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ISOLATION AND IDENTIFICATION OF ANAEROBIC BACTERIA

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THE ISOLATION AND IDENTIFICATION OF ANAEROBIC BACTERIA FROM VETERINARY CLINICAL SPECIMENS

By

Mary Fraser Sit

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ABSTRACT

THE ISOLATION AND IDENTIFICATION OF ANAEROBIC BACTERIA FROM VETERINARY CLINICAL SPECIMENS

By

Mary Fraser Sit

Little is known about the role of anaerobic bacteria in diseases of domestic animals at the present time. Therefore, the present investigation was undertaken to isolate and identify anaerobic bacteria in veterinary clinical specimens. Specimens were collected aseptically in anaerobic transport tubes and processed using Hungate anaerobic techniques (65) and an anaerobic glove chamber. Most isolates were identified to the species level according to the procedures described in the VPI Anaerobe Laboratory Manual (63).

A total of seventy-one clinical cases, primarily from dogs, cattle, horses, sheep, and swine, were examined for anaerobic organisms. Of these, forty-five cases (64%) were positive for one or more anaerobic species. Seventy-five strains of anaerobic bacteria were isolated, of which 50.7% were C. perfringens; 17.3% other Clostidium sp.; 9.3% Gram-negative, non-sporulating rods; 9.3% Gram-positive anaerobic cocci; 6.7% Gram-positive, non-sporulating rods; and 6.7% Actinomyces sp. While some of these organisms have previously been associated with various disease conditions in animals, many are being reported here for the first time.

The clostridial species isolated in this study included: C. perfringens, C. sphenoides, C. sordellii, C. carnis, C. barati, C. butyricum, C. glycolicum, C. tertium, C. botulinum non-proteolytic BEF, C. bifermentans, C. ramosum and C. acetobutylicum. The clinical conditions from which these organisms were isolated included: eye, ear and skin infections, allergic sinusitis, a thoracic effusion, mastitis, enterotoxemias malignant edema, diarrhea, and infertility.

The non-sporulating anaerobes isolated included: Bacteroides sp., B. clostridiiformis subspecies clostridiiformis, Fusobacterium varium, F. necrogenes, F. necrophorum, Eubacterium sp., E. cylindroides, Propionibacterium sp., Actinomyces sp., A. bovis and Lactobacillus sp. These organisms were isolated from such disease conditions as: eye, skin and joint infections, fistulous wounds, mastitis, allergic sinusitis, lymphadenitis and an abscess.

The results showed conclusively that various types of sporulating and non-sporulating anaerobes occur much more commonly in a wide variety of animal infections than has previously been reported.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LEGEND OF SYMBOLS FOR TABLES, FIGURES

+ = positive reaction

- = negative reaction

A = strong acid, pH 5.5 or below

a = <1 meq/100 ml acetic acid; A = $\geq$ 1 meq/100 ml acetic acid

b = <1 meq/100 ml butyric acid; B = $\geq$ 1 meq/100 ml butyric acid

B = growth enhanced by bile

c = curd (milk)

C = chloroform

d = digestion (milk, meat)

E = ether

f = <1 meq/100 ml formic acid; F = $\geq$ 1 meq/100 ml formic acid

g = gas (milk)

H = growth enhanced by heme

ib = <1 meq/100 ml isobutyric acid

ic = <1 meq/100 ml isocaproic acid

iv = <1 meq/100 ml isovaleric acid

l = <1 meq/100 ml lactic acid; L = $\geq$ 1 meq/100 ml lactic acid

p = <1 meq/100 ml propionic acid; P = $\geq$ 1 meq/100 ml propionic acid

P = propionate produced from (lactate, threonine)

py = <1 meq/100 ml pyruvic acid

s = <1 meq/100 ml succinic acid; S = $\geq$ 1 meq/100 ml succinic acid

S = stimulated (Tween-80)

W = weak acid, pH 5.6 to 6.0

2 = ethanol

3 = propanol

5 = isopentanol

α = alpha hemolysis

β = beta hemolysis; $\partial\beta$ = double zone hemolysis

γ = no hemolysis

I. INTRODUCTION

Anaerobic bacteria constitute a predominant portion of the normal flora in the human colon, vaginal tract, oral cavity, and on the skin (31, 46, 47, 48, 50, 76, 77, 87, 88). These organisms are being isolated with greater frequency from many different clinical conditions in humans (12, 35, 43, 44, 45, 71, 111) due in part to improved techniques for the isolation and identification of anaerobes and in part to the growing awareness of clinicians as to the significance of anaerobes in various clinical conditions. With the use of stringent anaerobic culture methods, 70% to 95% of human thoracic, intra-abdominal, obstetrical-gynecological and other deep tissue infections have been found to contain anaerobes in pure or mixed culture (35). Anaerobes are known to occur in deep tissue infections of man as frequently or more so than facultatively anaerobic bacteria (35, 111).

In contrast, little information is available concerning the occurrence of anaerobes in the normal flora or their role as causative agents of disease in animals. This is undoubtedly because, until recently, strict anaerobic techniques have not been employed in veterinary diagnostic laboratories. Clostridial diseases such as tetanus, botulism and gas gangrene have long been recognized in animals. Infections by this group of organisms are readily

identified by their distinctive symptoms and their relative aerotolerance in comparison to most other anaerobes. However, very little is known about the role of anaerobic, non-sporeforming bacteria in disease conditions in animals, except for certain actinomyces, fusobacteria and bacteroides which cause animal diseases of great significance to the livestock industry. Reports by Biberstein (10) and a recent report by Berkhoff and Redenbarger (9) indicate that a wide variety of non-sporing anaerobes may play an important role in animal infections also.

The present study was, therefore, undertaken to isolate and identify anaerobic bacteria present in selected clinical specimens from a number of animal species over a one-year period. Specimens were selected for the likelihood of yielding anaerobic bacteria or for having failed to yield microbial growth by routine bacteriological methods (20). Samples were collected aseptically and anaerobically and were processed using strict anaerobic techniques. The results of this investigation constitute this thesis.

II. LITERATURE REVIEW

A. Anaerobiosis

It has been recognized that anaerobes are very sensitive to oxygen and that even a brief exposure to oxygen is fatal to many anaerobic species. The mechanisms of oxygen toxicity for anaerobes are not clearly understood, however various explanations have been advanced. Some of the more prominent of these theories are discussed below.

1. Molecular Oxygen

A number of studies have been conducted demonstrating the toxic effects of even limited exposure to oxygen on the recovery of anaerobes from specimens (1, 2, 114). From the initial selection of specimen through isolation and identification procedures, preventing exposure to oxygen results in a consistently higher rate of recovery of anaerobes. Aranki, et al. (2) found that the brief exposure of specimen and media to air during plating, despite subsequent anaerobic incubation, reduced the extent of recovery of anaerobes 3 to 4 times compared to that obtained when all manipulations were carried out under continuous anaerobic conditions.

Loesche (72) systematically studied the oxygen sensitivities of selected anaerobic organisms. Agar plates streaked within an

anaerobic glove chamber, were placed in anaerobic jars connected via tubing to a vacuum line outside the chamber. The jars were evacuated and filled with a mixture of oxygen-free gases and oxygen varying in concentration from 0 to 12%. Based on his results, Loesche defined two categories of anaerobes: strict anaerobes which were incapable of agar surface growth at oxygen levels greater than 0.5% and moderate anaerobes capable of growth at oxygen levels as high as 2 to 8% which could be exposed to room atmosphere for one hour or longer without appreciable loss in viability. Loesche (72) concluded that molecular oxygen intefered with the multiplication of anaerobes and that anaerobes differed in their sensitivity to oxygen.

2. Organic Peroxides

The possible toxicity of organic peroxides that accumulate in oxidized media was first suggested by Barry and associates (6). Commonly used reducing agents such as cysteine, thioglycollate, or glucose react with atmospheric oxygen to produce hydrogen peroxide in quantities sufficient to inhibit many anaerobes. Further, the hydrogen peroxide may react with other organic substances in the medium to form peroxides that may be even more inhibitory (103, 104).

3. Catalase

Callow (19) suggested that the oxygen sensitivity of anaerobes was due to their inability to produce catalase, as most aerobic organisms do produce catalase. Catalase functions as a catalyst in the following reaction:

$$H_2O_2 \rightarrow H_2O + 1/2\ O_2$$

It was suggested that lethal concentrations of hydrogen peroxide accumulate when catalase-negative anaerobes are exposed to air (91) The production of detectable amounts of hydrogen peroxide was experimentally verified by Gordon, Holman, and McLeod (52) using aerated washed suspensions of clostridia. However, later studies showed that factors other than hydrogen peroxide production may be involved in explaining oxygen sensitivity of anaerobes. For example, the addition of crystalline catalase to aerated cultures of Clostridium had little or no stimulative effect on growth (79). Also, some anaerobes that do produce catalase or enzymes of similar function were still incapable of aerobic growth (122). Furthermore, exposure to air did not lead to detectable production of hydrogen peroxide by all species of anaerobes. Therefore, there appears to be no definite correlation between catalase production and the ability of an organism to grow under aerobic or anaerobic conditions.

4. Oxidation-Reduction Potential

The oxidation-reduction potential (O.R. potential) of the environs plays an important role in influencing growth of anaerobes. Oxidation-reduction potential is a measure of the tendency of a system to accept or donate electrons. O.R. potential is measured in volts or millivolts and is expressed as Eh, signifying that the normal hydrogen electrode is the standard used as comparison. The more reduced the medium, the lower the Eh value and vice versa. O.R. potential is influenced by the pH of the system under study. In general a decrease of one pH unit causes the Eh to become more positive by 57.7 mv (24). Many anaerobes have an upper Eh limit beyond which they will not grow.

Most clinically important anaerobes grow well at an Eh of -150 mv at pH 7.0. Certain anaerobes have been shown to grow over a wide range of Eh values, dependent upon experimental conditions. Generally, clostridia grow well at an Eh of +50 to +100 mv, being relatively aerotolerant anaerobes. However certain clostridial species (C. carnis), are known to initiate vegetative growth, under entirely aerobic conditions on routine bacteriologic media with an Eh of approximately 225 mv (63).

The Eh level of culture media can be readily adjusted by the addition of oxidizing or reducing agents. Thioglycollate, cysteine, and sodium sulfide are probably the most widely used reducing agents in the preparation of anaerobic media. Although the O.R. potential of a medium can most accurately be measured by electrical means, it is more convenient to incorporate a redox dye active over the desired Eh range. These dyes are usually colored in the oxidized state and colorless in the reduced form, and give a visible indication of the oxidation-reduction potential of the medium. The oxidation-reduction potentials at pH 7.0 of some common indicators are listed in Table 1.

Resazurin and methylene blue are the Eh indicators most commonly used in anaerobic bacteriology. Resazurin (bluish-purple) undergoes an irreversible change upon heating to resorufin. Resorufin is pink in the oxidized state and can be reversibly reduced to dihydroresorufin (colorless).

Various researchers have determined Eh levels above which specified anaerobes are unable to grow (67, 119). In these studies, the redox potential of the medium was adjusted with a mixture of nitrogen and air. The validity of the "absolute" potentials determined

TABLE 1. The O. R. Potential at pH 7.0 of Some Common Indicators

Indicator	Eh
Oxygen electrode	+ 810 mv
Chlorophenol indophenol	+ 233 mv
2,6-Dichlorophenol indophenol	+ 217 mv
Methylene blue	+ 11 mv
Janus green	- 11 mv
Resazurin	- 42 mv
Phenosafranine	- 252 mv
Viologen dyes	- 320 to - 420 mv
H_2 electrode	- 420 mv

is questionable due to the concomitant exposure to oxygen. Studies have also been made in which the adjustment of potential was accomplished through electrical means (53). Several species of anaerobes were able to grow in media through which air was continuously bubbled, provided the potential of the medium was maintained at a sufficiently low level (5, 57). This suggested that O.R. potential is of primary importance in the growth of anaerobes. Others challenged this view by showing inhibition of growth of several anaerobes in the presence of oxygen, even when the O.R. potential was very low (95, 120).

5. Oxidation of Intracellular Components

O'Brien and Morris (95) recently studied the effect of molecular oxygen on the enzymatic activities of Clostridium acetobutylicum. In living cells, the reduced forms of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) function as electron donors. O'Brien and Morris found that the activity of NADH oxidase increased dramatically in cells of C. acetobutylicum when exposed to oxygen, while other enzyme activities were not affected. NADH oxidase catalyzes the reaction:

$$NADH + H^+ + 1/2O_2 \rightarrow NAD^+ + H_2O$$

Thus, at high oxygen concentrations the supply of NADH would rapidly be depleted and the metabolic and biosynthetic functions dependent on NADH would be interrupted. It was postulated that, at low O_2 concentrations, NADH oxidase could serve as a reductive defense mechanism for the organism by using up limited quantities of oxygen.

6. Superoxide Dismutase

One of the more recent theories to gain attention has been the superoxide dismutase theory of anaerobiosis proposed by McCord and associates (89). Superoxide (O^-_2) is thought to be a possible intermediate in the reduction of oxygen via the flavoenzyme system. It is a highly reactive free radical form of oxygen which may accumulate in toxic amounts in anaerobes grown in the presence of oxygen. Superoxide dismutase catalyzes the conversion of superoxide radicals to less reactive molecules in the following manner:

$$O^-_2 + O^-_2 + 2H^+ \rightarrow O_2 + H_2O_2$$

McCord, et al. (89) based their theory on observations of selected species of aerobes, aerotolerant anaerobes and strict anaerobes. All aerobic bacteria displayed both superoxide dismutase and catalase activity. The aerotolerant anaerobes lacked catalase but exhibited superoxide dismutase at approximately 30% of the aerobic level. The strict anaerobes lacked superoxide dismutase and showed little or no catalase activity. These results suggested that the primary physiological function of superoxide dismutase in aerobes and aerotolerant anaerobes is to protect these bacteria from the highly reactive and potentially harmful superoxide radical. However, a few anaerobes have been shown to produce superoxide dismutase in quantity without being able to grow in the presence of oxygen (95, 111).

The above mentioned theories are, to paraphrase Smith (111), "in some measure satisfactory and in some measure incomplete." It is most likely that a combination of factors is involved in the oxygen sensitivity or oxygen tolerance of different anaerobes.

Experimentation with oxygen sensitivity has been primarily *in vitro*, presenting an array of parameters which are difficult to evaluate independently and are difficult to extrapolate to the *in vivo* conditions.

B. Anaerobic Methodology

Anaerobic organisms have been recognized for over a century. Pasteur introduced the terms "aerobies" and "anaerobies" to denote microorganisms living with and without oxygen, respectively (113). As in any discipline, contemporary technology is to a great extent a synthesis of earlier, proven techniques. A review of the historical breakthroughs is essential in understanding present methods in anaerobic bacteriology. A chronological listing of developments in anaerobic bacteriology is given in Table 2.

1. Surface Culture

Isolation in pure culture can most conveniently and reliably be carried out by streaking on solid plated media. Early researchers relied on observations made on liquid cultures, lacking the means for anaerobic surface culture. It is likely that some of the anomalous findings of these workers were based on studies of mixed or contaminated cultures. The development of techniques allowing for surface growth was prerequisite to extending knowledge of anaerobic organisms.

The first apparatus providing for surface cultivation of obligate anaerobes was the McIntosh-Fildes anaerobic jar introduced in 1916 (90). The removal of oxygen from the sealed jar was accomplished by a reaction with hydrogen, mediated by a heated catalyst such as platinum, palladium, or palladinized asbestos. Many

TABLE 2. Evolution of Anaerobic Culture Methods[a]

Deep shake and fluid cultures	1861-1915	Early attempts Evacuation Inert gases Roll tube (1886) Novy jar (1893)
Reducing agents		
In medium	1890	
In chamber		
Pyrogallol	1888	
Phosphorus	1904	
	1916	Cooked meat
Surface cultures		
McIntosh-Fildes jar	1916-1921	
	(1898)-1925	Cysteine
Brown, Brewer jars (heated catalyst)	1921-1939	
	1940	Thioglycollate and agar
	1950	Hungate Roll tube, pre-reduced media
Room temperature catalyst	(1933)-1958	
Selective media	1965	VPI
Glove box		Modifications of Hungate method

[a]From Ref. (113).

modifications of the anaerobe jar followed. One of the most popular of these modifications was the Brewer jar, utilizing an electrically heated catalyst. Baird-Tatlock and Torbal anaerobe jars were successive modifications using a catalyst active at room temperature. All of these anaerobe jars had the disadvantage of requiring gas cylinders, vacuum pumps, valves and gauges for operation.

The Gaspak system (Baltimore Biological Laboratories, Baltimore, Maryland) has gained rapid acceptance since its introduction in 1966 (15). It consists of a simple jar with a specially designed, tight-fitting lid and uses a disposable anaerobic gas-generating envelope. The lid is fitted with a holder for the catalyst, palladium coated alumina pellets, which is active at room temperature. The catalyst must be regenerated and replaced on a regular basis (depending on usage) to assure peak activity. The disposable gas generating envelope contains a sodium borohydride tablet and a tablet of citric acid plus sodium bicarbonate which release hydrogen and carbon dioxide respectively, on addition of distilled water. In the presence of active catalyst, the hydrogen generated reacts with oxygen in the jar to produce water. Carbon dioxide, a growth requirement of some anaerobes (111), constitutes approximately 8 to 10% of the final volume. A disposable methylene blue indicator can be included in the system as a visual reference of the anaerobic conditions being generated. The Gaspak is a safe, self-contained system that eliminates the need for tanks, pumps and gauges. No special technical skills are required for operation and results comparable to more demanding anaerobic methods can be obtained.

The greatest problem associated with the Gas Pak system is the failure on the part of the user to take anaerobic precautions in the handling of specimens and media prior to anaerobic incubation (35). Specimens processed in the presence of atmospheric oxygen may be markedly reduced in numbers and kinds of anaerobes present, while media prepared and stored under aerobic conditions (even with subsequent reduction) may be reduced in quality (6). Further, the opening of the container to obtain one plate, exposes all plates to oxygen. Even a limited exposure to oxygen (5-10 min.) may kill or inhibit sensitive organisms (72). In spite of these drawbacks, the Gas Pak system has been widely used, with good results in clinical situations. It has not been as widely used in normal flora studies owing to the very stringent anaerobic conditions required for the culture of some indigenous anaerobes.

2. Roll Tube Methods

The study of strict anaerobes requires a more stringent anaerobiosis. The roll tube technique of Hungate (65) was originally devised for the study of strictly anaerobic rumen bacteria. This technique or various modifications thereof has been used in cultivating a great variety of anaerobes (17, 82, 84).

Hungate used media containing a reducing agent, 0.01% cysteine HCl, and an Eh indicator, 0.0001% resazurin. Media were prepared, tubed and inoculated in the complete absence of oxygen. All manipulations were performed under a stream of oxygen-free gas, introduced into the container via a bent Pasteur pipette connected by rubber tubing to its source. Anaerobic gases (CO_2, N_2, etc.) were freed of

oxygen by passing them through a chromous acid solution. Transfer of the pre-reduced, anaerobically sterilized (PRAS) medium to sterile roll-tubes was accomplished without exposure to oxygen. A sterile, cotton-plugged pipette was fitted with a section of tubing as a mouthpiece. Prior to uptake of the medium, anaerobic gas was drawn into the pipette. The medium was then withdrawn and rapidly transferred from the flask to the tube. A continuous flow of anaerobic gas was maintained in both the tube and the flask during this manipulation. Filled tubes were sealed and rotated so as to form an even layer of agar on the inner surface. These tubes, referred to as roll-tubes, maintained an independent anaerobic environment when sealed. Subsequent manipulations such as inoculation, picking of isolated colonies and transfer of pure cultures, were likewise carried out under a continuous flow of anaerobic gas, using PRAS media.

The main advantages of the Hungate method were: (1) Media were sterilized, inoculated and incubated under strict anaerobic conditions hence oxidized medium constituents that might be inhibitory were not formed; (2) Anaerobic bacteria were not exposed to oxygen during isolation or transfer procedures; (3) The oxidation-reduction potential of the medium was favorable for the growth of most anaerobes (<-150 mv); (4) Ability to examine or manipulate an individual culture tube without exposing it or other cultures to air; and (5) Long shelf life of PRAS media.

The roll tube technique as first described has been modified by W.E.C. Moore and colleagues at the Virginia Polytechnic Institute and State University at Blacksburg, Virginia (60, 63, 84). The techniques used at the VPI Anaerobe Laboratory have essentially been adaptations

made for the easy isolation and identification of anaerobes on a large scale (87) for taxonomic studies (61, 85) and for efficient use in a clinical diagnostic laboratory (63, 86). Other modifications have been made by other workers to suit particular groups of organisms, such as the very strictly anaerobic methanogenic bacteria (17).

The syringe method for anaerobiosis was a modification of the original Hungate technique that is worthy of note. Macy, Snellen, and Hungate (74) first made use of injectable roll tubes in their laboratory. The tube (available through Bellco Glass Co., P.O. Box B, Vineland, N.J. 08360) has a slightly constricted neck into which a butyl rubber stopper fits. The stopper was held in place by an open-holed, screw-on cap. Material could be injected by syringe through the rubber seal into the tube without altering the gas phase contained therein. The tubes have been employed for specimen transport; isolation, including dilution and quantitation (74); enumeration (82); and biochemical testing (82). Anaerobic media were prepared according to standard Hungate technique. Prior to autoclaving, the medium was reduced and tubed anaerobically. The tubes remained sealed during autoclaving as the screw cap held the rubber stopper in place.

3. Anaerobic Chamber Techniques

Several researchers (30, 101) have described anaerobic glove chambers that provided a large enclosed space free of oxygen and were equipped with interchange locks. These chambers had the advantage that conventional bacteriological techniques could be employed, and in combination with an oxygen-free atmosphere and freshly prepared media, could give good surface growth of strict anaerobes. However, the

chambers were rather expensive, complex, and were not designed for routine or prolonged use in small to medium sized clinical diagnostic laboratories.

Aranki and associates (2) developed a chamber constructed of clear vinyl plastic, equipped with neoprene gloves. Mixtures of H_2, CO_2, and N_2 were used to inflate the chamber. Open trays of palladium-coated alumina pellets promoted the reduction of any O_2 entering the chamber. A temperature of 37°C was maintained so that the chamber served as anaerobic incubator as well. Materials were introduced to the glove box via a steel airlock with inner and outer doors. Two valves on the top of the airlock led to a vacuum pump and gas cylinders. Material was placed within the airlock and the outer door sealed. Evacuation and flushing of the lock eliminated most of the oxygen. Objects could then be passed through the inner door to the chamber. Withdrawals from the chamber were essentially a reverse of this procedure with the exception that no gas need be expended (provided the atmosphere within the lock was reduced).

The glove chamber was found to match the roll tube method in attaining a low O.R. potential in the media, and in the efficiency of isolating obligate anaerobes from the mouse cecum (2). The main advantages of the glove box were: (1) It required no special technical skills for operation; (2) Standard bacteriological techniques could be used within the chamber; (3) Media could be prepared in the conventional manner; and (4) The chamber could be constructed in any desired shape or size to suit the needs of a given laboratory.

C. Role of Anaerobes in Health and Disease

1. Indigenous Microflora

Bacteria colonize the skin and mucosal surfaces of man and animals alike. The upper respiratory, alimentary and genitourinary tracts harbor a multitude of species (31, 46, 47, 48, 50, 61, 62, 76, 77, 86, 87, 88) (see Table 3). Numbers and types of bacteria vary from one locale to another as differences in a particular micro-environment enhance or inhibit growth of certain organisms. In a healthy state, the host and normal flora represent an ideally balanced ecological system (55). When this balance is upset (as a result of abnormalities in gastro-intestinal function, dietary insufficiencies, etc. [29, 78]), disease results. Anaerobic infections can often be traced to some pre-disposing factor (see Table 4). Under these debilitating conditions, certain components of the normally harmless, natural flora assume a pathogenic role (86).

The great majority of anaerobic infections are endogenous in origin. Moore, Cato, and Holdeman (86) found that of approximately 40 different species of anaerobes isolated from human infections, only 3 or 4 were not found in the normal human intestinal tract. While the circumstances leading to pathogenesis are not completely understood, it has been established that some indigenous anaerobic bacteria can, under the right conditions, become invasive and destructive. Not all flora becomes invasive under conditions favoring pathogenesis. It appears only selective members of the flora, and not necessarily the most predominant, will become pathogenic (43, 44, 45). Alternatively, not all anaerobes present in clinical specimens can be considered

TABLE 3. Incidence of Anaerobes as Normal Flora in Humans.[a]

	Clostridium	Non-Sporulating Bacilli									
		Gram-Positive					Gram-Negative			Cocci	
		Actinomyces	Bifidobacterium	Eubacterium	Lactobacillus†	Propionibacterium	Bacteroides	Fusobacterium	Vibrio	Gram-Positive	Gram-Negative
Skin	0	0	0	U	0	2	0	0	0	1	0
Upper Respiratory Tract*	0	1	0	±	0	1	1	1	1	1	1
Mouth	±	1	1	1	1	±	2	2	1	2	2
Intestine	2	±	2	2	1	±	2	1	±	2	1
External Genitalia	0	0	0	U	0	U	1	1	0	1	0
Urethra	±	0	0	U	±	0	1	1	±	±	U
Vagina	±	0	2	U	2	0	1	±	1	1	1

* = includes nasal passages, nasopharynx, oropharynx and tonsils

U = unknown ± = irregular 2 = usually present in large numbers

0 = not found or rate 1 = usually present † = includes anerobic, microaerophilic and facultative strains

[a]From Ref. (35).

TABLE 4. Conditions Pre-Disposing to Infection with Anaerobes[a]

1. Injury to tissue, as in surgery or wounding.
2. Reduction of blood supply (shock, edema, frostbite, etc.).
3. Diabetes mellitus, any chronic debilitating disease.
4. Steroid, immunosuppressive, or cytotoxic therapy.
5. Malignancy.
6. Dental or gingival disease.

[a]Adapted from Ref. (35).

pathogens. For example, Propionibacterium acnes encountered in blood cultures is most often a skin contaminant picked up in sampling (35). Therefore, a knowledge of the normal anaerobic flora is important in evaluating the significance of anaerobes isolated from pathologic specimens.

The indigenous anaerobic flora of man has been extensively investigated (31, 47, 48) owing to the obvious applications to human medicine. There have also been several studies of the gastrointestinal flora of various animal species (75, 81, 109, 123). Where parallel studies were made in humans and animals (11, 83, 110) there were many similarities in the types of organisms found in the different species, with differences mainly in the relative proportions of these organisms.

a. Normal Aerobic Flora in Humans

Oral: The human oral cavity is an ideal habitat supporting the growth of a wide variety of anaerobic organisms (34, 66). The distribution of bacteria appears to be a function of their ability to adhere to the various oral surfaces (42, 70, 117). It was shown by VanHoute et al. (117) that lactobacilli were not able to adhere to tooth and vestibular mucosal surfaces as well as Streptococcus sanguis although both species attached equally well to the surface of the tongue.

The indigenous bacterial flora of the mouth changes as the human host matures from infant to adult. McCarthy et al. (88) found that in general, lactobacilli and streptococci are the first to colonize the newborn mouth; rapidly followed by staphylococci, veillonella and neisseria. As the first teeth erupt, at about six

months of age, actinomyces, nocardia and fusobacteria became established. Later in the first year, bacteroides, candida, leptotrichia and corynebacteria were acquired. The adult mouth has been found to contain a wide variety of anaerobes; however, the relative numbers of these bacteria differ considerably from one person to the next. Gordon and Jong (50) isolated 373 strains of bacteria from the sputum of six individuals. Presumably, microorganisms in the saliva were derived from areas of bacterial colonization on the tongue, tooth surface, etc. Of the bacteria isolated, 13% were gram-positive anaerobic cocci, with 4.8% each of gram-negative and gram-positive anaerobic rods and 1.2% gram-negative cocci.

Respiratory: Watson et al. (121) surveyed nasal washings from 5 healthy adults for both aerobic and anaerobic flora. The anaerobic counts were found to be consistently and significantly higher. Although no attempt was made to identify all the anaerobes of the nose, the most commonly encountered bacteria were anaerobic, catalase-forming diptheroids resembling Corynebacterium (Propionibacterium) acnes. This is consistent with the findings of Smith (111) in which P. acnes was found to be the most frequent isolate from the nasal passages of 22 subjects.

In the throat, Smith (111) found Peptostreptococcus intermedius and Veillonella parvula to be the most commonly encountered organisms. The lower respiratory tract has not been systematically examined for anaerobic flora. However, it appears that the normal healthy bronchi and lung tissue are sterile.

Gastrointestinal: Very soon after birth, the gastrointestinal tract of the infant is colonized by bacteria. Bacteria are present in the first stool passed, the meconium. As with other indigenous flora, the gastrointestinal flora can vary greatly, even between individuals of the same age and cultural background, eating the same diet (47).

The human stomach appears to be without a resident bacterial population. Nelson and Mata (93) found that human gastric and duodenal mucosa could not be identified as having an autochthonous flora by either histologic or bacteriologic techniques. The stomach is not sterile however as it has long been thought. It is constantly seeded with bacteria from upper respiratory and salivary secretions. Franklin and Skorna (37) cultured the gastric contents and oral/pharyngeal specimens from 149 individuals. Each subject was fasted for 12 hours prior to sampling with a nasogastric tube. Bacteria were cultured in 82% of the gastric samples. Positive specimens most often contained Staphylococcus epidermidis, Streptococcus mitis or Streptococcus salivarius. Other organisms were found less frequently, but all bacteria found were also present in corresponding oral or pharyngeal specimens. Organisms thus swallowed do not appear to persist long in the stomach (27).

The ingestion of food was found to cause a wave-like increase in the microbial population of the gastrointestinal tract (31). This was particularly apparent in the stomach, where few bacteria can be detected to begin with. Draser et al. (32) found a transient flora in the stomachs of 42 subjects of counts of up to 10^5 bacteria per ml, following a meal. The organisms found were primarily lactobacilli and

enterobacteria. One hour later, no bacteria could be detected in gastric samples.

The small intestine, unlike the stomach, did appear to have a resident flora, albeit sparse in comparison with that of the large intestine. The bacteria in the proximal portion of the small intestine consisted mainly of lactobacilli and enterococci. In the ileum, the microflora shifted towards the more variable population found in the large intestine. The bacteria increased in concentration as well as in variety. Bacteroides, bifidobacteria and coliforms first began to appear in significant numbers in the ileum.

The intestinal tract was also shown to experience an increase in bacterial numbers after the ingestion of food. Moore et al. (86) examined the contents of the small intestine taken from human and animal subjects (hogs, dogs, and rats) both prior to and after feeding. In the full small intestine, approximately 10^6-10^8 bacteria/g were found. Fasting for 12-24 hours prior to sampling resulted in less chyme and fewer bacteria (10^4-$.0^6$ bacteria/g).

Moore et al. (86) found that in the large intestine, the lactobacilli and enterococci are present in approximately the same concentration as in the small intestine, but account for only 0.1% of the colon population. Moore et al. (86) reported that 5 to 10% of the colon bacteria found were facultative while 90 to 95% were obligate anaerobes; however, later studies (62, 87) indicated an even greater predominate of strict anaerobes. Types of anaerobes predominating in the large intestine included bacterioides, bifidobacteria, eubacteria, fusobacteria, peptostreptococci, and clostridia (3, 30, 31, 48). The incidence of clostridia has been linked to the diet and age of

the individual (27). The older the individual or the more meat consumed in the diet, the greater the incidence of clostridia.

The feces, readily available for study, reflect to some extent the bacterial populations of the lower intestine. The most thorough investigation of human fecal flora was made by Moore and Holdeman (87). A total of 1147 random isolates yielded 113 different types of organisms. Two-thirds of the total number of isolates belonged to just ten species (see Table 5). A greater variety of bacteria, more than one hundred species, were isolated with far less frequency, and accounted for only about a third of the total isolations.

It has been pointed out that fecal material does not wholly correlate with intestinal contents as there are significant differences between the two, such as osmolarity, pH and redox potential (29). Problems in methodology have interfered in obtaining reliable information about the exact numbers and kinds of bacteria actually inhabiting the various portions of the intestinal tract. Attebery, Sutter, and Finegold (3) found deficiencies in many publications purporting to have studied the bacteriology of intestinal or fecal material. In some cases anaerobic culture was omitted completely while in others, anaerobic techniques proven to be unsatisfactory for the cultivation of strict anaerobes were employed. Even with the use of strict anaerobic techniques, there have been many technical difficulties in obtaining reliable samples. Individual variation, transport, diluent, correction for moisture content, and irregular distribution of bacteria in specimens were some of the problems involved. Wide differences in total counts of bacteria from various portions of the intestinal tract have resulted from differences in sampling technique. Aspiration

TABLE 5. Relative Frequency of Bacterial Species of the Normal Fecal Flora of 20 Japanese-Hawaiians[a]

Rank	Count[b]	% Flora[c]	Organism
1	5.76×10^{10}	12.1	Bacteroides fragilis ss vulgatus
2	3.40×10^{10}	7.15	Fusobacterium prausnitzii
3	3.07×10^{10}	6.45	Bifidobacterium adolescentis
4	2.86×10^{10}	6.02	Eubacterium aerofaciens
5	2.65×10^{10}	5.58	Peptostreptococcus productus - II
6	2.11×10^{10}	4.45	B. fragilis ss thetaiotaomicron
7	1.74×10^{10}	3.67	E. eligens
8	1.58×10^{10}	3.32	P. productus - I
9	1.53×10^{10}	3.23	E. biforme
10	1.16×10^{10}	2.45	E. aerofaciens - III

[a]From Ref. (87).

[b]The estimated viable count per gram of fecal dry matter.

[c]The percentage of total fecal population.

techniques that sample lumenal contents alone could not be adequate, considering the close association of some bacteria with the intestinal mucosa.

Genitourinary: In healthy individuals, the kidneys, ureters and bladder were found to be sterile. The relative sterility of the bladder was most likely due to the mechanical clearing of contaminants by voiding and the innate antimicrobial action of the bladder wall (94).

Finegold et al. (36) determined that the bacteria found in normal urine were derived from the flora of the urethra. Voided urine from male subjects, both initial flow and midstream, contained anaerobic bacteria at counts of up to 10^2-10^4 organisms/ml. Bacteria found included Bacteroides fragilis, B. melaninogenicus, Bacteroides sp., Fusobacterium sp., anaerobic gram-positive cocci and others. Urine obtained by suprapubic puncture directly from the bladder of healthy humans was uniformly sterile. Urethral flora of females was found in a study by Bran et al. (13) to contain Fusobacterium gonidiaformans, Bacteroides, sp., anaerobic diptheroids and others.

As many infections of the female genital tract have been shown to be caused by anaerobic organisms (71, 116), several studies have been made on the anaerobic flora of the lower female genital tract (13, 26, 46). Although there was considerable variation in the flora of individuals, a number of different anaerobes have been consistently isolated.

Lactobacilli were most frequently isolated by Mead and Louria (81) and de Louvois et al. (26) in studies of the vaginal flora.

Other commonly encountered anaerobes included: bifidobacteria, propionibacteria, bacteroides, fusobacteria and anaerobic cocci.

b. Normal Anaerobic Flora in Animals

The indigenous anaerobic flora of animals or of even one particular species of animal, has not been investigated in full. The use of strict anaerobic technique in veterinary bacteriology and research has been woefully lacking. Studies of indigenous flora in animals often omit anaerobic culture entirely or include relatively insensitive anaerobic culture methods. Anaerobic isolates from animals are often not characterized with precision. Studies may not indicate the criteria by which they have identified the bacteria they report. "Bacteroides" for example, may connotate any anaerobic gram-negative, rod-shaped organism. Such information as is available concerning indigenous anaerobes in animals, must be gleaned from those normal flora studies that included some sort of anaerobic technique. Very little is known about the anaerobic flora of sites other than the gastrointestinal tract in animals.

The microflora of the dog, pig, sheep, cattle, and horse will be reviewed as these species were the most commonly encountered in the course of the present work.

Canine: The indigenous gastrointestinal flora of the dog has been investigated more often because of its value as an experimental animal.

Smith (109) made a study of the development of the gastrointestinal flora in the young of several animal species, including the dog. As the contents of the stomach and various portions of the

intestines were removed aseptically and processed, in the presence of atmospheric oxygen, the results may differ from those obtained by strict anaerobic techniques. Media were inoculated and incubated in anaerobic jars. A total of 21 pups were examined, from 6 hours to 18 days of age. In the first day of life, C. perfringens, streptococci, Staphylococcus aureus and Escherichia coli colonized the gastrointestinal tracts of the puppies. Lactobacilli did not become established until day 4 and bacteroides until day 6. By day 18, bacteroides, at levels of 10^9-10^{10} organisms/g, were predominant in the colon and feces. Lactobacilli, streptococci, and E. coli were also significant in the colon and feces, with S. aureus and C. perfringens present to a lesser extent. Anterior to the colon, E. coli and C. perfringens were found in moderate amounts in all portions of the alimentary tract examined, with streptococci, S. aureus and lactobacilli usually present also. The investigation by Smith revealed that individual variation is as much a factor in animal flora as in humans.

Smith and Crabb (110) using methods similar to those described above, examined the fecal flora of ten adult animals in eleven species (see Table 6). They found bacteroides to be the predominant group in the feces of dogs, closely followed by C. perfringens, streptococci and E. coli. Lactobacilli were generally found in significantly lower concentrations. Matsumoto (80), in a 1972 study of canine gastrointestinal flora, reported the following to be significant in rectal contents: bacteroides, streptococci, clostridia, enterobacteria, lactobacilli and bifidobacteria. In the duodenum, the most commonly encountered bacteria were streptococci, lactobacilli and clostridia. Although infrequently isolated in the small intestine, bacteroides

TABLE 6. The Number of Viable Bacteria Found in the Feces of Ten Adult Animals of Each of Eleven Species[a]

Species	Logarithm of Viable Count per g faeces[b]				
	E. coli	*C. perfringens*	Strepto-cocci	Bacter-oides	Lacto-bacilli
Cattle	4.3 (1.7-5.8)	2.3 (0-3.3)	5.3 (4.3-6.4)	0 (0)	2.4 (0-5.6)
Sheep	6.5 (5.2-8.0)	4.3 (1.7-5.9)	6.1 (5.0-7.5)	0 (0-6.4)	3.9 (0-5.4)
Horses	4.1 (3.0-5.4)	0 (0-2.3)	6.8 (3.7-8.3)	0 (0-7.4)	7.0 (5.7-8.0)
Guinea pigs	0 (0-2.7)	0 (0-2.3)	0 (0-5.7)	0 (0)	8.2 (7.4-9.3)
Pigs	6.5 (5.3-7.6)	3.6 (2.8-5.7)	6.4 (5.7-8.2)	5.7 (0-8.0)	8.4 (6.0-9.2)
Chickens	6.6 (4.0-7.6)	2.4 (0-4.4)	7.5 (0-8.7)	0 (0-9.0)	8.5 (6.5-9.1)
Rabbits	2.7 (0-6.7)	0 (0)	4.3 (0-5.6)	8.6 (7.3-9.1)	0 (0-6.4)
Dogs	7.5 (6.2-8.9)	8.4 (4.7-9.0)	7.6 (5.7-10.1)	8.7 (6.5-9.6)	4.6 (0-9.0)
Cats	7.6 (5.2-9.0)	7.4 (5.5-9.0)	8.3 (4.1-8.9)	8.9 (8.0-10.0)	8.8 (4.3-10.0)
Mice	6.8 (4.8-8.9)	0 (0-2.6)	7.9 (6.4-8.9)	8.9 (0-10.3)	9.1 (7.8-10.9)
Man	6.7 (5.7-8.5)	3.2 (0-5.9)	5.2 (3.4-9.4)	9.7 (8.3-10.1)	8.8 (7.3-10.0)

[a]From Ref. (110).

[b]The median count for 10 animals of each species. The range of counts for the individual animals is given in parentheses.

showed a sharp increase in numbers in the large intestine. In a later study, Mitsuoka (83) reported the principal organisms in the fecal flora of 5 adult dogs to be: bacteroides, peptostreptococci, streptococci, clostridia, lactobacilli, corynebacteria and enterobacteria. Organisms found in lower concentrations include bifidobacteria, veillonella, staphylococci, bacilli and yeasts.

In canines, very little is known as to the normal flora of skin and mucosal surfaces other than the alimentary tract. Clapper and Meade (23) studied the normal flora of the rectum, nose and throat of 25 beagles. Stricter anaerobes were not detected by the inadequate anaerobic techniques employed; however, C. perfringens and Lactobacillus sp. were found in all three locales, with the incidence being highest in the rectum. C. septicum the only other species of anaerobe to be detected, was found in low incidence in the nose. It is to be expected that as the comparable sites favoring growth of anaerobes in man are examined in animals, similar anaerobic organisms will be found.

Swine: Smith and Crabb (110) investigated the fecal flora of seven piglets aged 1 day to 23 weeks. E. coli was found in high levels on the first day, with the numbers of C. perfringens and streptococci also being significant. Bacteroides and lactobacilli appeared later but soon became predominant in the fecal flora, as the numbers of E. coli, C. perfringens and streptococci declined. In pigs of 3 weeks of age and older, lactobacilli remained the predominant fecal isolates, followed by streptococci and E. coli, while the numbers of bacteroides and C. perfringens declined markedly. Bacteroides were, occasionally,

isolated in high numbers, while C. perfringens was usually isolated but in greatly reduced numbers. The overall numbers of fecal bacteria declined with age.

In examining the fecal flora of adult pigs, Smith and Crabb (110) noted the predominance of lactobacilli, followed by streptococci and E. coli. When present, bacteroides appeared to be a major component in the flora, illustrating the extent of individual variation. C. perfringens was present in all animals tested, but in low concentrations.

In a later study, Smith (109) examined 27 piglets aged 3 hours to 49 days. In piglets under 1 day old, the stomach and small intestine contained large numbers of E. coli, C. perfringens and streptococci. After 1 day, the gastric acid production increased and the numbers of the bacteria in the stomach and small intestine decreased sharply. The numbers of E. coli, C. perfringens and streptococci remained high in the large intestine for a longer time, but gradually decreased as well. This confirmed the earlier observation by Smith and Crabb (110) that the overall numbers of fecal bacteria decline with age. Lactobacilli colonized the gastrointestinal tracts of piglets later, but once established, became the predominant organism in the stomach and small intestine. Bacteroides, became established by the second day as the predominant organism in the flora of the large intestine. Veillonella were occasionally isolated from the large intestine of older animals.

In a 1964 study by Fuller and Lev (40), a selective medium containing neomycin and crystal violet was used to screen for gram-negative anaerobes in the swine alimentary trace. The validity of

these results is questionable as no precautions were taken to exclude oxygen prior to incubation in anaerobe jars and the selective media used are known to be toxic for certain Gram-negative anaerobes. Furthermore, the identification of genera was inadequate as it was based solely on oxygen intolerance, Gram-reaction and morphology. However, two types of bacteroides were noted, one associated with young pigs and one with older animals, both occurring with greater frequency in the distal portion of the intestinal tract. Fusobacterium and Veillonella were also associated with older animals and found primarily in the large intestine. Peptostreptococcus was isolated on rare occasions.

Sheep: Smith and Crabb (110) followed the development of fecal flora in 10 lambs from 1 day to 1 year. Initially, large numbers of E. coli and bacteroides (~10^{10} organisms/g) were present in the feces, followed by C. perfringens, streptococci and lactobacilli (~10^{9} organisms/g). The overall numbers of bacteria present in the feces declined as the lambs matured. In animals past 10 weeks of age, the numbers of bacteroides in the feces decreased dramatically, with sporadic isolations in significant numbers. By the end of the first year, E. coli predominated in the feces, followed by streptococci, lactobacilli and C. perfringens. In the same study, Smith and Crabb (110) noted the predominance of E. coli and streptococci, at levels of about 10^{6} organisms/g, in the feces of adult sheep. Lactobacilli and C. perfringens were also commonly isolated, while bacteroides continued to be isolated in significant numbers only sporadically.

Smith (109) in a later study, commented on the similarity of lambs and calves in the development of alimentary flora. E. coli, C. perfringens and streptococci were most numerous in the alimentary tract just after birth. Although later in appearing, lactobacilli soon became the principal inhabitant of the anterior portion of the intestinal tract as the numbers of other bacteria dwindled. A few days after birth, bacteroides and lactobacilli predominated in the large intestine.

Cattle: Smith (109) examined 25 calves, aged 8 hours to 12 days, to determine the sequential development of bovine alimentary flora. Eight hours after birth, E. coli, C. perfringens and streptococci were found in the gastrointestinal tracts of calves. At 24 hours, lactobacilli could be detected and at 48 hours, bacteroides appeared in the caeca and feces. Lactobacilli remained at consistently high levels throughout the gastrointestinal tract, increasing in concentration towards the caecum to compete with bacteroides as the predominating organism. E. coli and streptococci likewise appeared throughout the GI tract, but at somewhat lower counts. C. perfringens, initially present thoughout, experienced a decline in numbers in the intestinal tract anterior to the caecum after 3 days. Variation in numbers and types of bacteria present, was evident in the calves, despite being of the same age and breed, and living under similar conditions.

In an earlier study on the feces of calves from birth to 9 months, Smith and Crabb (110) noted the same general trends. E. coli, C. perfringens and streptococci were present initially, with

lactobacilli and bacteroides establishing a day or two later. The total viable count of ~10^{10} organisms/g apparent in the first few weeks, gradually decreased to counts as low as 10^6 organisms/ after 6 months.

Maki and Picard (75) in a study of 15 cattle, examined various portions of the gastrointestinal tract to characterize the flora of animals on a high-roughage diet. Samples from the duodenum, ileum, caecum and proximal colon were aseptically collected and rapidly processed, using strict anaerobic techniques. It was indicated by the authors that species of the genera Bacillus, Mucor and Streptomyces were probably being ingested with food material in the spore state and not germinating extensively in the intestinal tract. In general, they found E. coli and Streptococcus bovis predominating throughout the intestinal tracts of most of the animals. Most of the anaerobes isolated were found in the ileum: Lactobacillus bifidus, C. perfringens, C. butyricum, Clostridium sp. and Sphaerophorus sp. An unidentified gram-positive anaerobic rod was isolated from a duodenal sample and clostridia were also isolated from the caecum and colon.

Fecal flora of cattle was investigated by Smith and Crabb (110) and Mitsuoka (83). Mitsuoka reported high levels of bacteroides in the feces which was not confirmed by Smith and Crabb. This may be due in part to the poor selective media employed by the latter for enumeration of anaerobic gram-negative organisms. The prepondernance of streptococci, followed closely by enterobacteria; and the relatively low concentration of lactobacilli were reported by both (83, 110). Clostridium and Veillonella were shown to be present in high numbers in an occasional animal.

Horse: Less information is available on the development of equine alimentary flora. Mitsuoka (83) and Smith and Crabb (110) reported a preponderance of lactobacilli and streptococci, and the low numbers of enterobacteria in the fecal flora of equines; however, their results were in sharp disagreement about the numbers of clostridia and bacteroides found. Differences in experimental conditions and anaerobic culture methods, as well as individual variation between animals, may account for the disparate results.

A number of investigations have been conducted on the normal flora of mice (51, 68, 83, 105), chickens (4, 83, 102), rats, guinea pigs, rabbits and cats (83, 109, 110) due to their experimental and/or commercial value. However these species will not be reviewed here as clinical specimens from these animals were not significant in this study.

2. Anaerobes Associated with Disease

a. Human--Diagnostic Considerations

Infections with anaerobic organisms tend to be serious, often life-threatening and progress rapidly. It is important to recognize the involvement of anaerobes in a given disease condition early so that appropriate therapy can be initiated promptly. Fortunately there are clues that aid in the recognition of anaerobic infections. Certain clinical and bacteriologic findings that may be suggestive of infection with anaerobes appear in Table 7. Further, certain types of infections which have so often been associated with anaerobic bacteria in the past, that their presence should be suspected appear in Table 8.

TABLE 7. Clincial Hints Suggesting Infection with Anaerobes[a]

1. Foul-smelling discharges; black discoloration of bloody exudates.
2. Necrotic tissue, gangrene, gas in tissues or discharges.
3. Bacteremias, septicemias, any suppurative or inflammatory processes in internal organs.
4. Septic abortion; septic thrombophlebitis; infections following gastrointestinal surgery, puncture wounds, or compound fractures.
5. Classical clinical picture presented: actinomycosis, tetanus, clostridial myonecrosis.
6. Infections with negative routine bacterial cultures.
7. Failure to cultivate organisms seen on original gram stain by the usual aerobic methods.

[a]From Ref. (35).

TABLE 8. Human Infections Commonly Involving Anaerobic Bacteria[a]

1. Wound infections following surgery or trauma.
2. Abscesses of the brain, breast, lung, liver, and other internal organs.
3. Post-abortal sepsis and other gynecological infections.
4. Appendicitis, peritonitis, endometritis, chronic otitis media, thrombophlebitis, endocarditis, and other inflammatory processes.
5. Septicemias and bacteremias.
6. Dental and oral infections; aspiration pneumonia.
7. Gas gangrene.

[a]From Ref. (35).

It is of interest to note that of the great variety of anaerobic flora indigenous to humans, relatively few species are encountered with any great frequency in clinical infections. In fact, just five organisms or groups of organisms account for two-thirds of all clinically significant anaerobic isolates (35): Bacteroides fragilis, B. melaninogenicus, Fusobacterium nucleatum, anaerobic Gram-positive cocci and Clostridium perfringens.

b. Veterinary Medicine

Strict anaerobic culture is expensive and time consuming, but where a human life is at stake, more than worthwhile. As a result, research into the role of anaerobes in human diseases has advanced significantly in the past decade. In contrast, relatively little is known about anaerobes in animal diseases. Except for a few well-documented illnesses such as tetanus, gas gangrene, black leg, etc., caused by clostridia, the role of anaerobic bacteria in veterinary medicine is to a great extent, unknown. Diseases caused by anaerobes are often isolated incidents and the loss of a single animal may be written off with no more than routine investigation. In non-fatal diseases, if treatment is initiated, it is often based on case history and symptoms before even a routine bacteriologic report is returned. As anaerobes may be as sensitive to chemotherapeutics as aerobic or facultative organisms, the presence of anaerobes may go unrecognized in such cases.

Some anaerobic diseases in animals have been identified because of characteristic features of the disease (stiffness in tetanus, sulfur granules in actinomycosis) and the occurrence of parallel diseases

man (115). The more oxygen-tolerant anaerobes such as clostridia and actinomyces are more readily cultivated and even without culture, Gram stains of specimens containing these organisms may be distinctive. Often a diagnosis can be made on the basis of clinical history, symptoms and Gram stain alone.

Much less is known about the involvement of gram-negative, non-sporing anaerobic rods or anaerobic cocci in veterinary disease (16). As foot-rot in sheep and cattle is a contagious anaerobic infection causing considerable economic losses, a greater effort has been made to identify the causative organisms.

Clostridial Infections: Domestic animals are more likely to contract clostridial infections than man owing to the comparative squalor in which they often must live (115). Clostridia in soil and feces are an ever present source of gas gangrene, enterotoxemias, necrotic hepatitis, bacillary hemoglobinuria, tetanus, botulism and other diseases.

The genus Clostridium encompasses a number of large, rod-shaped, Gram-positive sporulating organisms that vary widely in their degree of aerotolerance. On the basis of their pathogenesis, clostridia may be divided into two groups (16): (1) organisms with little or no invasive capacity, in which case the disease is due primarily to the elaboration of powerful exotoxins, and (2) invasive organisms that rapidly multiply, invade, and progressively destroy tissue.

Of the first group, C. tetani and C. botulinum are prime examples. Tetanus, an affliction of man as well as animals, has been recognized for centuries by the distinctive symptoms of the disease (35).

Infections results from the contamination of wounds with spores of C. tetani. The spores germinate and proliferate locally in damaged tissues, generating a powerful toxin, tetanospasmin. The toxin spreads from the site of infection to the central nervous system where it acts on the motor neurons to produce spastic muscular contractions. Of domestic animals, the horse is most often affected, followed by sheep, cattle and swine (16).

Botulism is a disease condition of man and a wide variety of animals that is due to the ingestion of a pre-formed toxin elaborated by C. botulinum. There are several recognized strains of C. botulinum; these range from harmless, non-proteolytic constituents of the normal GI flora to highly fatal, toxigenic strains (18, 115). Some of the more commonly occurring animal botulisms caused by strains of C. botulinum are noted in Table 9.

The second group of clostridia, characterized by invasive, tissue-destroying organisms, includes many species (Tables 10 and 11). The diseases caused by some of these organisms (e.g., black leg by C. chauvoei) may be infectious, posing considerable threat to the healthy animals in the herd. Infections may result from the contamination of wounds or from proliferation in the intestinal tract. Toxins are often elaborated but these are much less potent than botulinum and tetanus toxins and not solely responsible for the production of disease (16). Extracellular proteins elaborated by this group are varied and include hemolysins, proteases, lecithinases, lipases, hyalases, amylases, deoxyribonucleases, ribonucleases and neuraminadases. These contribute to the breakdown of tissues and

TABLE 9. Neurotropic Intoxications Caused by Clostridia[a]

C. botulinum Type A	Food poisoning of man, often fatal. Occasionally of poultry and mink.
C. botulinum Type B	Food poisoning of man, less deadly than Type A. Has been noted in mink and poultry and reported in horses.
C. botulinum Type C	Widespread in animals and birds. Occurrence in man doubtful.
C. botulinum Type D	Almost entirely confined to cattle. Widespread in some areas. Very toxic.
C. botulinum Type E	Virtually confined to man, but has been reported in mink. Intermediate in toxicity between Types A and B.
C. tetani	A wound infection, often fatal, of man and a wide variety of animals. Is a particular hazard of primitive surgical and obstetrical procedures in man, and of surgical procedures on the farm in animals.

[a]Condensed from Ref. (115).

TABLE 10. Enterotoxemias and Infections of Hepatic Origin Caused by Clostridia (often associated with liver fluke infestation)[a]

C. novyi Type B	Necrotic hepatitis (black disease; bradsot) of sheep and rarely, cattle.
C. novyi Type D (*C. hemolyticum*)	Bacillary hemoglobinuria ("red water disease") of cattle.
C. sordellii	Found occasionally in infections of the liver of cattle and sheep.
C. perfringens Type A	A rare cause of enterotoxemia in unweaned lambs (the yellows) in the U.S.A., and of a similar condition in newborn alpacas.
C. perfringens Type B	The usual cause of necrotic enteritis in very young lambs (lamb dysentery) and occasionally of a similar condition in foals.
	A sub-type causes enterotoxemia of adult goats and sheep in the Middle East.
C. perfringens Type C	Enterotoxemia of sheep (struck) associated with liver fluke infestation.
	Enterotoxemia of very young lambs and calves in the U.S.A.
	Enterotoxemia (necrotic enteritis) of young piglets.
C. perfringens Type D	Very widespread and important cause of enterotoxemic conditions of sheep of all ages.
C. septicum	Causes "braxy," an enterotoxemia of sheep commencing in the abomasum. May cause considerable losses in unprotected flocks.

[a]Condensed from Ref. (115).

TABLE 11. Gas Gangrene and Wound Infections Caused by Clostridia[a]

C. chauvoei	Endogenous gas gangrene (blackquarter, blackleg) of cattle and sheep, and rarely, pigs.
	Fulminating gas gangrene of cattle after the use of contaminated syringes or needles.
	Parturient gas gangrene; fatal wound infection after docking, shearing, castration, and inoculations of sheep.
C. novyi Type A	Causes "big head" in rams. This follows infection of tissues bruised in fighting.
C. perfringens Type A	Found occasionally in traumatic gas gangrene in animals.
C. septicum	A cause, occasionally, of malignant edema in cattle and sheep. Very rarely a cause of endogenous gas gangrene (blackquarter) in cattle.
	The most usual cause of endogenous gas gangrene (blackquarter) in pigs.
C. sordellii	Infection in animals after inoculations with contaminated syringes.

[a]Condensed from Ref. (115).

facilitate the spread of infection. Some of the more significant species are discussed below.

C. perfringens

Toxigenic strains of C. perfringens cause a variety of enterotoxemias in man and animals. These range from an uncomplicated food poisoning in man (Type A) to an often fatal necrotic enteritis of man, piglets, calves and lambs (Types B and C; see Table 11). Sterne and Batty (115) found overeating a common factor in precipitating enterotoxemias caused by this organisms. For example, a change from poor to rich pasture may initiate an outbreak of enterotoxemia in sheep due to C. perfringens, Type D. Type A C. perfringens may also be a cause of gas gangrene in man and animals following trauma or gynecological manipulation (see Table 11).

C. chauvoei

C. chauvoei is most often associated with black leg in cattle and sheep, although it has been found in other species as well. It is thought that the bruising of the large muscle groups in the quarters of these animals provide favorable conditions for the development of C. chauvoei spores circulating in the blood (115). The spores are presumed to have entered the bloodstream from the liver or intestinal tract. Although its occurrence is usually sporadic, it is recognized that once the soil of a pasture or grazing ground becomes laden with spores, the disease will reappear regularly (16). Fatal wound infections with C. chauvoei are also known to occur, particularly in sheep following lambing, docking and shearing (see Table 11).

C. novyi

C. novyi is pathogenic for both man and a wide variety of animals. C. novyi Type A is most often associated with gas gangrene in man, but also causes "big head" in rams. Type B causes a necrotic hepatitis (black disease), primarily in sheep, that has been associated with liver fluke infestation (16). The organism apparently multiplies in liver tissue damaged by the fluke (115).

C. septicum

C. septicum commonly causes diseases such as braxy in sheep and black leg in pigs (18, 115). It has also been reported as a rare cause of black leg and malignant edema in cattle and sheep (115).

Others

A number of other clostridia may become involved in gas-gangrene type infections following trauma, vaccination or gynecological-obstetrical procedures. C. sordellii has been recovered from cattle dead of bacillary hemoglobinuria, atypical black-leg and fatal post-vaccination infections (118). Although it is often found together with C. perfringens, C. sordellii alone is toxigenic and pathogenic, both for man and a number of animal species (35, 115). Saprophytic clostridia may also participate in an infection by enhancing the invasiveness of other organisms. For example, C. sporogenes is considered non-pathogenic and non-toxigenic (115) but is frequently cited in clinical infections of both man and animals (63).

Non-Spore Forming Anaerobes: Of the non-sporeforming anaerobes, only actinomyces and several gram-negative rods have been identified as causing disease in animals.

Actinomycetes are gram-positive, non-acid fast, branching organisms that are microaerophilic to anaerobic. Actinomyces bovis is the causative agent of "lumpy jaw" in cattle, a common infection of the mandible (16). It may cause other chronic infections characterized by granulomatous lesions that break down to form fistulous tracts, usually in the facial, pharyngeal or thoracic region (16). Pus from these lesions usually contains characteristic sulfur granules which appear as a tangled mass of filaments surrounded by acidophilic capsular material when stained and magnified (63). A. bovis has also been reported to cause infection in deer, swine, dogs, and horses. A. suis has been reported from mastitis in swine (16). A. viscosus has been reported in a number of cases of canine actinomycosis (24, 38) and from suppurative lesions in pigs and goats. A. israelii and A. naeslundii are primarily pathogenic to humans and are not known to be important in animal infections.

The gram-negative anaerobic rods most frequently identified in pathologic conditions of animals are Fusobacterium necrophorum, Bacteroides nodosus, and Bacteroides melaninogenicus. F. necrophorum, also known as Sphaerophorus necrophorus, is found in a number of necrotizing infections, including foot-rot of cattle, sheep and pigs, as well as oral and hepatic necrobacillosis and foot abscesses of those species (106). In bovine liver abscesses, pure cultures of F. necrophorum have been isolated (107) and there is little doubt as to the pathogenicity of the organism. In other infections such as foot-rot, the etiology of the disease is less clear. In foot-rot, pure cultures of F. necrophorum are never obtained and the introduction of a pure culture alone does not duplicate the disease. Bacterial synergy is most likely the key. Synergy between F. necrophorum, Bacteroides

nodosus and Corynebacterium pyogenes was shown by Roberts and Egerton to result in ovine foot-rot (99). Berg and Loan (8) demonstrated that F. necrophorum and B. melaninogenicus could act synergistically to produce foot-rot in cattle.

B. nodosus

B. nodosus (also known as Fusiformis nodosus) has been identified in the lesions of foot-rot in sheep, only. Although there are reports of a mild foot-rot in cattle due to this organism (16) these findings have not been well documented. B. nodosus alone cannot produce the typical lesions of ovine foot-rot unless F. necrophorum, and possibly other facultative organisms, are present (99). B. nodosus facilitates the growth of F. necrophorum through its proteolytic action on tissues and the production of a stimulatory cofactor (99). The organism has been implicated, but not proven to occur, in human clinical conditions (111).

B. melaninogenicus is associated with inflammatory processes other than foot-rot. It is rarely recovered in pure culture and most often found in association with C. pyogenes, Pastuerella multocida and Escherichia coli (10). Abscesses, regardless of location, and inflammatory exudates from or adjacent to areas normally populated with anaerobes, most often yielded B. melaninogenicus. Isolations were most frequent from feline specimens but were also made from cattle, dogs, horses, sheep, pigs and others (10).

Information concerning the role of other anaerobic organisms in diseases of animals is as yet, unavailable. The isolation of an organism does not necessarily indicate its pathologic significance;

further investigative work is necessary. Interest in this area is sure to continue as veterinarians become increasingly aware of the significance of these types of organisms in animal infections.

III. METHODS AND MATERIALS

Source of Specimens

Specimens for anaerobic culture were obtained from the staff and associates of the Veterinary Clinical Center, Michigan State University, East Lansing. Specimens submitted by the Large Animal Surgery and Medicine Division included material from cattle, horses, sheep, swine and a goat. The Small Animal Surgery and Medicine Division submitted specimens primarily derived from dogs, but also included a cat, a rabbit and a chicken. Specimens were taken from both clinical and necropsy cases.

Type of Specimens

Anaerobic specimens were selected with care to avoid contamination with natural anaerobic flora. Specimens from sites not normally populated with bacteria were generally considered acceptable. This category included: (1) Body fluids such as blood, bile, bone marrow, and synovial, ascitic, pericardial, pleural and cerebrospinal fluids; (2) Pus aspirated from deep wounds or abscesses; and (3) Biopsy specimens from surgical procedures (as on the appendix, gall bladder, etc.). Sampling of such sites as the respiratory, genito-urinary and gastrointestinal tracts necessitated precautions for the exclusion of normal flora. Suprapubic puncture was recommended for the

collection of urine, while transtracheal aspiration or bronchial brushings were suggested for respiratory samples. Gastric contents were not usually recommended for anaerobic culture in pathologic conditions but were screened in cases of suspected enterotoxemia for Clostridium species.

Specimen Collection and Transport

As the proper collection and transport of specimens is essential for optimal recovery of anaerobes, special anaerobic transport containers were prepared. These were of three different types, for the collection of liquid, swab and tissue specimens.

Liquid

Collection tubes for liquid specimens were prepared by flushing Hungate screw-capped tubes with CO_2 gas (74). The tubes were then sealed and sterilized. Liquid specimens such as pus were aseptically aspirated into a sterile syringe (previously made anaerobic by flushing with CO_2) and were injected through the rubber septum (pre-swabbed with alcohol) into the collection tube.

Swab

For collecting swabs, a two-tube system using 18 x 150 mm test-tubes stoppered with butyl rubber stoppers, was employed. Approximately 0.5 ml anaerobic dilution solution (63) was added to both the tubes under a continuous flow of CO_2 gas. One tube was sealed with a stopper to which a swab had been affixed while the second tube had a stopper only. Many sets of such paired tubes were prepared as above and autoclaved in a press (Bellco Glass Inc., 340 Edrudo Rd., Vineland,

N.J.). The collection prodedure for swabs was as follows: the tube with a swab affixed to the stopper was opened and the sample taken. The stopper and swab were then replaced in the second tube (with the second stopper being discarded). The anaerobic atmosphere of the second tube tended to be retained despite opening because CO_2 is heavier than air.

Tissue

Tubes for the collection of solid tissue specimens, such as small sections of muscle, tumor, or organs, were prepared using standard 18 x 150 mm test-tubes. Approximately 0.5 ml anaerobic dilution solution was added to each tube, under a continuous flow of CO_2 gas. The tubes were then sealed and autoclaved in a press. Specimens were collected by aseptically dropping into the collection tube. As tissue specimens required liquefaction before bacteriologic processing could be continued, a 10 ml volume of anaerobic dilution solution was added to facilitate homogenization. Specimens were blended aseptically and anaerobically under a gas mixture of 95% CO_2 and 5% H_2 in a Waring blender for approximately 1 min.

Preparation of Media

Anaerobic conditions were maintained from the time specimens were collected through the isolation and identification procedures necessary for each anaerobic isolate. Exposure to oxygen was minimized during all manipulations by employing the Hungate anaerobic techniques (65) as modified by Moore and colleagues (63, 84). Unless sealed in an anaerobic atmosphere, anaerobic conditions were maintained by providing a continuous stream of oxygen-free gas. Gases were supplied

from commercial tanks (Matheson Gas Products, P.O. Box 96, Joliet, Ill.) connected via rubber tubing to a heated copper catalyst terminating in gassing cannulae. The copper catalyst (Sargent-Welch Scientific Co., 8560 West Chicago Ave., Detroit, Mi.) trapped any contaminating oxygen. Gas flow was checked by a regulator attached to the gas tank. Cannulae were fashioned from bent 3 inch, 18 gauge needles attached to glass 2 ml luer-lok tip syringes (Beckton-Dickenson, Rutherford, N.J.) plugged with cotton. Unless otherwise noted, a gas mixture containing 85% nitrogen (N_2), 12% carbon dioxide (CO_2) and 3% hydrogen (H_2) was used.

The media used during the course of this project were of two basic types: (1) Solid media, reducible in a glove chamber or anaerobic jar; and (2) Media that were pre-reduced and anaerobically sterilized (PRAS). The medium formulas and preparations used were as described in the VPI Anaerobe Laboratory Manual (63) unless otherwise specified. Media used most often included: (1) Blood agar plates (BAP); (2) Chopped meat-glucose broth (CMG); and (3) Peptone-yeast broth (PY) and modifications thereof.

Blood Agar Plates (BAP)

Supplemented brain-heart infusion agar (BHIA-S) was used as a basal medium (63) and augmented with 5% defibrinated sheep blood plus 0.033% (W/V) palladium chloride (33). Although the blood agar was prepared, sterilized and plated aerobically (as described by Ellner, Granato and May, 33) the inclusion of palladium chloride in the medium facilitated subsequent reduction of the plates in an anaerobic atmosphere (96). If refrigerated in plastic bags, plates could be

stored aerobically up to 4 weeks without any loss in efficiency in cultivating anaerobes. Blood agar plates were reduced for a minimum of 24 hours prior to use by placing in the anaerobic glove chamber.

Chopped Meat Glucose (CMG)

Cooked meat phytone medium (Bio Quest, 1825 N. Lincoln Plaza, Chicago, Ill.) was substituted for fresh meat in the formulation of CMG Broth (63). The medium was otherwise prepared as described by Holdeman and Moore (63) in standard 18 x 150 mm tubes.

Peptone-Yeast Extract

Peptone-yeast extract (PY) broth supplemented with vitamin K-heme solution was used as the basal medium for conducting a wide range of biochemical tests as described in the VPI Anaerobe Laboratory Manual (63). Slight modifications in procedure allowed dispensation of media prior to sterilization rather than after. This was necessary to assure the absolute sterility of the medium and because of the use of injectable screw-cap Hungate tubes. The concentrations of various carbohydrates or other substrates added to the basal medium were the same as those recommended by Holdeman and Moore (63). All substrates, except gelatin were added before boiling. After carefully weighing out and reconstituting the ingredients, the pH of the medium was adjusted, with 10% NaOH, to the level recommended by Holdeman and Moore (63) for different media. The medium was then placed in a round-bottomed flask that was only slightly larger than the volume of the ingredients in it and heated, while under a continuous flow of anaerobic gas, to drive off the dissolved oxygen. Cysteine HCl

(0.5 g/liter) was added anaerobically to reduce the medium. When the medium became colorless, the flask was sealed and allowed to cool to approximately 50°C. Unless indicated otherwise, sterile, CO_2-equilibrated sodium carbonate (Na_2CO_3) was then added aseptically and anaerobically to the medium to give a final concentration of 0.4% (W/V). The medium was then anaerobically dispensed in 5 ml amounts to Hungate tubes, which were sealed and autoclaved at 12°C for 15 min.

Glove Chamber

An anaerobic glove chamber was used for the reduction and incubation of solid plated media. The glove chamber used in this study was made by Coy Manufacturing Co., 1393 Harpst St., Ann Arbor, Mi. It consisted of a clear vinyl plastic chamber fitted with an air lock, equipment entry porthole and two sets of plastic sleeves with gloves for working in the chamber. Shelving and equipment too large to enter via the air lock were placed in the chamber through an opening in the end prior to creating an anaerobic atmosphere. This equipment entry porthole was then sealed and not used further while the chamber was in use. While in operation, the air lock provided the only entry to the chamber. An atmosphere of 85% N_2, 12% CO_2 and 3% H_2 was maintained in the chamber. Gas tanks and an evacuation pump, with regulating valves, were connected to the air lock. Entry to the chamber without atmospheric contamination was achieved by evacuating the air lock and filling with anaerobic gas twice. Trays of palladium-coated alumina pellets on two thermostatically controlled heating units catalyzed the conversion of any oxygen present in the chamber to water. These pellets were regenerated twice weekly by

heating at 160°C for two hours. As the hydrogen sulfide gas generated by some anaerobic organisms acts as a poison on the catalyst, a solution of silver nitrate in a glass beaker was placed in the chamber to minimize poisoning of the catalyst. The pellets were routinely replaced every 3 months. The humidity was maintained at approximately 30-50% and temperature at 37°C. An incandescent flaming device with an exterior foot switch was used for sterilizing transfer loops. Electrical connections led into the chamber through a small opening plugged with a rubber stopper.

Isolation Procedures

The processing of specimens began within an hour after receipt. Liquid and swab specimens were processed in the form received, while solid specimens were homogenized as noted above. A loopful of liquid specimen or a specimen swab was streaked on an anaerobic BAP (anBAP) in an anaerobic glove chamber. Platinum or stainless steel, rather than nichrome, transfer loops or needles were used in all cases to minimize oxidation of the medium. Streaked anBAP plates were incubated in the chamber until growth was observed. Plates were discarded after a week if growth was not apparent. The remainder of the specimen was used for Gram staining and inoculation of PRAS CMG broth for enrichment. The latter was incubated at 37°C until growth was observed. If growth was not apparent after 7 days (as determined by Gram staining) CMG cultures were discarded as negative. All bacterial morphotypes and their relative numbers observed in the original Gram stain, were noted for comparison with subsequent isolations. If, on Gram-staining the CMG broth culture,

bacterial morphotypes not present as isolated colonies were observed, the CMG culture was restreaked on an anBAP.

Representative colony types of bacteria growing on anBAPs were Gram-stained and streaked again on fresh anBAPs at least once to check for purity. Isolated colonies from the latter anBAPs were picked to CMG broths. Aerotolerance of each pure isolate was checked by streaking on a conventionally prepared aerobic blood agar plate (ABAP). Only those isolates failing to grow aerobically were studied further.

Identification

Isolates were identified on the basis of their Gram reaction, cellular and colonial morphology, motility, spore formation, metabolic end products, and on the basis of a series of biochemical tests as recommended by Holdeman and Moore (63). Isolates were numbered according to the chronological order in which positive cases were received. When more than one isolate was recorded per case, they were designated by case number and consecutive letters. A sterile, glass 2 ml syringe fitted with a disposable needle was flushed with anaerobic gas prior to aspirating a portion of the pure CMG culture. Approximately, 0.2-0.3 ml of this pure culture, grown in CMG broth for 24-48 hrs, was then injected into various biochemical test media contained in screw-capped Hungate tubes. To prevent contamination, the tops of the tubes were swabbed with 95% ethyl alcohol just prior to injection of the inoculum (74). Tubes were incubated at 37°C until good growth was observed, usually 48 hrs.

Acid production from carbohydrates or other substrates was determined by measuring the final pH of the medium using a pH meter (Model No. 265, Beckman Instruments, Inc., 2400 Wright Ave., Richmond, CA). A pH of 6.0 to 5.6 was read as weak acid, and a pH of 5.5 or below was read as acid production. Esculin and starch hydrolysis, indol production and nitrate reduction were all determined by addition of the appropriate reagents (63). Gelatin cultures were chilled to 4°C along with uninoculated control tubes; gelatin hydrolysis was indicated by the liquefaction of the medium in culture tubes but not in control tubes. Inoculated milk cultures were observed up to 7 days for development of acidity, coagulation and/or digestion. Lecithinase and lipase reactions were checked on modified McClung-Toabe egg yolk agar (EYA). Opacity under and surrounding bacterial growth indicated lecithinase activity, while an iridescent appearance to growth indicated lipolytic action (63).

All anaerobic isolates were inoculated into PY broth supplemented with glucose (PYG) for analysis of fermentation end products. A Model 15C-3 Dohrmann gas chromatograph (Dohrmann Division-Envirotech, 1062 Linda Vista Ave., Mountain View, CA) was used to separate and quantitate volatile and non-volatile acids. Helium was used as a carrier gas to sweep the sample through a resoflex separating column at a flow rate of 120 cc/min. The column and detector temperatures were maintained at 118-120°C while the injection port was maintained at 145°C. Samples, for analysis of volatile fatty acids (VFA) and non-volatile organic acids (NVOA), were prepared according to the chromatographic procedures detailed in the VPI Anaerobe Laboratory Manual (63). A Series 2400 Varian Aerograph chromatograph (Varian

Associates, Walnut Creek, CA) was used for the detection of alochol metabolic end products expected to be produced by certain strains. The temperatures at which the column, injector and flame ionization detector were set, were 170°C, 205°C and 200°C respectively. Nitrogen (30 ml/min), hydrogen (30 ml/min) and air (300 ml/min) were used collectively as carrier gases. The detector was set at different attenuations depending upon the amounts of various alcohols present. Samples for injection were prepared by distilling 50 ml PYG cultures, according to the procedures of Neish (92).

IV. RESULTS

Of the approximately seventy clinical specimens submitted for culture, forty-five (64%) were positive for one or more anaerobic organisms. Thirty-four specimens (48%) were positive for one or more Clostridium species. The total incidence of NSF bacteria in the clinical specimens examined was 30%, with NSF bacteria present as the only anaerobes in 16% of the cases.

Of the total number of seventy-five anaerobic isolates, 50.7% were C. perfringens; 17.3% other Clostridium sp.; 9.3% Gram-negative NSF rods; 9.3% Gram-positive anaerobic cocci; 6.7% Gram-positive NSF rods; and 6.7% Actinomyces sp.

Clostridial Isolates

C. perfringens

Thirty-eight strains of C. perfringens were isolated from thirty-one clinical specimens positive for this organism. Fifty percent (50%) of the C. perfringens strains were isolated from canine specimens (19 strains), 26.3% from bovines (10 strains); 10.5% from equines (4 strains) and 13.2% from miscellaneous species (two swine, a rabbit, chicken and cat). C. perfringens was isolated from a variety of clinical conditions in canines. It was the only anaerobe

isolated from cases of pododermatitis and thoracic effusion in dogs. C. perfringens was also isolated from a number of disorders of the canine eye, ear, and skin (Table 12). As shown in Table 13, all isolations of C. perfringens from bovines came from cases of enterotoxemia, mastitis, infertility, malignant edema and an aborted fetus. In horses, C. perfringens was isolated from cases of diarrhea, infertility, dermatitis and corneal opacity (Table 13). One strain of C. perfringens was isolated from a case of feline conjunctivitis. Other strains of C. perfringens were isolated from necropsy specimens (two swine, a rabbit, and a chicken) and the cause of death in these cases was not known (see Table 13).

There were some variations in the colonical morphology of the C. perfringens isolates. In some cases, discreet, round, convex colonies, semi-opaque and greyish in color were observed. More often, large, spreading, irregular colonies were seen that were translucent and brownish in color. The cellular morphologies of all C. perfringens isolates were markedly similar. All isolates were large, Gram-positive rods, although some strains decolorized more readily than others (Table 14).

All C. perfringens isolates were positive for acid in starch medium, gelatin hydrolysis and lecithinase, and were negative for mannitol, xylose, indol and lipase. The isolates showed minor variations in other biochemical tests, but the variations observed were within the range of results noted for this organism in the VPI Anaerobe Laboratory Manual (63). However, most of the C. perfringens strains isolated in this study hydrolyzed esculin, while previous reports (18, 63) indicated that the majority of strains were negative for this characteristic.

TABLE 12. Isolation of Clostridial Species from Various Clinical Conditions in Canines

Case Number	Clinical Condition	Anaerobic Isolates[a]	Aerobic and Facultative Isolates[b]
1	Post-surgical descemetocele	C. perfringens	Not tested
10	Extracted tooth	C. perfringens C. sphenoides	E. coli (H) S. aureus (H) α Streptococcus sp (H)
12	Bilateral Mass (lacrimal sac)	C. perfringens	α Streptococcus sp (L) Pasteurella sp (L) Micrococcus sp (L)
16	Pododermatitis	C. perfringens	No growth
18	Pyoderma	C. perfringens E. cylindroides	Acinetobacter sp (L)
20	Meibomian glands O.S.	C. perfringens (2) C. sordellii	No growth
21	Conjunctivitis O.S.	C. perfringens	P. aeruginosa (M) Streptococci (L)
24	Chronic ocular discharge	C. perfringens (2)	E. coli (M)
25	Ear infection left: right:	C. barati C. perfringens (2)	Not tested Not tested

TABLE 12. Continued

Case Number	Clinical Condition	Anaerobic Isolates[a]	Aerobic and Facultative Isolates[b]
26	Chronic dermatitis	C. perfringens (2) C. carnis	S. aureus (L) E. coli
28	Chronic dermatitis / ear:	C. perfringens	Bacillus sp (L)
30	Allergic sinusitis	C. perfringens P. intermedius	S. aureus (L) Moraxella sp (H) M. phenylpyruvica (L)
41	Dyspnea (thoracic effusion)	C. perfringens	No growth
45	Dermatitis	C. perfringens (2)	Not tested

[a]Number in parentheses refers to the number of biotypes of the same species isolated from a specimen.

[b]H = heavy growth; M = moderate growth; L = light growth.

TABLE 13. Isolation of Clostridia from Different Clinical Conditions in Various Species of Domestic Animals

Animal Species	Case No.	Clinical Conditions	Anaerobic Isolates[a]	Aerobic and Facultative Isolates[b]
Bovine	3	Mastitis	C. perfringens Eubacterium sp. (E. limosum)	γ E. coli (H)
	4	Enterotoxemia	C. perfringens	E. coli (H) Enterobacter agglomerans (H)
	5	Enterotoxemia	C. perfringens	Not tested
	9	Black leg Malignant Edema	C. perfringens	E. coli (H) γ Streptococci (H) Micrococci (H)
	11	Infertility	C. perfringens (2)	E. coli (L)
	14	Aborted fetus	C. perfringens C. butyricum P. anaerobius	Pastuerella sp. (H) Streptococci (L)
	17	Mastitis	C. perfringens (2) C. glycolicum	Not tested
	44	Enterotoxemia	C. perfringens C. tertium C. botulinum (non-prot BEF) C. bifermentans	E. coli (H) γ Streptococci (H) Enterobacter sp. (L)
Equine	8	Diarrhea	C. perfringens	E. coli (H) γ Streptococci (L)

TABLE 13. Continued

Animal Species	Case No.	Clinical Conditions	Anaerobic Isolates[a]	Aerobic and Facultative Isolates[b]
Equine (Cont.)	13	Corneal opacity with vascular congestion	C. perfringens	Bacillus sp. (L)
	15	Dermatitis	C. perfringens P. intermedius	S. aureus (H) β Streptococci (H)
	22	Infertility	C. perfringens	Bacillu sp. (L) α Streptococci (L)
	34	Infertility	C. beijerinckii	No growth
	36	Infertility	C. botulinum BEF	No growth
Swine	37	Necropsy/Hock:	C. perfringens C. ramosum	No growth
	38	Necropsy/Hock:	C. perfringens	No growth
Rabbit	2	Necropsy/	C. perfringens	Bacillus sp (H)
Chicken	7	Necropsy/intestine:	C. perfringens	E. coli (H)
Cat	35	Conjunctivitis	C. perfringens	Micrococcus sp (L)
Goat	29	Blind	C. acetobutylicum	No growth

[a]Number in parentheses refers to the number of biotypes of the same species isolated from a specimen.

[b]H = heavy growth; M = moderate growth; L = light growth.

TABLE 14. Cellular and Colonial Morphology of Clostridial Isolates.

Organism	Colonial Morphology	Cellular Morphology
C. perfringens	Varied: round, convex, with entire margin (~2.0 mm) to large, spreading, irregular colonies (≥5.0 mm), translucent to semi-opaque, greyish to brownish in color.	Large Gram-positive rods, approx. 1.0 x 6.0 µm non-motile, spores are oval, subterminal, non-swollen and rarely observed on usual media.
C. sphenoides	Round, convex, with entire margin (~2.0 mm) glossy, translucent, greyish in color.	Large Gram-positive rods, approx. 0.5 x 4.0 µm motile, spores subterminal, swollen.
C. butyricum	Round, flat, with entire margin (~2.0 mm) matt surface, opaque, white.	Large Gram-positive rods, approx. 1.0 x 6.0 µm, motile, spores subterminal, swollen. Single or in short chains.
C. glycolicum	Irregular, flat (~2.0 mm) matt surface, translucent, greyish in color.	Large Gram-positive rods, approx. 0.5 x 4.0 µm motile, spores subterminal, non-swollen.
C. sordellii	Round, flat (~2.0 mm) matt surface, translucent, greyish in color.	Large Gram-positive rods, approx. 1.0 x 4.0 µm motile, spores not observed. Single or short chains.
C. carnis	Round, convex (~2.0 mm) glossy, translucent, greyish in color.	Large Gram-positive rods, approx. 0.5 x 4.0 µm non-motile, spores terminal, swollen.
C. barati	Round, convex (~2.0 mm) glossy, translucent, greyish in color.	Large Gram-positive rods, approx. 0.5 x 4.0 µm motility not observed, spores subterminal, swollen.
C. acetobutylicum	Iregular, flat (3.0-5.0 mm) glossy, semi-opaque, greyish-white.	Large Gram-positive rods, approx. 1.0 x 4.0 µm motile, spores subterminal, swollen.
C. beijerinckii	Round, flat (~2.0 mm) glossy, translucent, greyish in color.	Large Gram-positive rods, approx. 0.5 x 4.0 µm motile, spores not observed.
C. botulinum	Irregular, raised (~3.0 mm) matt surface, translucent, brownish in color.	Large Gram-positive rods, approx. 0.5 x 4.0 µm motile, spores not observed.
C. ramosum	Circular, peaked (~2.0 mm) glossy, opaque, white.	Medium-sized Gram-positive rods, approx. 0.25 x 3.0 µm non-motile, spores terminal, swollen.
C. tertium	Round, flat (~2.0 mm) matt surface, opaque, white.	Large Gram-positive rods, approx. 0.5 x 4.0 µm motile, spores terminal, swollen.
C. bifermentans	Irregular, flat (~2.0 mm) glossy, opaque, white to creamy in color.	Large Gram-positive rods, approx. 1.0 x 4.0 µm motile, spores subterminal, non-swollen.

This may be due to the fact that these organisms were isolated from animals and they may represent a different sort of population than that observed in humans. All strains produced acetic and butyric acids as their major fermentation end-products, with variable amounts of lactic acid. When present, pyruvic and succinic acids were found in trace amounts only. A typical gas chromatogram for C. perfringens is shown in Figure 1, with the range of variation in butyric, pyruvic and lactic acid peaks included. The biochemical characteristics and metabolic products for each of the isolated are presented in Table 12.

Other Clostridia

In addition to C. perfringens, thirteen other Clostridium species were isolated from eleven different clinical cases (Tables 12 and 13). Many of these species had never been reported from clinical conditions in animals before. C. sphenoides, C. sordellii, C. carnis and C. barati were isolated from canines, all in association with at least one strain of C. perfringens. C. tertium, C. botulinum (non-prot. BEF) and C. bifermentans were isolated from a single case of bovine enterotexmia. In addition, C. butyricum and C. glycolicum were isolated from an aborted bovine fetus and from a case of bovine mastitis, respectively. In all three of the above bovine cases, the organisms were isolated in conjunction with C. perfringens. Two organisms most resembling C. beijerinckii and C. botulinum (non-prot. BEF) were isolated in pure culture from separate cases of equine infertility. C. ramosum was isolated, in addition to C. perfringens, from a swine hockjoint and C. acetobutylicum was isolated in pure culture from a case of blindness in a goat.

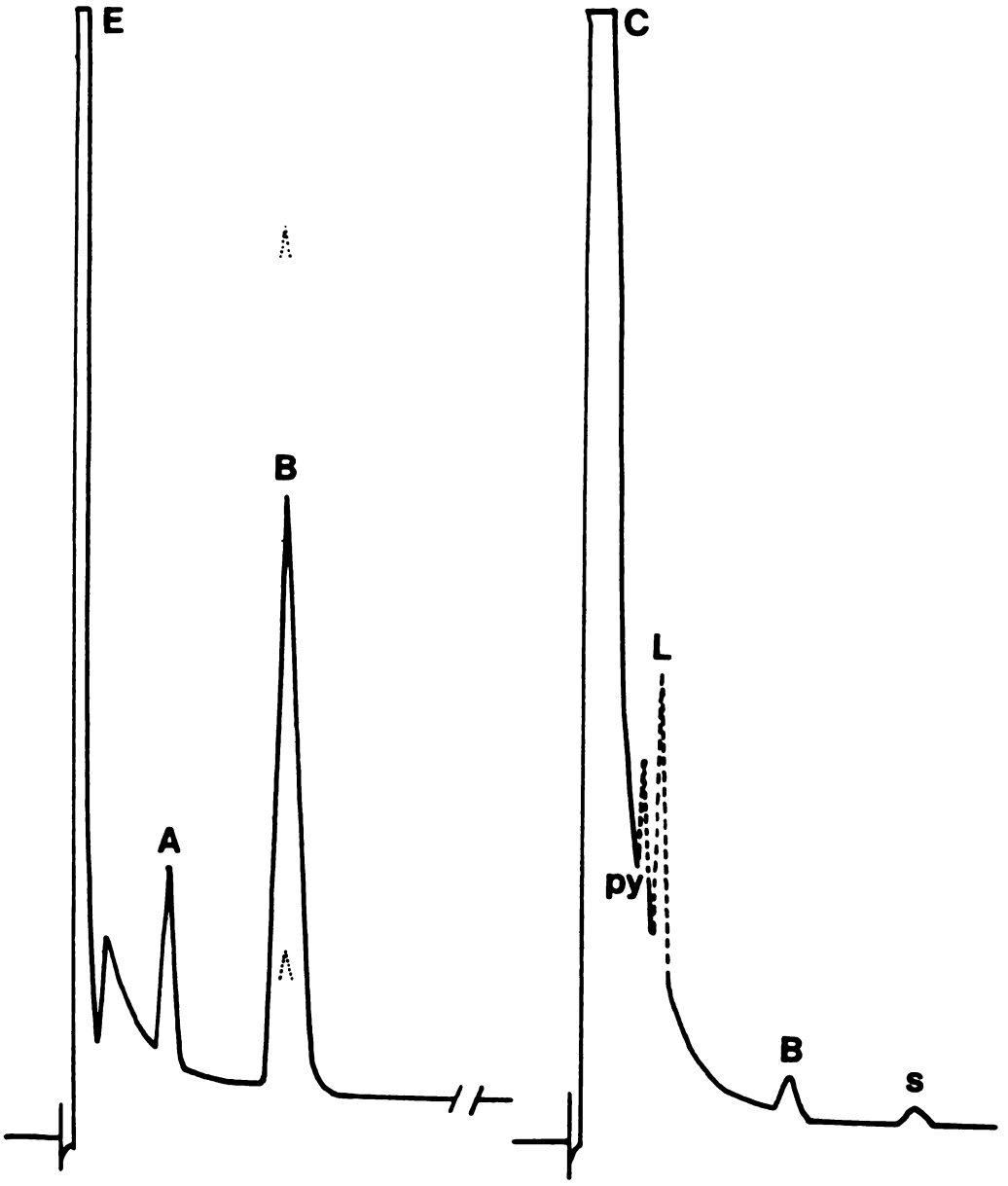

Fig. 1. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of a typical strain of C. perfringens. The extent of variation in acid production between strains is indicated by the dotted lines. For explanation of symbols, refer to p. x.

TABLE 15. Biochemical Characteristics and Acid Metabolic Products of C. perfringens Isolates.[a]

Animal Species	Isolation Number	Esculin Hydrolysis	Fructose	Glucose	Lactose	Maltose	Mannose	Melibiose	Sucrose	Milk	Meat	Hemolysis	Acid End Products
Canine	1	+	A	A	W	A	A	-	A	cg	d	2β	ABpyLs
	10A	A	A	A	W	A	A	-	A	cg	d	2β	AB
	12	+	A	-	-	A	A	-	A	cd	d	β	ABl
	16	+	A	A	W	A	A	-	W	cg	-	2β	ABpyls
	18a	+	A	A	A	A	A	-	A	c	-	2β	AB
	20a	-	A	A	A	A	A	-	A	cgd	d	2β	ABpyls
	20b	+	A	A	A	A	A	-	W	cg	d	2β	AB
	21	-	A	A	A	A	A	-	A	cg	d	β	ABpyls
	24a	+	A	A	A	A	A	W	A	cg	d	2β	Ab
	24b	+	A	A	W	A	A	-	A	cg	d	-	AB
	25a	+	A	A	A	A	A	-	W	cg	d	2β	AB
	25b	+	A	A	A	A	A	-	A	cg	d	2β	ABLs
	26a	+	A	A	A	A	A	W	W	d	d	β	ABs
	26b	+	A	A	A	A	A	-	W	cg	d	2β	AB
	28a	+	A	A	A	A	A	-	W	ct	d	β	ABls
	30a	+	A	A	-	A	A	-	A	cd	d	2β	AB
	41	+	A	A	A	A	A	-	W	cg	d	2β	AB
	45a	-	A	A	A	A	A	-	A	cg	-	2β	ABs
	45b	+	A	A	A	A	A	-	A	cd	d	-	ABl
Bovine	3a	-	-	A	A	W	A	-	W	cg	-	2β	ABL
	4	+	A	A	W	A	A	-	W	cg	d	2β	ABls
	5	+	A	A	A	A	A	-	W	cg	d	2β	ABLs
	9	+	A	A	A	A	A	-	W	cg	d	2β	ABLs
	11a	+	A	A	A	W	A	-	-	cg	d	2β	AB
	11b	+	A	A	W	A	A	-	W	cg	d	2β	ABLs
	14a	+	A	A	W	W	W	-	W	cg	d	β	AB
	17a	-	-	A	A	W	A	-	W	cg	-	2β	Ab
	17b	+	A	A	A	A	A	-	A	cd	d	2β	AB
	44a	-	A	A	A	A	A	-	A	cg	d	2β	ABls
Equine	8	+	A	A	W	A	A	-	A	cg	d	β	AB
	13	+	A	A	W	A	A	-	A	cg	d	2β	AB
	15a	+	A	A	A	A	A	-	W	cg	d	2β	AB
	22	-	A	A	A	W	A	-	A	cg	d	β	AB
Swine	37a	+	A	A	A	A	A	-	A	cg	d	2β	ABs
	38	+	A	A	A	A	A	-	A	cg	d	β	AB
Rabbit	2	+	A	A	A	A	A	-	W	-	d	β	AB
Chicken	7	+	A	A	W	A	A	-	W	-	d	2β	AB
Cat	35	+	A	A	A	A	A	-	A	cg	d	2β	AB

[a]For explanation of symbols refer to p. ix.

Except for C. ramosum, the cellular morphologies of these clostridial isolates were comparable to the C. perfringens isolates. However, considerable differences in motility and sporulation patterns were noted amongst the isolates. The cellular and colonial morphologies, motility and sporulation patterns of these organisms are given in Table 14. Spores were not observed in the strains of C. sordellii, C. botulinum non-prot. BEF, or C. beijerinckii isolated. However these organisms survived a heat test (63) which indicated that they were sporulating clostridia.

Each of the unusual clostridial isolates was identified on the basis of their biochemical characteristics (Table 16) and acid metabolic end products (Figures 2 through 13). The results showed that each of the clostridial species isolated in this study was very similar to previous descriptions (18, 63) with the exception of some minor variations. Some of the salient features of these species are noted below.

C. sphenoides was a large, Gram-positive, motile rod with swollen, subterminal spores that typically hydrolyzed gelatin and produced indol but was negative for lecithinase and lipase. A major amount of acetate and a minor amount lactate were produced from glucose. C. sordellii was similar to previously described strains of this organism; e.g., a large, Gram-positive motile rod that produced lecithinase and urease, but not lipase. However, meat was not digested. Acid end products included major amounts of acetate and formate, with minor amounts of propionate, isovalerate and lactate. Consistent with previous reports, C. carnis was able to initiate growth on conventional aerobic media but produced spores only under anaerobic conditions. The organism appeared as a large, Gram-positive,

TABLE 16. Biochemical Characteristics and Metabolic Products of Clostridial Species Other than C. perfringens.[a]

	C. sphenoides (10b)	C. sordellii (20c)	C. carnis (25c)	C. barati (26c)	C. butyricum (14b)	C. glycolicum (17c)	C. tertium (44b)	C. botulinum (non-prot BEF) (44c)	C. bifermentans (44d)	C. beijerinckii (?) (34)	C. botulinum (?) (non-prot BEF) (36)	C. ramosum (37b)	C. acetobutylicum (29)
	Canine				Bovine					Equine		Swine	Caprine
Esculin hydrolysis	+	+	+	+	+	+	+	-	+	+	-	+	+
Fructose	A	W	A	A	A	A	A	A	W	A	A	A	A
Glucose	A	W	A	A	A	A	A	A	A	A	A	A	A
Lactose	A	-	A	A	A	-	A	-	-	A	-	A	A
Maltose	W	W	W	A	A	A	A	A	W	A	A	A	A
Mannitol	W	-	-	-	-	-	W	-	-	-	-	-	A
Mannose	A	W	A	A	A	-	A	A	-	A	A	A	A
Melibiose	-	-	-	-	A	-	A	-	-	A	-	A	-
Starch pH	-	-	-	-	A	-	A	A	-	W	A	-	A
Sucrose	W	-	A	A	A	-	A	A	-	A	A	A	W
Xylose	-	-	-	-	A	A	A	-	-	W	W	W	-
Gelatin	-	+	-	-	-	-	-	+	+	-	+	-	W
Milk	c	cd	-	-	c	-	-	-	d	-	-	c	cd
Meat	-	-	-	-	-	-	-	-	d	-	-	-	-
Indol	+	+	-	-	-	-	-	-	+	-	-	-	-
Lecithinase	-	+	-	+	-	-	-	-	+			-	-
Lipase	-	-	-	-	-	-	-	+	-			-	-
Hemolysis	-	-	α	-	-	-	-	β	β	-	-	-	β
Motility	+	+	-	-	+	+	+	+	+	+	+	-	+
Urease		+							-				
GMB[b]					+								
Acid products	Al	AFpivl	BAFL	BALs	BAFls	Apibivl	AFbls	BAls	AFpibbivic	BALs	BAFl	La	BALs
Alcohol products		i5(2)			i5 (2,3)	(2,3, i5)			2(3)				(2,3,i5)

[a]For explanation of symbols refer to p. ix.

[b]Glucose-Minimal Salts-Biotin medium.

[c]Parentheses indicate minor alcohol production.

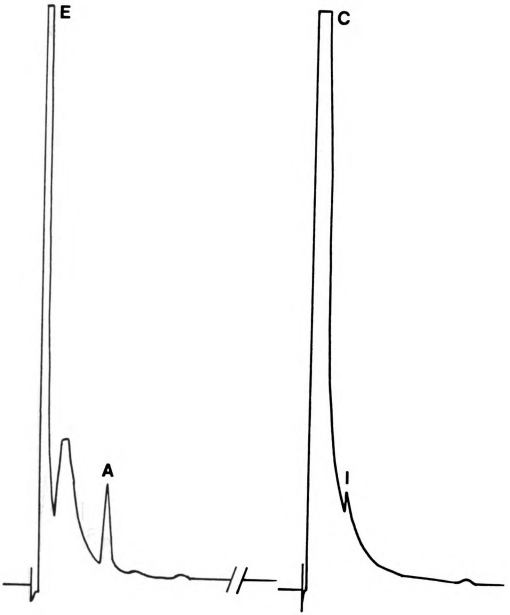

Fig. 2. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. sphenoides (#10b). For explanation of symbols, refer to p. x.

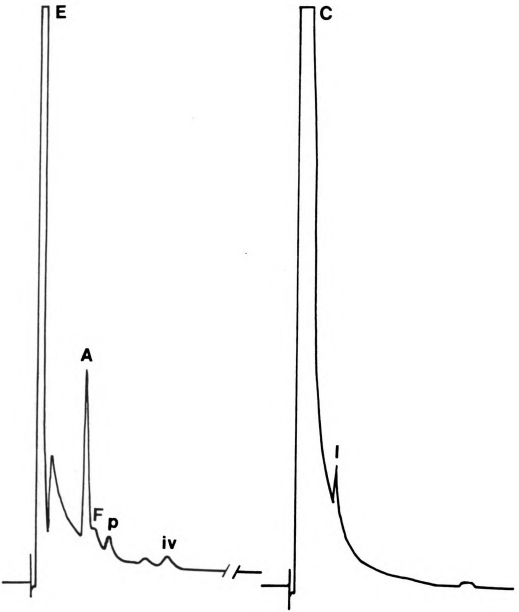

Fig. 3. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. sordellii (#20c). For explanation of symbols, refer to p. x.

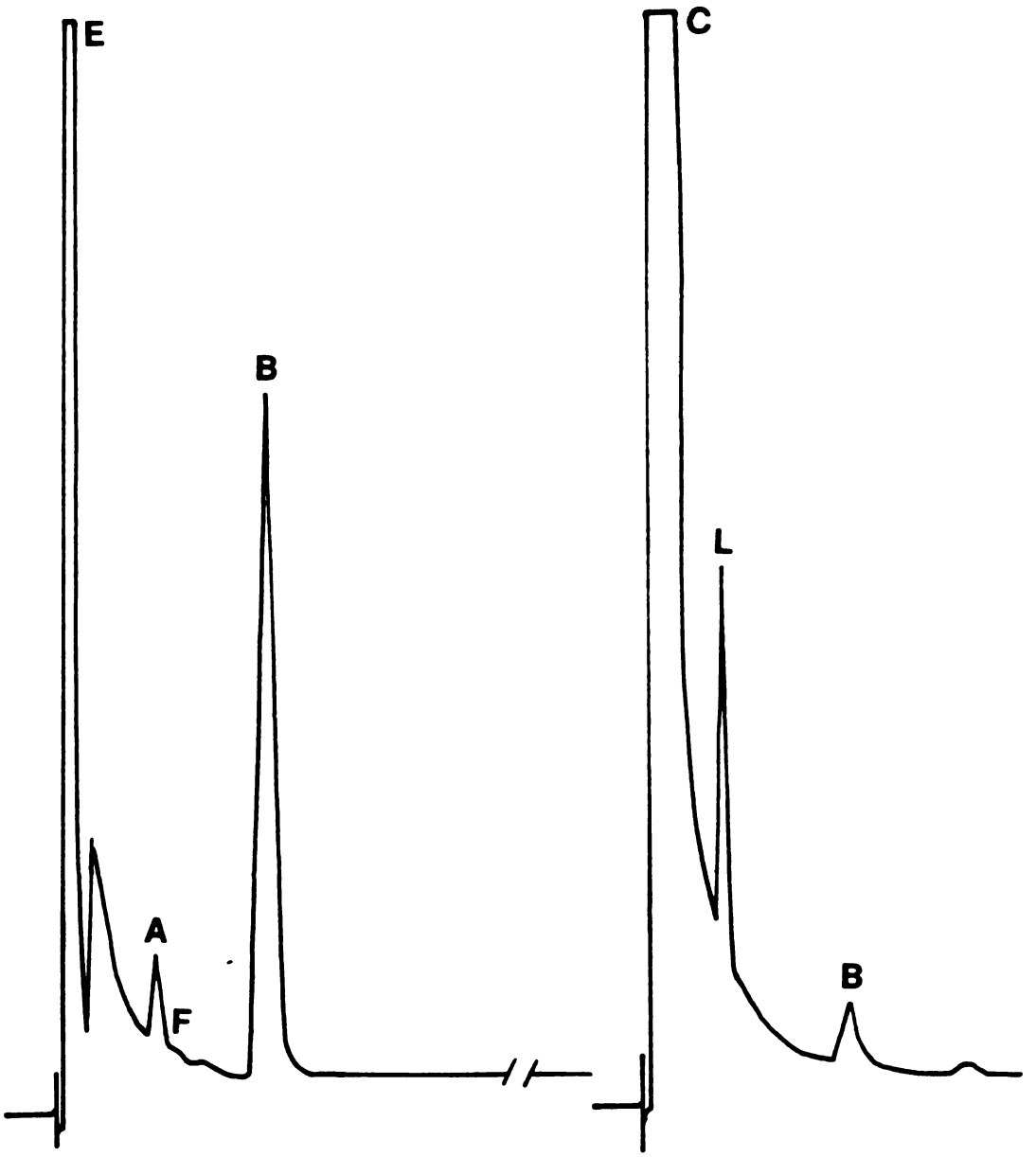

Fig. 4. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. carnis (#25c). For explanation of symbols, refer to p. x.

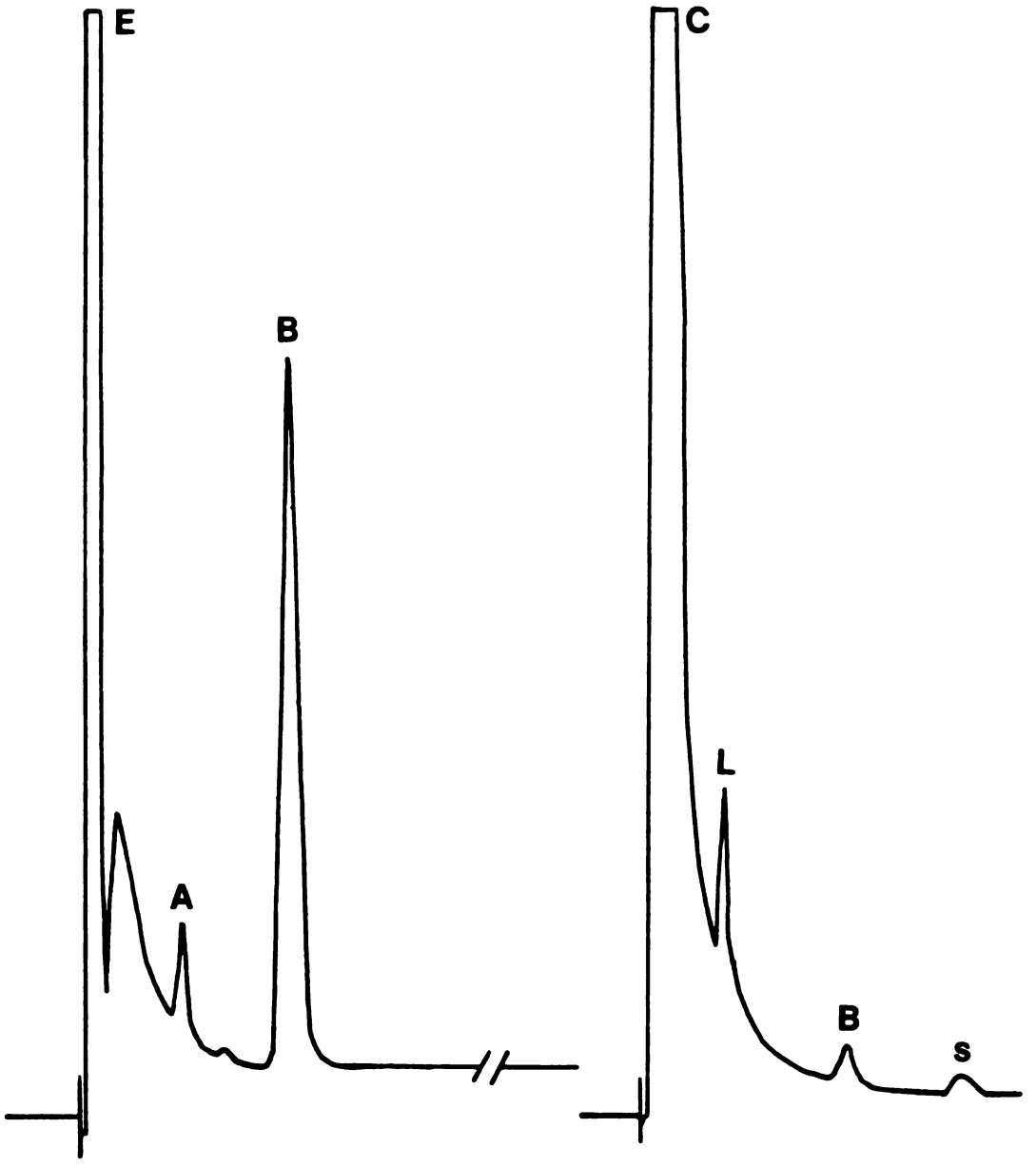

Fig. 5. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. barati (#26c). For explanation of symbols, refer to p. x.

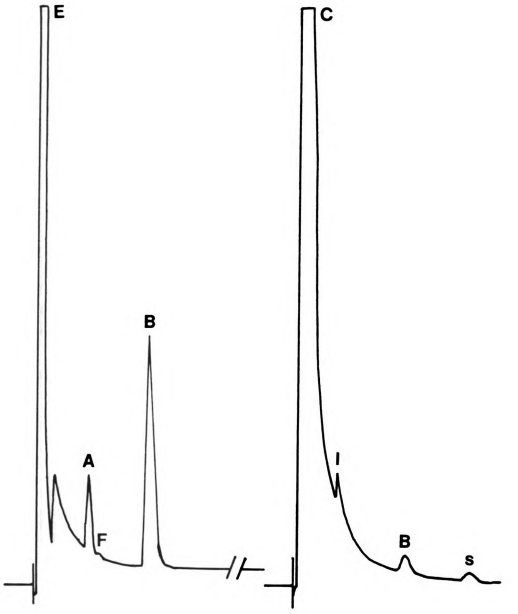

Fig. 6. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. butyricum (#14b). For explanation of symbols, refer to p. x.

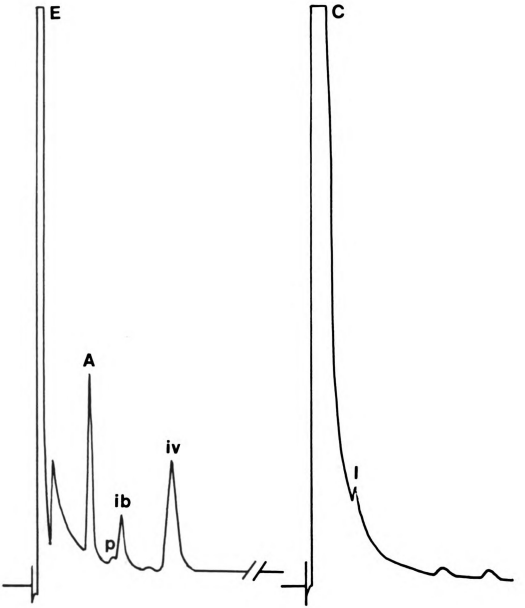

Fig. 7. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. glycolicum (#17C). For explanation of symbols, refer to p. x.

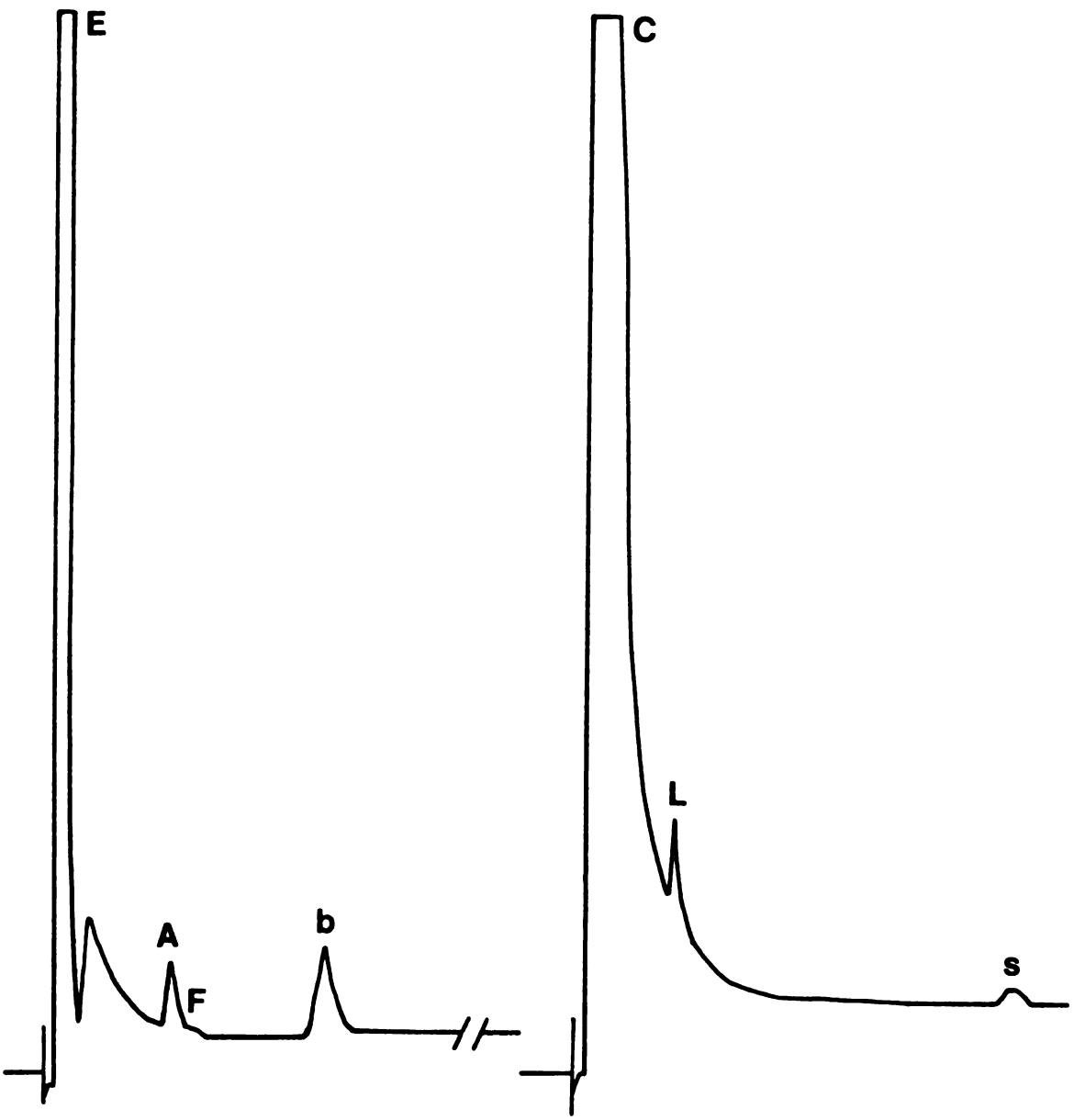

Fig. 8. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. tertium (#44b). For explanation of symbols, refer to p. x.

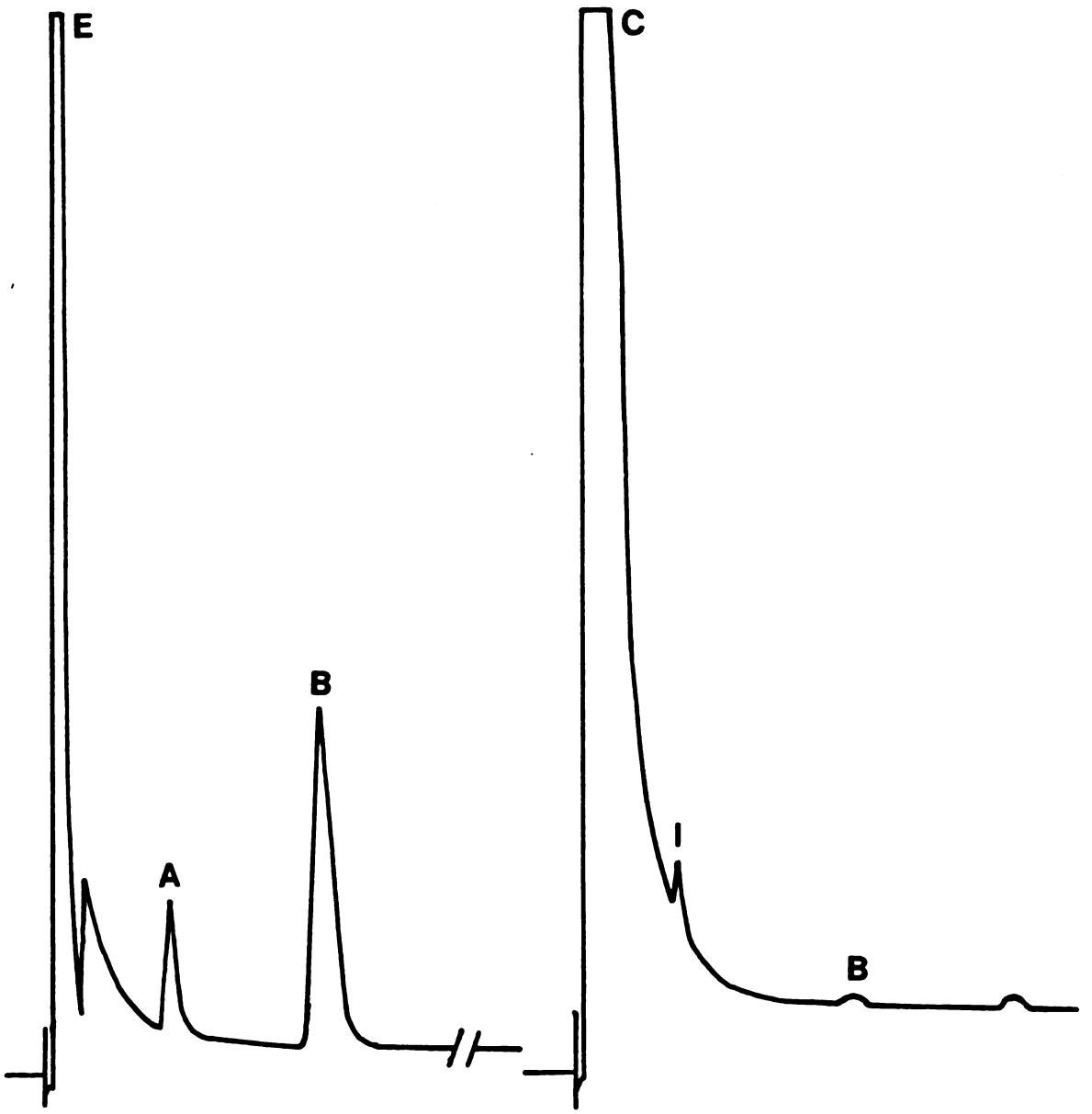

Fig. 9. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. botulinum non-prot. BEF (#44c). For explanation of symbols, refer to p. x.

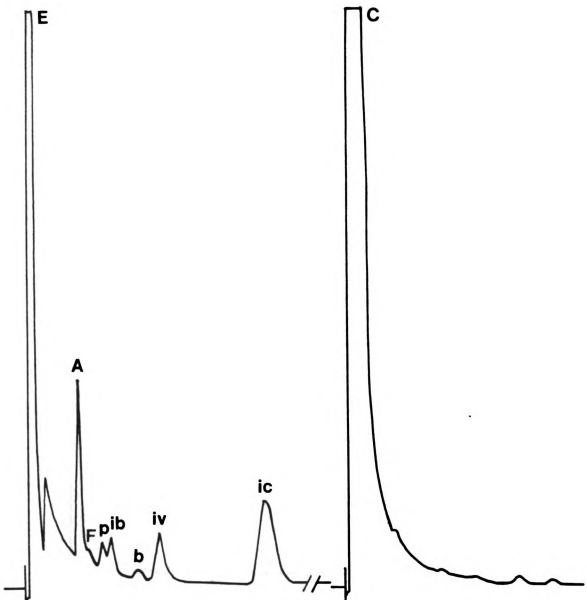

Fig. 10. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. bifermentans (#44d). For explanation of symbols, refer to p. x.

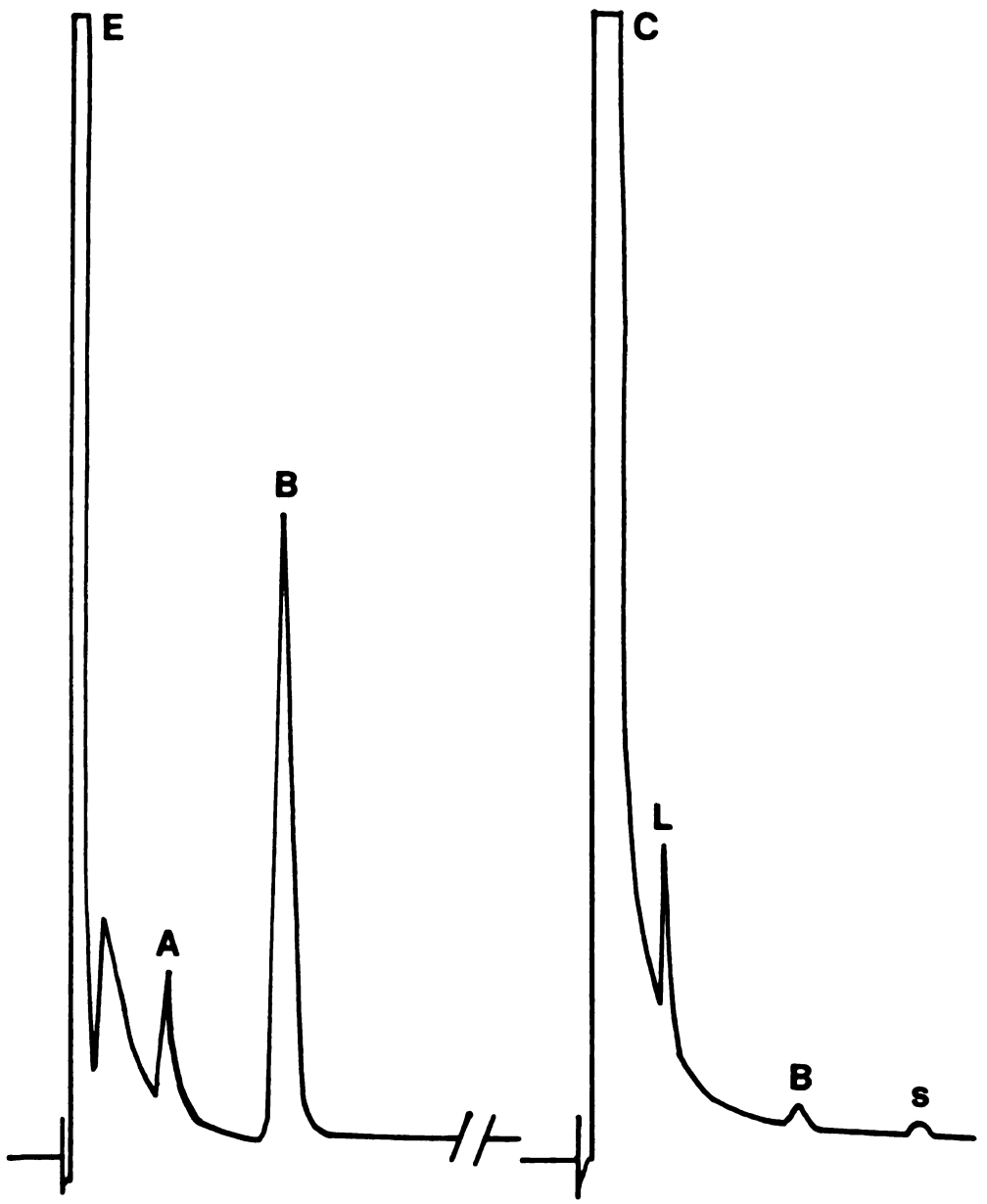

Fig. 11. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. beijerinckii (#34). For explanation of symbols, refer to p. x.

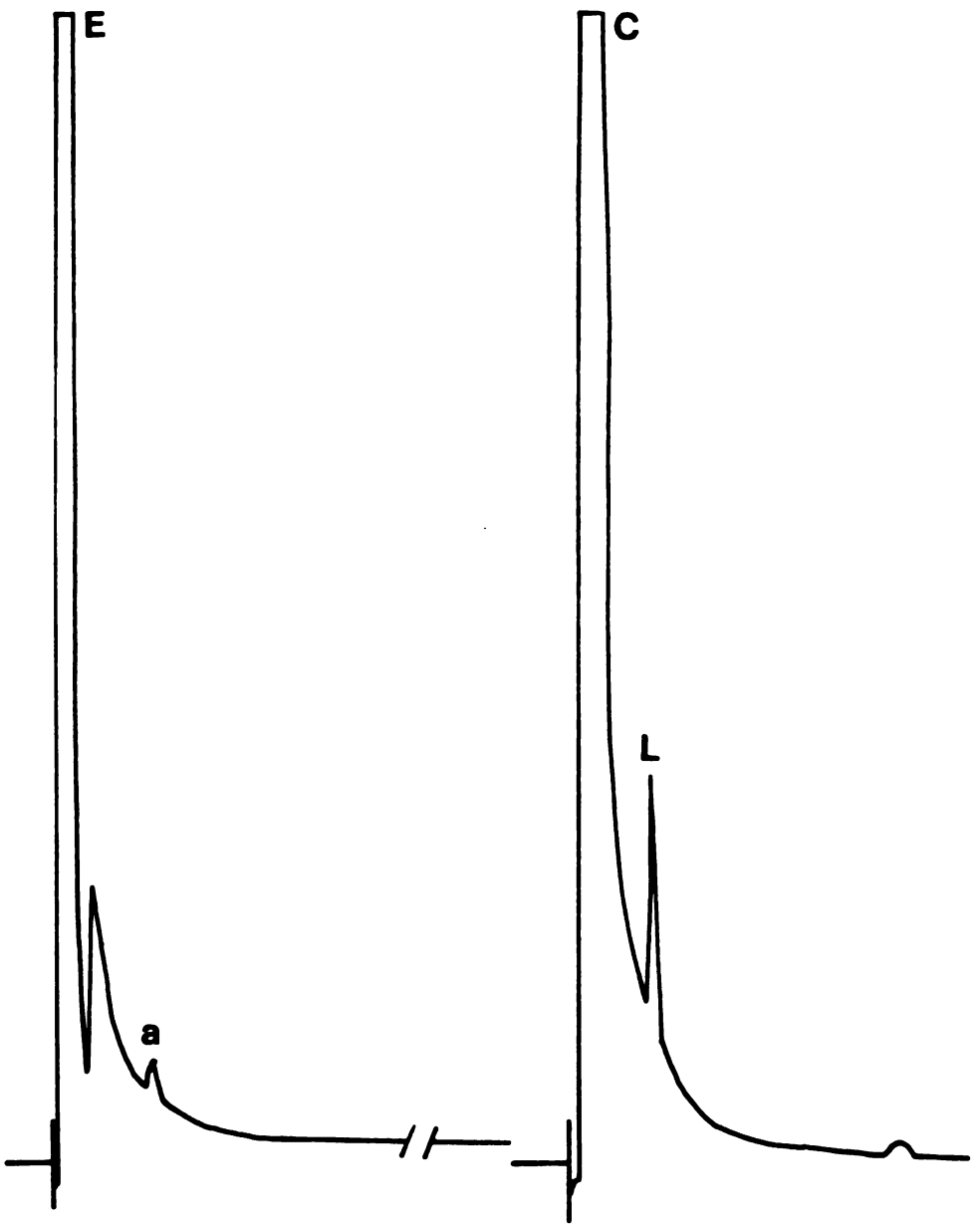

Fig. 12. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. ramosum (#37b). For explanation of symbols, refer to p. x.

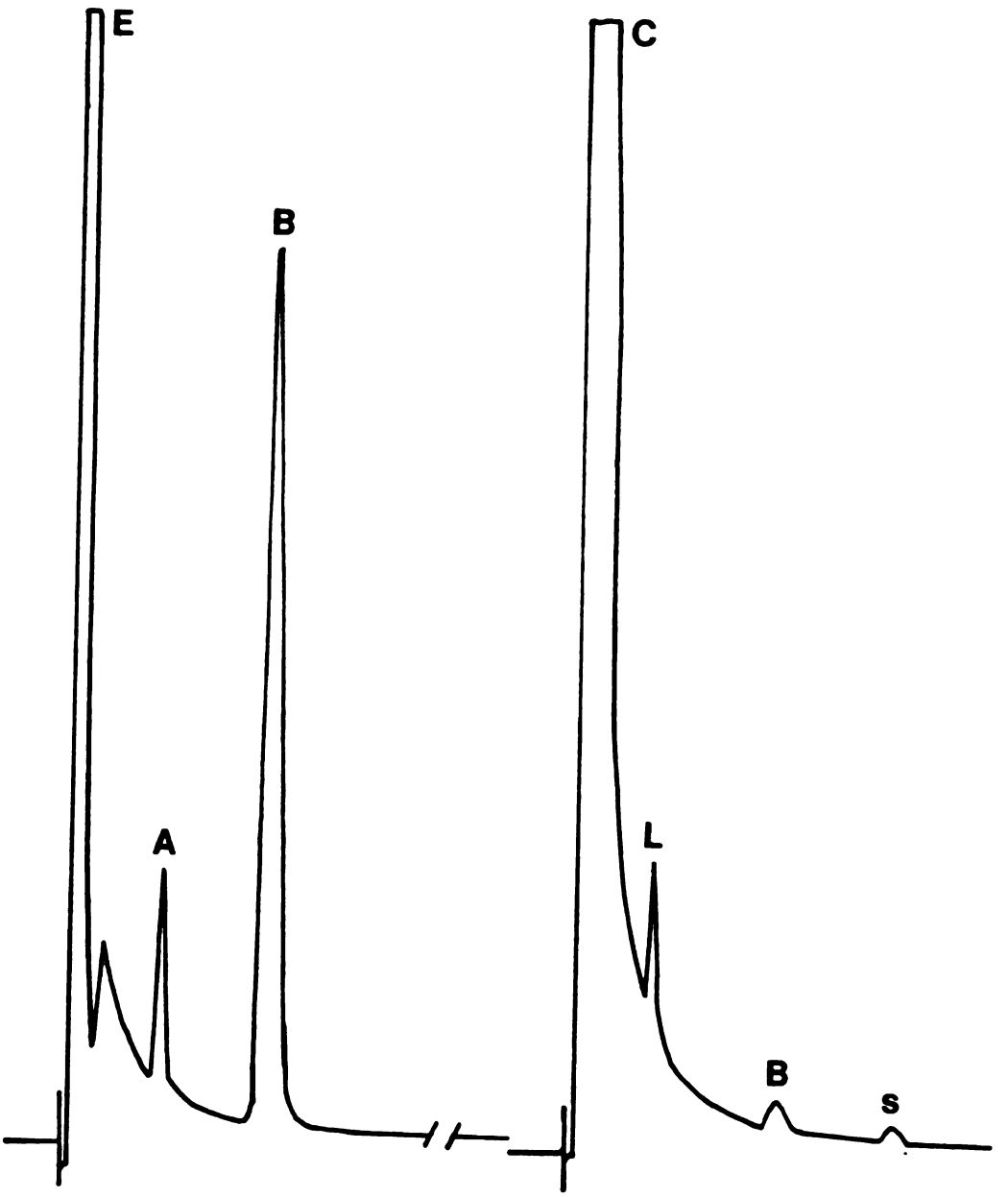

Fig. 13. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of C. acetobutylicum (#29). For explanation of symbols, refer to p. x.

non-motile rod with swollen, subterminal spores. Acetate, formate, butyrate and lactate were produced in major amounts. C. barati was a large, Gram-positive, motile rod with swollen, subterminal spores that was positive for lecithinase and negative for lipase and gelatin hydrolysis. Atypically, the organism produced no change in milk. Major amounts of acetate, butyrate and lactate with a trace amount of succinate were produced from glucose. C. butyricum was a large, Gram-positive, motile rod with swollen, subterminal spores that closely resembled the classical description of this species. Lecithinase, lipase, indol and gelatin hydrolysis were all negative, while growth in glucose-minimal salts-biotin (GMB) medium was positive (distinguishing this species from C. beijerinckii). C. glycolicum appeared as a large, Gram-positive, motile rod with subterminal spores. Gelatin hydrolysis, indol, lecithinase and lipase were all negative, with major amounts of acetate and minor amounts of propionate, isobutyrate, isovalerate and lactate produced from glucose. C. tertium also grew aerobically but produced spores only under anaerobic conditions. The organism was a large, Gram-positive, motile rod with swollen, terminal spores. Atypically, milk was not coagulated. Acid end products included major amounts of acetate and formate with trace amounts of butyrate, lactate and succinate. The two strains of C. botulinum non-prot. BEF (#36, #44c) were large, Gram-positive rods that produced major amounts of acetic and butyric acids with trace amounts of formic and/or lactic acids. Strain #36 was lost prior to completion of testing, however the isolate was tentatively identified as C. botulinum non-prot. BEF on the basis of gelatin hydrolysis, fermentation of glucose and negative indol and lactose results. Strain #44 typically produced lipase and

lacked lecithinase. Both strains failed to coagulate milk unlike the majority of strains previously described. C. bifermentans was a large, Gram-positive, motile rod with subterminal spores that was positive for lecithinase and negative for lipase and urease. A major amount of acetate and formate, with minor amounts of propionate, isobutyrate, butyrate, isovalerate and isocaproate were produced from glucose. Strain #34 was tentatively identified as C. beijerinckii on the basis of the acid end products produced, weak acid production in starch and xylose, and negative results for gelatin hydrolysis and indol. C. ramosum appeared as a medium-sized, thin Gram-positive rod with swollen, terminal spores. The organism was negative for indol, lecithinase and lipase, and produced a major amount of lactate with a trace of acetate. No formate was detected, contrary to what was expected. C. acetobutylicum was a large, Gram-positive, motile rod with swollen, subterminal spores. The organism was very typical of this species with the exception that gelatin was weakly hydrolyzed and milk was both coagulated and digested.

Non-Sporulating Isolates

Gram-Negative Non-Sporing Bacteria

Seven strains of Gram-negative NSF bacteria were isolated from five clinical cases (see Table 17). A Bacteroides sp. was isolated from a case of canine conjunctivities. A fistulous tract infection in a dog yielded B. clostridiiformis ss. clostridiiformis, Fusobacterium varium and F. necrogenes, in addition to P. intermedius and several facultative organisms. A post-surgical follow-up yielded an organism most resembling F. necrogenes. An infected bovine stifle

TABLE 17. Isolation of Non-Spore Forming Bacteria from Clinical Conditions in Various Domestic Animals

Animal Species	Case No.	Clinical Condition	Anaerobic Isolates[a]	Aerobic and Facultative Isolates[b]
Canine	6	Conjunctivitis	Bacteroides sp.	S. aureus (H) Micrococcus Sp. (H)
	18	Pyoderma	E. cylindroides C. perfringens	Acinetobacter sp. (L)
	19	Subepithelial keratopathy (eyes)	(OD) Actinomyces bovis (OS) no growth	(OD) S. aureus (OS) No growth
	23	Fistulous tract	B. clostridiiformis ss clostridiiformis F. varium F. necrogenes P. intermedius	γ E. coli (H) S. aureus (L)
	28	Chronic dermatitis Ear: Conjunctivitis:	C. perfringens Eubacterium sp. (E. lentum) Lactobacillus sp. (L. fermentum)	Bacillus sp (L) P. aeruginosa (L)
	30	Allergic sinusitis	P. intermedius C. perfringens	No growth
	33	Fistulous tract	Fusobacterium sp. (F. necrogenes)	E. coli (L) α Streptococci (L)
Bovine	3	Mastitis	Eubacterium sp. C. perfringens	γ E. coli (H)

TABLE 17. Continued

Animal Species	Case No.	Clinical Condition	Anaerobic Isolates[a]	Aerobic and Facultative Isolates[b]
Bovine (cont.)	14	Aborted fetus	P. anaerobius C. perfringens C. butyricum	Pastuerella sp (H) α Streptococci (L)
	27	Stifle joint infection	Fusobacterium sp (F. necrophorum) P. intermedius C. perfringens	No growth
Equine	15	Dermatitis		S. aureus (H) β Streptococci (H)
	32	Fistulous withers	Propionibacterium sp. (P. acnes)	γ E. coli (L) α Streptococci (L)
	40	Sterility	P. intermedius	No growth
Ovine	39	Lymphadenitis: Lung	Actinomyces bovis	P. hemolytica (L) γ Streptococci (L) (L) C. psuedotuberculosis (L)
	42	Necropsy: Intestine	Actinomyces sp.	γ Streptococci (H) (H) C. pyogenes (H) γ E. coli (H)
Swine	43	Abscess	P. anaerobius F. necrophorum Actinomyces sp (2)	No growth
Feline	31	Chronic cystitis	P. intermedius	No growth

[a]Number in parentheses refers to the number of different biotypes of the same species isolated from a specimen.

[b]H = heavy growth; M = moderate growth; L = light growth.

joint yielded an organism most resembling F. necrophorum. The culture, received for identification, was somewhat attenuated from aerobic exposure and was difficult to grow. Pus from a swine abscess also yielded F. necrophorum, in addition to other anaerobic and facultative organisms.

F. necrogenes and F. necrophorum were very similar in cellular morphology, appearing as long, thin cells with tapered ends, but differed slightly in colonial morphology. The cell size of F. varium was significantly smaller, more on a par with that observed for B. clostridiiformis subsp. clostridiiformis and B. fragilis. However, the cells of both Bacteroides species were more pleomorphic; increasingly so with age. Generally, colonial morphologies in this group of organisms were not distinctive (see Table 18).

Gas chromatograms for the five different species of Gram-negative, NSF anaerobes are shown in Figures 14 through 18. The biochemical characteristics and metabolic products for each of the seven isolates appear in Table 19. Some of the key features of these organisms are noted below.

Strain #6 was lost prior to completion of testing but was identified as a Bacteroides sp. on the basis of cellular morphology and the production of major amounts of acetic and succinic acids. While this organism resembled B. fragilis in many respects, a number of essential tests for distinguishing this species were lacking. B. clostridiiformis ss. clostridiiformis was a small, non-motile, Gram-negative rod that produced minor amounts of acetate, propionate, isovalerate, lactate and succinate. Acid was produced from fructose but not from melezitose and amygdalin. F. varium appeared as a very

TABLE 18. Cellular and Colonial Morphology of Non-Spore Forming Bacteria.

Organism	Colonial Morphology	Cellular Morphology
Peptrostreptococcus anaerobius	Small domes colonies (~1.0 mm) translucent, grey in color.	Gram-positive cocci in chains, non-motile, readily decolorized when aged.
Peptostreptococcus intermedius	Small domes colonies (~2.0 mm) opaque, white to greyish in color.	Gram-positive cocci in pairs, chains, non-motile not readily decolorized.
Bacteroides sp. (fragilis)	Small convex colonies (~1.0 mm) translucent, brownish in color.	Small Gram-negative rods occurring singly, in pairs or short chains, non-motile, pleomorphic when aged.
Bacteroides clostridiiformis ss. clostridiiformis	Small convex colonies (~1.0 mm) translucent, greyish in color.	Small spindle-shaped Gram-negative rods, singly or short chains; non-motile.
Fusobacterium varium	Very small colonies (<1.0 mm) translucent, colorless.	Small Gram-negative rods, occurring singly, non-motile.
Fusobacterium necrogenes	Very small colonies (<1.0 mm) translucent, greyish in color.	Long thin Gram-negative rods with tapered ends, non-motile.
Fusobacterium necrophorum	Small convex colonies (~1.0 mm) opaque, white.	Long thin Gram-negative rods with tapered ends, non-motile, pleomorphic when aged.
Eubacterium sp. (limosum)	Small convex colonies (~1.0 mm) translucent, colorless.	Gram-positive rods, non-motile, pleomorphic when aged.
Eubacterium cylindroides	Irregular wrinkled colonies (~3.0 mm) translucent, greyish-grown in color.	Thin Gram-positive rods in chains, non-motile, readily discolorized.
Eubacterium sp. (lentum)	Small convex colonies (~1.0 mm) translucent, colorless.	Very small Gram-positive rods, non-motile.
Propionibacterium sp. (acnes)	Very small colonies (<1.0 mm) opaque, white.	Short Gram-positive rods (coccobacilli) non-motile.
Actinomyces bovis	Small flat colonies (~1.0 mm) opaque, white.	Gram-positive, highly pleomorphic rods non-motile.
Actinomyces sp.	Small flat colonies (~1.0 mm) opaque, white	Gram-positive rods, highly pleomorphic, non-motile.
Lactobacillus sp. (fermentum)	Small flat colonies (~1.0 mm) translucent, colorless.	Thin Gram-positive rods, non-motile.

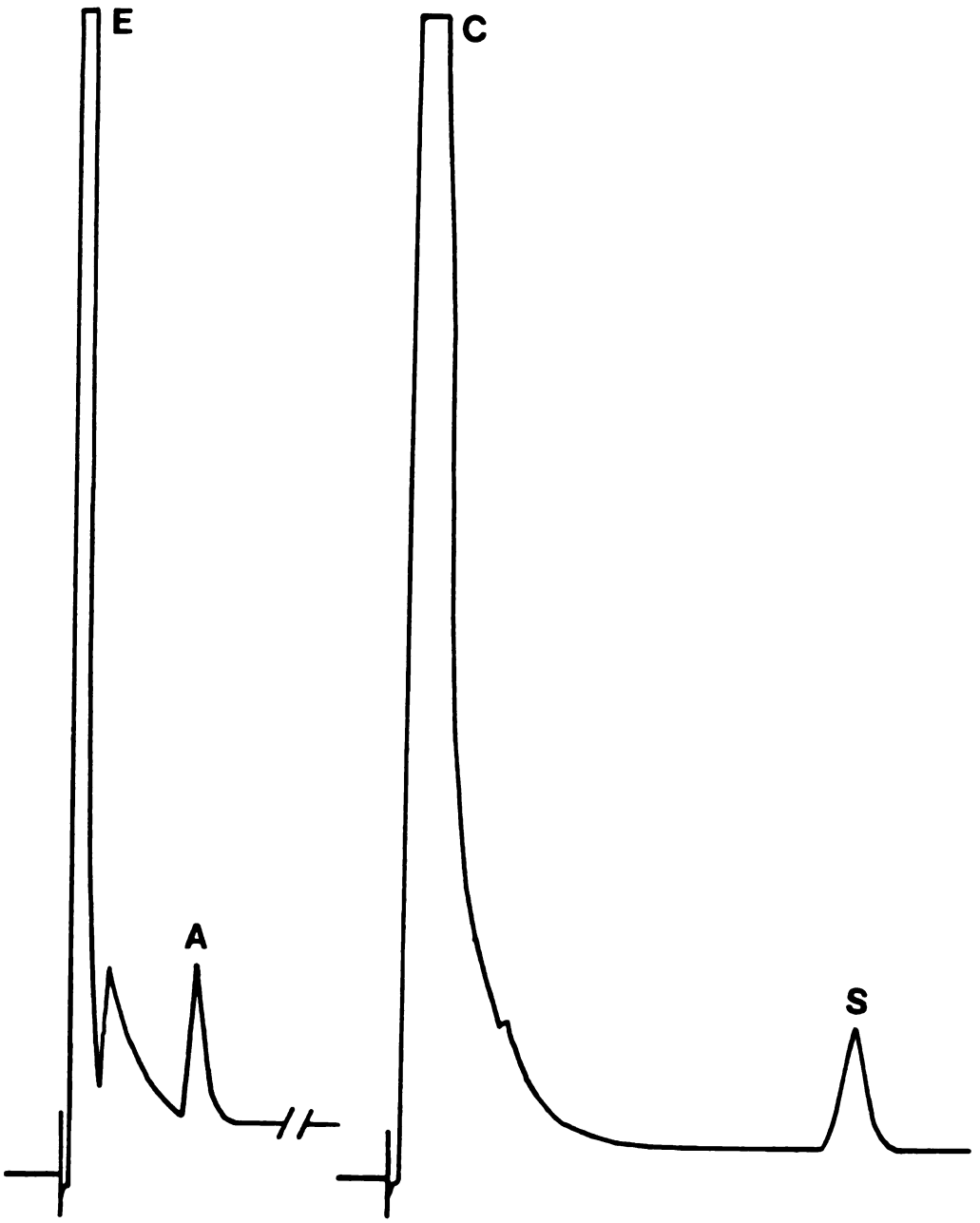

Fig. 14. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of Bacteroides sp. (6). For explanation of symbols, refer to p. x.

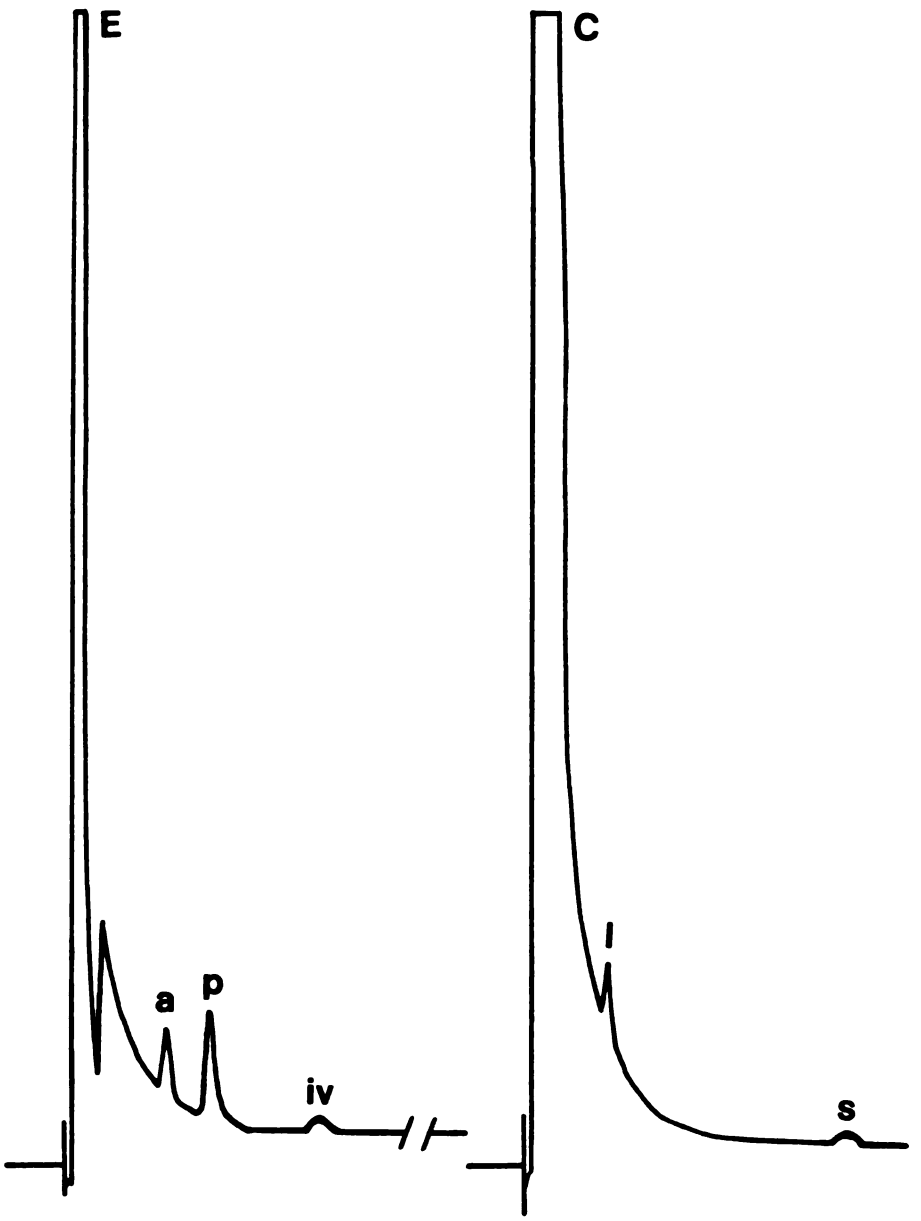

Fig. 15. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of B. clostridiiformis ss clostridiiformis (23a). For explanation of symbols, refer to p. x.

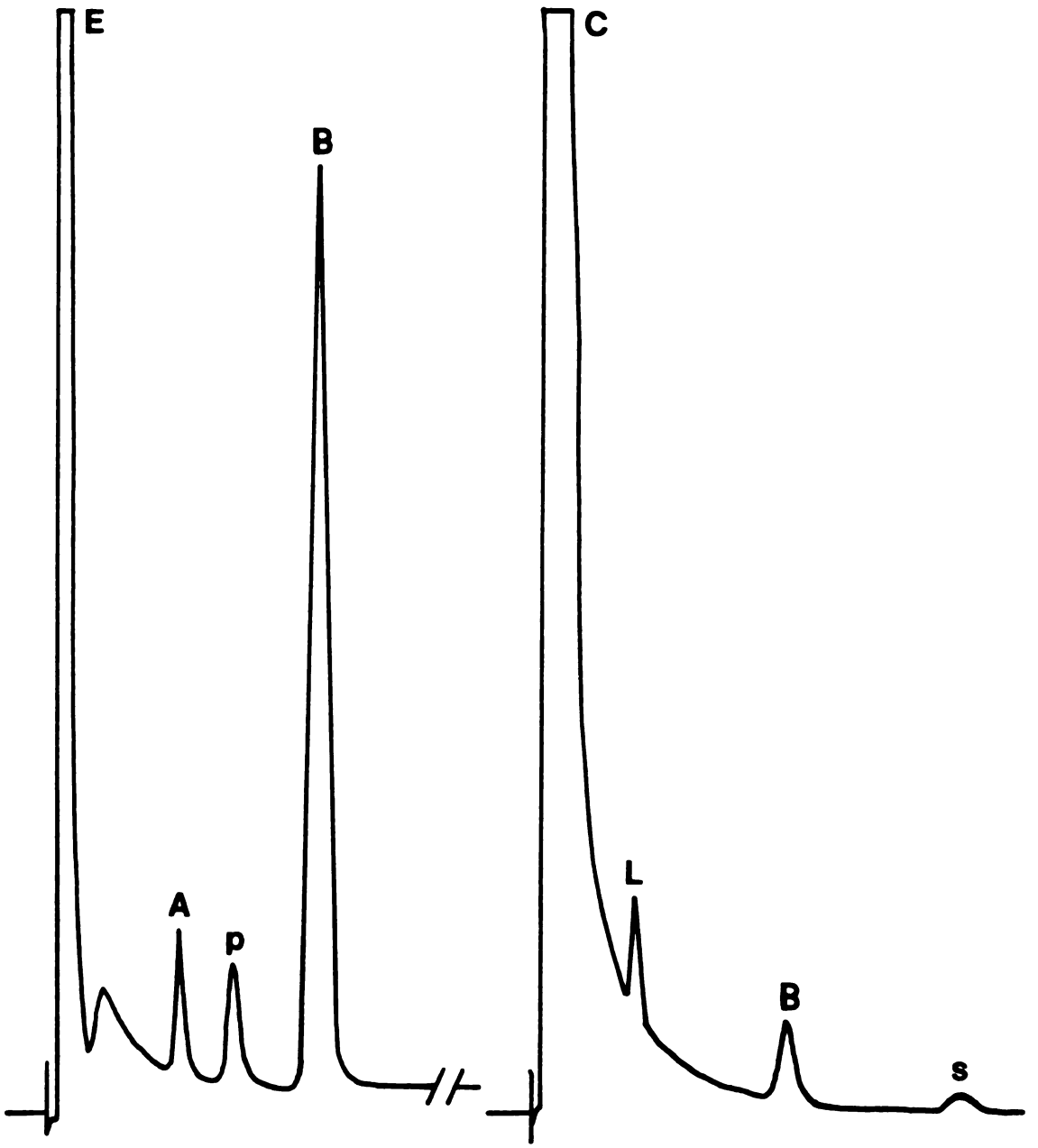

Fig. 16. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of F. varium (23b). For explanation of symbols, refer to p. ix.

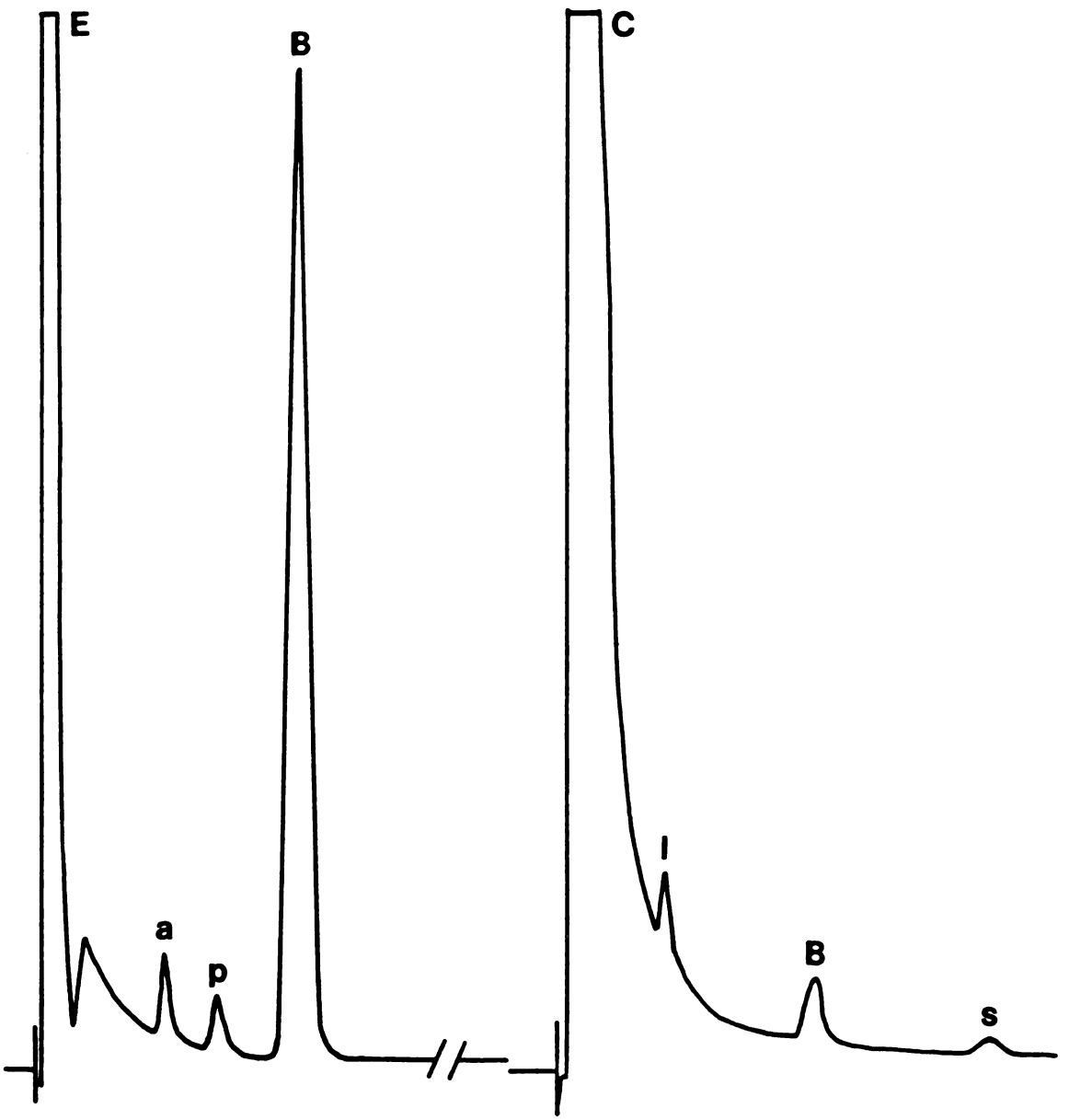

Fig. 17. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of F. necrogenes (#23c). For explanation of symbols, refer to p. x.

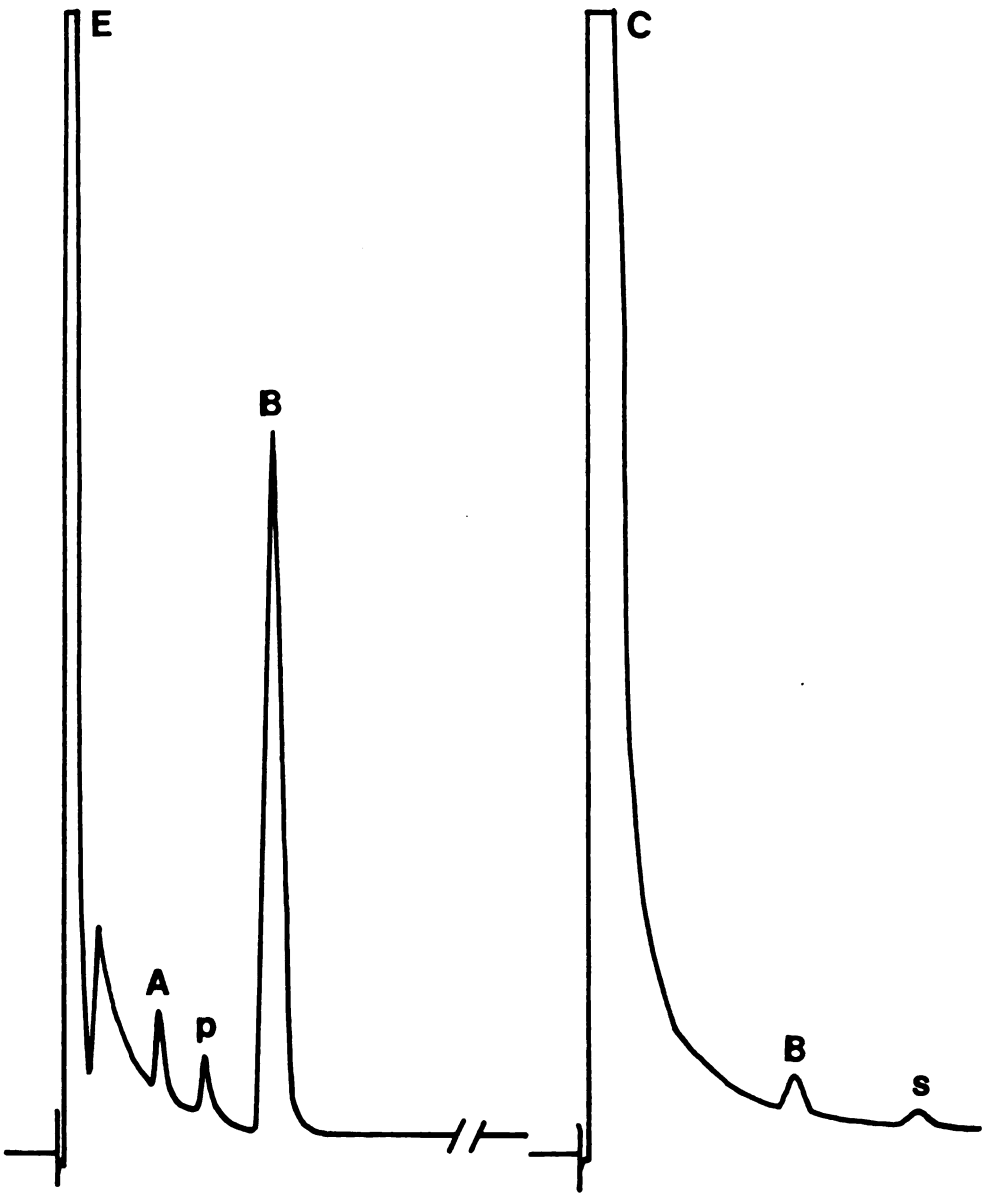

Fig. 18. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of F. necrophorum (#27). For explanation of symbols, refer to p. x.

TABLE 19. Biochemical Characteristics and Acid Metabolic Products of Gram-Negative, Non-Sporing Bacteria.[a]

	Bacteroides sp. (6)	B. clostridiiformis ss clostridiiformis (23a)	Fusobacterium varium (23b)	F. necrogenes (23c)	Fusobacterium sp. (necrogenes) (33)	Fusobacterium sp. (necrophorum) (27)	F. necrophorum (43b)
	Canine					Bovine	Swine
Py Growth		-	-	-	-	-	-
Amygdalin		W	-	-	-	-	W
Arabinose		W	-	-	-	-	-
Cellobiose		A	-	-	-	-	-
Esculin pH	-	-	-	-	-	-	-
hydrolysis	+	+	+	+	+	-	-
Fructose	W	W	A	W	W	-	-
Glucose	A	A	A	W	A	W	A
Lactose	A	A	-	-	-	-	-
Maltose	W	A	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-
Mannose	A	A	A	W	W	-	-
Melezitose		-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-
Raffinose		-	-	-	-	-	-
Rhamnose		W	-	-	-	-	-
Starch pH	W	-	-	-	-	-	-
hydrolysis	+	+	-	-	-	-	-
Sucrose	A	-	-	-	-	-	-
Trehalose		-	-	-	-	-	-
Xylose	A	-	-	-	-	-	W
Gelatin	-	-	-	-	-	-	-
Milk	C	-	-	-	-	C	-
Meat	-	-	-	-	-	-	-
Indol	-	-	+	-	-	+	+
Nitrate	-	-	-	-	-	-	-
Bile growth		-	+	+	+	-	-
Lecithinase		-	-	-			-
Lipase		-	-	-			+
Hemolysis	-	β	-	-	-	α	α
Motility	-	-	-	-	-	-	-
Lactate		-	-	-			P
Threonine		-	P	P			P
Growth stim.		H	BH	BH			
Acid end products	AS	apivls	ApBLs	apBls	apBls	apBs	apBs

[a]For explanation of symbols refer to p. ix.

small, non-motile, Gram-negative rod that produced acetate, butyrate and lactate in major amounts with minor amounts of propionate and succinate. Indol was positive and propionate was produced from threonine, but not from lactate. The two strains of F. necrogenes (#23c, #33) were long, slender, Gram-negative rods with tapered ends that produced large amounts of butyrate with trace amounts of acetate, propionate, lactate and succinate. Propionate was produced from threonine but not from lactate and indol was negative. The two strains of F. necrophorum (#27, #33) both appeared as long, thin, Gram-negative rods that became increasingly pleomorphic with age. Butyrate was the sole major acid produced, along with minor amounts of acetate, propionate and succinate. Strain #27 was lost prior to completion of testing but was tentatively identified as F. necrophorum on the basis of a positive indol test; negative results for esculin hydrolysis, lactose and mannose; and coagulation in milk. Strain #43b characteristically produced propionate from lactate and threonine, and was positive for lipase.

Gram-Positive Non-Sporing Bacteria

Eight clinical cases were positive for Gram-positive NSF bacteria, yielding a total of 10 strains; (5) Actinomyces , (3) Eubacterium, (1) Lactobacillus, and (1) Propionibacterium (Table 17).

Only one strain of Actinomyces, isolated from a canine eye disorder, fitted the classical description of A. bovis. On the basis of starch hydrolysis, a second strain (isolated from ovine lymphadenitis) was also identified as A. bovis, although the biochemical test results were skewed due to apparent utilization of nutrients in

the basal medium. Three other strains of Actinomyces, from sheep intestine (1) and swine abscess (2), did not key to any known species. They were designated Actinomyces on the basis of the characteristic acid end products produced and on the distinctive cellular morphology observed. E. cylindroides and an organism resembling E. limosum were isolated from cases of canine pyoderma and bovine mastitis, respectively, both in conjunction with strains of C. perfringens. Organisms very similar to E. lentum and L. fermentum were isolated from a case of conjunctivitis in a dog. A strain of Propionibacterium, most similar to P. acnes, was isolated from a case of fistulous withers in a horse, in addition to several facultative organisms.

The cellular morphology of Actinomyces, as noted previously, was very distinctive; appearing as a tangled mass of Gram-positive rods. The colonial morphology was similar for all strains of Actinomyces isolated. E. cylindroides and E. limosum were similar in cellular morphology (thin Gram-positive rods) but were distinguished by very different colonial morphologies (see Table 18). E. lentum was similar to E. limosum in colonial appearance, but was easily distinguished from the latter by its very small cell size. The P. acnes isolate was a Gram-positive cocco-bacillus while L. fermentum was a medium-sized, thin, Gram-positive rod. All were non-motile.

The biochemical characteristics and metabolic products of the isolates are given in Table 20. Typical gas chromatograms for the different species are presented in Figures 19 through 24. Salient characteristics of these organisms are noted below.

The Actinomyces isolates were distinguished on the basis of cellular morphology as well as on their production of acetic, formic,

TABLE 20. Biochemical Characteristics and Acid Metabolic Products of Gram-Postive, Non-Sporing, Rod-Shaped, Bacteria.[a]

	Eubacterium cylindroides (18b)	*Actinomyces bovis* (19)	*Eubacterium* sp. (Tentum) (28b)	*Lactobacillus* sp. (fermentum) (28c)	*Eubacterium* sp. (Timosum) (36)	*Propionibacterium* sp. (acnes) (32)	*Actinomyces bovis* (39)	*Actinomyces* sp. (42)	*Actinomyces* sp. (43)	*Actinomyces* sp. (43d)
	Canine				Bovine	Equine	Ovine		Swine	
Py Growth	-	-	-	-	-	-	W	-	-	-
Amygdalin	-	-	-	-		-	W	W	W	-
Arabinose	-	-	-	-		-	W	A	W	A
Cellobiose	A	-	-	-	W	-	W	-	W	A
Erythritol	-	-					W	W	A	W
Esculin pH	A	-	-	-	W	-	W	W	A	W
hydrolysis	+	-	-	-	+	-	-	+	+	-
Fructose	A	A	-	-	A	-	W	A	W	A
Glucose	A	A	-	A	A	W	A	A	A	A
Glycogen	-	-					A	A	A	A
Inositol	-	-					W	A	A	A
Lactose	-	-	-	A	-	-	A	A	W	W
Maltose	-	W	-	A	-	-	A	A	A	A
Mannitol	-	-	-	-	A	-	W	A	-	A
Mannose	A	A	-	-	-	-	W	W	W	W
Melezitose	-	-	-	-		-	W	W	W	W
Melibiose	-	-	-	-	-	-	W	W	W	W
Raffinose	-	-	-	A		-	A	W	W	-
Rhamnose	-	-	-	-		-	A	-	-	W
Ribose	A	W					W	W	A	A
Salicin	A	-					W	A	A	A
Sorbitol	-	-					W	A	A	A
Starch pH	-	-	-	-	W	-	A	A	A	-
hydrolysis	-	+	-	-	-	-	+	-	-	-
Sucrose	-	-	-	-	-	-	W	-	W	W
Trehalose	-	-	-	-		-	A	-	W	W
Xylose	-	-	-	A	W	-	W	W	A	A
Gelatin	-	-	-	-	-	-	+	-	-	-
Milk	-	c	-	-	-	-	c	c	-	-
Meat	-	-	-	-	-	-	-	-	-	-
Indol	-	-	-	-	-	-	-	-	-	-
Nitrate	+	+	+	-	-	+	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-
Lecithinase	-	-					-	-	-	-
Lipase	-	-					-	-	-	-
Hemolysis	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-
Gas	2+	-	-	-	-	-	-	-	-	-
Acid end products	AB	AFLS	al	aLs	AbLs	aP	AFLS	AFLS	AFLS	AFLS

[a]For explanation of symbols refer to p. ix.

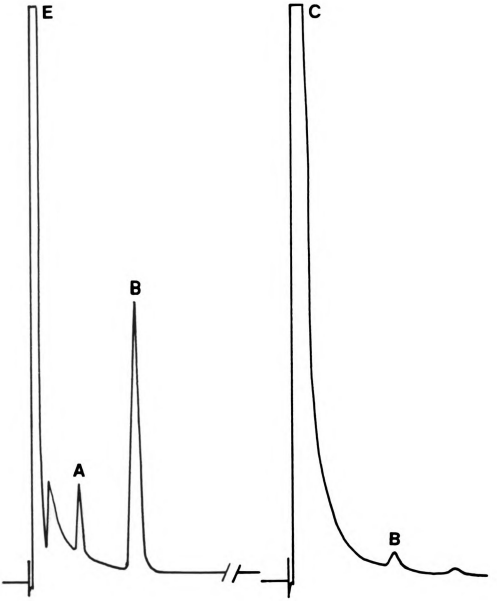

Fig. 19. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of E. cylindroides (#18b). For explanation of symbols, refer to p. x.

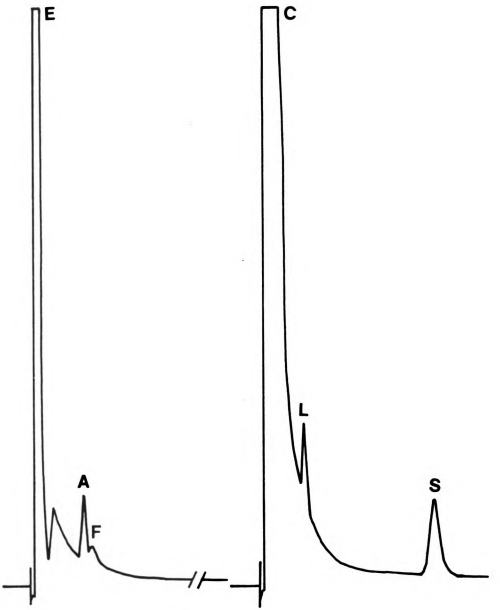

Fig. 20. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of A. bovis (#19). For explanation of symbols, refer to p. x.

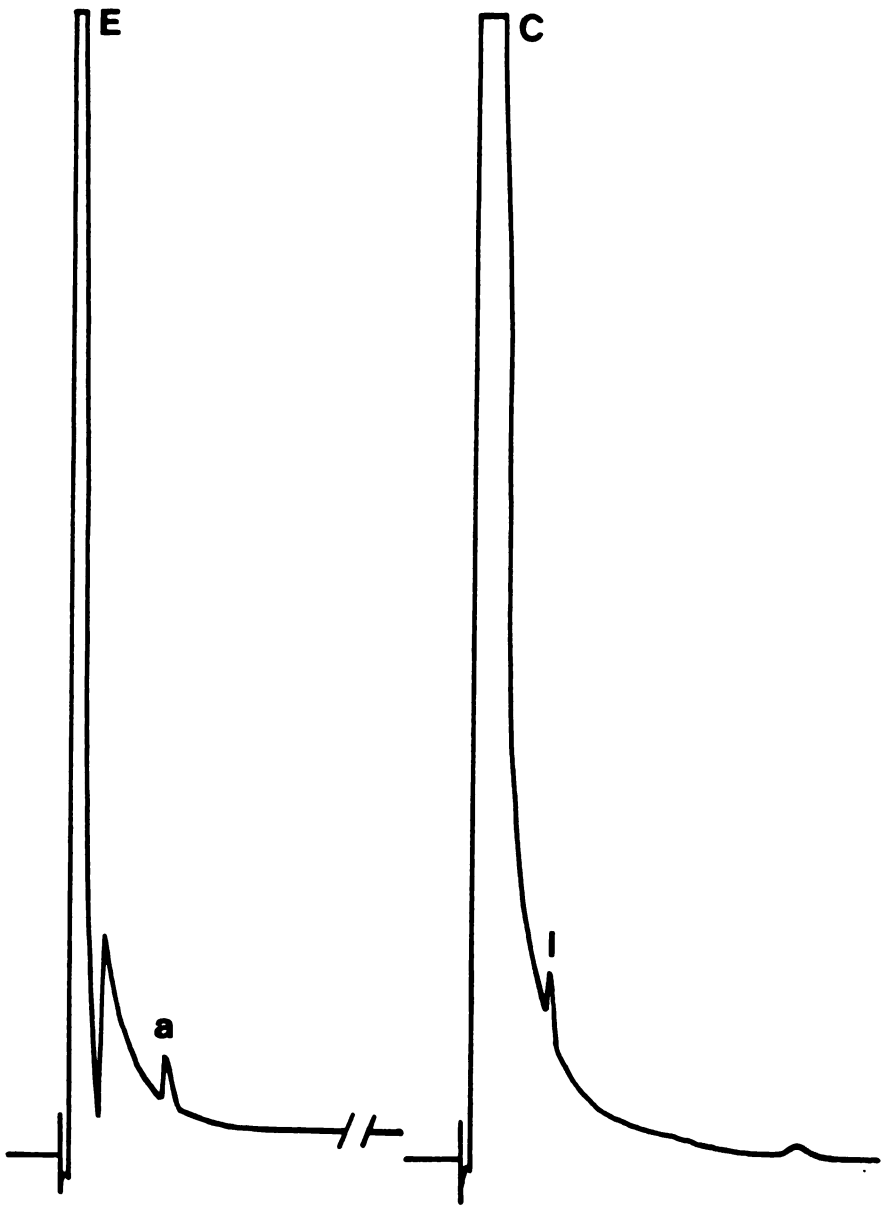

Fig. 21. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of E. lentum (#28b). For explanation of symbols, refer to p. x.

Fig. 22. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of L. fermentum (#28c). For explanation of symbols, refer to p. x.

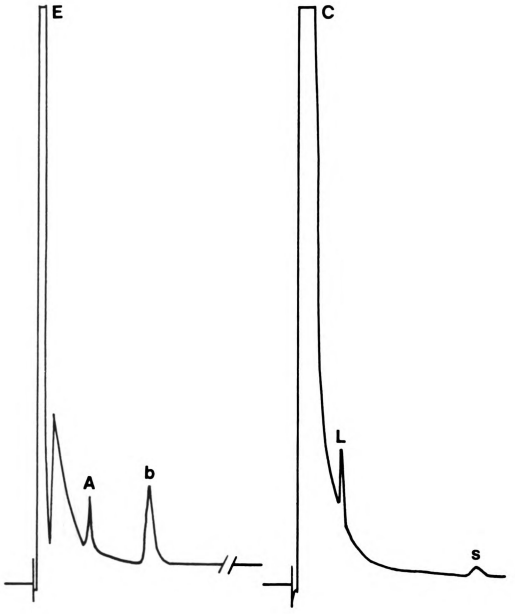

Fig. 23. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of E. limosum (#36). For explanation of symbols, refer to p. x.

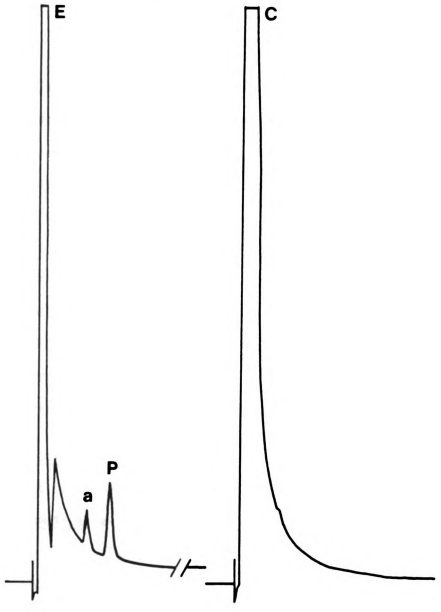

Fig. 24. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of P. acnes (#32). For explanation of symbols, refer to p. x.

lactic and succinic acids. E. cylindroides was a small, readily decolorized, Gram-positive rod that produced major amounts of acetate and butyrate only. The organism was negative for lactose, maltose, mannitol and indol, and was positive for esculin hydrolysis, fructose and mannose. Strain #28b was tentatively identified as E. lentum on the basis of cellular morphology, the production of trace amounts of acetate and lactate only, and its failure to ferment fructose. Strain #36 was identified as E. limosum on the basis of the acid end products produced (major amounts of acetate and lactate, minor amounts of butyrate and succinate), the production of acid in mannitol and failure to produce any change in milk. Strain #28c was tentatively identified as L. fermentum on the basis of the acid end products produced (large amounts of lactic acid with trace amounts of acetate and succinate), and negative results for esculin hydrolysis, mannose and sucrose. Strain #32 was tentatively identified as P. acnes because it produced major amounts of propionate and minor amounts of acetate and failed to utilize most substrates tested.

Anaerobic Cocci

Seven clinical cases yielded five strains of Peptostreptococcus intermedius, and two strains of P. anaerobius. P. intermedius was isolated from a fistulous tract infection in a dog (as noted earlier), along with a number of other anaerobic and facultative organisms. It was isolated, in association with strains of C. perfringens, from cases of canine sinusitis and equine dermatitis. P. intermedius was isolated in pure culture from cases of equine sterility and chronic cystitis in a cat. P. anaerobius was isolated

from an aborted bovine fetus and a swine abscess. In both cases, it was found in association with several other anaerobic organisms (see Table 17).

The two strains of P. anaerobius were similar in morphology (relatively large Gram-positive cocci in chains) while the P. intermedius isolates showed considerable variation in cell size. One P. intermedius strain (#40) occurred as clusters of cells rather than in chains. All five P. intermedius strains were obligate anaerobes on primary isolation although they became aerotolerant on subculture (see Table 18).

The biochemical and chromatographic test results are shown in Table 21 and Figures 25 and 26. Although strain #40 varied from the other P. intermedius isolates in weakly fermenting esculin and melezitose, this was still within the range of results previously reported for this species (63). Strain #43a of P. anaerobius fermented maltose, which is atypical, but has been observed previously (63).

TABLE 21. Biochemical Characteristics and Acid Metabolic Products of Gram-Positive, Anaerobic Cocci.[a]

	Peptrostreptococcus intermedius (23d)	P. intermedius (30b)	P. anaerobius (14c)	P. intermedius (15b)	P. intermedius (40)	P. anaerobius (43a)	P. intermedius (31)
	Canine		Bovine	Equine		Swine	Feline
PY Growth	-	-	-	-	-	-	-
Cellobiose	A	A	-	A	W	-	A
Esculin pH	-	-	-	-	W	-	-
hydrolysis	+	+	-	+	+	-	+
Fructose	A	A	W	A	W	W	A
Glucose	A	A	A	A	W	A	A
Lactose	A	A	-	A	A	-	A
Maltose	A	A	-	A	W	A	A
Melezitose	-	-	-	-	W	-	-
Sucrose	A	A	-	A	W	-	A
Gelatin	-	-	-	-	-	-	-
Indol	-	-	-	-	-	-	-
Nitrate	-	-	+	+	-	-	-
Catalase	-	-	-	-	-	-	-
PYG-Tween	-	S	S	S	S	-	-
Threonine	-	-	P	-	-	P	-
Acid End Products	aL	aL	Apibbiv icls	aL	aL	Apibbiv icls	aL

[a]For explanation of symbols refer to p. ix.

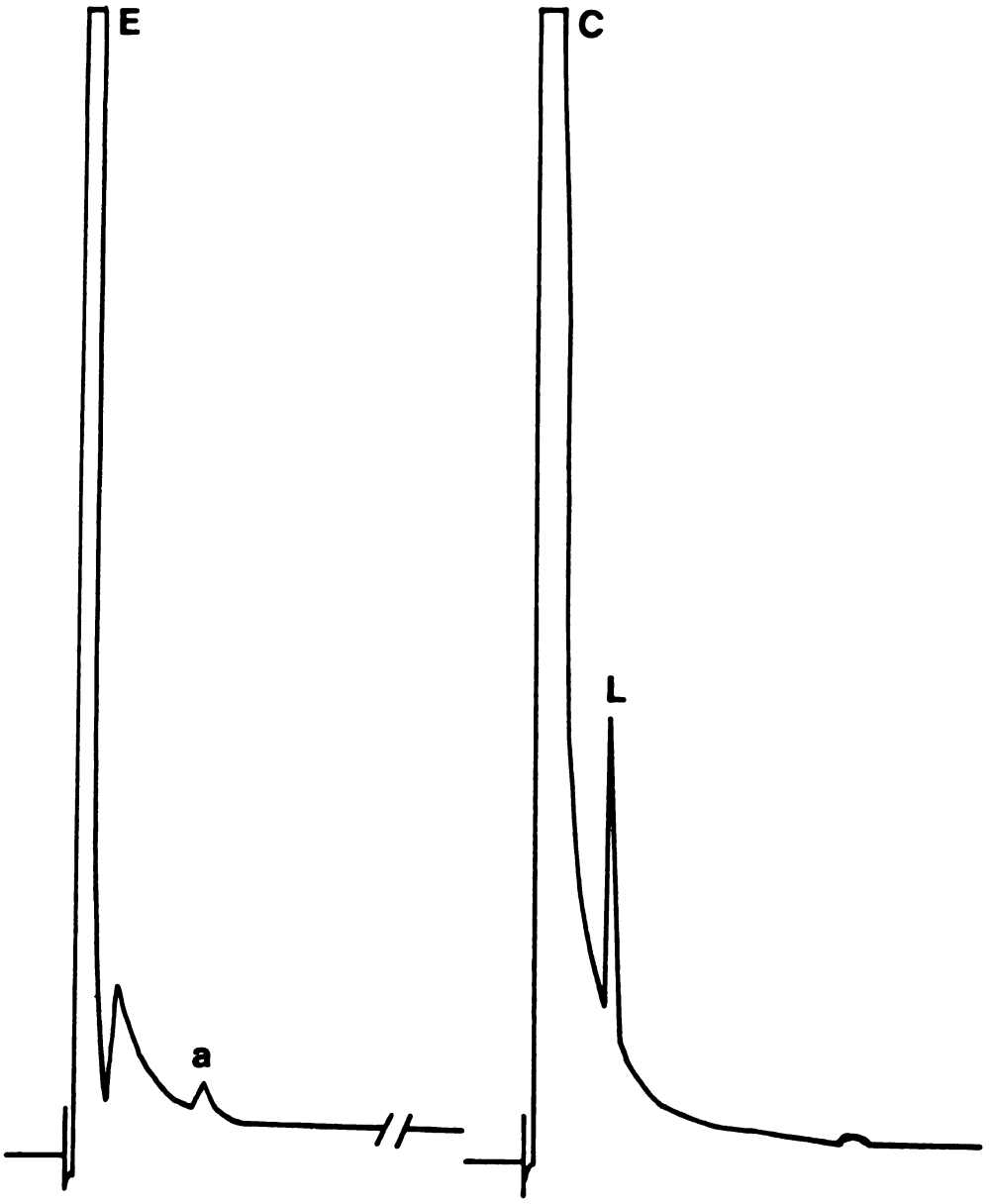

Fig. 25. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of P. intermedius (#23d). For explanation of symbols, refer to p. x.

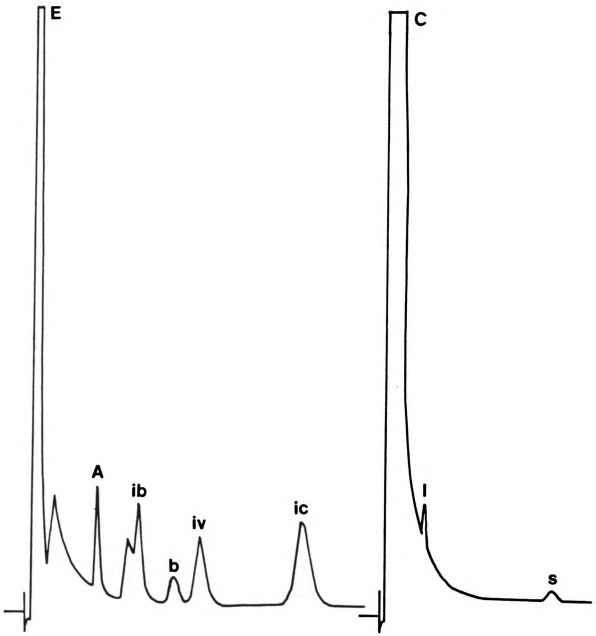

Fig. 26. A gas chromatogram showing the volatile (left) and non-volatile (right) acid end products of P. anaerobius (#30b). For explanation of symbols, refer to p. x.

V. DISCUSSION

Recently there have been several studies pertaining to the isolation and identification of anaerobes in diseases of animals (7, 9, 38, 39). Berg and Fales (7) investigated the anaerobic flora of selected canine lesions. They reported that anaerobic bacteria of the following genera were commonly isolated: Actinomyces, Bacteroides, Clostridium, Fusobacterium, Peptostreptococcus, and Propionibacterium. While they did not report precise numbers, they did report a relatively high incidence of Clostridium species. Berkhoff and Redenbarger (9) in a similar study, investigated the incidence of anaerobes in a variety of veterinary clinical specimens obtained primarily from dogs, cattle, horses, and swine. Anaerobes were isolated in the following proportions: Clostridium sp., 50%; Gram-negative NSF rods, 19%; Gram-positive NSF rods, 19%; Actinomyces sp., 10%; and anaerobic cocci, 1%. However, in this report no correlations were drawn between the animal source, the type of specimen, the kinds of anaerobes isolated, and whether they were present in pure or mixed culture.

In agreement with the above studies, the results of the present investigation revealed a higher incidence of Clostridium sp. in infections of animals than has been found in man (35, 49, 111). Further, the incidence of anaerobic Gram-negative rods (particularly

B. fragilis) was found to be much lower in animal infections than in humans. Many of the anaerobic species isolated during the course of this study have previously been reported to be a part of the normal gastrointestinal flora of these animals (18, 63) and have also been shown to be associated with human infections (35, 43, 44, 45, 111).

Clostridial infections generally occur more frequenly in animals than in man because clostridia are ubiquitous in soil and animal quarters, and many animals harbor clostridial spores on their hair or skin (115). The clostridia may invade through breaks in the skin or open wounds and cause various superficial infections.

C. perfringens was frequently isolated from a number of different clinical disorders in animals during the course of this study. This organism has also been the most common clostridial isolate from a variety of infections in humans (35, 111). C. perfringens has been associated with a number of relatively harmless and self-limiting infections and has often been isolated in the absence of disease entirely (49). However, this organism has also produced serious and often fatal illnesses in both humans and animals (35, 111, 115).

Dogs appear particularly susceptible to C. perfringens dermatitis, conjunctivitis, and otitis. There were no previous reports of isolation of C. perfringens from these sites in dogs or in other animals. Interestingly, veterinarians specified anaerobic culture in these cases, with screening for C. perfringens often requested. In a preliminary sampling, neither C. perfringens nor any other anaerobe could be detected in fresh swabs from the conjunctivae of three healthy dogs. C. perfringens was previously isolated from human cases of

pyodermatitis and conjunctivitis and was shown to be frequently associated with otitis media and mastoiditis (35). The results suggest that C. perfringens may be very significant in infections of the eyes, ears and skin of dogs.

C. perfringens was isolated in pure culture, with no other anaerobic or facultative organisms present, in cases of pododermatitis and thoracic effusion in dogs. The isolation of C. perfringens in canine pododermatitis may have been significant as the organism could well have gained access by minor trauma, into the animal's foot. In the case of thoracic effusion, the isolation of C. perfringens may be of more interest. C. perfringens has been isolated from a number of thoracic infections of man, particularly those infections following surgical or accidental wounds (35, 111). There was nothing in the clinical history of this animal to indicate whether trauma or some other pre-disposing factor was involved. However, considering the pathogenic potential of this organism in humans and animals, it s isolation in this case appears significant. As no other organisms were cultured in either of these cases, it is unlikely that the C. perfringens isolates were picked up in sampling as contaminants.

Of the other clostridial isolates in dogs, C. carnis and C. sordellii have previously been reported in animal infections (18, 115). C. sordellii is more commonly found in liver infections of cattle and sheep, but has been found in contaminated wounds of these species as well (115). C. sphenoides has not been reported in animals, but was isolated from infected wounds in man (18). The significance of its presence in extracted tooth material of a dog cannot be determined at the present time, although it is generally believed that clostridia are

not present as a significant portion of the normal flora in the mammalian oropharynx (35). C. barati, also known as C. paraperfringens, has never been reported in clinical conditions in man or animals. Isolation of this organism in pure culture from an ear infection in a dog suggests that this organism may occur as a natural pathogen in canines.

Consistent with previous reports (18, 115), C. perfringens was found in several cases of suspected bovine enterotoxemia and in one case of malignant edema. From one case of suspected enterotoxemia (#44), C. botulinum (non-prot. BEF), C. tertium, and C. bifermentans were isolated, in addition to C. perfringens, of which no one organism predominated in the specimen. The isolation of C. perfringens is of dubious significance in this case, as an apparently normal spectrum of clostridia and facultative anaerobes was found in the intestinal contents of this animal. C. perfringens and C. botulinum (non-prot. BEF) are both found in the normal intestinal flora of a wide variety of birds and mammals (18, 63). C. tertium has been found in human feces (18) and may well be present in the normal flora of other animal species. However, this is the first reported isolation of this organism from the GI tract of an animal. C. bifermentans is known to occur in the normal flora of ruminants, and has been isolated from several human and animal infections (63).

C. perfringens was also isolated from several cases of bovine mastitis. Bovine mastitis due to C. perfringens has not previously been reported. This organism appears particularly significant in case #17. In this case, anaerobic culture alone was requested and clostridial infection was strongly suspected by the clinician.

Although C. perfringens predominated in the specimen, C. glycolicum was also isolated. C. glycolicum has never been reported from infections in animals although it has been isolated from soil and certain human clinical infections (18). In this case, it is not clear whether C. glycolicum was acting as a pathogen, opportunist, or a contaminant picked up in sampling.

The significance of the isolation of C. perfringens from an aborted bovine fetus (#14); is difficult to assess because it was present in association with C. butyricum, P. anaerobius, and large numbers of Pasteurella sp. C. perfringens has been found in the normal flora of the human female genitourinary tract, as well as in numerous genital infections (46, 49, 71). C. butyricum has been found both in human wounds of traumatic origin and in animal feces (18). P. anaerobius is commonly found in the normal human vaginal flora and (less often) in infections of the female genital tract (26, 35, 36). The pathogenicity of this organism for animals has not been documented.

The isolation of several clostridial species (C. perfringens, C. beijerinckii, and C. botulinum non-prot. BEF) from cases of bovine and equine infertility may or may not be significant depending upon the care with which specimens were taken. Several clostridial species have been found in the normal human vaginal and cervical flora, as well as in uterine infections (26, 35, 49). Normally, the human uterus is devoid of bacterial contamination and the same should be true of animals. Although C. perfringens has not previously been isolated from cases of human infertility, a low-grade infection with this organism might preclude conception in animals. C. beijerinckii, often found in soil, has also been reported as a rare clinical isolate in

humans (18). The significance of C. botulinum (non-prot. BEF), isolated from a case of equine infertility, is not clear as this has been the only reported isolation from an animal so far. The organism is not known to be associated with any human infections.

C. perfringens has been reported to be a cause of enterotoxemia in foals (115) and in this study was isolated from a young horse suffering from diarrhea. C. perfringens was also found in an eye disorder and dermatitis in older horses. This probably reflects a susceptibility of equines to skin and eye infections by C. perfringens, similar to that noted earlier in dogs; however, it is conceivable that the isolates were soil contaminants acquired during specimen collection.

C. perfringens was isolated twice from the hock joints of two necropsied swine. In the first case, C. ramosum was also isolated but no aerobic growth was obtained in either case. C. perfringens is known to occur in a number of soft tissue, muscle, and bone infections in man and animals, particularly following trauma (35, 104, 108). C. ramosum is second only to C. perfringens in the frequency with which it is isolated from clinical cases in humans (35). Reportedly, this organism also occurs in animal infections (18) and in the normal flora of swine (63). Therefore, the isolation of C. perfringens in pure culture in one case and in mixed culture with C. ramosum in a second case may be of considerable pathogenic significance. It is also possible, however, that both of these organisms were post-mortem invaders.

The isolation of C. perfringens from a case of feline conjunctivitis may reflect the predisposition of cats to eye infections by this organism, similar to that observed in dogs and horses.

In the rabbit and chicken necropsy cases, the significance of the isolation of C. perfringens cannot be determined. The isolates may represent post-mortem invaders which were originally a part of the normal flora or may have caused a fatal septicemia or toxemia in these species. Throughout the course of this study, typing of C. perfringens was not done due to a lack of facilities here. Toxin typing would have been of considerable help in determining the pathogenicity of the C. perfringens isolates.

The anaerobic NSF bacteria isolated in this study included organisms previously isolated in conjunction with animal disease (63, 115) as well as several species which have never before been reported to occur in animals.

B. fragilis is reported to be the most frequently isolated anaerobe from soft tissue infections of humans (35). A similar Bacteroides sp. was isolated from a case of canine conjunctivitis. Berkhoff and Redenbarger (9) reported isolations of several Bacteroides sp. in their study, but did not indicate the source animals, the clinical conditions or the biochemical characteristics for any of their isolates. As the recognition of anaerobic infections in animals increases and stricter anaerobic techniques are utilized, more isolations of this organism may be reported from animals. Regrettably, however, veterinary diagnostic laboratories still identify anaerobic rods found in clinical specimens primarily on the basis of morphology. Gram-negative cells with pointed ends are usually reported as Fusobacterium sp., while those with rounded ends are reported as Bacteroides.

Several anaerobic, Gram-negative NSF rods (B. clostridiiformis ss. clostridiiformis, F. varium, and F. necrogenes) were isolated from a fistulous tract in a dog. The infection apparently stemmed from a blow to the side, which resulted in a swelling that later opened and began draining. When surgically debrided, the fistulous tract was traced back to the abdominal cavity. F. necrogenes was isolated again from a post-operative specimen collected from this animal. B. clostridiiformis ss. clostridiiformis (now thought to belong in the genus Clostridium [18]) has been found in abscesses and the lower intestinal tract of various animal species as well as man (18, 63). F. varium has been reported from a number of purulent infections in man (notably necrotizing fascitis) but this is the first time that it has been reported from an animal infection. F. necrogenes was first isolated from a necrotic abscess in a chicken but has not been reported in any animal infection. Both of the above fusobacteria have reportedly been found in the intestinal flora of man and several animal species (18, 63).

The cultivation of F. necrophorum from a bovine stifle joint and a swine abscess (#43), is consistent with the kinds of infection reportedly caused by this organism in man and lower animals. F. necrophorum is found in many necrotic lesions in a variety of warm-blooded animals, with occasional joint involvements (16). It is also a part of the normal GI flora in ruminants and swine (63). Characteristic infections include bovine liver abscesses and foot-rot in cattle and sheep (8, 99, 106, 107); although this organism causes a number of different animal infections collectively termed "necrobacilloses." In man, F. necrophorum is the most common anaerobic isolate in joint

infections, although it is also associated with a number of other purulent, necrotizing and/or metastatic diseases of man (35).

P. anaerobius and two strains of Actinomyces were also isolated from the above mentioned swine abscess (#43). The location of the abscess was not stated in the clinical history; however, a large volume of pus was submitted for examination, indicating an abscess of substantial size. The known pathogenic role of P. anaerobius in purulent infections of both man and animals (35, 63), as noted above, makes the isolation of this organism significant. Actinomyces species are known for the production of chronic low-grade infections. These are characterized by granulomatous lesions that break down to form draining abscesses (16). Actinomyces have been isolated from suppurative processes in humans in association with a number of microaerophilic and/or anaerobic organisms; one of the more common being Fusobacterium sp. (98). In swine, Actinomyces commonly localize in the mammary gland, although pulmonary actinomycosis has also been reported (16).

A. bovis, isolated as the predominant organism from a case of subepithelial keratopathy in a dog, may have been significant in the pathogenesis of this condition. This animal was reported to be immunologically deficient and subsequently responded to a combined steroid-antibiotic therapy. Actinomyces have not previously been reported from eye infections in animals; however, this organism has been reported in lacrimal canal infections in man (35). A. bovis was also isolated from a case of lymphadenitis in sheep in this study. Actinomyces are known to cause chronic nodular bronchopneumonias in sheep, which may have contributed to the lymphadenitis observed (16). The significance of the isolation of an Actinomyces sp. from

the intestinal contents of a necropsied sheep could not be determined as no clinical history was available.

The eubacteria isolated in this study were all found in association with C. perfringens. E. cylindroides, isolated from a case of canine pyoderma, has occasionally been reported in human clinical material (35, 63). This organism has been reported in the canine GI tract, as a part of the normal flora (63). Berkhoff and Redenbarger (9) reported that 2.6% of their anaerobic isolates from animal infections were eubacteria but did not mention the type or source of specimen or the characteristics of the organisms isolated.

An organism similar to E. lentum was isolated from a case of canine conjunctivitis, in conjunction with L. fermentum. The animal suffered from chronic facial dermatitis and a strain of C. perfringens was isolated from the animal's ear. Neither species, E. lentum or L. fermentum, has been reported from any animal infections before. E. lentum commonly occurs in a number of purulent, inflammatory processes of man, as well as in the normal human flora (35), but has not been reported in any animal species. L. fermentum is a rare isolate in human disease and has been found in the normal flora of man and several animal species (35, 63). Considering the pathogenicity of these organisms in man, their isolation in this case is significant, despite the fact they have not previously been reported in conjunctivitis.

E. limosum, isolated from a case of bovine mastitis, normally occurs in the rumen. This is the first reported occurrence of this organism in animal infections, although it has been reported from a number of cases of human peritonitis and other abdominal disorders

(35). In this particular case of mastitis, C. perfringens and large numbers of E. coli (non-hemolytic) were also isolated. The significance of the isolation of E. limosum is uncertain at this time since E. coli by itself has previously been shown to be a causative agent of bovine mastitis (16).

A case of fistulous withers in a horse yielded an organism similar to P. acnes as the sole anaerobic isolate. Small numbers of non-hemolytic E. coli and alpha-hemolytic streptococci were also isolated from this specimen. Later, the animal was found to have brucellosis and was euthanized. These results are in agreement with those of Smith (111) who reported that P. acnes is almost always found in association with a Brucella sp. from fistulous withers in horses.

All five strains of P. intermedius isolated during the course of this study were strictly anaerobic on primary isolation, although they rapidly became aerotolerant on subculture. Aerobic streptococci were not detected in any of these cases. In only one case (#15), a beta-hemolytic streptococcus was isolated by routine methods while a non-hemolytic P. intermedius strain was isolated anaerobically. The taxonomic validity of P. intermedius has been questioned (18), as the development of aerotolerance and variation in strains would seem to indicate a diverse group of anaerobic to microaerophilic streptococci. However, the designation may have clinical value considering the inadequacy of aerobic techniques for primary isolation.

Although P. intermedius is often detected in human clinical material, its pathogenicity to humans has not been satisfactorily demonstrated. The organism is ubiquitous in the normal human flora and has been reported in animals as well (35, 63). As P. intermedius was

found in mixed culture with other potential pathogens in three cases (canine fistulous tract infection and sinusitis, and equine dermatitis) the significance of the organism in these cases is not clear. The organism may or may not have contributed to the pathologic conditions cited and could have been present as a contaminant from the normal flora only. In two cases, equine sterility and feline chronic cystitis, P. intermedius was isolated in pure culture and thus may be of greater significance. In the case of feline chronic cystitis, several aerobic cultures were negative while the anaerobic culture promptly yielded a pure culture of P. intermedius.

The high incidence of Clostridium species, specifically of C. perfringens, noted in this study may be more a reflection on the types of specimens received for study than on the anaerobic techniques used. Specimens that would ordinarily be excluded from anaerobic culture, such as gastrointestinal contents, were examined at the request of clinicians. The anaerobes found in the specimens processed are certainly valid isolations. However, where a single anaerobe was isolated it is not certain whether the organism was present as a pure culture or whether some of the more strictly anaerobic organisms died in transport. In spite of this possible deficiency, a number of strictly anaerobic and possibly significant organisms were isolated from the specimens submitted. However, as is true of most anaerobic isolates from humans, the pathogenic significance of the isolates obtained in this study is not clear. Further serologic and toxigenic studies would be necessary to determine the pathogenicity of these isolates. As many of these organisms are presently being maintained as stock cultures, these studies could be pursued further. A continuing

study of eye, ear and skin infections in dogs due to C. perfringens may be particularly worthwhile.

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