacteria in abscesses and pathogenic factors elaborated there, are not known. Since it was not clear from our data (Table 4, Fig. 10) whether a synergistic interaction actually occurred between \textit{B. fragilis} and \textit{E. coli} during abscess formation, possible mechanisms by which these two organisms might interact were investigated in vitro. One such mechanism by which \textit{B. fragilis} and \textit{E. coli} might interact is the production by one or both organisms of substances that are stimulatory to the growth of the other. To test this possibility, the in vitro growth of these organisms in mixed culture was examined.

The data obtained from in vitro experiments clearly indicates that a unilateral stimulation of \textit{B. fragilis} is occurring. When \textit{B. fragilis} was inoculated alone in certain media, it would not grow (Fig. 13, 14, 17). However, when cultured together, \textit{E. coli} was found
to stimulate the growth of *B. fragilis* (Fig. 13, 14, 17). In fact, the *E. coli* inoculum required for stimulation of *B. fragilis* could be reduced to as low as $10^1$ cells/ml (Fig. 16). As discussed above, this finding is significant in that even low numbers of *E. coli* might be sufficient to cause mixed anaerobic infection.

The stimulation of *B. fragilis* growth by *E. coli* could be demonstrated in two carefully selected growth media; stored Bacto-peptone (Fig. 13, 14, 16) and semi-synthetic (Fig. 17) medium. In both media, initial growth of *E. coli* appears to be essential for subsequent growth of *B. fragilis*.

Data obtained from the in vitro study appears to indicate that at least three different mechanisms are involved in the stimulation of *B. fragilis* growth by *E. coli*. 1. Dissolved oxygen might be removed from the media by growth of *E. coli*. This reduction in oxygen level is necessary before growth of the anaerobe, *B. fragilis*, can be initiated. 2. Substances that are toxic or inhibitory to *B. fragilis* might be present in the media. If *E. coli* could metabolize or alter these substances to a nontoxic form, *B. fragilis* might be able to grow. 3. *B. fragilis* might require some unknown factor(s) for its growth. It is possible that on growth of *E. coli*, these stimulatory factors are derived from breakdown products of media components, or are formed by *E. coli* de novo. Each of the above possibilities will be discussed below.

When $10^5$ cells of *E. coli* were inoculated in Bacto-peptone media and incubated aerobically at $37^\circ$C, the oxygen level was reduced from 9.1 to a minimum of 1.03 μmoles/l within a 2 h time period (Fig. 13).
During this time, *B. fragilis* continually lost viability. We have shown that if *E. coli* was selectively inhibited by an antibiotic during the first few hours of growth (Fig. 16), *B. fragilis* growth was not initiated. This indicates that high levels of dissolved oxygen might indeed be partially responsible for the inability of *B. fragilis* to grow in such a medium. This view is further supported by the facts that stored boiled peptone partially allowed the growth of *B. fragilis* (Table 10) and that the introduction of oxygen to peptone by vortexing rendered such a medium unsuitable for *B. fragilis* growth (Table 10). It is quite probable that the rapid scavenging of oxygen by *E. coli* would modify physiologic conditions of infected sites, creating an environment more conducive to the growth of *B. fragilis*. However, the elimination of oxygen is not alone sufficient to account for the observed stimulation of *B. fragilis* growth in vitro, since (a) reduction in oxygen level in stored peptone by anaerobic incubation (Table 10) did not allow *B. fragilis* growth or (b) in spite of the relatively high level of dissolved oxygen in other stored peptones (Table 8), *B. fragilis* was able to grow under certain conditions. In addition, other facultative microorganisms that also rapidly reduce oxygen level varied in their ability to enhance *B. fragilis* growth (Table 11).

Substances toxic to *B. fragilis* might be present in the stored peptone. When the medium was adsorbed with activated charcoal to nonspecifically remove certain substances, and then inoculated with *B. fragilis*, slight growth occurred (Table 13). Carlsson et al. (1978) recently demonstrated that a variety of anaerobic broth media,
including Brain Heart infusion (BBL), Trypticase soy broth (BBL), and thioglycollate medium (Difco), accumulated hydrogen peroxide and superoxide radicals on storage under aerobic conditions. Since *B. fragilis* contains little or no catalase (Flen and Rosenblatt, 1976) or superoxide dismutase (Tally et al., 1977), the accumulation of such toxic substances is certainly detrimental to the survival and growth of *B. fragilis*. However, the data presented in Table 12, which demonstrated that superoxide dismutase had little effect, render this possibility less likely, although it is still possible that toxic substances other than superoxide radicals are metabolized or destroyed by co-existent *E. coli*.

Growth of *B. fragilis* might be stimulated by substances released by *E. coli*. If the stimulatory substances were cellular components of the *E. coli*, addition of high concentrations of lysed cells should enhance growth of *B. fragilis*. Washed *E. coli* cells, as concentrated as $10^8$ cells/ml, were lysed by various treatments. Addition of these lysates, to *B. fragilis* cultures under anaerobic conditions did not stimulate its growth (data not shown). This indicates that enhancement of *B. fragilis* growth was not due to structural components of *E. coli*.

Materials, stimulatory to *B. fragilis* growth, might be actively produced and excreted by *E. coli* into the medium. We showed (Fig. 15, 17) that if *E. coli* in a mixed culture was selectively inhibited at early stages (less than 2 h), growth of *B. fragilis* would not occur. It appears that the accumulation of a threshold level of stimulatory material is necessary to trigger *B. fragilis* growth, and that this
level is achieved before 2 h of growth (Fig. 16).

It is significant to note that the initial presence of only small numbers of *E. coli* (Fig. 17) is sufficient to produce this substance, and that such substances are produced only by certain other enterobacteriaceae, but not by *Staphylococcus aureus* (Table 11). Vitamins or nutrients produced by one organism have been shown to stimulate another microorganism in various mixed culture interactions. In the mutualistic interaction between *Proteus vulgaris* and *Bacillus polymyxa*, biotin produced by *P. vulgaris* stimulated *B. polymyxa* and niacin produced by *B. polymyxa* stimulated *P. vulgaris* (Yeoh et al., 1968). Another study demonstrated *S. cerevisiae* stimulation of a riboflavin requiring *Lactobacillus casei* (Megee et al., 1972).

*B. melaninogenicus* was shown (MacDonald et al., 1963) to be essential for the pathogenicity of mixed cultures of organisms isolated from periodontal disease. However, another microorganism capable of supplying it with required vitamin K was also necessary. The microenvironment where *B. fragilis* is normally found (either in the intestine or other infected sites) or the organisms with which they are associated, may be capable of providing this organism with essential growth stimulatory compounds. Vitamins, such as vitamin K, breakdown products of blood, such as hemin, (Varel and Bryant, 1974; Sperry et al., 1977), amino acids (methionine may substitute for the cyanocobalamin requirement, Bryant, 1974), and peptides and ammonia (Pittman and Bryant, 1964) are known growth stimulatory compounds. If *E. coli* could provide these substances, *B. fragilis* growth might be initiated. However, the data obtained from the present study seem to
indicate that stimulatory compounds produced by *E. coli* are not hemin, vitamin K, ammonia, or amino acids, since no stimulation of *B. fragilis* in stored peptone medium has been observed by the addition of these substances (Table 14).

Iron is known to be a virulence factor for most microorganisms (Calver et al., 1976; Wheeler and Hanks, 1965; Weinberg, 1974; Weinberg, 1978). Hill demonstrated that iron-containing compounds such as blood and hemoglobin enhanced the infection caused by mixed cultures of *B. fragilis* and *Peptostreptococcus anaerobius*, and that the infection enhancing effect could be duplicated by ferric ammonium citrate. Our data (Fig. 12) show that a plasma clot impregnated with *B. fragilis* or *E. coli* and implanted into the peritoneal cavity of rats will not induce abscess formation. However, a blood clot containing the same organisms encapsulated and begins to form an abscess by 2 days. In contrast to this finding, Bornside and Cohn (1968) could find no enhancement of virulence when hemoglobin as added to inocula of non-sporing anaerobic bacteria.

The human body normally prevents bacterial use of iron by the production of iron-binding compounds such as lactoferrin and transferrin (Bornside and Cohn, 1968; Weinstein, 1978). In spite of this host mechanism to deny iron to bacterial invaders, some bacteria, such as *Salmonella typhimurium* (Luckey et al., 1972) and certain strains of *E. coli* (Payne and Finkelstein, 1977) have the capacity to retrieve iron from the iron-lactoferrin complexes by the use of siderophores (Wilkins and Lankford, 1970). In fact, the siderophores produced by *E. coli* were able to assist other microorganisms in
acquiring needed iron. *E. coli* might be performing this function when in mixed infections with *B. fragilis*, although this possibility was not investigated.

Another mechanism by which *E. coli* might help prepare the area for the growth of *B. fragilis* is by its production of SOD. As discussed earlier, this is one of the means by which facultative organisms protect themselves from byproducts of oxygen metabolism, such as superoxide radicals. *B. fragilis* is normally killed under aerobic conditions. If *E. coli* destroys these toxic radicals as they are formed, *B. fragilis* might be protected from their lethal effects and be allowed to grow. Once initiated, the growth of *B. fragilis*, as well as *E. coli* growth, would act to keep the area in a reduced state.

We have demonstrated that the stimulatory substances accumulated in spent media are dialyzable (data not shown) and quite stable (Table 17). By means of an agar diffusion method, it was further demonstrated that these compounds are able to withstand heat (121°C for 15 min) and treatment to acid and alkaline conditions (Table 17). The stimulatory material produced by *E. coli* was not ether extractable, (Table 17, Fig. 20), and therefore, probably not lipid in composition. It was removed by both anionic and cationic exchange resins (Table 17, Fig. 20), implying that the material contains both positive and negative charges. Further studies must be performed before any conclusions concerning the exact nature of this (these) stimulatory material(s) can be made. The spent semi-synthetic medium should be treated with enzymes that selectively destroy certain bacterial components, such as proteins, sugars, or nucleic acids to further identify
the general composition of the material. Extraction procedures could then be used to concentrate and purify the stimulatory compounds. In addition, fractionation procedures would then be used to separate actual components participating in the stimulation phenomenon. Various electrophoretic and chromatographic techniques would then be employed to assist in the identification of these materials.

From our data (Table 17), it is unlikely that the stimulatory compounds are proteinaceous in nature, although certain extracellular enzyme proteins such as SOD or iron chelating proteins elaborated by one organism may stimulate the growth of bacteria deficient in such proteins (Luckey et al., 1972; Payne and Finkelstein, 1978). More extensive discussions on these aspects of microbial interactions in the pathogenesis of infectious agents are found elsewhere in this section.

The outcome of microbial entry into the peritoneal cavity will be determined by type and dose of each organism spilled. When the number of microorganisms is small, they may be rapidly killed by the host defense mechanisms and no serious infections occur. When a large number of bacteria, especially E. coli or other facultative anaerobes are introduced, severe peritonitis usually develops. B. fragilis, even in high numbers, does not cause severe peritonitis, for still undetermined reasons. When, however, B. fragilis and E. coli are introduced into the peritoneal cavity together with solid or semisolid intestinal contents, the organisms may survive and multiply in the localized area which is surrounded by the omentum. It appears that the initial growth of E. coli may eventually render the area
suitable to the growth of *B. fragilis* by (a) removing available oxygen, thereby lowering the overall oxidation reduction potential, (b) metabolizing or altering materials, such as superoxide radicals, that are toxic to the anaerobes, and/or (c) providing *B. fragilis* with substances required to, or stimulatory for, the growth of the anaerobe.

Once *B. fragilis* begins to grow, it may excrete various extracellular enzymes. Heparinase, presumably produced by *B. fragilis*, could assist in this process by causing clot formation and resultant occlusion of capillaries. The infection thus becomes a rather closed system, further protecting *B. fragilis* from dissolved oxygen in blood. Enzymes produced by *B. fragilis*, during its growth, such as hyaluronidase, collagenase, protease, or lipase, could conceivably then destroy host tissue and extend the infection. During this process, bacterial cell components and tissue damage could elicit the inflammatory reaction, attract numerous phagocytic cells, and eventually result in intra-abdominal abscess formation.
The interactions between *Bacteroides fragilis* and *Escherichia coli* were investigated in vivo and in vitro. It was shown that both *B. fragilis* and *E. coli* could independently produce intra-abdominal abscesses in rats, provided that bacteria were implanted together with simulated fecal material. In this study, no strong evidence was obtained which indicates that a synergistic interaction between obligate and facultative anaerobes plays an important role in intra-abdominal abscess formation. The formation of intraperitoneal abscesses was found to be influenced by several factors, such as the nature of materials in which organisms were impregnated, type of bacteria used, and the inherent pathogenicity of each organism. The presence of solid or semi-solid material was found to be necessary for the induction of intra-abdominal abscesses. If properly inoculated, even low numbers of bacteria were able to produce abscesses. The number of bacteria inoculated did not affect the ultimate weight of the abscess. However, if too high a number of *E. coli* was inoculated (> $10^8$ microorganisms) death from peritonitis invariably resulted. In contrast to this, no animals died from peritonitis even when very large doses of *B. fragilis* were used ($10^{10}$ microorganisms).

When *B. fragilis* and *E. coli* were incubated together in media that would not allow the growth of *B. fragilis*, growth of the anaerobe was stimulated. Under our experimental conditions, approximately 2 h of *E. coli* growth was required before an increase in viable number of
B. fragilis was observed. Other enteric organisms, such as Proteus vulgaris and Klebsiella pneumoniae, were also found to enhance the growth of B. fragilis, although Staphylococcus aureus would not. At least three mechanisms appear to be responsible for the stimulation of B. fragilis growth by E. coli: 1. Dissolved oxygen is removed from the media by E. coli, lowering the overall oxidation-reduction potential. 2. Substances that are toxic to B. fragilis are detoxified by E. coli. 3. E. coli provides some as yet unidentified compound(s) required for, or stimulatory to, the growth of B. fragilis. The stimulatory compound(s) accumulated in media by the growth of E. coli was dialyzable and quite stable to heat (121°C for 15 min) or mild acid and alkali treatments. It was not extractable by ether, but was removed by both anionic and cationic resins. Based on the data obtained in this investigation and those reported in the literature, a possible mechanism of anaerobic intra-abdominal abscess formation was proposed.
LITERATURE CITED


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The dissertation submitted by James C. Hagen has been read and approved by the following committee:

Tadayo Hashimoto, M.D., Ph.D., Director
Professor, Microbiology, Loyola

H. J. Blumenthal, Ph.D.
Professor and Chairman, Microbiology, Loyola

William W. Yotis, Ph.D.
Professor, Microbiology, Loyola

Walter S. Wood, M.D.
Professor and Chairman, Community and Family Medicine, Loyola

James P. Filkins, Ph.D.
Professor and Chairman, Physiology, Chairman

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

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology.

December 6, 1978
Tadayo Hashimoto, M.D., Ph.D.
Director