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Enumeration and Low Temperature Storage of Obligately Anaerobic Bacteria in Convenience Foods

Craig Edward Bremmon

ENUMERATION AND LOW TEMPERATURE STORAGE
OF OBLIGATELY ANAEROBIC BACTERIA IN CONVENIENCE FOODS

CRAIG EDWARD BREMMON

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser $\cup$ Date

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Head, Microbiology Department   Date

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INTRODUCTION

As reported by the Food Protection Committee of the National Academy of Sciences, the cause of about half of the foodborne illnesses recorded since 1957 have been dismissed as unknown (30). In the 1976 issue of Foodborn and Waterborne Disease Outbreaks by the Center for Disease Control, it was stated that of 458 foodborne disease outbreaks reported in 1974, no specific etiological agent was determined for 255 or 56% of the cases (6). It is thought that agents not detectable by conventional laboratory methods may be responsible for these undetermined illnesses. Previous work in food microbiology deals with the presence of aerobes, facultative anaerobes, and the two sporeforming obligate anaerobes Clostridium perfringens and Clostridium botulinum. There has been no known previous work in the field of food microbiology which deals with other species of Clostridium and any of the non-spore-forming obligate anaerobes as responsible for foodborne illnesses.

It has long been known that the major source of the facultative anaerobes and Clostridium responsible for the known forms of bacterial food poisoning is the human intestine at levels of 10^{10} cells/g. Within the last three years, obligate anaerobes also have been found in the intestine at levels of 10^{11} cells/g. These obligate anaerobes have been implicated as the pathogens responsible for many of the "sterile abcesses" in humans in the past. These obligate anaerobes have been found to resist 1 hr exposures to room air with little loss in viability thus, demonstrating their ability to be aerobically transferred from feces to food. Studies have shown that the traditional bacterial food poisoning

agents are not destroyed by freezing and are present after thawing in high numbers in many food samples. This might also be true for the sporeforming and non-sporeforming obligate anaerobes as well.

The present study was undertaken to determine the kinds and numbers of obligate anaerobes possibly present in various frozen and refrigerated commercial convenience foods. Also, known numbers of pathogenic species of obligate anaerobes were added to various convenience foods and stored at refrigeration and freezing temperatures to determine their die-off or loss in viability. The conditions investigated included the effects of different organisms, different foods, different temperatures, and various storage time periods.

The results of this investigation demonstrate to food producers as well as consumers that the possibility does exist for the presence of non-sporeforming obligate anaerobes in frozen or refrigerated convenience foods.

LITERATURE REVIEW

According to the Food Protection Committee of the National Academy of Sciences, no specific etiological agent has been determined in about half of the foodborne illnesses recorded since 1957. In a 1976 issue of Foodborne and Waterborne Disease Outbreaks by the National Center for Disease Control, of 458 foodborne disease outbreaks reported, 255 were attributed to unknown causes. This is approximately 56% of the total (6). Although lack of appropriate samples and inadequate laboratory support may be contributing factors, bacterial or other agents not detectable by conventional laboratory procedures are probably responsible for many foodborne illnesses (30).

Centralization of industry which results in mass production and large-scale marketing involves potential microbiological risks on a considerable scale. The increased production of precooked foods which are not subject to cooking by the consumer and of mildly processed products stored for long periods of time at temperatures at or above freezing is the source of these risks (30).

There are at present no published reports in the field of food microbiology dealing with non-sporeforming obligate anaerobes as bacteria responsible for foodborne illnesses. There have however, been a large number of publications about foodborne illnesses in regard to various species of the anaerobic sporeforming genus Clostridium (7, 8, 9, 12, 13, 28, 34, 39). The cause of the second highest recorded number of food poisoning cases in the United States is the sporeforming, obligate anaerobe Clostridium perfringens (19). Although this organism

has been known for some time to be responsible for the pathological condition known as gas gangrene, it is only within the past 30 years that Clostridium perfringens was shown to be a very important food poisoning agent (25).

Pathogenicity of obligate anaerobes. Many of the non-sporeforming anaerobes are pathogenic in that they are responsible for about 4 to 10% of the bacterial abesses and septicemias with a high morbidity rate in man (11, 3). Patients exhibit necrotizing cellulitis and symptoms of generalized toxemia (2). Endotoxic lipopolysaccharides (LPS) have been isolated and purified from genera of non-sporeforming anaerobes including: Veillonella, Fusobacterium, Bacteroides, Leptotrichia, and Sphaerophorus. LPS from Bacteroides melaninogenicus were found to be pyrogenic and produced a local Shwartzman phenomenon in rabbits. Rabbits responded with fever to submicrogram quantities of Veillonella LPS. Purified LPS from Fusobacterium proved to be a potent endotoxin when it was assayed for the capacity to alter the dermal reactivity in rabbits to epinephrine. Evidence also suggests that these bacteria interfere with vitamin B_{12} absorption in the lumen of the small intestine of man (2). Other studies have shown that various anaerobes may actively penetrate the intestinal wall. Thus the organisms presented are pathogenic in several ways. If a food is assumed to be heavily contaminated with one or more non-sporeforming obligate anaerobes, it may be possible that these organisms might be responsible for a foodborne illness.

Obligate anaerobes in the human intestine. Recent studies have shown that large percentages of both sporeforming and non-sporeforming

obligate anaerobes normally reside in the lower human intestinal tract (2, 18, 26, 27, 32). Bacteroides fragilis was the non-sporeforming anaerobe most frequently isolated from human feces (32). Other genera of anaerobic bacteria which were present at 5.0% or less of the total cell count included Eubacterium, Lactobacillus, Butyrivibrio, Fusobacterium, Clostridium, Peptostreptococcus, and Peptococcus (26). Subspecies of Bacteroides fragilis were found to number approximately 10^{11} cells/g (dry weight) (26). The genus Eubacterium numbered 10^9 cells/g, Clostridium 10^{10} cells/g, Bifidobacterium 10^9 cells/g, anaerobic cocci 10^9 cells/g, Escherichia 10^{10} cells/g, enterococci 10^9 cells/g, and other streptococci 10^9 cells/g (26). Thus, it is evident that these pathogenic bacteria are present in enormous numbers in the lower intestine of man.

Intestinal bacteria in convenience foods. Previous studies reported the presence of coliform and other fecal aerobic and facultatively anaerobic organisms in frozen foods (4, 7, 12, 13, 16, 19, 20, 24, 30). Total bacterial counts ranged from 5 x 10^4 to 10 x 10^4/g of frozen meat pies. In most cases, fecal streptococci outnumbered coliform bacteria (20). In a study involving breaded onion rings, coliform bacteria ranged from 90 to 28,000 cells/g with a mean of 3000 cells/g (24). Precooked, fresh-frozen foods including chicken chow mein and chicken salad were analyzed and counts of coliform bacteria were found as high as 782,000 cells/g with a median of 3000 cells/g and staphylococci as high as 20,000 cells/g (13). When studying poultry pies, Ercole and Ordal found coliform bacterial counts up to 2,100 cells/g, enterococci

up to 160,000 cells/g, and staphylococci up to 390,000 cells/g (4).

All of the above publications cited mishandling of food as the primary cause of bacterial contamination. In effect, actual handling of the food contributes a great amount of contamination. Thus, since coliform bacteria, fecal streptococci and other fecal originated organisms are introduced in foods in significant numbers, it may be that non-sporeforming obligately anaerobic bacteria are also introduced in significant numbers.

It has already been pointed out that the obligate anaerobe Clostridium perfringens, discovered only 30 years ago, is the second most frequent organism to cause food poisoning. This organism is also present at levels of 10^{10} cells/g in feces of man (26). Thus, it is evident that non-sporeforming obligately anaerobic bacteria might also pose a significant problem.

Survival of microorganisms in frozen convenience foods. Since the vast majority of commercial convenience foods are packed, stored and shipped while frozen, it is important to consider the effect of freezing and storage on microorganisms.

Although the effect of freezing on microorganisms varies somewhat from genus to genus, several factors seem to be common to all. These include: 1) a sudden mortality observed immediately on freezing, which varies with the species, 2) the proportion of cells surviving immediately after freezing is nearly independent of the rate of freezing, 3) the cells which are still viable immediately after freezing die gradually when stored in the frozen state, and 4) the decline in

numbers is relatively rapid at temperatures just below the freezing point. This is especially true at -2 C, less so at lower temperatures, and it is usually very slow below -20 C (19). Thus, freezing should not be regarded as a means of destroying foodborne microorganisms (19).

Experiments with Clostridium perfringens and Clostridium botulinum in frozen foods verified the above statements for these obligate anaerobes (12, 15, 16, 39). Clostridium perfringens was found to increase spore production about 2 logarithms in 16 hours in vacuum sealed samples of hamburger, roast beef, and turkey that had been heat shocked and held at 37 C (9). Samples of chicken meat after freeze-dehydration and rehydration were shown to support a survival of 81% for Clostridium sporogenes. This study pointed out the natural selection for anaerobic sporeforming bacteria and a need for consumer education in product handling for safety purposes (7).

Some new problems caused by convenience foods. The utilization of commercially prepared foods in homes and in chain food-service establishments raises new problems not thoroughly investigated by microbiologists (30). Microwave heating of precooked chicken has been found to warm the center of an average sized piece of chicken from 49 C to 84 C, at a power setting of 2 KW, for a 45 to 90 second exposure. Differences in the conformation and composition of pieces accounted for the presence of cold spots. This heat treatment is not considered to be adequate to eliminate the possibility of Clostridium perfringens food poisoning (8). Vacuum sealed meats provide an environment suitable for Clostridium perfringens to grow and sporulate (9). It may be

possible that other non-sporeforming obligate anaerobes may also survive microwave heating, or become introduced into a vacuum sealed meat package. Poultry pot pies baked at 425 F for 40 minutes had a maximum center internal temperature which ranged from 165 F to 189 F (4). These temperatures were sufficient to destroy non-sporeforming organisms, such as coliform bacteria, enterococcus, Salmonella and paracolons, but if the baking temperature was not as high as 425 F or if the baking time was shortened to 30 minutes, some bacteria survived. A baking time of only 30 minutes allowed a coagulase-positive staphylococcus count of 1,300 cells/g and an enterococus count of 330 cells/g to occur in many samples (4).

Environmental tolerance of obligate anaerobes. It has been shown that non-sporeforming obligate anaerobes are somewhat aerotolerant and might be found in unexpected places. Marples and McGinley found the most common microorganisms resident on the skin of the head and upper trunk of man are anaerobic diphtheroids, most of which were characterized as the obligate anaerobe Propionibacterium acnes (23).

In studies on the effects of oxygen and of oxidation-reduction on obligate anaerobes, Hentges concluded that oxygen tension rather than oxidation-reduction potential is the critical factor regarding inhibition of the growth of Bacteroides fragilis, Peptococcus magnus, Clostridium perfringens and Clostridium acetobutylicum (14, 22, 41). Loesche, in studies on the oxygen sensitivity of various anaerobic bacteria, found that anaerobes can be ranked into three major groups according to their sensitivity to oxygen in shallow agar plates (22).

Strict anaerobes were those with no growth at oxygen tensions greater than 0.5% oxygen. This group contains the genus Treponema and various Lachnospira, Selenomonas and Butyrivibrio. Moderate anaerobes grew routinely at oxygen tensions greater than 0.5% and up to about 3.0%. This group included most of the genera of Bacteroides, Fusobacterium, Clostridium, and Peptostreptococcus. Microaerophiles that grew in from oxygen tensions 5.0% to 10% were represented by the genus Vibrio. Some overlapping occurred in these last two ranges by Bacteroides fragilis and Fusobacterium nucleatum. In some cases there was a large variation even between strains (22).

Another interesting study made by Loesche involves exposure of strict and moderate anaerobes to an aerobic room atmosphere after they had been streaked on agar plates. He found that Bacteroides fragilis could achieve 100% maximal growth after a 300 minute exposure, Fuso-bacterium nucleatum, a 79% growth after 100 minutes, and Treponema macrodentium, a 75% growth after 40 minutes (22). Thus, it is evident that various obligate anaerobes have the capacity to multiply in an environment containing as much as 3.0% molecular oxygen and to survive open exposure to room atmosphere for at least one hour with almost no loss in viability. Work by Foster on Clostridium botulinum has shown that this anaerobe can grow well under a shallow layer of meat gravy that is continually exposed to room atmosphere. Facultatively anaerobic organisms, such as Escherichia coli in a mixed culture with an obligate anaerobe, have been found able to consume oxygen so that even the most oxygen sensitive anaerobes such as Methanobacillus omelianskii were

provided with a very suitable environment (35). This again may be illustrated in work done by Post, on Bacteroides in sewage (33). It was found that once sewage enters a treatment plant, the decline in numbers of Bacteroides more or less parallels the decline in coliform bacterial counts. Counts on Bacteroides in raw sewage environments were found to be at least 2.2×10^3 cells/ml.

One might conclude that obligate anaerobes alone or with the assistance of facultative anaerobes to remove molecular oxygen, could grow wherever there were suitable nutrients.

Sporeforming obligate anaerobes in soils. It is evident that many vegetable foods may pick up clostridial species from the soil in which they grow in as indicated by studies on the clostridial content of 21 soil samples. Two hundred thirty-two strains of sporeforming obligate anaerobes were isolated from random soil samples. Species of Clostridium including subterminale, sordellii, sporogenes, bifermentans, perfringens, idolis and mangenati were found with counts which ranged from 2.7×10^6 to 3.3×10^2 cells/g (36).

Research objectives. Since pathogenic bacteria of fecal origin may readily be found in foods, and since pathogenic obligately anaerobic bacteria are present in the human intestine in numbers as high as 10^{11} cells/g, it is possible that these anaerobes may also be found in and present a health hazard in foods. There are at present no published reports dealing with studies of this nature. To investigate the possibility of anaerobic bacteria existing in convenience foods, research in this study was conducted in two phases.

In the first phase, experiments were conducted to detect the presence of any obligate anaerobes in convenience foods. In effect, the market quality of selected commercially prepared convenience foods such as frozen meat, poultry and fish pot pies or TV dinners was determined with regard to kinds and numbers of anaerobic non-sporeforming and sporeforming bacteria.

The second phase of research in the present study was conducted to determine the effect of freezing and storage on known pathogenic anaerobes when added to foods. Selected obligate non-sporeforming anaerobes were added to convenience food samples in known quantities to determine the degree of bacterial hazard to consumers after controlled freezing and storage. Factors affecting die-off of anaerobic pathogens which were determined include the effect of kind of food, type of organism, and the time and temperature of storage.

MATERIALS AND METHODS

Source of cultures. The species of obligately anaerobic bacteria used in this study were acquired from the stock culture collection of the Anaerobe Laboratory at the Virginia Polytechnic Institute, Blacksburg, Virgina. The cultures were maintained on prereduced anaerobically sterilized (PRAS) chopped meat peptone broth at 24 C. Monthly transfers of the cultures were made by aseptic anaerobic inoculation into PRAS chopped meat peptone and incubated for 2 days at 37 C. Peptone yeast medium was also inoculated to confirm growth.

The three main cultures used in this study, Bacteroides fragilis ss. fragilis, Peptostreptococcus intermedius and Peptococcus constellatus, were tested for validity by the VPI method of gas liquid chromatography. The chromatography spectra appear in the appendix (Fig. 14, 15).

Preparation of pre-reduced media. Pre-reduced, anaerobically sterilized (PRAS) media were prepared as described in the Anaerobe Laboratory Manual by the Virginia Polytechnic Institute (17). The steps in PRAS media preparation are as follows:

1. A boiling apparatus was constructed using a 500 ml erlenmyer flask with a 24/40 standard tapered top on which was placed a reflux chimney constructed of a 200 ml round bottomed flask with a 24/40 standard tapered neck affixed to the bottom of the flask. To the top of this reflux chimney, was added a plastic bottle fitted with a rubber stopper. A 1.0 cm hole was cut in the top of the plastic bottle to allow outflow of steam.

2. The dry ingredients recommended by VPI were placed in the boiling apparatus with distilled water, salts solution, resazurin, vitamin K_1 and hemin. The vitamin K_1 (stock no. 103283) and hemin (stock no. 101924) were purchased from International Pharmaceuticals, Inc. (17).

3. This assembly was placed on a magnetic stirring hot plate and the medium boiled for 20 minutes.

4. Before the boiling assembly was removed from the hot plate, a cannula delivering a stream of oxygen-free CO_2 was inserted into the top of the boiling apparatus to prevent entrance of air.

5. The boiling apparatus containing the reduced medium was then placed in an ice water bath and allowed to cool to room temperature. As the medium was cooling, granular cysteine-HCl was added to form a 0.05% solution. The boiling chimney was removed and a cannula was inserted into the neck of the flask to bubble CO_2 into the medium to exclude oxygen and to add CO_2 to the medium.

6. The medium was adjusted to the recommended pH by the insertion of a single electrode into the medium and by the dropwise addition of 8N NaOH or 10N HCl.

7. After the medium reaches room temperature, the gas was switched to oxygen-free N_2 and the flow was adjusted to exclude air.

8. A Cornwall syringe (10 ml Becton, Dickinson Co.) was affixed in the manner suggested by VPI, and adjusted to deliver the desired amount of medium to an anaerobic culture tube, either 18 x 142 mm or 25 x 142 mm (Bellco Glass, Inc. stock no. 2046-18142 and 2046-25142). Amber gum rubber tubing was allowed to stand in a 0.1% solution of cysteine-HCl when not in use on the Cornwall syringe to remove O_2 from the rubber.

9. A cannula combined with the media cannula was used to inject both the medium and oxygen-free N_2 into the anaerobic culture tube at the same time. Here again, the flow of N_2 was adjusted to flush air from the tube.

10. The anaerobic culture tubes were individually stoppered with butyl rubber stoppers (Bellco Glass, Inc. stock no. 1926-00001 and 1926-00004) as the double cannula was carefully withdrawn from each tube to prevent the entrance of air.

11. Each rack of anaerobic culture tubes was placed in an aluminum clamp assembly, similar to that produced by Bellco. (Bellco Glass, Inc. stock no. 7790-55555). The clamp assemblies were then tightened to prevent the rubber stoppers from blowing out during autoclaving and cooling.

12. Sterilization was carried out by autoclaving at 121 C for the recommended time required for each variety of medium.

PRAS roll tubes. In preparing PRAS roll tubes, the agar was weighed and added to each tube separately prior to the tubing of the broth. This was required because agar solidifies at 42 C, thus it could not have been added to the broth which was cooled to room temperature.

PRAS chopped meat media. Chopped meat PRAS media was prepared as stated in the general preparation of PRAS media except that the hamburger was initially steamed in the presence of NaOH as discussed in the VPI manual to remove most of the fat by saponification. The broth was then strained from the hamburger using cheese cloth and to it were added the recommended amounts of dry and liquid ingredients. The meat chunks were

added to each tube separately prior to the tubing of the broth.

PRAS dilution blanks. Ten ml PRAS dilution blanks were made as des-
cribed in the general preparation of PRAS media. Dilution blanks of
180 mls were reduced in the previously stated manner and the ingredients
were included as specified in the VPI manual. Diluent was dispensed
into 200 ml screw capped dilution bottles by the use of volumetric
pipets made anaerobic by drawing into them a quantity of oxygen-free
CO_2. A separate cannula was placed in the dilution blank bottle for
flushing. After 180 mls had been delivered, the bottle was stoppered
with a no. 3 rubber stopper. The bottles were then placed in a clamping
apparatus and steamed for further reduction of the solution and to
eliminate bacterial contamination.

Preparation of carbohydrate concentrates for addition to basal media.
Since it is unfeasible to prepare and keep on hand all of the most basic
carbohydrate media, 23 in number, for biochemical tests in our labora-
tory, it was decided to make a concentrate of each that could easily be
added dropwise to a tube of basal PRAS medium to transform it into a
carbohydrate medium suitable and comparable to those used by VPI in
fermentation tests.

Five hundred mls of distilled water was placed in the boiling
apparatus along with 2 mls of resazurin solution and boiled for 20 min-
utes. After cooling, 0.25 g cysteine-HCl was added as the system was
kept air-free by the flushing of oxygen-free CO_2. This reduced water
was then anaerobically added in 10.0 ml aliquotes, by the use of the Corn-
wall automatic pipetting apparatus previously mentioned, to 13 x 125 mm

screw capped test tubes. Each of the 13 x 125 mm tubes contained the predetermined amount of dry carbohydrate to raise the concentration of 10 mls of distilled water to a concentration where by delivering a 10-drop quantity from a pasteur pipette would introduce the required amount of carbohydrate into 5.0 mls of basal peptone yeast PRAS medium to render it comparable in composition to that used by VPI, for example, a carbohydrate concentration of 1.0% for glucose. The 13 x 125 mm screw capped tubes kept air out to keep the solutions reduced (17).

Kopeloff modified gram staining method. As advised by the VPI manual, the Kopeloff-Beerman modification of the gram stain was followed in all gram staining. This procedure may be found on page 16 of the Manual of Microbiological Methods (37, 17).

Gas liquid chromatography. The gas chromatograph used was the Dormann Anaerobic Bacteriology System (ANABAC) distributed by the Clinical Analysis Products Company (CAPCO), Sunnyvale, California. This system employs a dual thermal conductivity detection system with a built in strip chart recorder. The prepacked columns used were 0.25 in x 6 ft stainless steel packed with 15% CPE 2225 on 45/60 mesh chromosorb W-AW.

Volatile fatty acids, non-volatile fatty acids and alcohol standards were provided by the CAPCO company.

Conditions of the runs were as follows: Approximately 105 C column, 140 C injection, 113 C detector, 95 ma. detector current, and a helium gas flow rate of 100 ml per minute at 18 lbs.

All gas chromatography was run according to the established VPI procedure as follows:

Volatile fatty acids and alcohols.

1. Cultures of the organism to be tested were grown in PRAS peptone yeast glucose for eight hours at 37 C.

2. The cultures were acidified to a pH of 2.0 or below using about 0.1 ml of 50% aqueous H_2SO_4 (v/v) per 6.0 ml of culture. This is done to change all fermentation acids to the free acid form so they are soluble in ether.

3. To a stopperable container, 2.0 mls of the acidified culture were pipetted and to this, 1.0 ml of ethyl ether was added.

4. Excess NaCl was added to form a saturated solution for salting out fatty acids.

5. The solution was then mixed 20 times by inversion. The ether layer was then pipetted with a pasteur pipette into a stoppered container containing about 0.5 g anhydrous granular $MgSO_4$ to remove water.

6. After 10 minutes, 14 μls were injected into the gas chromatograph column using a Unimetrics Corporation teflon tipped plunger type 50 μl glass syringe (model no. 4050).

Analysis of non-volatile fatty acids.

1. To a stopperable container, 1.0 ml of the original culture was pipetted and to it were added 2.0 mls methanol and 0.4 mls of 50% aqueous sulfuric acid.

2. This solution was then held at 55 C by an aluminum temperature block for 30 minutes.

3. After heating, 1.0 ml of water and 0.5 ml of chloroform were added and the solution mixed by inversion 20 times.

4. The 14 μl syringe was filled from the chloroform extract which lies directly under the aqueous layer.

Transfer cannula apparatus used. A three place swing-type cannula was constructed as described in the VPI manual (17).

Methods for the determination of facultative anaerobic growth in PRAS media with low carbohydrate concentrations. Selected pure cultures of facultative anaerobes were inoculated into a tube of BHI (Difco) and of non-reduced peptone yeast media to acclimate the bacteria to the medium and incubation temperature. The BHI tubes were used as a control and to prepare an active culture as an inoculant.

These cultures were incubated for 48 hours at 37 C. Only tubes with a visible turbidity were used for the next inoculum. Cultures that did not grow well in peptone yeast (PY) medium were discarded. Two drops of PY inoculum from a pasteur pipette, from the peptone yeast turbid tubes were added to the corresponding tube of PRAS peptone yeast. This medium was exactly the same as what the inoculant was grown in except that it was reduced by boiling and had resazurin and cysteine-HCl as a reduction indicator and to keep it reduced. These tubes were then incubated at 37 C for 48 hours and was visually examined for quantity or turbidity of growth.

Procedure for the immediate recovery of known numbers of obligate anaerobes from a food sample. Samples of salisbury steak with gravy were purchased locally and used throughout these experiments. In our anaerobic hood, 20 g of the food sample were added to 180 mls of anaerobic

dilution blank in the Osterizer blender jars. Anaerobic bacterial cultures were grown for 24 hours at 37 C and added in 5.0 ml quantities to the food sample plus the dilution blank. To another Osterizer blender jar containing 180 mls of anaerobic diluent was added 5.0 mls of the same cultures but no food sample. The jars were removed from the anaerobic hood and blended for three minutes at alternating high and low speeds on the Galaxey Osterizer. These diluted foods were then serially diluted with 0.1 ml inoculum in 9.9 mls of anaerobic dilution blank and added to duplicate BHIA roll tubes in the manner previously stated. Anaerobic dilutions were also made directly from 1.0 ml of the initial inoculum and added serially to BHIA roll tubes. All BHIA roll tubes were incubated at 37 C for 48 hours and the colonies counted.

Procedure for anaerobic colony counting. Roll tubes were marked with spiral lines and counted at 40 X under an American Optical stereo dissection microscope lighted from both above and below the stage. A wire support was used to hold the roll tube at a 30° angle to prevent the washing of colonies by the free water at the bottom of the tubes. Tubes with counts between 30 and 300 colonies per tube were used to estimate the cultural counts.

Methods for isolation and enumeration of anaerobic bacteria in commercial convenience foods using the roll tube technique. This investigation of convenience foods involved both the roll tube technique and the anaerobic hood method. The hood used in our laboratory was constructed of a flexible plastic chamber, 85 cm x 50 cm x 50 cm, fitted with a 35 cm x 30 cm cylindrical air lock. A pair of flexible rubber gloves were

fitted into the side of the plastic chamber to allow manipulation of material. The atmosphere of the plastic chamber consisted of 45% N_2, 45% CO_2, and 10% H_2. The atmosphere was kept anaerobic by the action of H_2 with O_2 in the presence of palladium coated catalyst, producing water.

Foods to be tested were placed in the anaerobic hood along with a triple beam balance and Oster jars containing 180 mls of sterile anaerobic diluent. These jars and diluent were prepared as described previously. Twenty grams of each food were randomly collected and weighed into the 180 ml diluent. After removing them from the hood, the samples were blended at alternating high and low speeds for 3 minutes on a Galaxie Osterizer (model no. 457). Anaerobic dilutions were made by placing a 9.9 ml anaerobic dilution blank under one of the cannulas of the three place swing-type cannula system already described in materials and methods, and transferring 0.1 ml of the diluted food sample (also under a gassing cannula) with 1.1 ml pipette, to the dilution blank. The pipette was initially flushed with CO_2 by sucking the atmosphere from the inside of the dilution blank under the gassing cannula. After the proper dilutions were made, 0.1 ml or 1.0 ml aliquotes of the dilutions were anaerobically transferred to duplicate fluid-state BHIA roll tubes. The roll tubes and inoculum were then anaerobically stoppered, as stated in the VPI manual, and the agar plus inoculum rolled under a stream of cold running water to deposit a thin agar layer on the inside of the roll tube glass. The roll tubes were then incubated at 37 C for 4 days and counts were estimated.

Methods for enumeration of fecal streptococci. The decision was made early in this project to run fecal streptococci counts in parallel to any anaerobic enumerations of food. The multiple-tube technique given in Standard Methods for the Examination of Water and Wastewater 13th Ed., 1971 page 689, was followed closely as follows (1):

1. Azide dextrose broth (Difco) and ethyl violet azide broth (Difco) were made according to directions, added in 10.0 ml portions to loose capped 16 x 150 mm test tubes and autoclaved at 121 C for 15 minutes.

2. Dilutions of food samples to be tested were made by adding 1.0 ml of the 1:10 dilution of food to a 99 ml sterile aerobic dilution blank and mixed by shaking vigorously 20 times.

3. Appropriate quantities of the dilutions were pipetted into five replicate tubes of azide dextrose broth.

4. Incubation was carried out at 35 C for 48 hours.

5. The tubes were then checked for turbidity and positive cultures were transferred with two sterile swab sticks to tubes of ethyl violet azide broth.

6. These ethyl violet azide tubes were then incubated for 24 hours at 35 C and checked for positive tubes, a purple button at the bottom of the tube or dense turbidity.

7. Negative tubes were reinoculated and incubated in the same manner and again checked for positive growth.

8. Positive tubes were recorded in their corresponding dilution and converted into the most probable number index (MPN) to be used in the MPN table in determining estimated counts.

Total aerobic plate count method. Serial dilutions which were made during roll tube enumeration of food samples were used simultaneously for the preparation of duplicate aerobic plate counts. Warm fluid BHI agar was poured over the sample solution in each plate and mixed by a figure eight motion. After cooling, the plates were inverted and incubated at 37 C for 4 days. Colonies numbering 30 to 300 per plate were used to estimate the count of aerobic bacteria. The standard Quebec colony counter was used to make direct plate counts.

Methods for the detection of obligate anaerobes among facultative anaerobes. Roll tubes containing colonies isolated from food samples were placed under a cannula delivering oxygen-free CO_2. Using a picking needle (Bellco Glass, Inc. stock no. 7790-99999), all colonies of different morphological type were picked and streaked on aerobic BHIA petri plates and inoculated into chopped meat medium. At the same time the colonies were smeared on glass slides and gram stained according to the Kopeloff modification. The aerobic plates were inverted and incubated at 37 C for 4 days and observed for growth.

Coulter Counter. Much work has already been published involving the Coulter Counter with the counting of aerobic and facultatively anaerobic microorganisms (21, 10, 38). However, no reports were found dealing with the counting of obligate anaerobes with a Coulter Counter.

The Coulter Counter determines the number and size of particles suspended in an electrically conductive liquid. The electrically conductive liquid, also called an electrolyte or Isotone, containing the particles (or bacteria) to be counted is forced through a small orifice

in a 50 µl metered quantity. There is a difference in electrical resistance between the electrolyte and the particles suspended in it. When a particle passes through the orifice, there is a change in resistance between two electrodes, one located on either side of the orifice. This produces a voltage pulse corresponding to a single particle. All voltage pulses are recorded and added on a decade counter. The pulses may also be viewed on an oscilloscope screen.

The Coulter Counter used in our laboratory was Coulter Counter Model F with a 70 µ orifice nosepiece, manufactured by the Coulter Electronics Inc. Hialeh, Florida.

Methods used in finding Coulter Counter settings. Three settings must be made on the Coulter Counter to count particles. These are aperture, attenuation, and threshold. The aperture setting is the actual voltage between the two electrodes, the attenuation is the particle size sensitivity, and the threshold is an adjustable level to eliminate the counting of interference, background, or relative particle size.

According to the Coulter Counter manual, one must experiment with the material to be counted by observing it at various settings on the oscilloscope screen. The best aperture and attenuation combination for a particular particle will give a readout of one-half the height of the oscilloscope screen. The combination which was found to be best was an aperture of eight and an attenuation of one. This corresponds to the findings of Parikh (31). According to Naeve, this setting combination will allow the counting of a range of particles from 5 to 75 cubic microns, the volume range in which bacterial particles fall (29).

For use in finding the proper threshold setting, BHI broth was prepared by straining it with a 0.45 μ Millipore filter and tubing it anaerobically. Several tubes containing this medium were inoculated with obligate anaerobes and incubated at 37 C for 16 to 18 hours. One culture was measured at both 16 and 18 hours. The cultures were diluted 1:100 with Isotone. Triplicate counts were made on each culture at threshold settings from 1 to 13. A control count was also made on the broth alone.

Method of computer assisted graphing. All graphing in this investigation was accomplished through the use of a fourth degree polynomial interpolation and graphing program recorded in the memory of a Hewlett Packard model 9830A mini-computer.

Growth curves of twelve anaerobic bacteria. Because the Coulter Counter is a particle counter and not a viable cell counter, it was necessary to determine the approximate mid-point in the growth curve of the anaerobes to be tested, to prevent the counting of dead cells at the late logarithmic phase or stationary phase of the growth curve.

Twelve anaerobes were chosen, gram stained according to the Kopeloff modification for purity and inoculated into 5.0 mls of Millipore filtered anaerobic BHI broth. These cultures were serially transferred three times at 12-hour intervals to limit the number of dead cells. These cultures were then used to inoculate anaerobically prepared side arm flasks each containing 100 mls of Millipore filtered PRAS BHI broth under an oxygen-free atmosphere of N₂. These flasks were 300 ml size screw topped erlenmyer side arm flasks, the top being stoppered with a

no. 6 rubber stopper and the side port plugged with a Vacuutainer tube stopper. The flasks were placed in a clamp and sterilized by auto-claving for 15 minutes at 121 C. After inoculation the flasks were incubated at 37 C and absorbance readings taken at 1-hour intervals and in some cases every 30 minutes at 500 mμ on a Bausch and Lomb Spectronic 20.

Determination of correlation between Coulter Counter counts and roll tube counts. To obtain a better comparison between roll tube counts and Coulter Counter counts, a conversion factor was needed which could only be secured by doing actual counts on the same organism at least three times.

Three selected obligate anaerobes were each inoculated into 5.0 mls of Millipore filtered PRAS BHI broth and serially transferred it three times at the predetermined mid-growth curve time for each cul-ture, to eliminate most of the dead cells. One ml of each culture was then diluted 1:100 in Isotone to be counted with the Coulter Counter. Another 1.0 ml aliquot was serially diluted at the same time in 9.9 mls of PRAS dilution blanks and added in duplicate to BHIA roll tubes which were then mixed by swirling and rolled under cold running water. The roll tubes were then incubated at 37 C for 48 hours, and counts made.

Methods for the preparation of inoculated foods stored at -18 C and +4 C. Cultures of selected obligate anaerobes were inoculated into BHI broth (Gibco) and incubated at 37 C for 24 hours and gram stained using the Kopeloff modification to establish their purity. One ml of each was anaerobically transferred to a 250 ml erlenmyer flask containing

20 g of a food sample overlaid by an oxygen-free atmosphere. Flasks stored for 1 week were overlaid with CO_2 while flasks stored for longer periods were overlaid with N_2. All containers were sealed with no. 6 rubber stoppers and mixed by vigorously shaking for 2 minutes. Samples to be frozen were placed in a -18 C freezer, while refrigerated samples were stored at +4 C. The counts at zero time were made before freezing by adding 180 mls of PRAS diluent and transferring this solution to a sterile Oster blender jar. All transferring was done under the three-place swing-type cannula delivering CO_2. These samples were then blended for three-fourths of a minute at high speed. Because the blender jars had been under the cannula, its atmosphere permitted blending to be accomplished essentially anaerobically. In some instances the oxygenated food would turn the indicator of the poorly poised PRAS diluent to a pink color. After blending, the samples were anaerobically serially diluted in 9.9 ml PRAS dilution blanks and aliquotes were anaerobically transferred to PRAS BHIA roll tubes and rolled under cold water. The tubes were then incubated at 37 C for 48 hours and counts estimated.

Each set of frozen samples were, at 2 week intervals, thawed at room temperature until no visible signs of frozenness remained (usually 1.5 hours). After thawing, the samples were anaerobically diluted and incubated in roll tubes. All samples were treated in the same manner for the steps of inoculation, freezing, thawing and enumeration.

RESULTS AND DISCUSSION

The objective of this study was to determine if non-sporeforming pathogenic obligate anaerobic bacteria could be linked as a possible causitive agent for some of the unknown 56% of the foodborne disease outbreaks. This was accomplished in two phases. First, selected samples of frozen or refrigerated convenience foods were analyzed for kinds and numbers of sporeforming and non-sporeforming obligate anaerobes.

The second phase involved the addition of several known pathogenic obligate non-sporeforming anaerobes to various types of food in order to determine their loss in viability at freezing and refrigeration temperatures over extended storage times. Thus, the potential microbiological hazard for the consumer in regard to non-sporeforming obligate anaerobes in convenience foods was estimated through experimentation.

Growth of facultative anaerobes in PRAS media. An experiment was conducted to investigate the possibility that a completely reduced basal medium such as peptone yeast (PY) with no added carbohydrate might be inhibitory against the growth of a facultative anaerobe, while at the same time actively supporting the growth of an obligate anaerobe. Twelve facultative anaerobes were selected from our laboratory culture collection for this study. As the results show (Table 1.), the facultative anaerobes including Escherichia coli, Salmonella typhi, Staphylococcus aureus and others, grew well in the aerobically prepared BHI and

TABLE 1. Growth of facultative anaerobes in PRAS peptone yeast broth
as compared to growth in aerobically prepared peptone yeast
and BHI broths

ORGANISM	BHI BROTH	PY BROTH	PRAS PY BROTH
Corynebacterium xerose	++++ [a]	++++	++
Enterobacter aerogenes	++++	++++	++++
Escherichia coli	++++	++++	++++
Gaffkya tetragena[b]	++	++	++
Proteus morganii	++++	++++	+++
Salmonella typhi	++++	++++	++++
Shigella flexneri	+++	++++	+++
Staphylococcus aureus	+++	++++	+++
Streptococcus bovis	++	+++	+++
WINE YEAST # 2	++	+++	+
COORS YEAST	++	++	+
Saccharomyces fragilis	++	++	+

a Turbidity visually compared using ++++ for best growth, +++ for
good growth, ++ for fair growth, and + for poor growth

b Named according to Bergey's Manual of Determinative Bacteriology
7th Ed.

PY broths. In PY medium with the same ingredients but produced anaerobically by boiling, flushing with oxygen-free N_2, and the addition of cystein-HCl, it is evident that growth was almost as good as in the non-reduced aerobically prepared medium. The wine and beer yeasts and <u>Saccharomyces</u> <u>fragilis</u> show some inhibition by anaerobically prepared medium but not to a great extent.

It was concluded, therefore, that a low carbohydrate isolation media would not inhibit the facultative anaerobes normally isolated from foods; therefore, an alternative method must be found to differentiate facultative anaerobes from obligate anaerobes. The decision was then made to adopt an anaerobic modification of the plate count method to enumerate bacteria in food samples. Random colonies were picked and tested for aerotolerance, thus an obligate anaerobe count was estimated along with obtaining isolates.

It was determined by Vervaeke and Van Nevel that of the three methods tested, the Gas Pack anaerobic jar by Bioquest, the roll tube method of Hungate, and the anaerobic ringed plate technique of Van Der Hyde, the roll tube method was determined to be the best of the three for the examination and isolation of the predominant bacteria in the digestive tract of pigs (40). The roll tube technique was therefore adopted for enumeration and isolation of bacteria in samples of commercial convenience foods.

<u>Experimental recovery of added anaerobic organisms using roll tube counting techniques</u>. In these experiments, the objective was to determine the actual loss of obligate anaerobes in the anaerobic dilution

blanks, blender jars, and roll tube techniques to be used for food sample analysis.

As shown in the table (Table 2), in the particular experiment involving Fusobacterium nucleatum and Clostridium perfringens type D, there was a loss in count of about one-half a logarithm when the organisms were added to the dilution blank plus 20 g of food and blended. This loss was probably due to a number of factors including the inhibition by the particular food of the organisms used and the physical damage caused by the blender. In tests using Bacteroides fragilis ss. fragilis and Lactobacillus catenaforme, the loss in viability was not nearly as much, a drop from 11.5×10^8 cells/ml to 9.4×10^8 cells/ml. Again the activity of the blender and the inhibition by the food may be considered responsible. In both experiments, it is obvious that the greatest drop occurs not because of the dilution blank alone, but because of the combined action of the food sample and the blender. Essentially, these experiments were conducted to show that if obligate anaerobes were present in large numbers in a food sample, the recovery technique used would detect the majority of them.

Bacterial counts on commercial convenience food samples. These experiments initially involved two procedures for the analysis of the bacterial content of food samples. These were the total anaerobic roll tube count including the obligate anaerobes and facultative anaerobes, and the total fecal streptococci. Later, aerobic plate counts were added in parallel to the other two counts.

TABLE 2. Immediate recovery of known numbers of obligate anaerobes
from Salisbury Steak

	VIABLE CELL COUNT
Fusobacterium nucleatum + Clostridium perfringens TYPE D AS INOCULUM	23.3×10^6 [a]
DILUTION BLANK + INOCULUM	19.9×10^6
DILUTION BLANK + INOCULUM + 20g. FOOD	10.0×10^6
Bacteroides fragilis ss. fragilis + Lactobacillus catenaforme AS INOCULUM	11.5×10^8
DILUTION BLANK + INOCULUM	11.9×10^8
DILUTION BLANK + INOCULUM + 20g. FOOD	9.4×10^8

[a] All values are at 10^6/ml

No obligate anaerobes were detected using the technique of random picking of colonies from diluted samples in roll tubes. The values in the anaerobic count column (Table 3) include facultative anaerobes as well as possible obligate anaerobes. All colonies tested proved to be facultative anaerobes by aerotolerance tests. This, however, does not rule out the possibility that obligate anaerobes may be present, but it does indicate that if they are present, they are in such low numbers that they could not be found by the random picking of colonies. It is also evident that in such low numbers, they probably would not cause a foodborne illness problem. Emphasis must be made on the fact that if obligate anaerobes were present at levels of 10^2 cells/g of food sample, they would be diluted to extinction at dilutions of 10^3 to 10^{10}, the count levels at which many facultative bacterial counts actually existed in these foods.

It is interesting to note that most of the frozen convenience foods tested for a total anaerobic count ranged from 10^3 cells/g to 10^8 cells/g. One exception to this was a brand of frozen prepared lasagna. In each method used to get a total count, the counts went well over the dilution used of 10^{10} for anaerobic counts, 10^8 for total aerobic counts, and 10^{10} for total fecal streptococci. The procedures used in processing the macaroni of this product were suggested as the possible source of contamination.

The refrigerated hamburger and frozen fish bacterial counts were higher than other frozen convenience foods, but this is to be expected due to the large surface area, and storage and processing conditions of these products.

TABLE 3. Evaluation of various commercial convenience foods for anaerobic, facultatively anaerobic, and aerobic bacteria

FOOD	TOTAL ANAEROBIC COUNTS	TOTAL AEROBIC COUNTS	TOTAL STREPTOCOCCUS COUNTS
BEEF POTPIE	3.1×10^{4} [a]	b	4.6×10^{2}
CHICKEN POTPIE	7.0×10^{4}	b	b
BEEF POTPIE	2.5×10^{3}	b	1.7×10^{3}
CHICKEN POTPIE	7.3×10^{4}	b	1.4×10^{2}
LASAGNA	$>3.0 \times 10^{6}$	b	$>3.0 \times 10^{7}$
SALISBURY STEAK	6.0×10^{3}	1.5×10^{4}	7.0×10^{1}
TURKEY	6.7×10^{4}	1.2×10^{5}	4.9×10^{3}
LASAGNA	$>3.0 \times 10^{10}$	$>3.0 \times 10^{8}$	$>3.0 \times 10^{10}$
HAMBURGER (REFRIGERATED)	1.8×10^{6}	7.7×10^{6}	5.0×10^{3}
FISH	$>3.0 \times 10^{8}$	2.7×10^{4}	3.3×10^{2}
EGG ROLL	$<3.0 \times 10^{4}$	$<3.0 \times 10^{4}$	$<3.0 \times 10^{2}$
TURKEY (REFRIGERATED)	$<3.0 \times 10^{4}$	$<3.0 \times 10^{5}$	1.1×10^{3}
BROCCOLI	1.9×10^{5}	3.5×10^{3}	2.3×10^{2}

[a] All values are viable counts/g

[b] Counts not made

The fecal streptococci counts in most cases were 1 or 2 logarithms less in numbers in comparison to the total anaerobic counts. It is evident in the food samples tested that the fecal streptococci count does not necessarily correlate with the total anaerobic count as indicated in the samples of frozen fish and refrigerated hamburger where the total anaerobic counts were higher but the fecal streptococci count remained the same.

Gram stains of the various anaerobic colonies picked at random showed cellular morphology of small, medium, or large gram positive rods, of varying lengths, gram positive cocci in tetrads and staphylo-cocci like clusters, and small, medium, and large gram negative rods of varying lengths. The colonial morphologies covered the complete range of diversities.

Thus in phase one, no obligately anaerobic bacteria were isolated or enumerated in the food samples tested using the method of random picking of colonies from roll tubes at countable dilutions. It was then decided to move in this study to phase two, which involves the addition of known numbers of pathogenic obligately anaerobic bacteria to com-mercially prepared foods stored from 2 to 6 weeks.

Since this second phase of addition of anaerobes to foods involved or required the enumeration in viable cell count of many inocula, the Coulter Counter counting method was examined as a time saving and more accurate method of enumerating cell counts in the inocula to be used.

Growth curves of twelve anaerobic bacteria. Growth curves of twelve selected anaerobes were run specifically to determine the midpoint in

the growth curve of each organism (Fig. 1, 2, 3, 4). These midpoints were needed for Coulter Counter counting. In effect, it was important to know at what point in their growth curve would be best to count a culture to prevent the non-viable cells produced in the stationary phase from being included in the count. It is evident that obligate anaerobes conform to the typical growth curves of other bacteria.

Coulter Counter counts. It was found, through counting several different anaerobic bacteria plus a control at various theshold settings (Fig. 5), that the greatest number of cells were counted at threshold three with the least amount of background interference.

In an experiment comparing Coulter Counter counts with roll tube counts for 12 obligate anaerobes (Table 4), it is evident that there is no direct relationship between the two counting methods when considering the species tested.

As seen in the results (Table 5), organisms counted with the Coulter Counter compared with roll tube counts in triplicate also does not show a direct relationship. The reader should note that each pair of counts between the Coulter Counter and roll tube method are separate experiments.

A growth curve comparison experiment between Coulter Counter counts and roll tube counts may be seen (Fig. 6). Here Bacteroides fragilis ss. vulgatus has a higher roll tube count through the entire curve. The counts are identical at 0.9 hr of growth, but the difference rapidly increases with the roll tube count more than a logarithm higher at 6.2 hrs. In effect the curves are not parallel.

Absorbance
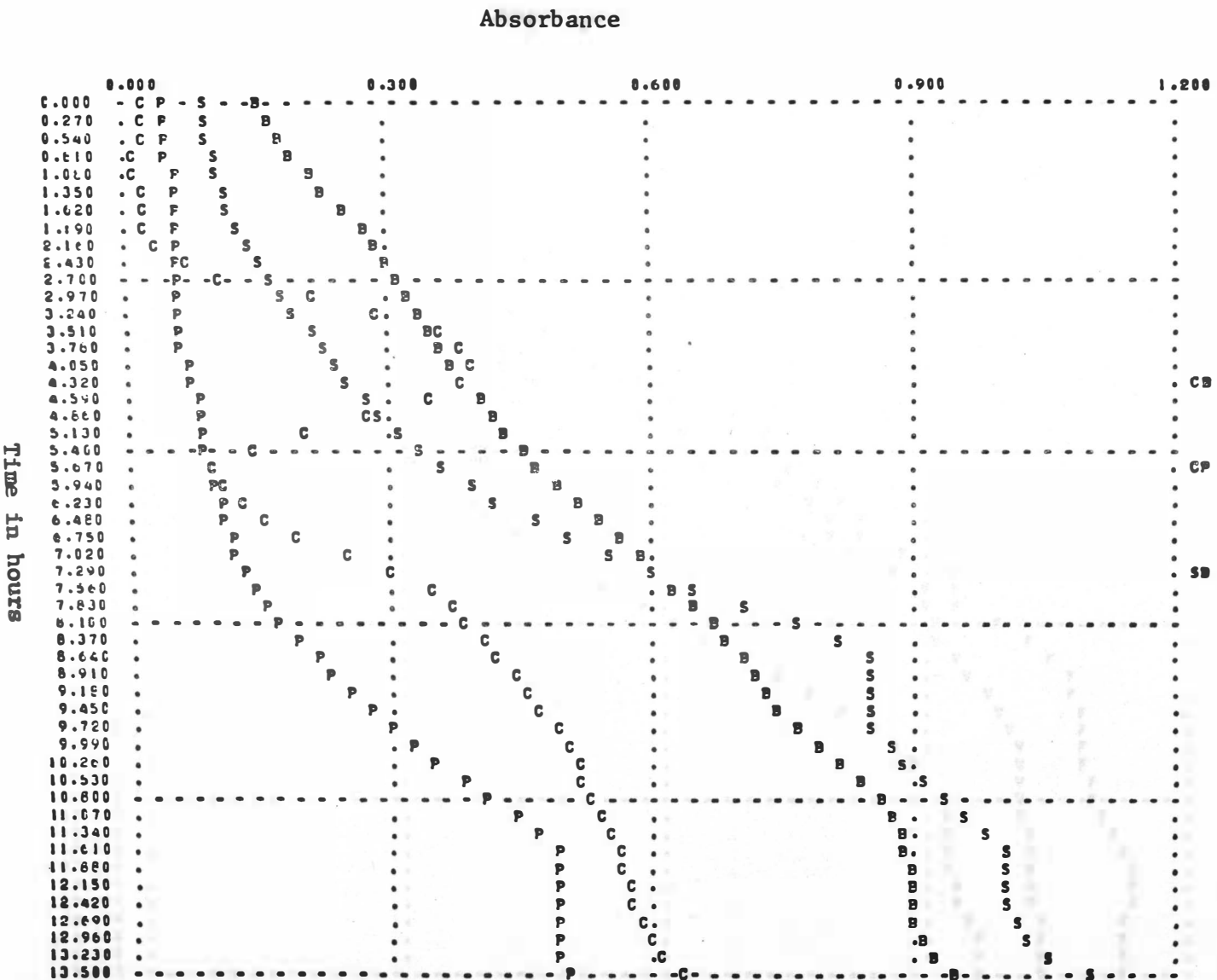
Fig. 1. Growth curves of selected anaerobic bacteria. C. perfringens = C, C. sporogenes = S, P. constellatus = P, B. fragilis ss. thetaiotaomicron = B.

Time in hours

```
            0.000              0.300              0.600              0.900              1.200
C.000    - C P  - S  - -B- .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
0.270    . C P    S      B    .                   .                   .
0.540    . C P    S       B   .                   .                   .
0.810    .C  P    S        B  .                   .                   .
1.080    .C    P   S        B .                   .                   .
1.350    . C    P   S         B .                 .                   .
1.620    . C    P      S        B .               .                   .
1.890    . C    P      S         B.               .                   .
2.160    .  C  P       S         B.               .                   .
2.430    .    FC       S          B               .                   .
2.700    - - -P- -C- - B - - - - -B- - - - - - - -.- - - - - - - - - -.- - - - - - - -
2.970    .     P       S C        .B              .                   .
3.240    .     P         S     C. B               .                   .
3.510    .     P          S      BC               .                   .
3.760    .     P           S    B C               .                   .
4.050    .    P             S    B C              .                   .
4.320    .    P              S    C               .                   .  CB
4.540    .    P             S .  C   B            .                   .
4.660    .    P             CS .      B           .                   .
5.130    .    P       C      .S        B          .                   .
5.400    - - -P- -C- - - - - -S- - - - B- - - - - .- - - - - - - - - -.- - - - - - -  CP
5.670    .     C              . S       B         .                   .
5.940    .    PC              .   S      B        .                   .
6.230    .     PC C           .    S      B       .                   .
6.480    .     P   C          .     S      B      .                   .
6.750    .     P     C        .      S      B     .                   .
7.020    .     P      P    C  .       S   B.      .                   .  SB
7.290    .      P      P      C       .    S      .                   .
7.560    .       P      P     . C     .   B S     .                   .
7.830    .       P       P    . C     . B    S    .                   .
8.100    - - - - -P- - - -P- -.- -C- - - B - - - S- - - - - - - - - - .- - - - - - -
8.370    .        P       P   .   C     . B       S                   .
8.640    .          P       P .    C    . B       S                   .
8.910    .          P       P .    C C  . B       S S                 .
9.190    .            P      P.     C    . B       S                  .
9.450    .             P     P.      C   . B        S                 .
9.720    .             P     P.      C   . B        S                 .
9.990    .              . P   P      C   . B        S .               .
10.260   .                P    P     C   . B        S.                .
10.530   .                  P  P     C   . B         S                .
10.800   - - - - - - - - - -P- - - - C- - B- - - - - S- - - - - - - - .- - - - - - -
11.070   .                  P     P  C   . B.        S                .
11.340   .                   P    P  C   . B.          S              .
11.610   .                    P  P   C C . B            S             .
11.880   .                      P  P C   . B            S             .
12.150   .                      P    C   . B             S            .
12.420   .                      P    C . . B             S            .
12.690   .                      P    C.  .B              S            .
12.960   .                      P     C.  .B             S            .
13.230   .                      P     .C   .B           S   S         .
13.500   . - - - - - - - - - - -P- - - C- - - - - - - - B- - - - - S- - .
```

Fig. 2. Growth curves of selected anaerobic bacteria. B. fragilis ss. vulgatus = V, B. fragilis ss. fragilis = F, P. intermedius = P.

Absorbance

Fig.3. Growth curves of selected anaerobic bacteria. P. morbillorum = O, P. magnus = A, E. lentum = E.

Time in hours

```
              0.000              0.060              0.120              0.180              0.240
 0.000   .  -  .  -  C  -  .  -  E- .  -  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .  .  CA
 0.460   .        C           E                   .                   .                   CA
 0.920   .        AO          E                   .                   .                   .
 1.380   .        AO          E E                 .                   .                   .
 1.840   .        A O         E E E               .                   .                   .
 2.300   .        A  C        E E E               .                   .                   .
 2.760   .        A     O     E E                 .                   .                   .
 3.220   .        A       C   E E                 .                   .                   .
 3.680   .        AA        C        E            .                   .                   .
 4.140   .        AA          C                   .                   .                   .
 4.600   .  -  -  AA  -  -  -  O  -  .  -  E-  -  .  -  .  -  .  -  .  -  .  -  .  -  .  -  .
 5.060   .         AA          C         E        .                   .                   .
 5.520   .          AA           C        E       .                   .                   .
 5.980   .           A A            O     E       .                   .                   .
 6.440   .             A A           O  .     E E .                   .                   .
 6.900   .              A A            C          E E                 .                   .
 7.360   .               A  A          C  O       E E                .                   .
 7.820   .                 A  A          O  O       E               .                   .
 8.280   .                    A  A         O   O     E              .                   .
 8.740   .  -  -  .  -  -  -  A  -  .  -  O  -  .  -  E- .  -  .  -  .  -  .  -  .  -  .  -  .
 9.200   .                       A       O    O    E E              .                   .
 9.660   .                         A       C    O   E E E           .                   .
10.120   .                          A A     O  O      E E E         .                   .
10.580   .                           A A      O  O       E E        .                   .
11.040   .                            A A      O      OE            .                   .
11.500   .                             A A        EO               .                   .
11.960   .                              A        .E  O             .                   .
12.420   .                               A A      .E  O   O        .                   .
12.880   .                                A        E  O   O        .                   .
13.340   .                                 A  A     .  E   O        .                   .
13.800   .  -  -  .  -  -  -  .  -  .  -  O-A-  .  -  .  -  .E- O  -  .  -  .  -  .  -  .  -  .
14.260   .                                  A A .       E  O   C     .                   .
14.720   .                                  A   .       E E   O  O   .                   .
15.180   .                                  A A .       E E     O  O .                   .
15.640   .                                  A A .       E E       O  O                   .
16.100   .                                  A A .       E          O .                   .
16.560   .                                  A  .             E    O   .OE               .   .
17.020   .                                  A A .                   .OE      C           .
17.480   .                                  A A .                   OEE            C     .
17.940   .                                  A A.                    EEE                C  .
18.400   .  -  -  .  -  -  -  .  -  .  -  -  -A-  .  -  .  -  .  -  .EEE- .  -  .  -  C C  O- .
18.660   .                                 .A A                     EEE               C O   .
19.320   .                                  A  A                    EEE               C O   .
19.780   .                                     A                    EEE               C O   .
20.240   .                                      A                   EEEE              C O C  .
20.700   .                                      A A                 EEEE              C O C  .
21.160   .                                      AAA                 EEE               C O C  .
21.620   .                                      AAAA                EEE               C O C  .
22.080   .                                      A A                 EE                C O C  .
22.540   .                                      A                   EE                 C C  .
23.000   .  -  -  .  -  -  -  .  -  .  -  -  -  -A-  .  -  .  -  .  -  E-  -  .  -  .  -  C  - .
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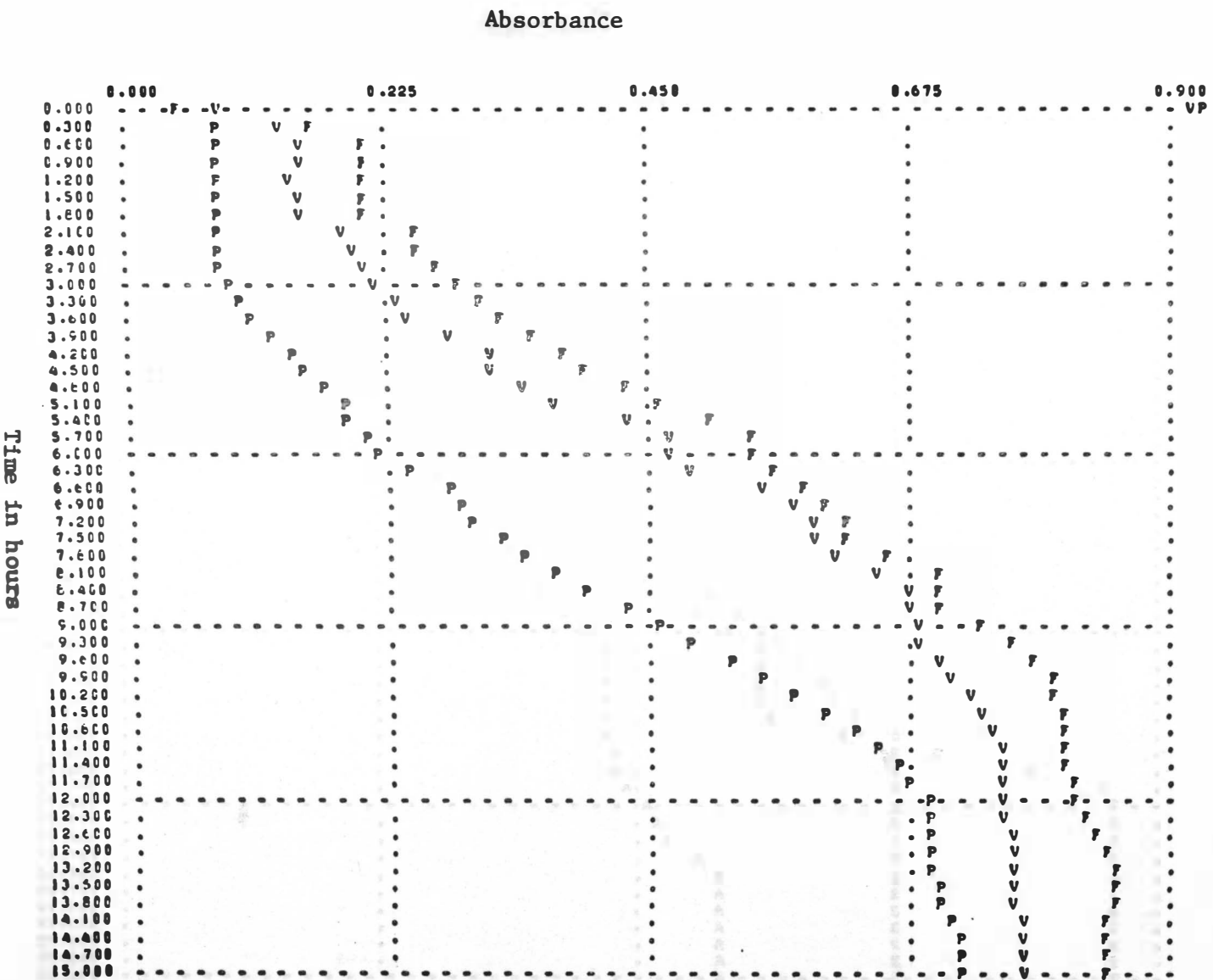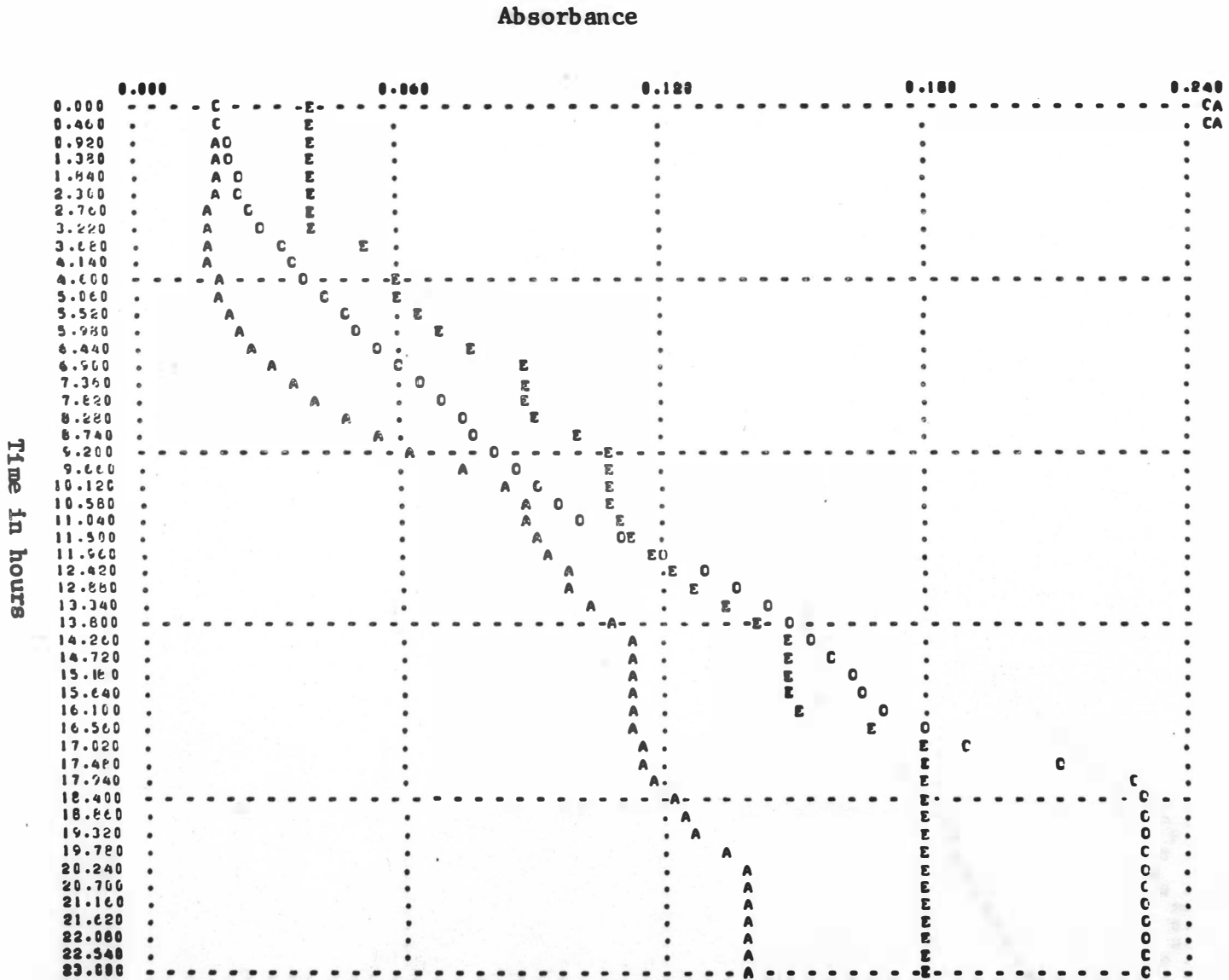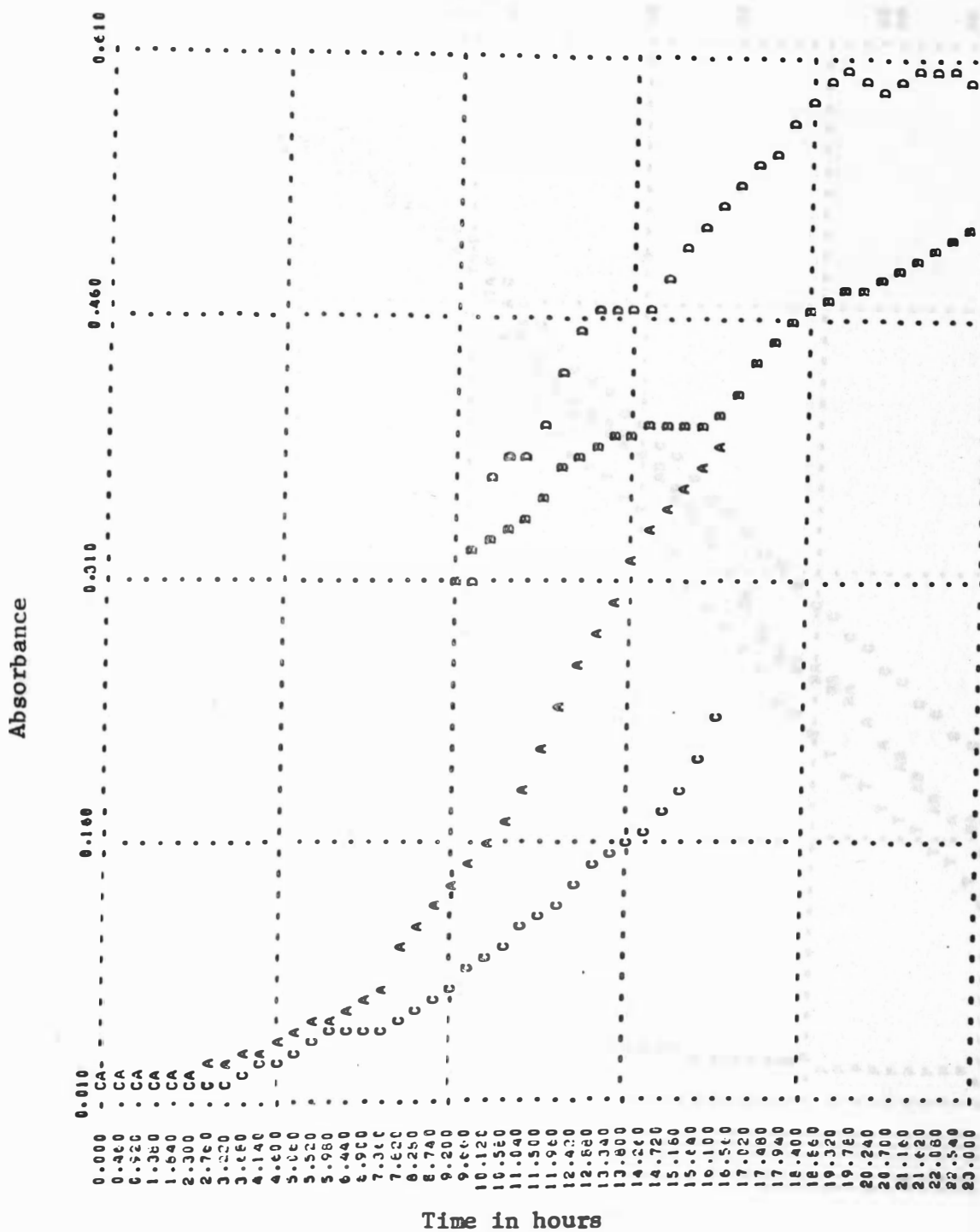
Fig. 4. Growth curves of selected anaerobic bacteria. P. acnes: 0-16
         hrs = A, P. acnes: 9-23 hrs = B, F. nucleatum: 0-16 hrs = C,
         F. nucleatum: 9-23 hrs = D.

Fig. 5. Cell count vs. threshold number for selected anaerobic bacteria.
Control = 0, B. fragilis ss. fragilis at 16 hrs = A, B. fragilis
ss. thetaiotaomicron at 18 hrs = T, B. fragilis ss. fragilis at
18 hrs = B, C. sporogenes at 18 hrs = C, P. acnes at 18 hrs = P.

TABLE 4. Comparison between Coulter Counter counts and roll tube
counts using twelve obligate anaerobes

ORGANISM	COULTER COUNTER COUNTS	ROLL TUBE COUNTS	NUMERICAL DIFFERENCE
Bacteroides fragilis ss. fragilis	90.8 [a]	27.0	63.8
Bacteroides fragilis ss. thetaiotaomicron	93.2	3.1	90.1
Bacteroides fragilis ss. vulgatus	90.8	2.5	88.3
Clostridium perfringens	81.1	19.0	62.1
Clostridium sporogenes	92.8	25.0	67.8
Eubacterium lentum	5.0	15.0	10.0
Fusobacterium nucleatum	7.5	1.5	6.0
Peptococcus constellatus	36.9	11.0	25.9
Peptococcus magnus	8.7	0.4	8.2
Peptococcus morbillorum	5.3	3.8	1.5
Peptostreptococcus intermedius	51.1	29.0	22.1
Propionibacterium acnes	1.8	4.9	3.1

[a] All values are at 10^6/ml

TABLE 5. Reproducibility of triplicate Coulter Counter counts compared
with roll tube counts using three obligately anaerobic
organisms

ORGANISM	COULTER COUNTER COUNTS [a]	ROLL TUBE COUNTS
Bacteroides fragilis ss. fragilis	90.3 [b]	2380.0
	90.8	27.0
	91.6	339.0
Peptococcus constellatus	18.5	130.0
	36.9	11.0
	64.7	70.3
Peptostreptococcus intermedius	51.1	29.0
	64.1	231.0
	67.8	113.0

[a] Each value represents a separate culture

[b] All numbers are viable counts at 10^6/ml
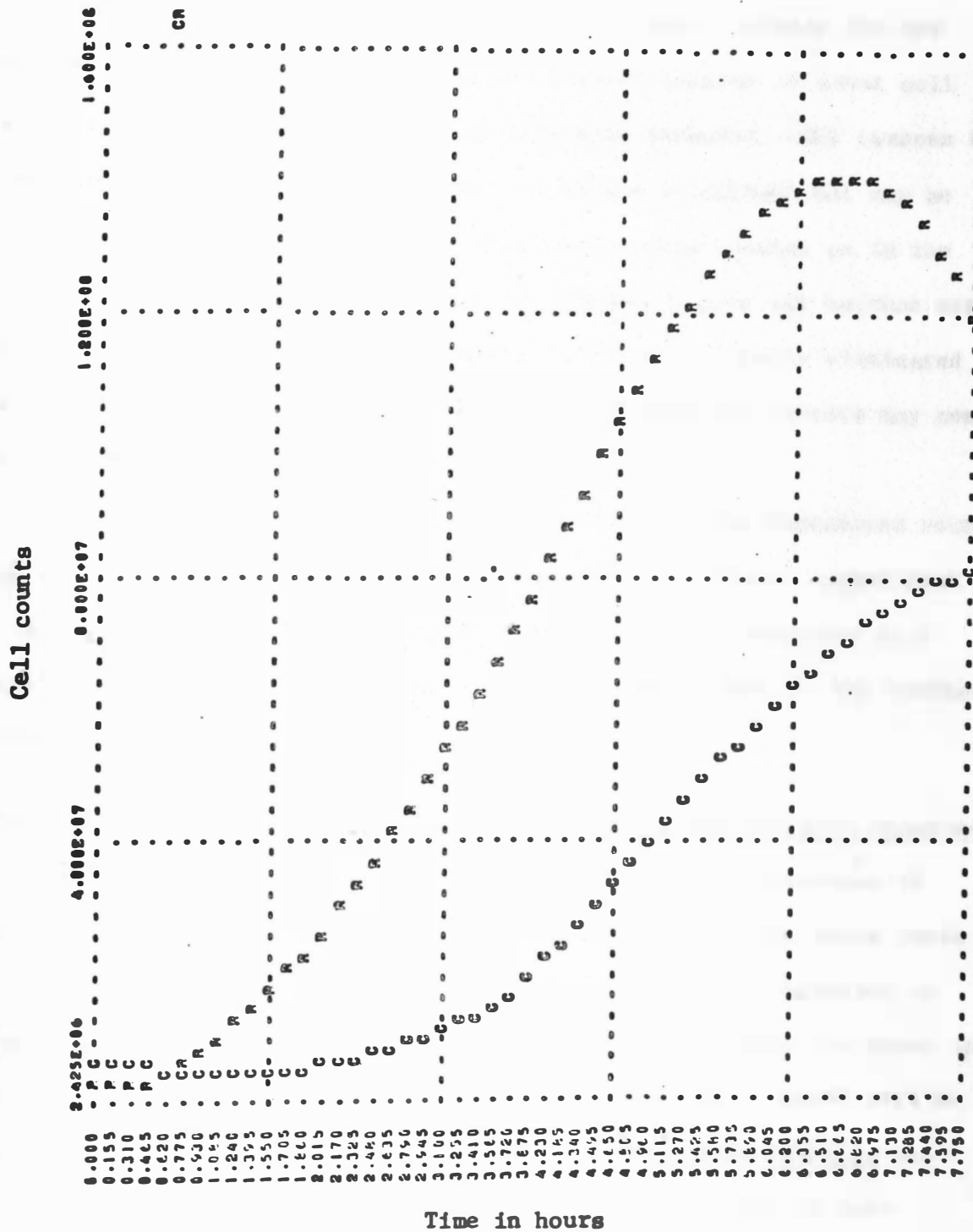
Fig. 6. Comparison between the Coulter Counter method and roll tube method for cell counting in the growth curve of B. fragilis ss. vulgatus. Coulter Counter counts/ml = C, roll tube counts /ml = R.

Because of these great discrepancies in counts between the two methods, it was decided not to use the Coulter Counter to count cell inocula for freezing experiments as initially intended. The reasons for the large discrepancies between the two counts is unknown but may be attributed to a presence of non-viable cells being counted as in the case of higher Coulter Counter counts. Another reason may be that with the threshold set at three, a number of cells are probably eliminated along with the background. A combination of these two factors may contribute to intermediate differences.

In all experiments using the Coulter Counter, the background count of the control (0.45 μ Millipore filtered PRAS BHI broth) ranged from 0.3×10^6 to 0.5×10^6 particles/ml. Although these particles were smaller than bacteria, they were included in counts made by the Coulter Counter.

Preliminary one week freezing experiments with twelve obligate anaerobes in hamburger. The purpose of these experiments was to determine if there were any particular organisms or groups of organisms among these 12 selected anaerobic pathogens that were exceptionally resistant or sensitive to freezing and thawing in a 1-week time period. As shown in the results (Table 6), percent loss ranged from 96.8% to 34.0% with no apparent resistance groups. Two organisms, Bacteroides fragilis ss. fragilis and Eubacterium lentum had the lowest percentage of loss.

From these results were selected three obligately anaerobic pathogens to be used in later experiments involving three different foods stored at refrigeration temperature for 2 weeks and freezing temperatures

TABLE 6. Viability of twelve obligately anaerobic bacteria stored at – 18 C for one week in hamburger

ORGANISM	INITIAL ROLL TUBE COUNT	FINAL ROLL TUBE COUNT	NUMERICAL DIFFERENCE	PERCENT LOSS
Bacteroides fragilis ss. fragilis	27.0^a	26.00	1.0	43.7
Bacteroides fragilis ss. thetaiotaomicron	3.1	0.06	3.0	96.8
Bacteroides fragilis ss. vulgatus	2.5	0.13	2.4	94.8
Clostridium perfringens	19.0	0.30	18.6	97.9
Clostridium sporogenes	25.0	0.007	25.0	99.9
Eubacterium lentum	15.0	9.90	5.1	34.0
Fusobacterium nucleatum	1.5	0.007	1.5	99.5
Peptococcus constellatus	11.0	2.80	8.2	74.8
Peptococcus magnus	0.4^b	8.80	8.4	b
Peptococcus morbillorum	3.8	0.90	2.9	76.2
Peptostreptococcus intermedius	29.0	3.20	25.8	88.9
Propionibacterium acnes	4.9^b	12.00	7.1	b

a All values are at $10^6/g$,   b Laboratory accident

for 6 weeks (Fig. 7). These organisms, <u>Bacteroides</u> <u>fragilis</u> ss. <u>fragilis</u>, <u>Peptostreptococcus</u> <u>intermedius</u> and <u>Peptococcus</u> <u>constellatus</u>, were chosen on the basis of their record of being highly pathogenic where anaerobic infections are involved and their resistance to freezing and thawing conditions as demonstrated in this experiment.

These studies have shown that all of the 12 obligate anaerobes tested can withstand storage at -18 C for 1 week, and thawing with a loss in viability within a range of 2 logarithms to less than one-half a logarithm.

<u>Viable counts of three obligate anaerobes stored at low temperature for 6 weeks in three foods</u>. These studies (Fig. 7) were conducted to investigate the possible bacterial hazards to the consumer as the result of the survival of large numbers of obligate anaerobes after freezing, storage, and thawing of convenience foods.

In the figures (Fig. 8, 9, 10, 11, 12, 13), the curves of cell die-off versus time in days for all cultures involved in each food tested, begin at a high level and then rapidly drop about 1 logarithm to a point at about 2 weeks from zero time. At this time the curves then level off with a less rapid decline in cell viability. During the next two sampling periods, or within 4 weeks, less than a logarithm drop in count is evident and a very noticable leveling off of all curves may be seen in the tabulated results (Tables 7, 8, 9). The actual percentage loss between sampling periods does not decrease but in most cases remains the same. Rather, what is occurring is that at each sampling period, a lower number of viable cells remains to be counted than the preceding period.
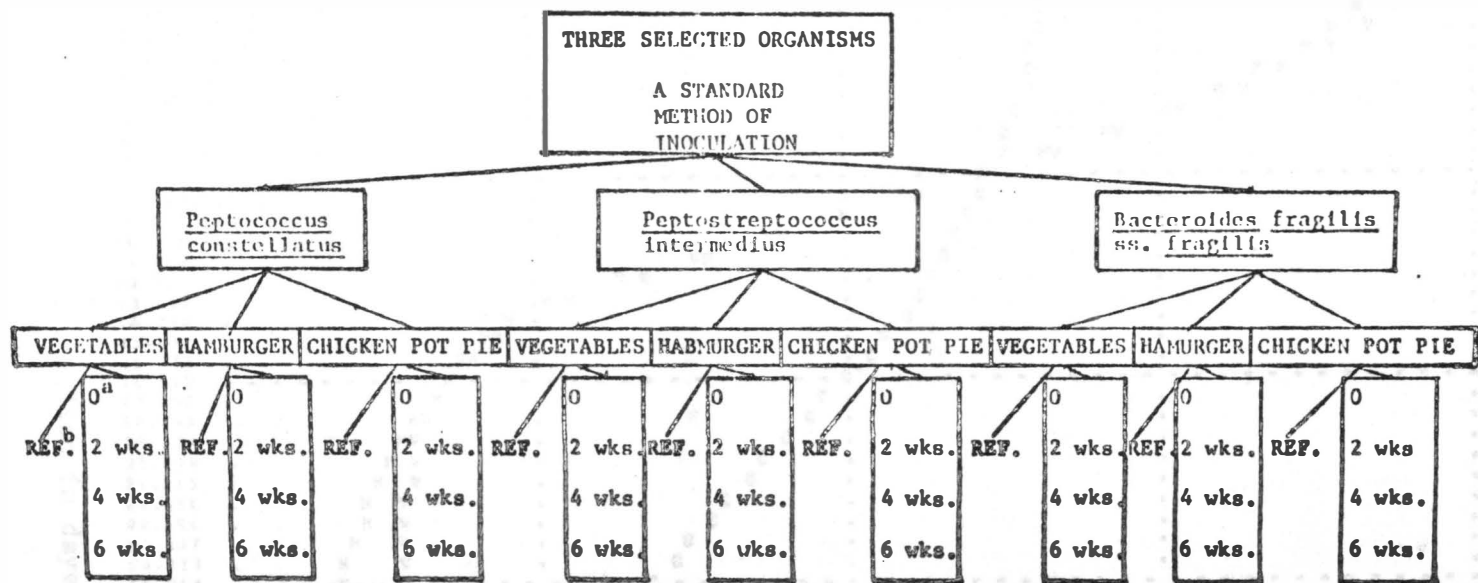
FIG. 7. A flow diagram involving three obligate anaerobes stored at – 18 C and + 4 C in three foods
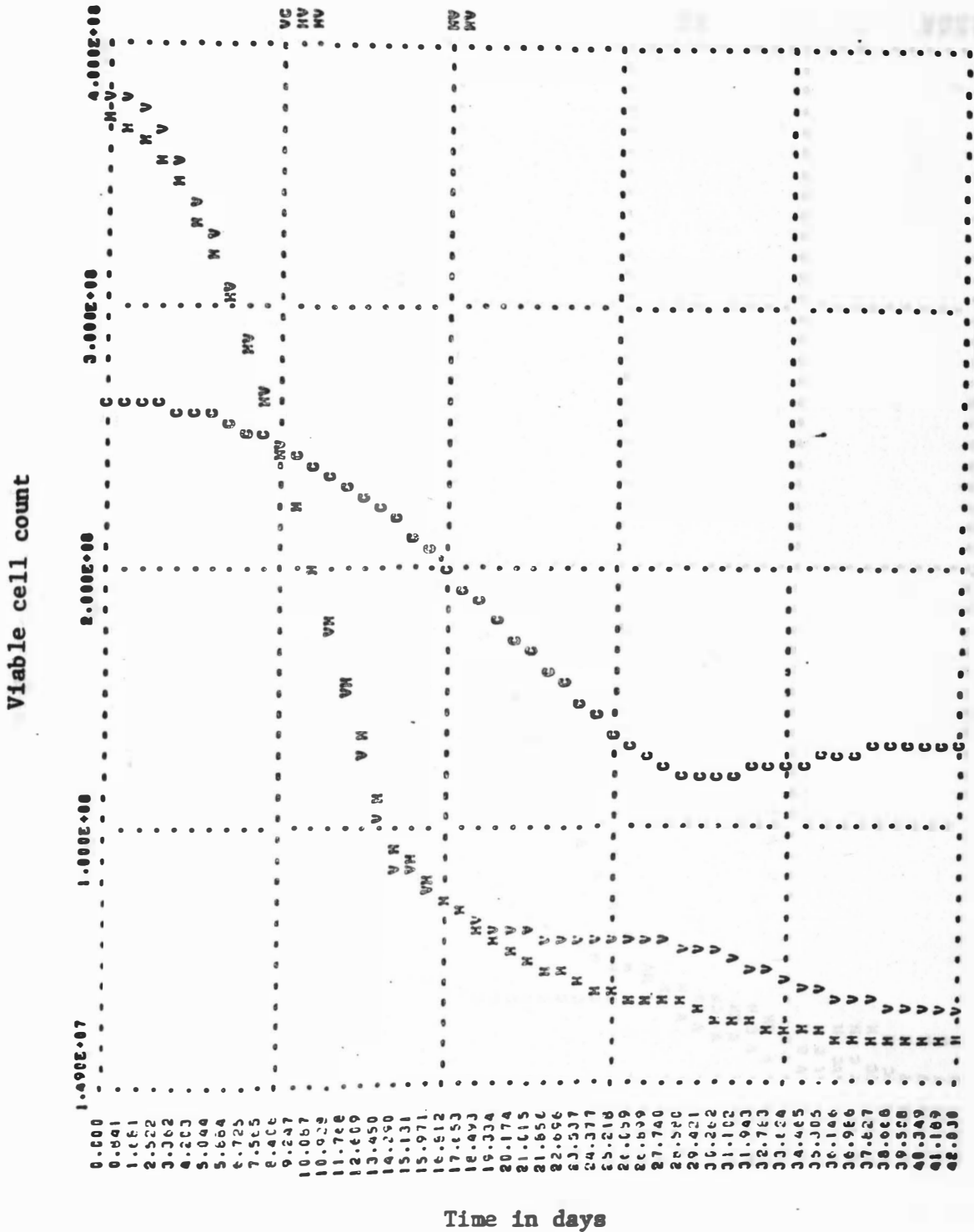
a Storage at – 18 C

b Storage at + 4 C

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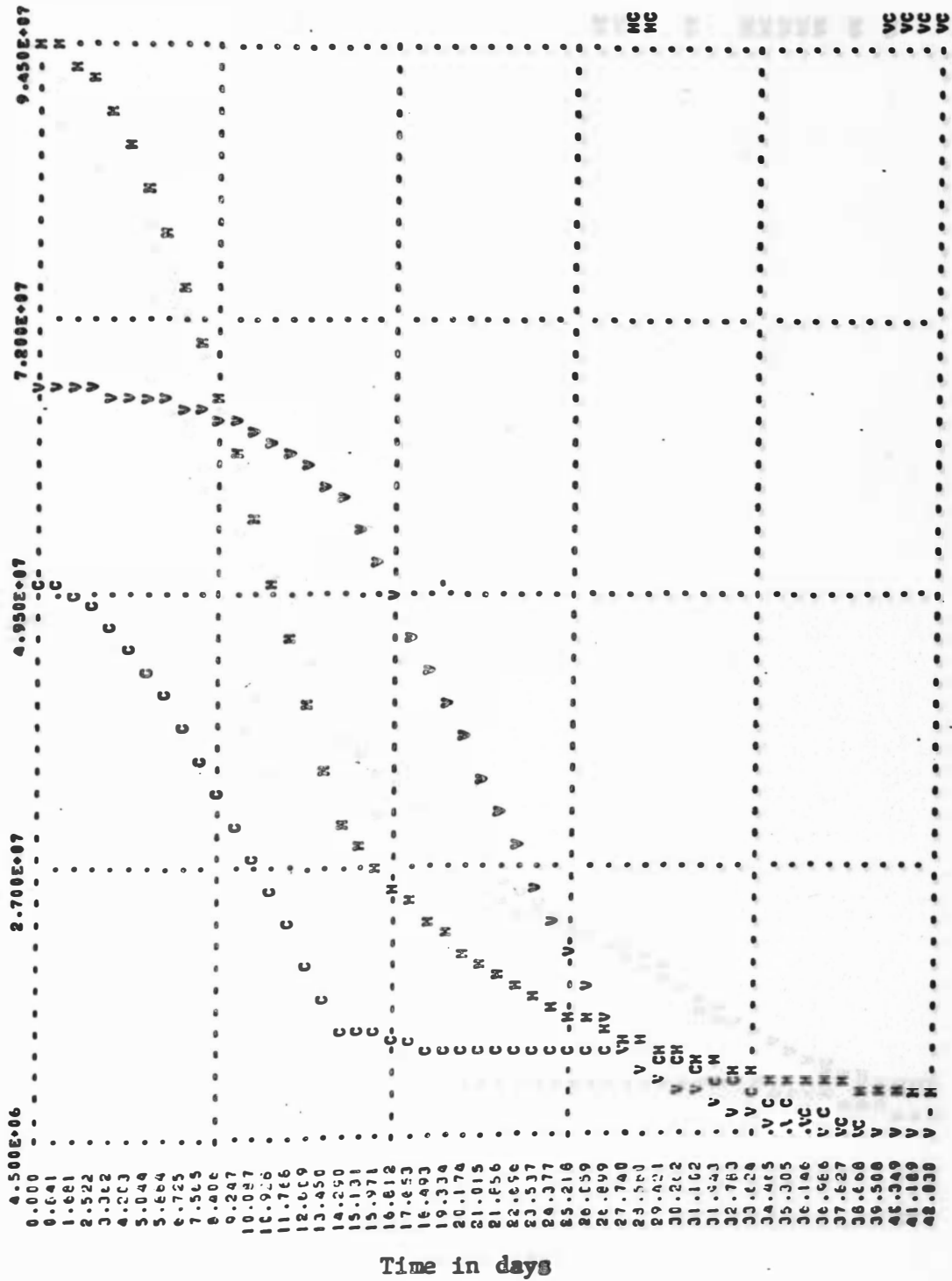Fig. 8. Viability of B. fragilis ss. fragilis stored at -18 C in various foods. Hamburger = H, mixed vegetables = V, chicken potpie = C.
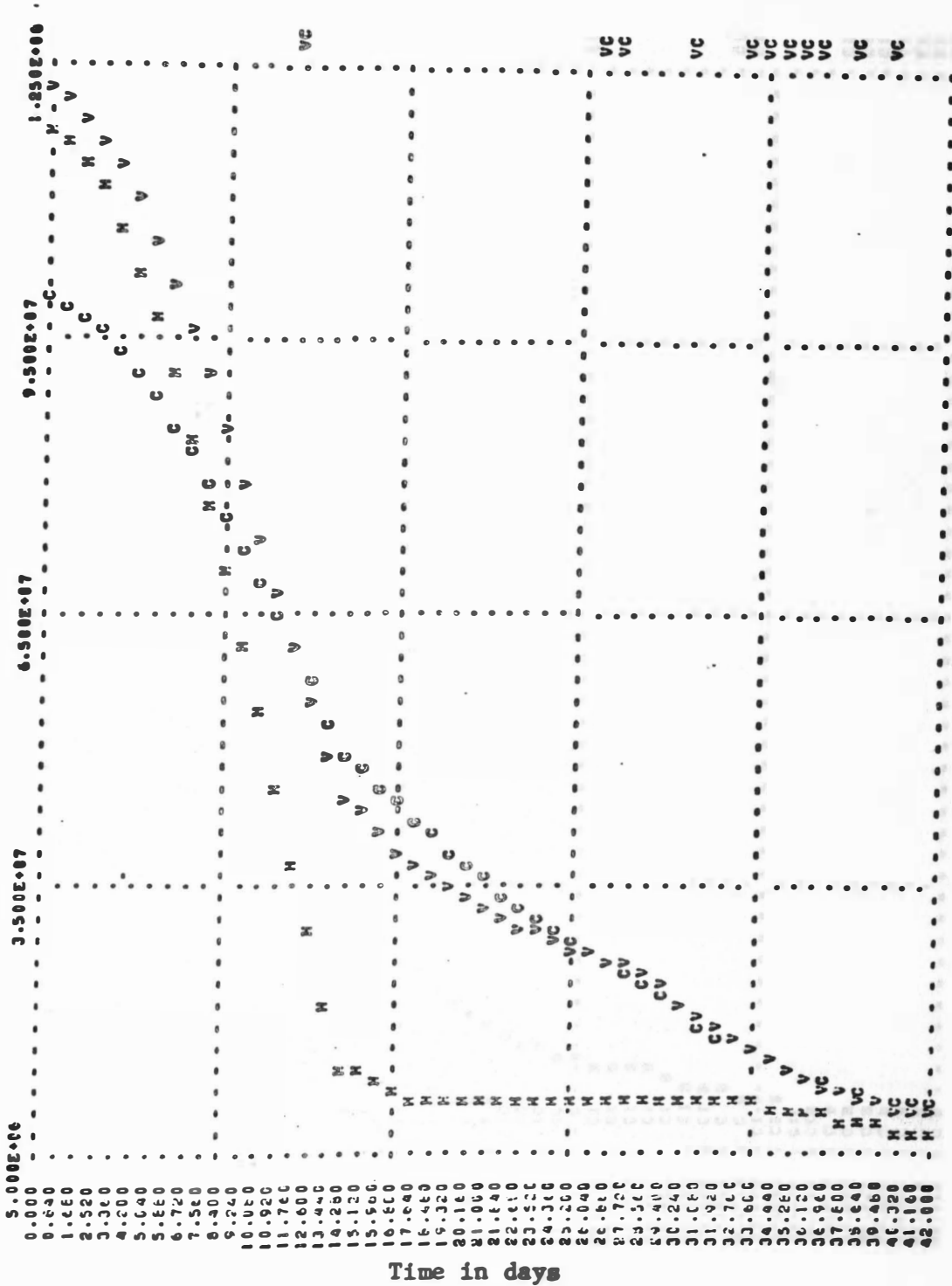
Fig. 9. Viability of P. constellatus stored at -18 C in various foods.
Hamburger = H, mixed vegetables = V, chicken potpie = C.

Fig. 10.   Viability of P. intermedius stored at -18 C in various foods.
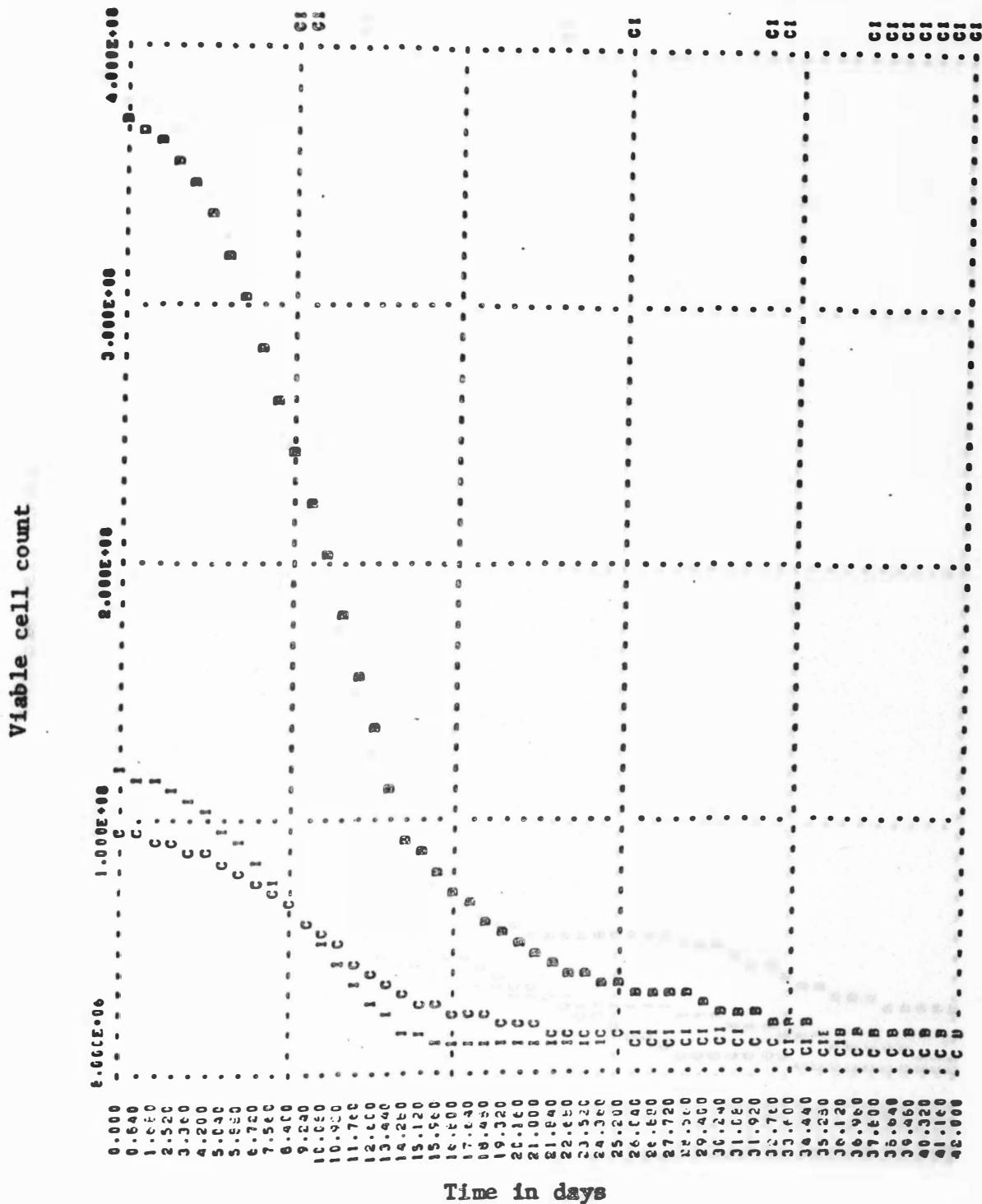Hamburger = H, mixed vegetables = V, chicken potpie = C.

Fig. 11. Viability of three anaerobes stored at -18 C in hamburger.
B. fragilis ss. fragilis = B, P. constellatus = C, P.
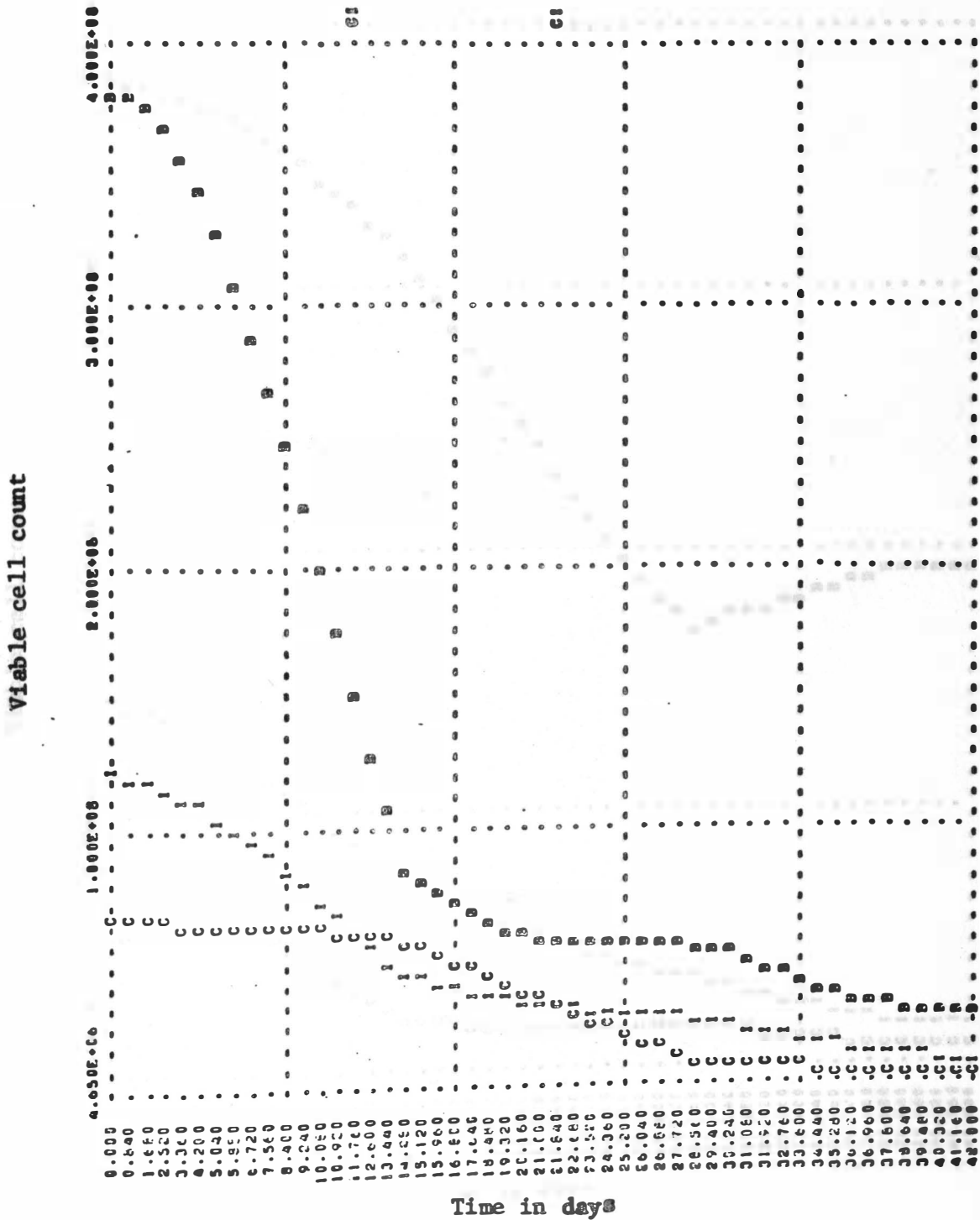intermedius = I,

Fig.12. Viability of three anaerobes stored at −18 C in mixed vegetables. B. fragilis ss. fragilis = B, P. constellatus = C, P. intermedius = I.
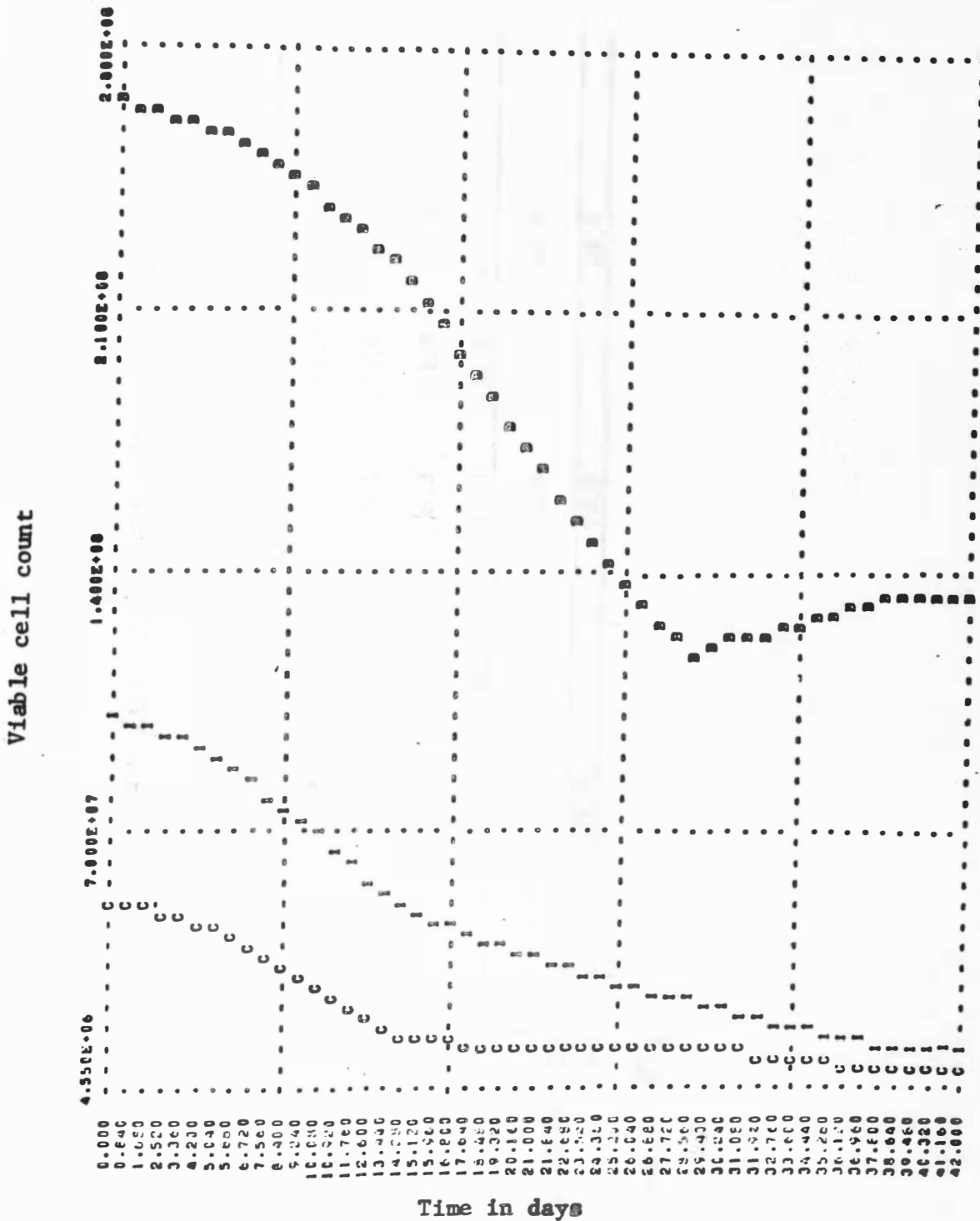
Fig 13. Viability of three anaerobes stored at -18 C in chicken potpie. B. fragilis ss. fragilis = B, P. constellatus = C, P. intermedius = I.

TABLE 7. Viability of Bacteroides fragilis ss. fragilis in three selected foods after storage at -18 C.

ORGANISM	TIME IN DAYS	HAMBURGER	% LOSS	MIXED VEGETABLES	% LOSS	CHICKEN POT PIE	% LOSS
Bacteroides fragilis ss. fragilis	0	370.0 [a]		381.0		265.0	
	14	98.0	73.5	91.0	76.1	221.0	16.0
	28	31.1	68.3	58.0	36.3	121.0	45.2
	42	14.9	52.1	26.5	54.3	134.0	+10.7
	AVERAGE % LOSS BETWEEN SAMPLING PERIODS		64.6		55.6		30.9
	TOTAL % LOSS		96.0		93.0		49.4

[a] All counts are at 10^6/g

TABLE 8. Viability of <u>Peptostreptococcus intermedius</u> in three selected foods after storage at -18 C.

ORGANISM	TIME IN DAYS	HAMBURGER	% LOSS	MIXED VEGETABLES	% LOSS	CHICKEN POT PIE	% LOSS
Peptostreptococcus intermedius	0	118.0[a]		122.0		100.0	
	14	17.1	85.5	46.0[b]	62.3	51.0[b]	49.0
	28	17.0	0.6	26.3	42.8	24.8	51.4
	42	7.6	55.3	9.6	63.5	10.9	56.0
AVERAGE % LOSS BETWEEN SAMPLING PERIODS			47.1		56.2		52.1
TOTAL % LOSS			93.6		92.1		89.1

[a] All counts are at 10^6/g

[b] From a single count

TABLE 9. Viability of Peptococcus constellatus in three selected foods after storage at – 18 C

ORGANISM	TIME IN DAYS	HAMBURGER	% LOSS	MIXED VEGETABLES	% LOSS	CHICKEN POT PIE	% LOSS
Peptococcus constellatus	0	94.5 [a]		66.4		50.0	
	14	32.0	66.1	58.0 [b]	12.6	14.7	70.6
	28	12.9	59.7	11.2	80.7	12.2	17.0
	42	8.2	36.4	4.6	58.9	4.5	63.1
	AVERAGE % LOSS BETWEEN SAMPLING PERIODS		54.0		50.8		50.2
	TOTAL % LOSS		91.3		93.1		91.0

[a] All counts are at 10^6/g

[b] From a single count

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Consequently there is a decrease in the actual numerical cell loss simply because there are a lower number of viable cells. This then explains why the first segment of the die-off curves are relatively steep and the second segment shows a leveling off effect.

Another contributing factor to the initial rapid loss in viability is the fact that the viable cell count taken at 0 time was an initial count of an unfrozen food sample. In other words, the combined effects of freezing, thawing, and storage are acting together to give the lowered second week count.

It is obvious that of the three foods tested, chicken pot pie gives the most protection to each of the three organisms tested (Tables 7, 8, 9). In the case of each food tested with each organism, the total percentage loss is less with chicken pot pie. Hamburger and mixed vegetables seem to be about equal in offering protection against loss of viability in freezing.

The calculated average percent loss in the three foods tested for Bacteroides fragilis ss. fragilis was 79.5%, for Peptococcus constellatus 91.8%, and for Peptostreptococcus intermedius a 91.6% loss. This indicates that Bacteroides fragilis ss. fragilis is the most resistant of the three organisms tested to freezing, thawing, and storage. The two genera of anaerobic cocci seem to be about equal in this respect.

The range in average percent loss between sampling periods was calculated to be 30.9% to 64.6%. The mean of the average percent losses between sampling periods was found to be 51.3% for all organisms tested at -18 C. The overall total 6 week percent loss was 87.6%.

The experiments involving three anaerobic organisms stored in the three foods and held at a refrigeration temperature of +4 C (Table 10) indicate that a high meat protein food, such as hamburger, offers the most low temperature storage protection as indicated by an average 78.8% loss for the three organisms. Chicken pot pie offers the least protection with a 96.3% loss average for the three organisms. For all three foods, the average calculated percent loss for Bacteroides fragilis ss. fragilis was 83.1%, Peptococcus constellatus was 93.2%, and Peptostreptococcus intermedius was 90.6%. Here again, Bacteroides fragilis ss. fragilis is most resistant to low temperature storage, while the two anaerobic cocci are about the same in resistance to low temperature refrigerated storage. The overall total percent loss for the three organisms in the three foods stored at +4 C for 2 weeks was 88.9%. It is interesting to note that the 2-week percent loss was found to average 51.3% for these same organisms stored in the same foods.

It is evident that at least one-eighth of the initial viable cells remain after 6 weeks of storage in a food at -18 C. If a certain food containing high numbers of obligate anaerobes was allowed to stand after thawing and a mild heat treatment, food poisoning or infection could result.

Summary. Essentially, this study was performed in two phases. In the first phase, various commercial convenience foods were analyzed for kinds and numbers of obligate anaerobes. No obligate anaerobes were found among the high counts of facultative anaerobes by the method of random picking of colonies in the 13 foods tested. This indicates that

TABLE 10. Viability of three anaerobes in three selected foods after storage at + 4 C

ORGANISM	TIME IN DAYS	HAMBURGER	% LOSS	MIXED VEGETABLES	% LOSS	CHICKEN POT PIE	% LOSS
Bacteroides fragilis ss. fragilis	0	370.0 [a]		381.0		265.0	
	14	164.0	55.7	20.4	94.6	3.0	98.9
Peptococcus constellatus	0	94.5		66.4		50.0	
	14	15.1 [b]	84.0	2.6 [b]	96.1	0.3	99.4
Peptostreptococcus intermedius	0	118.0		112.0		100.0	
	14	3.8	96.8	18.8	84.6	9.5	90.5
		AVERAGE % LOSS	78.8		91.8		96.3

[a] All counts are at 10^6/g

[b] From a single count

obligate anaerobes were not present in numbers as high as facultative anaerobes, but it is not known if they were present in low numbers, possibly less than 10^3/g. More work is needed using a different method of analysis to determine if any obligate anaerobes actually are present in low numbers in convenience foods. One suggested method of detecting obligate anaerobes in low numbers might be an anaerobic modification of the replica plating method of Lederburg and Lederburg.

In the second phase, known numbers of obligate anaerobes were added to different foods and stored at -18 C for 6 weeks and other samples at +4 C for 2 weeks. Here it was found that of the foods tested, chicken pot pie offered the greatest protection for the organisms used, at -18 C. At +4 C it was found that hamburger offers the most low temperature protection for the anaerobes tested. The die-off curves of the organisms tested in each food follow the same trend; there is initially a sharp decline in numbers that gradually levels off to an almost horizontal slope in 6 weeks. Of the anaerobes tested, it was found that Bacteroides fragilis ss. fragilis is the most resistant organism with respect to viability loss in frozen storage.

From the results of this study, it is evident that at least one-eighth of the original number of anaerobes are viable after 6 weeks of frozen storage. This should be adequate cause for concern for the producer as well as the consumer, because without proper precautions, a large number of obligate anaerobes could be ingested with a convenience food and cause a foodborne illness.

CONCLUSIONS

1. Facultative anaerobes were found to grow as well in low carbo-
hydrate PRAS basal medium as obligate anaerobes, thus low carbohy-
drate PRAS basal medium did not have a differential selective advan-
tage.

2. The method of random picking of colonies from serially diluted
food samples in roll tubes was inadequate for isolating and enumer-
ating obligate anaerobes in low numbers from convenience foods when
they are in the presence of higher numbers of facultative anaerobes.

3. Obligate anaerobes were not found in the 13 types of commercial
convenience foods tested at the dilutions used. However, facultative
anaerobes were found at various viable counts/g such as; beef pot pie
at 3.1×10^4, chicken pot pie at 7.0×10^4, hamburger at 1.8×10^6,
lasagna at greater than 3.0×10^{10}.

4. Although obligate anaerobes were not found in large numbers using
the roll tube count, the possibility is not ruled out that obligate
anaerobes may be present at viable counts of 10^2/g or less in certain
convenience foods.

5. Of the foods tested, there was no correlation found between the
total anaerobic counts and total streptococcus counts.

6. The deviations between Coulter Counter counts and roll tube counts
were too great to allow the use of the Coulter Counter for counting
anaerobic bacteria in PRAS BHI broth inocula. With more investigation
on this subject, a correction factor could be developed with confidence
limits to be applied to the differences between the two counting methods.

7. The growth curves of obligate anaerobes in PRAS BHI broth were found to be comparable in form to aerobic organisms.

8. Of the 12 obligately anaerobic bacteria tested for viability after storage at −18 C for 1 week in hamburger, Eubacterium lentum and Bacteroides fragilis ss. fragilis were found to be the most viable. The majority of the other anaerobic bacteria tested decreased approximately 2 logarithms in cell viability.

9. It was found that when obligate anaerobes were stored at −18 C in hamburger, mixed vegetables, and chicken pot pie for longer than 1 week, an average percent loss in viability of 51.3% was observed at each 2 week sampling period.

10. The overall loss in viable numbers of Bacteroides fragilis ss. fragilis, Peptostreptococcus intermedius, and Peptococcus constellatus stored for 42 days at −18 C in hamburger, mixed vegetables, and chicken pot pie was 87.6%.

11. The above bacteria stored for 2 weeks in the above commercial convenience foods at +4 C lost 88.9% of their viable cell count.

12. Of the three foods tested, chicken pot pie gives the most protection to each of the three organisms tested at −18 C.

13. At +4 C, it was found that hamburger offers the most protection against viability loss of the anaerobes tested and chicken pot pie the least.

14. Of the three obligately anaerobic bacteria tested, Bacteroides fragilis ss. fragilis was found to be the most resistant to both −18 C and +4 C storage temperatures.

15. It may be concluded from the above that at 2-week intervals the
viable cell count is reduced by approximately 50% for anaerobic bac-
teria stored at -18 C in convenience foods thus, after 6 weeks of
storage at -18 C in a food, one-eighth of the initial viable cell
count remains. This may be sufficient cell survival to be responsible
for food poisoning if the foods are inadequately prepared by the con-
sumer and producer.

APPENDIX

<u>Carbohydrate concentrates made in our laboratory as compared to carbo-</u>
<u>hydrate media used by VPI</u>. In an attempt to find a better method of
producing the 23 various carbyhydrate broths for biochemical differen-
tiation as described in the VPI manual, it was found that concentrates
of these carbohydrates are quite useful. These concentrates, which are
made separately from the PRAS peptone yeast basal medium, are added in
dropwise quantities to a 5.0 ml standard basal medium tube when that
particular carbohydrate test is desired. The advantage of this method
over the VPI method is that only the small tubes of concentrates need be
prepared and stored instead of 23 racks of different broths which must
be refrigerated. This method would be especially useful to a small
clinical laboratory. The comparison of results of the carbohydrate
concentrates used on three obligate anaerobes with the results of VPI
(Table 11) show very little difference. The inconsistencies that do
exist are due to strain variations.

TABLE 11. Comparison between our carbohydrate concentrate method and the VPI method for biochemical testing using three obligately anaerobic organisms

	Fusobacterium nucleatum			Bacteroides fragilis ss. fragilis			Clostridium sporogenes		
ARABINOSE	6.1	– (ours)	–(VPI)	5.8w	w	–^{w}	6.2	–	–
CELLOBIOSE	6.2	–	–	5.9	w	–^{w}	6.1	–	–
ESCULIN pH	6.1	–	–	5.7	w	–^{w}	6.4	–	–
ESCULIN HYDROLYSIS	–	–	–	+	+	+	+	+	+
FRUCTOSE	6.1	–	w^{-a}	5.1	a	a^w	6.0	–	–^{w}
GLUCOSE	5.9	w	–^{v}	5.1	a	a$^w{}_a$	5.7	w	w$^-$
GLYCOGEN	6.1	–	–	5.9	w	w^a	6.3	–	–
INOSITOL	6.1	–	–	5.8	w	–	6.3	–	–
LACTOSE	6.1	–	–	5.0	a	w^a	6.3	–	–
MALTOSE	5.9	w	–	5.0	a	a^w	5.8	w	–^{w}
MANNITOL	5.9	w	–	5.8	w	–	6.2	–	–
MANNOSE	6.1	–	–	5.2	a	a^w	6.2	–	–
RAFFINOSE	6.1	–	–	5.1	a	a^w	6.3	–	–
SALICIN	6.1	–	–	6.0	–	–	6.2	–	–
SORBITOL	5.9	w	–	5.8	w	–	6.0	–	–
STARCH pH	6.0	–	–	5.8	w	a^w	6.1	–	–
STARCH HYDROLYSIS	–	–	–	–	–	+	–	–	–
SUCROSE	6.1	–	–	5.0	a	a^w	6.3	–	–
XYLOSE	6.1	–	–	5.0	a	a^w	6.2	–	–
GELATIN	–	–	–^{w}	–	–	–^{w}	+	+	+
INDOL	–	–	+	–	–	–	–	–	–
NITRATE	–	–	–	–	–	–	–	–	–

CODE: pH 6.0 and above = NEGATIVE, pH 5.5 to 6.0 = WEAK ACID (w), pH 5.5 and below = STRONG ACID (a)

[a] A superscript symbol indicates some strains react differently

A = Acetic
F = Formic
P = Propionic
ISB = Isobutyric
B = Butyric
ISC = Isovaleric
V = Valeric
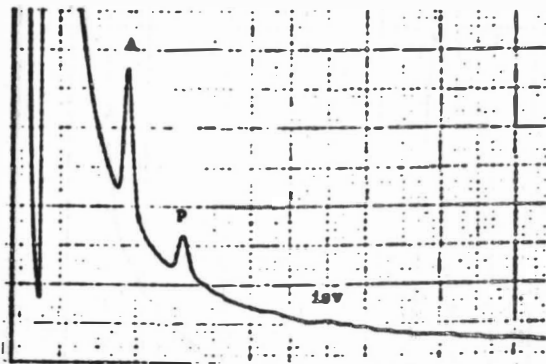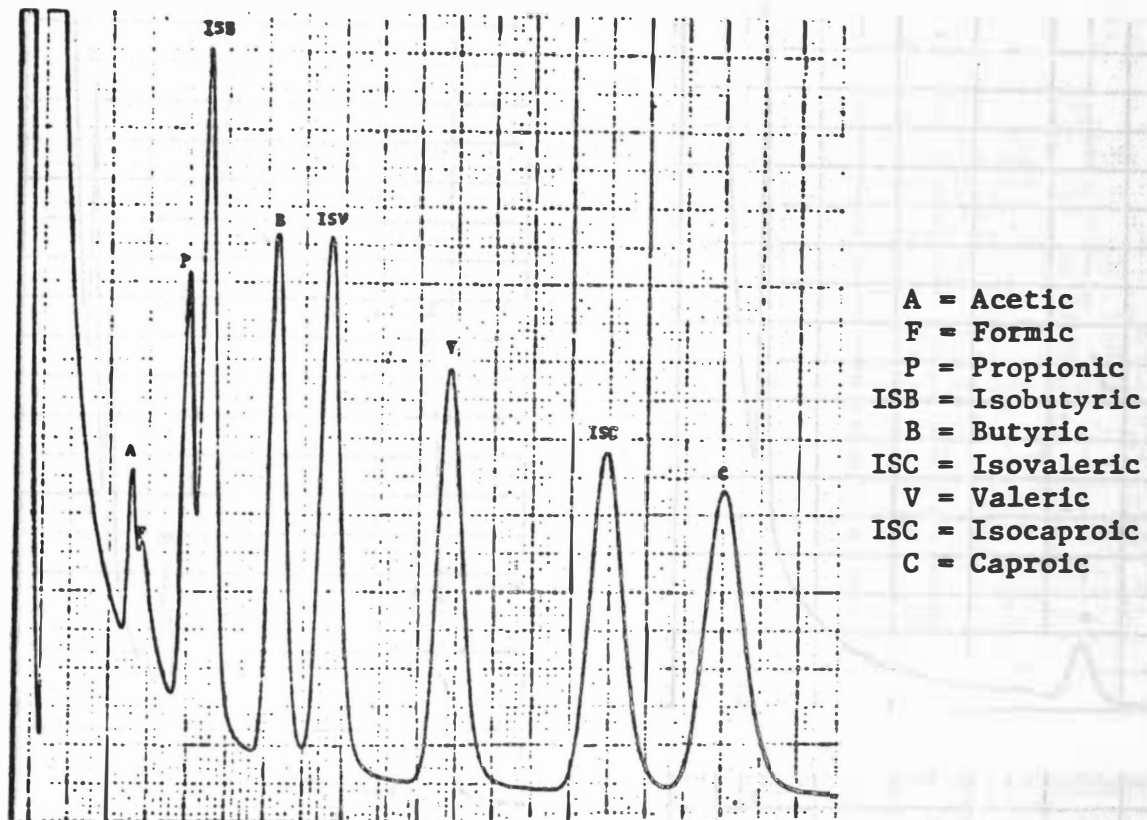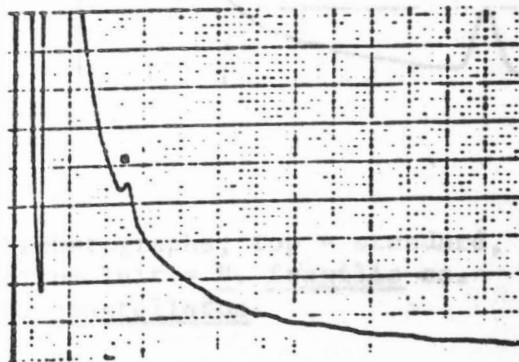ISC = Isocaproic
C = Caproic

Fig. 14. Volatile fatty acid chromatographs; top = standard, middle left = B. fragilis ss. fragilis, middle right = P. intermedius, bottom = P. constellatus.

P = Pyruvic
L = Lactic
OX = Oxalacetate
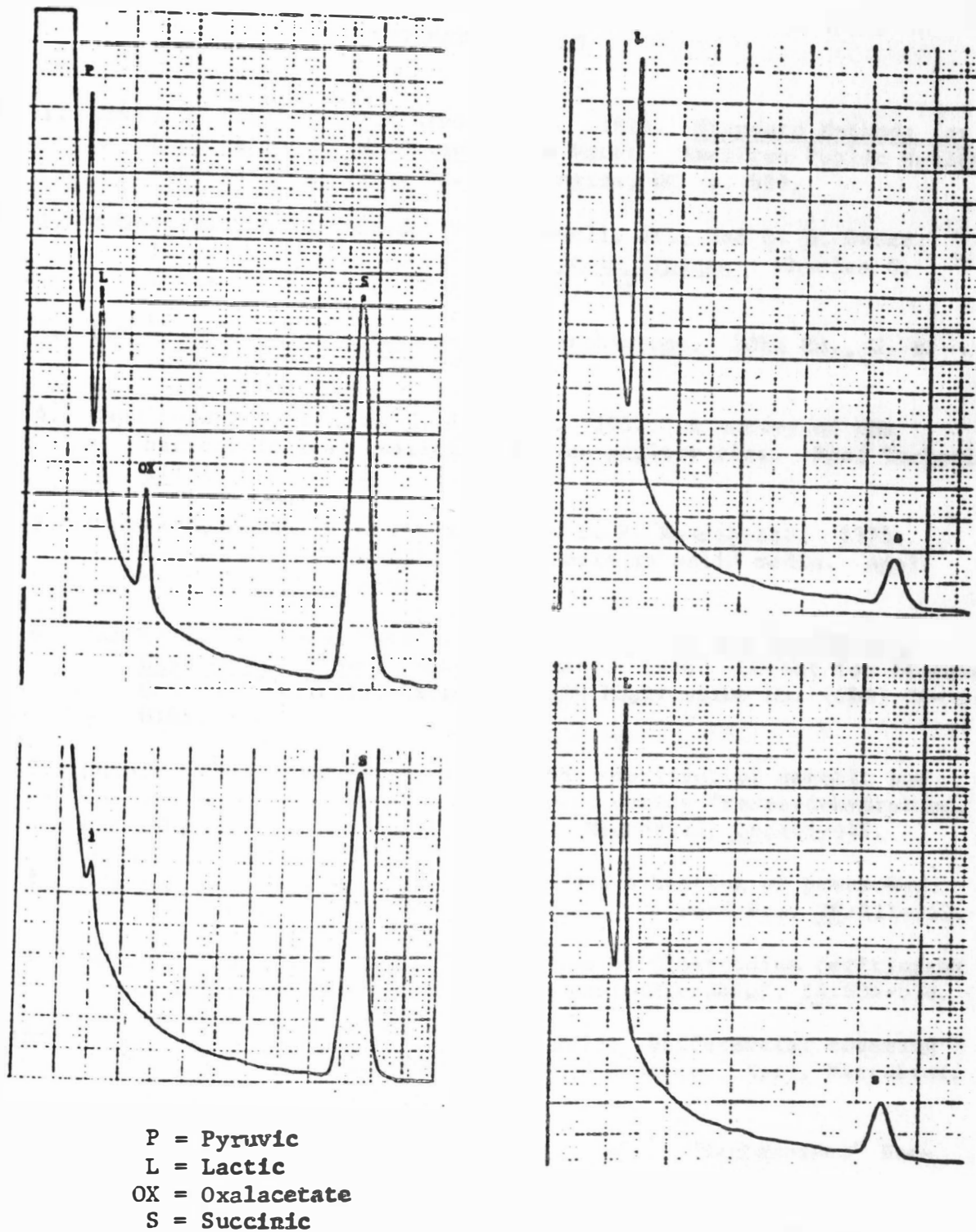S = Succinic

Fig. 15.   Non-volatile fatty acid chromatographs; top = standard, top
           right = P. intermedius, bottom left = B. fragilis ss.
           fragilis, bottom right = P. constellatus.

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