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Methods for Rapid Identification and Enumeration of Streptococcus Bovis from Water

Lawrence Robert Koupal

METHODS FOR RAPID IDENTIFICATION AND ENUMERATION OF *STREPTOCOCCUS BOVIS* FROM WATER

BY

LAWRENCE ROBERT KOUPAL

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OF *STREPTOCOCCUS BOVIS* FROM WATER

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor _____ Date _____

Head, Bacteriology Department _____ Date _____

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INTRODUCTION

Because water can be a carrier of enteric pathogenic microorganisms, water pollution from a fecal source is a serious problem to a community obtaining water from streams and rivers. Adequate methods for detection of the enteric pathogens are lacking, consequently, much attention has been given to the detection of other microorganisms as indicators of fecal pollution.

The microorganisms called the fecal streptococci are those streptococci which are commonly found in the intestinal tract of man and animals. The fecal streptococci have been divided into two basic groups within the Lancefield Group D streptococci. These two groups are the enterococcus group and the viridans group. Included in the enterococcus group are the Streptococcus faecalis strains and included in the viridans group are Streptococcus bovis and Streptococcus equinus.

The fecal streptococci are intestinal microorganisms of man and animals. Because of their normal habitat the fecal streptococci are being studied as possible indicators of enteric pathogenic microorganisms in water. The fecal streptococci have characteristics which make them desirable as indicators of water pollution. Croft (1959) placed these characteristics into four basic statements: 1) fecal streptococci are present in feces and sewage, 2) fecal streptococci are found in polluted water, 3) fecal streptococci are not found in pure water sites away from man and animals, and 4) the fecal streptococci do not multiply outside the host in water or soil.

In the past, the most common means of detecting fecal pollution has been the fecal coliform test and the total coliform test. However, the known inadequacies of the fecal coliform test and the total coliform test are significant enough to warrant a change in emphasis from the fecal coliform to the fecal streptococci.

The normal habitat of the fecal streptococci is the human or animal intestinal tract. The S. faecalis strains are predominant in humans while S. bovis and S. equinus are prevalent in animals. Because the distinction between animal and human fecal material can be made using these microorganisms more emphasis has been placed upon the fecal streptococci as pollution indicators. However, the literature is lacking reports of consistent isolations of S. bovis. Attempts by Tiede (1968) to isolate S. bovis in high numbers have shown the existing methods and media used to be ineffective.

The difficulty of isolation of S. bovis has given purpose to this study. This investigation was undertaken to find an isolation medium and a technique to make isolation of S. bovis less difficult and more consistent. It is also the intent of this work to gather more information as to the length of time S. bovis may be viable in a stream so that the value of this microorganism as a tracer for animal fecal pollution will be more clear.

LITERATURE REVIEW

Classification

An early classification of the streptococci dates back to 1906 when Andrews and Horder proposed seven groups of streptococci based upon their morphology, their fermentative ability, and their growth characteristics in milk. In their study Andrews and Horder reported isolating a new streptococcus from horse feces which did not ferment lactose and which grew well at 37 C. This isolate was named Streptococcus equinus. Later, Orla-Jensen (1919) classified the groups of the streptococci by basing the groups on fermentation characteristics, their tolerance to heat and to sodium chloride, and temperature limits of growth. In addition, Orla-Jensen isolated streptococci from bovine feces which possessed different physiological characteristics. Because of their characteristics, he proposed a new species named Streptococcus bovis.

In 1937 the classification of the genus Streptococcus which was accepted was made by Sherman. His scheme separated the genus into four groups (pyogenic, viridans, enterococcus, and lactic) based upon the growth at 10 C or 45 C, growth at pH 9.6 and growth in 6.5% sodium chloride. The streptococci which grew at 10 C and 45 C, and exhibited tolerance to 6.5% sodium chloride and pH 9.6 were placed into the enterococcus group. However, the enterococcus group excluded S. bovis and S. equinus as these microorganisms could not grow at 10 C or in a medium containing 6.5% sodium chloride. Therefore, Sherman

placed S. bovis and S. equinus into the viridans group.

It was found that all of the streptococci classified in Sherman's enterococcus group contained the Lancefield Group D antigen (Sherman, 1938) and that after culturing on 1% glucose at a pH of 4 S. bovis and S. equinus contained the group D antigen (Smith and Shattock, 1962; Medrek and Barnes, 1962). Consequently, S. bovis and S. equinus were included in the group D streptococci in spite of their physiological differences which separate them from the enterococci. The serological grouping of these organisms afforded another method of classification of the streptococci. All of the microorganisms found within the group D streptococci are referred to as the fecal streptococci.

There was no real separation of S. bovis and S. equinus into two distinct species until Hartman, Reinbold, and Saraswat (1966) divided them by the inability of S. equinus to ferment lactose and mannitol, and the inability of S. equinus to produce a dextran slime from sucrose. Because of the variable sugar fermentations of S. bovis a good classification scheme for these microorganisms was lacking.

Nutrition

The relatively simple nutritional requirements of S. bovis readily differentiate this organism from the rest of the streptococci. Almost all of the S. bovis strains studied require the vitamin biotin for growth. Thiamine, pantothenate, and nicotinic acid stimulate growth when this organism is incubated under aerobic conditions (Niven,

Washburn, and White, 1948). When S. bovis was cultured under anaerobic conditions with an increased carbon dioxide tension only a requirement for biotin was demonstrated, and when it was cultured under an increased carbon dioxide tension with oxygen no vitamin requirement was observed (Ford, Perry, and Briggs, 1958). However, strain variations were noticed.

All of the strains of S. bovis show an absolute requirement for carbon dioxide for their growth (Prescott and Stutts, 1955; Prescott, Ragland, and Stutts, 1957). In the absence of an increased carbon dioxide tension with amino acids as the sole source of nitrogen growth of S. bovis was limited. Addition of carbon dioxide allowed growth. A pathway for carbon dioxide fixation was shown for this microorganism by Wright in 1960. In the presence of biotin S. bovis will fix carbon dioxide to produce oxaloacetic acid from pyruvic acid. The oxaloacetic acid is then aminated to form aspartic acid. Wright also postulated a mechanism of the action of Tween 80 when it aids the growth of S. bovis. Tween 80 increased the permeation of the cell membrane of S. bovis to allow diffusion of exogenous aspartic acid into the cell.

No requirement for essential amino acids was demonstrated for the growth of S. bovis (Niven, Washburn, and White, 1948). The simple nitrogen requirements were observed by many researchers to be fulfilled by ammonium chloride or other ammonium salts (Wolin, Manning, and Nelson, 1959; Barnes, Seeley, and VanDenmark, 1961). It should be noted that no other Streptococcus species was capable of utilizing ammonium salts as sole source of nitrogen and because the growth of

S. bovis was very slow the use of ammonium salts was limited to taxonomic studies.

Origin and Occurrence

The first isolations of S. bovis and S. equinus were made from the feces of horses and cattle (Orla-Jensen, 1919; Andrews and Horder, 1906). Since that time more reports of isolations of S. bovis and S. equinus have been made by Ayers and Mudge (1923) and by Cooper and Ramadam (1955). No real correlation was made between the presence of S. bovis or S. equinus and animal feces until Seeley and Dain (1960) made a study of the starch hydrolyzing streptococci in the bovine rumen. From all of the isolates tested from the rumen Seeley and Dain indicated that S. bovis was the major starch hydrolyzing streptococcus while S. equinus and S. uberis played a lesser role in starch hydrolysis. Of the fecal streptococci the predominance of S. bovis in cattle and swine was shown to be 75 to 87% and 18 to 56% respectively (Kenner, Clark, and Kabler, 1960; Tilton and Litsky, 1967). Other streptococci that hydrolyze starch were described by Mann, Masson, and Oxford (1954) as atypical S. faecalis. The atypical S. faecalis was found in the rumen of sheep. Cooper and Ramadam (1955) also reported isolating atypical S. faecalis from cattle feces.

Reports of isolation of S. bovis from sources other than animal feces have been few and show no correlation with fecal pollution. Geldreich, Kenner, and Kabler (1964) reported the absence of S. bovis on vegetation and insects. Likewise, Mundt (1964) indicated that

isolations of S. bovis and S. equinus were not made from soil and plants even though the other fecal streptococci were isolated from the same sources. However, isolations of S. bovis were made from the laundry but not from feces of prison farm personnel who were associated with the dairy unit of the farm (Gledreich, Clark, and Huff, 1964). They also indicated that S. bovis was found in the cattle feces and sewage of the dairy unit. Attempts to isolate S. bovis from a cattle lagoon by Iwami (1967) were unsuccessful. Geldreich and Kenner (1969) isolated S. bovis infrequently and in low numbers from river water. No explanation of the infrequency of isolation from water was given other than the possibility of the chance discharge of animal wastes into water or the short life span of S. bovis in water.

Isolation Methods

From the time that the fecal streptococci have been suggested as indicator microorganisms of fecal pollution much emphasis has been placed on isolation media and techniques for the enterococci. The early acceptance of the fecal streptococci as fecal pollution indicators has been poor because of the relatively low recovery rates, the multiplicity of detection procedures, and poor agreement between various methods for their quantitative enumeration.

Undue emphasis has been placed upon the enterococci with little regard for other fecal streptococci present in the intestinal tract of animals. Some detection procedures gave excellent results with human feces but very poor quantitative results of the fecal streptococci

present in the feces of cattle or swine. Isolation media of this type have been the azide dextrose broth of Roth as tested by Mallmann and Seligmann (1950) with confirmation of presumptive positive tubes by the ethyl violet azide broth of Litsky, Mallmann, and Fifield (1953, 1955). A medium designed to isolate streptococci from the rumen was introduced in 1953 by Bryant and Burkey as rumen fluid glucose-cellobiose agar (RGCA medium). Other media and methods for isolation of fecal streptococci were presumptive counts in a Lab-Lemco peptone glucose broth and tetrazolium glucose agar which utilize thallium acetate (Barnes, 1956). The use of potassium tellurite medium has been proven to be of little success when isolating S. bovis (Ramadam and Sabir, 1963). Because of the difficulty of handling and poor quantitation many of these media have not been widely accepted.

As the membrane filter technique gained acceptance (Slanetz, Bent, and Bartley, 1955) a medium designed for more reliable isolation of enterococci was described by Slanetz and Bartley (1957) and called M-enterococcus medium. Another membrane filter medium described by Kenner, Clark, and Kabler (1961), Kenner fecal (KF) streptococcus medium, was accepted for its ability to increase the isolation frequency of enterococci and fecal streptococci which are common in animal feces. Even though isolations of the enterococci increased, the recovery of S. bovis and S. equinus was low when using KF streptococcus medium (Hall, Brown, and Angelotti, 1963). Since 1961, no other significant advances have been made in the techniques by which S. bovis may be isolated from sources in which this microorganism is prevalent.

Persistency and Significance

The presence of fecal streptococci in streams usually suggests fecal pollution and the absence of the fecal streptococci indicates little or no fecal pollution. Because the normal habitat of S. bovis and S. equinus is the rumen the presence of these microorganisms in water would suggest animal fecal pollution of that water (Deibel, 1964).

Streptococcus bovis and S. equinus have been suggested as being the most sensitive indicator organisms because of their rapid die-off once outside the animal intestinal tract. Laboratory studies by Geldreich and Kenner (1969) show the persistence of S. bovis in storm water was 1 to 2 days at 10 C and 8 to 9 days at 20 C. The storm water had a chemical composition similar to most fresh water streams. Geldreich and Kenner also indicated that the persistence of S. equinus was shorter than that of S. bovis. The results showed a significant enough decrease to warrant useage of S. bovis as a suitable indicator organism.

Experiments in a stream using dialyzing bags to suspend the microorganisms in fresh water and in salt water showed similar results (Slanetz and Bartley, 1965; Bartley and Slanetz, 1960). These findings point out that isolation of S. bovis and S. equinus from a source other than its normal habitat indicates recent pollution by animal feces.

MATERIALS AND METHODS

Source of Cultures

The known stock cultures used in this study were obtained from the American Type Culture Collection (ATCC) and were designated S. bovis ATCC 15351, S. bovis ATCC 9809, and S. faecalis ATCC 8043. A third S. bovis culture, designated I-104, was isolated during this study from the Big Sioux River to represent a typical isolate. The results obtained in this study were produced by both S. bovis ATCC 15351 and S. bovis ATCC 9809 unless otherwise indicated.

All the unknown cultures were obtained from four sources. These four sources are the Big Sioux River, Skunk Creek (a tributary of the Big Sioux River), cattle feces, and swine feces. Three sampling sites were chosen along the Big Sioux River: 1) a bridge at the intersection of the Big Sioux River and Highway 14 west of Brookings, South Dakota, 2) a bridge at the intersection of the Big Sioux River and Highway 38A northwest of Sioux Falls, South Dakota, and 3) Brandon Station east of Sioux Falls. Periodic samples were taken from these three sites from May to July, 1969. In July samples were collected from Skunk Creek which flows along a cattle feed lot. The cattle and swine fecal samples were taken from different animals at the John Morrell packing plant stockyards in Sioux Falls.

The fresh water samples were taken with a water sampler (Fig. 1) at the depth of 0.5 to 1.0 meter. This water sampler was used aseptically to obtain water samples which represented river water free from

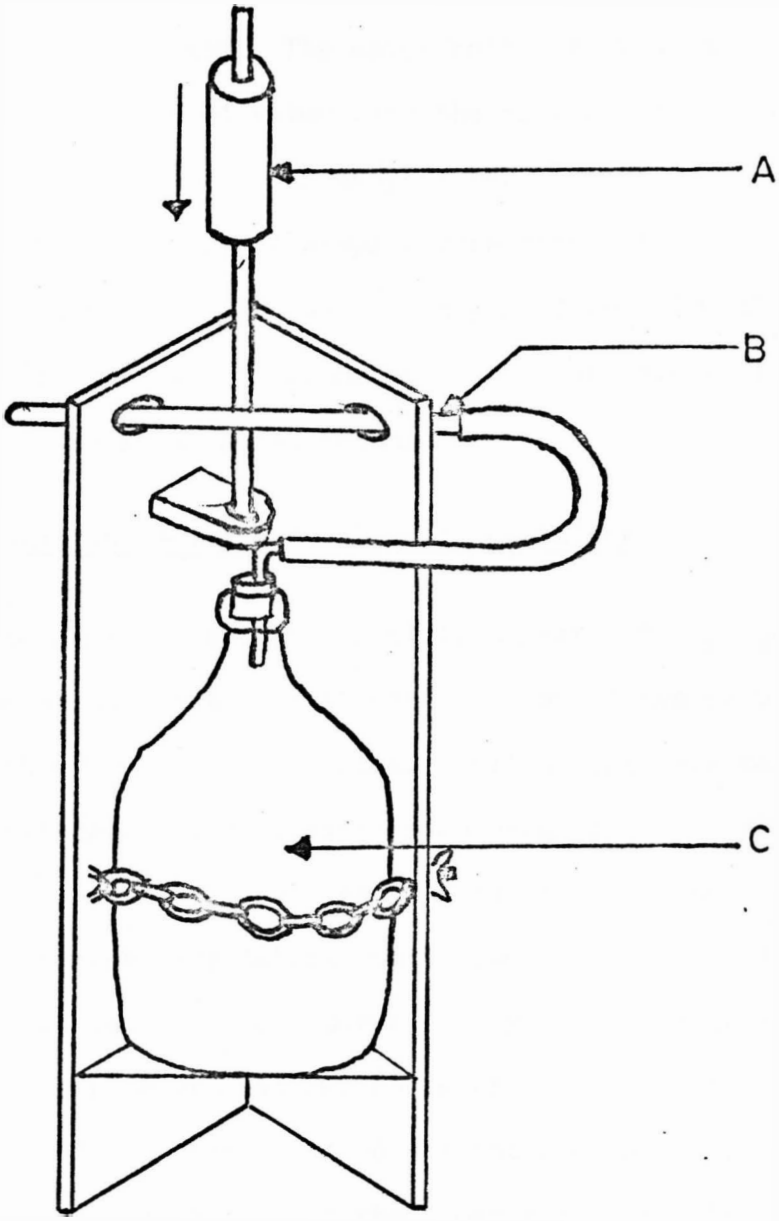

Fig. 1. Water sampler used for isolation study.
Key: A, sliding weight; B, glass tube;
C, 800 ml flask.

other sources of contamination. The water bottle of the sampler was evacuated to allow entrance of water into the bottle when the glass tube was broken. These fresh water samples were then transported to the laboratory. The animal fecal samples were obtained by rectal swabs of each animal. The rectal swabs were then placed into 100 ml of phosphate buffer (pH 7.4) and transported to the laboratory for immediate isolation of the fecal streptococci.

Isolation Medium and Cultural Conditions

In order to obtain a suitable isolation medium for S. bovis, studies were made of the best constituents and conditions required to support the growth of this microorganism. Evaluations were made of carbon sources, nitrogen sources, vitamin sources, optimium pH, atmospheric conditions, and concentrations of a selective agent.

The growth curves were determined in 300 ml flasks equipped with 16 mm side arm tubes and clean out ports (No. 5771 Bellco Glassware, Vineland, N. J.). For determinations using anaerobic atmospheres the flasks were closed with a screw cap and a Vacutainer stopper (Bectin and Dickensen, Rutherford, N. J.) on the clean out port. Each flask contained 100 ml of liquid medium which was autoclaved at 121 C for 15 min. The flasks were inoculated with 0.1 ml of a 24 hr brain heart infusion broth (BHI) culture of the test organism. For experimentation with an anaerobic atmosphere a vacuum pump was connected to the flask by a No. 21 needle inserted through the Vacutainer stopper. The flasks were evacuated and flushed with nitrogen three times before the

appropriate gas mixture was introduced. The final gas mixture was measured by a mercury manometer. A 10% negative pressure was left for accumulation of fermentation gases. When experiments were run under aerobic conditions, the above procedure was not necessary except to loosen the screw cap so that gas exchange could take place. The cultures were then incubated at 37 C on a New Brunswick rotary shaker model G-77 and shaken at 150 rpm to achieve a good gas exchange with the medium.

Growth of the organisms was determined turbidimetrically using a Bausch and Lomb Spectronic 20 spectrophotometer at 620 mμ. The absorbance was determined every 2 hr using a medium blank to set the spectophotometer. Growth was measured until the growth curve reached its maximum stationary phase. Duplicate samples and control flasks were run with every experiment. In one experiment, plate counts were used to make a viable count-growth curve. At 2 hr intervals counts were made by using a sterile needle and syringe to extract the sample from the flask. Dilutions were made and plated by a spread plate technique on growth medium No. 4 which had 1% agar added (described in Materials and Methods). The plates were incubated at 37 C in a humidified chamber.

Carbon Source. An isolation medium inhibiting the growth of S. faecalis without limiting the growth of S. bovis can be made by utilizing a carbon source which S. faecalis cannot ferment readily. Soluble starch and raffinose are two carbon sources which are readily used by S. bovis but not S. faecalis.

The carbon source experiment was set up using glucose, raffinose, and soluble starch (Difco Laboratories) as primary carbon sources. A growth medium contained the following ingredients:

Growth Medium No. 1

Carbon source	2 g
Bacto yeast extract	5 g
Bacto tryptose	20 g
Dipotassium phosphate	4 g
Water	1000 ml
pH	7.2

Aerobic flasks were set up in duplicate for turbidimetric growth determination of each organism tested.

Nitrogen Source. Of the fecal streptococci S. bovis has been the only microorganism which will utilize an inorganic nitrogen source as well as an organic nitrogen source (Wolin, Manning, and Nelson, 1959). If S. bovis can grow using an inorganic nitrogen source as well as it can an organic nitrogen source, the inorganic nitrogen source may be used to enhance the selectivity of the isolation medium.

To determine which nitrogen source would be the most effective for the growth of S. bovis, ammonium chloride (NH_4Cl) and Bacto tryptose were compared aerobically at different concentrations. For each microorganism tested growth medium No. 2 had Bacto tryptose (0.5, 1, 2, and 3%) or NH_4Cl (0.005, 0.01, and 0.02%) added.

Growth Medium No. 2

Yeast extract	5 g
Glucose	2 g
Dipotassium phosphate	4 g
Water	1000 ml
pH	7.2

Control flasks containing no nitrogen source other than yeast extract were also run. Turbidimetric determinations of the growth were made.

Selective Agent. A selective agent has to be used so that only a small group of microorganisms will grow on the isolation medium. The selective agent commonly used for the fecal streptococci has been sodium azide. A small amount of sodium azide inhibits aerobic bacteria by interfering with the enzyme, cytochrome oxidase. High concentrations of sodium azide also will inhibit the oxidation processes of anaerobic bacteria as well. The concentration of sodium azide used in the literature was high enough to effectively select for the enterococci but was too toxic to allow growth of S. bovis (Tiede, 1968).

The toxicity of varying levels of sodium azide were tested with S. bovis. Serial dilutions (1:2) were made of a 0.1% solution of sodium azide to obtain final solutions containing 0.1, 0.05, 0.025, and 0.0125% sodium azide. Each solution was made up to 100 ml. The constituents of growth medium No. 4 were added to each solution and placed into an aerobic flask. A flask containing 0% sodium azide was also set up in the same manner. Turbidimetric growth curves were determined as previously described.

To test the capability of sodium azide to inhibit the growth of coliform and spore-forming bacteria without inhibiting S. bovis, a 2 by 2 factorial experimental design was used. Sodium azide in several serial dilutions was one variable for the experiment while serial dilutions (1:10) of a mixture of the coliform bacteria and spore-forming bacteria was the other variable. The coliform and

spore-forming bacteria were obtained by inoculating BHI broth with river water and incubating for 24 hr. These microorganisms were used as representives of the flora of river water. A solution of sterile BHI broth containing 0.1% sodium azide was diluted (1:2) to duplicate the concentrations given above. A zero concentration of sodium azide was used as the control. A mixture of the coliform and spore-forming bacteria was diluted to 10^{-6}. Each dilution (1 ml) was inoculated into the medium containing sodium azide. The 2 by 2 design was repeated using S. bovis as the test microorganism. The tubes were incubated at 37 C and checked after 48 hr. Any trace of turbidity was counted as growth. The dilution extinction point used was the uninoculated control tube.

Optimum pH. Because the habitat of S. bovis was the rumen of animals one of the variables critical to the growth of S. bovis was pH. For any microorganism to exhibit its greatest possible growth an optimum pH must be maintained.

A series of side-arm flasks using the following medium were buffered at a pH range of 5 to 9. The pH of the medium was adjusted after sterilization using 1 molar concentrations of sterile monobasic and dibasic phosphate solutions.

Growth Medium No. 3

Bacto tryptose	20 g
Yeast extract	5 g
Glucose	2 g
Water	1000 ml
pH	7.2

Atmosphere. Duplication of the habitat in which S. bovis was normally found was the main objective of the atmosphere study. If an anaerobic atmosphere would enhance the growth of S. bovis more than an aerobic atmosphere, then the S. bovis isolate should be cultured under anaerobic conditions. The possibility of S. bovis being unable to adapt to aerobic conditions was taken into account.

Because carbon dioxide has been shown to favor the growth of S. bovis, various concentrations and combinations of carbon dioxide, air, and nitrogen were placed over S. bovis cultures. The medium was used as given below:

Growth Medium No. 4

Bacto tryptose	20 g
Yeast extract	5 g
Glucose	2 g
Dipotassium phosphate	4 g
Water	1000 ml
pH	7.2

Under increased carbon dioxide tension S. bovis will produce an insoluble dextran (Deibel, 1964). This dextran interfered with the absorbance readings obtained for a turbidimetric growth curve. For this reason, a plate count was run in conjunction with the turbidimetric growth curve as given on page 13.

Isolation Techniques

Isolation media used in this study were the M-enterococcus medium (Slanetz and Bartley, 1957) and a modification of the M-enterococcus medium. The modified M-enterococcus medium contained the following

constituents:

Modified M-enterococcus Medium

Bacto tryptose	20.0 g
Yeast extract	5.0 g
Dipotassium phosphate	4.0 g
Raffinose	2.0 g
Sodium azide	0.2 g
2,3,5-triphenyl tetrazolium chloride	0.1 g
Bacto agar	10.0 g
Water	1000 ml

The final pH of the modified M-enterococcus medium was 7.2 to 7.4. The modified M-enterococcus medium was autoclaved at 121 C for 15 min and was cooled before addition of the tetrazolium chloride. The media were dispensed into sterile 60 mm petri dishes in 10 ml amounts. The M-enterococcus medium obtained from Difco Laboratories in dehydrated form was prepared according to directions.

The samples included fresh water from the various sources, fecal swabs as described above from cattle and swine, and known cultures of S. bovis. Duplicate samples of the known cultures were diluted and filtered. The animal fecal swabs were immersed in 100 ml of phosphate buffer (pH 7.4) and diluted before filtration. The counts of the fecal swabs were expressed as number of bacteria per 100 ml of buffer or approximately 1 g of fecal material. The fresh water samples were filtered in 10 ml, 5 ml, 1 ml, and 0.1 ml amounts.

The membrane filter technique was used to isolate and enumerate S. bovis from the samples (APHA Standard Methods, 1965). The samples were filtered through Millipore HAWG filters. After filtration and placement of the membrane filters on the desired medium, 1.5 ml of

sterile starch agar at 50 C was aseptically layered over the membrane. The starch agar contained 1% soluble starch (Fisher Scientific Co., indicator grade) and 0.75% agar (Difco Laboratories). After the starch agar layer was allowed to solidify, the membrane plates were incubated at 37 C in an atmosphere of 25% carbon dioxide and 75% nitrogen for 24 to 48 hr.

Fecal coliform counts were run on each sample using the multiple tube EC confirmation test incubated at 44.5 C for 24 hr. Total fecal streptococci were determined by the azide dextrose and ethyl violet azide MPN technique (APHA Standard Methods, 1965).

After incubation, the membrane plates were flooded with a 1% iodine solution for 2 min before pouring off the iodine. Colonies showing zones of hydrolysis were counted as starch positive. Accurate counting was afforded by the use of an American Optical cycloptic stereoscope with a magnification of 70X. Photographs were taken to demonstrate the appearance of the zones of hydrolysis. A Pentax Spotmatic camera (Honeywell) with a 1.5X close up lense was mounted on a movable copy stand. A Wolf MG-7 illuminator was used to supply illumination from below while two No. 1 photoflood lamps were placed on either side above the Wolf illuminator. The petri plates to be photographed were placed on the Wolf illuminator and photographed with Pan X film (Eastman Kodak, Chicago, Ill.).

The colony responsible for producing the zone of hydrolysis was picked from the membrane with a needle and transferred to a tube of BHI broth. The isolates were incubated at 37 C for 24 hr before identification. A pure culture of the isolate was confirmed as a

streptococcus using a wet mount procedure with an American Optical dark contrast phase microscope at 1000X.

Stock cultures were obtained by inoculating the organisms into a stock culture maintenance medium described by Tiede (1968) as given below:

Maintenance Medium

Bacto tryptose	20 g
Yeast extract	5 g
Glucose	1 g
Dipotassium phosphate	4 g
Bacto agar	10 g
Water	1000 ml
pH	7.2

The organisms were incubated for 16 to 18 hr and then stored at 4 to 6 C.

Identification of streptococci was accomplished by biochemical tests. Because it has been indicated that S. bovis would not always follow any set taxonomic scheme, selected tests used by Hartman, Reinbold, and Saraswat (1966) were used. Growth at 10 C, growth at 45 C, bile sensitivity, salt tolerance, starch hydrolysis, and lactose fermentation were the criteria used in this study to identify S. bovis.

All of the streptococcal cultures confirmed by microscopic examination were inoculated into test media with a loop 2-3 mm in diameter and incubated at 37 C for 48 hr unless otherwise indicated. Turbidity and changes in color were recorded as positive tests. The following procedures and media were used to carry out these tests:

1. Growth at 10 C. Tubes containing 10 ml of BHI broth were pretempered to 10 C before inoculation. The BHI tubes

were then incubated at 10 C and checked for growth after 5 days.

2. Growth at 45 C. Tubes containing 10 ml of BHI broth were warmed to 45 C before inoculation. The tubes were incubated at 45 C for 48 hr and checked for growth by turbidity.
3. Bile sensitivity. The bile medium was prepared by adding 40 ml of sterile 10% Bacto oxgall to 60 ml of sterile BHI broth. This test was run to determine the ability of the microorganism to grow in the presence of bile. They were read at 48 hr.
4. Salt tolerance. Enough sodium chloride was added to BHI broth to obtain a 6.5% sodium chloride solution. Tolerance to sodium chloride was indicated by the presence of growth after 48 hr of incubation.
5. Starch hydrolysis. The test organism was inoculated into BHI broth containing 0.1% soluble starch (Difco). After incubation of the organism for 24 hr the ability to hydrolyze starch was tested by placing a drop of the inoculated starch medium on a spot plate containing a drop of Gram's iodine. The absence of a blue or rust color was taken as a positive test.
6. Lactose fermentation. The 1% lactose broth prescribed by Deibel (1964) was used. The production of acid from lactose was indicated by a color change from yellow to red by the methyl red indicator.

Bacteria which grew at 45 C but not at 10 C, which grew in bile but not in 6.5% sodium chloride, hydrolyzed starch, and fermented lactose were said to be S. bovis. However, variability of lactose fermentation and growth in 6.5% sodium chloride was noticed. The streptococci which grew at both 10 C and 45 C were of the enterococcus group (Sherman, 1937).

Persistency Study

In this study persistency was measured by percentage of survival which accurately represents the relative life span of a microorganism under adverse conditions (Geldreich and Kenner, 1969). Determinations of survival were measured by representing the initial bacterial count as 100% and reporting any growth or death of the microorganism in terms of percent. Persistency studies done in the laboratory were limited to effects of temperature, effects of aeration, and effects of organic nitrogen concentration in water. The results obtained were compared with the information obtained from a stream study.

A series of 100 ml samples of river water taken from a pooled sample and added to 250 ml flasks contained approximately 0.28 mg organic nitrogen per 100 ml. The flasks were sterilized at 121 C for 15 min to remove competition from other microorganisms in the water. The flasks were pretempered to the desired temperature before use. To inoculate the flasks 1 ml of an 18 hr BHI culture of S. bovis was used. The streptococci were counted by a spread-plate technique using the isolation medium described as modified M-enterococcus medium. A 1%

starch agar layer was used over the medium to aid in detection of *S. bovis*. All plates were incubated at 37 C and counted after 48 hr using the procedure described.

A series of duplicate flasks were set up at temperatures of 10, 15, 20, and 25 C to study the effects of temperature upon the persistency of *S. bovis* in fresh water. The flasks were incubated in the temperature study without aeration.

To study the effects of aeration upon the survival of *S. bovis*, duplicate flasks were run at 20 C in both static and aerated systems. Aeration was achieved by baffled flasks, fitted with four dents, shaken at 150 rpm on a New Brunswick rotary shaker Model G-77.

The nitrogen concentration of raw sewage and fresh water (from the Big Sioux River) was determined by the Kjeldahl technique (APHA Standard Methods, 1965). The river water was established as the lower limit while the sewage was established as the upper limit of organic nitrogen concentration. Peptone (Difco) was added to the river water to give total nitrogen concentrations of 0.28 mg to 2.9 mg of organic nitrogen per 100 ml. The flasks were inoculated and incubated at 20 C.

RESULTS AND DISCUSSION

Isolation Medium Development

Previous workers have developed selective media for isolation of enterococci which were not suitable for isolation of S. bovis, a viridans streptococcus. Studies in this laboratory have proven the isolation of S. bovis to be unsatisfactory when using media developed for isolation of the enterococci (Tiede, 1968). This study was undertaken to find a medium suitable for cultivation of S. bovis and to find cultivation conditions which would allow the best growth of S. bovis. In order to find an acceptable isolation medium the physiological requirements of S. bovis had to be met for a carbon source, a nitrogen source, and a vitamin source.

Because glucose can be readily used by S. bovis and many other streptococci, a substitute carbon source which only S. bovis could utilize had to be found. The hydrolytic capability of S. bovis for carbohydrates was not confined to polysaccharides such as starch. Disaccharides and trisaccharides were also susceptible to the amylases produced by S. bovis; however, S. faecalis did not have the capability to produce amylases as evidenced by its lack of starch hydrolyzing ability. Ideally, one should be able to use this lack of hydrolytic ability to effectively limit the growth of S. faecalis which in high numbers would mask the presence of S. bovis in an isolation medium.

The results of Figure 2 show the ability of S. bovis and S. faecalis to grow on glucose. Because the growth of S. faecalis was

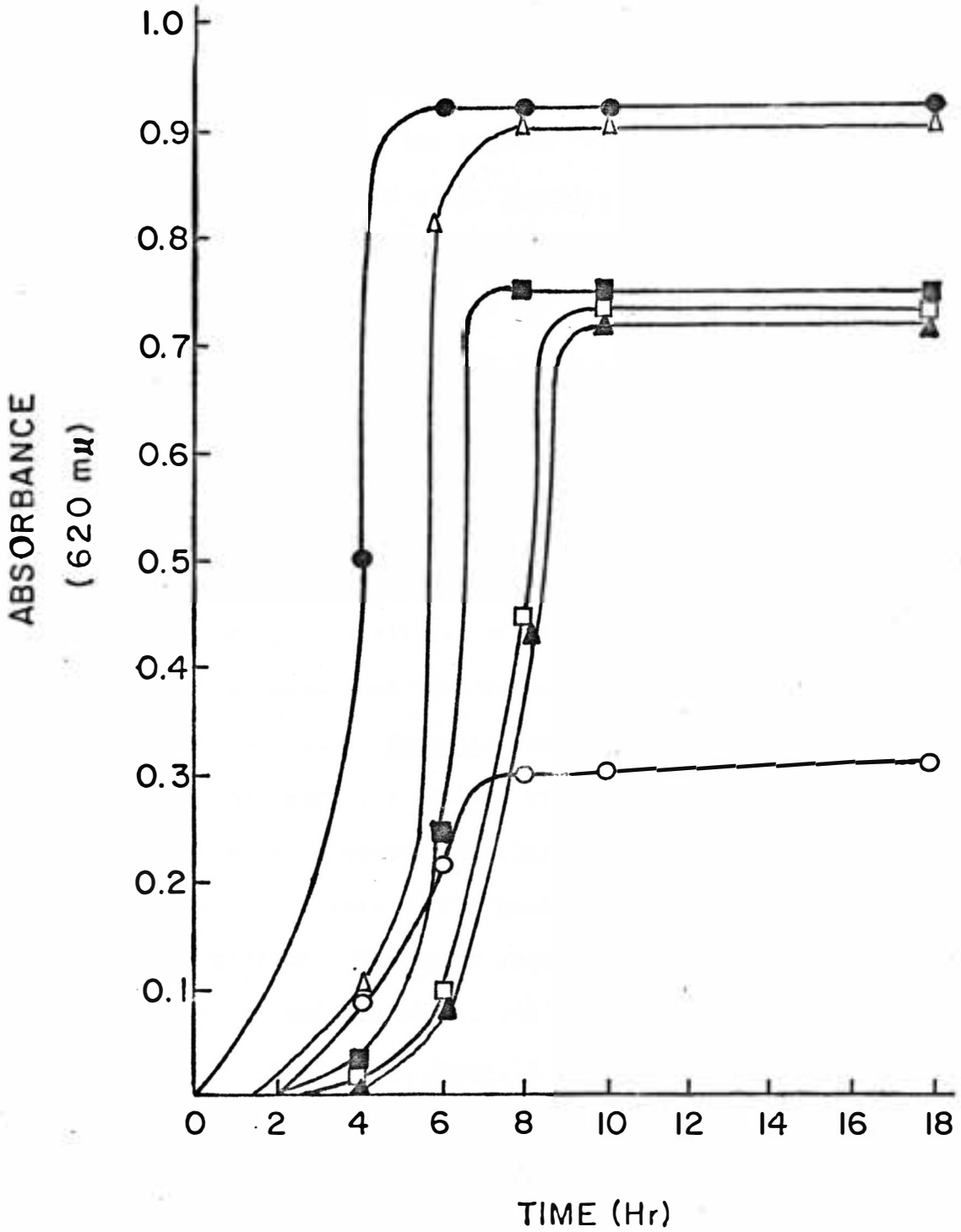

Fig. 2. Utilization of raffinose and soluble starch by Streptococcus bovis and Streptococcus faecalis. Symbols: ●, S. faecalis and glucose; ○, S. faecalis and raffinose; ■, S. bovis and glucose; □, S. bovis and raffinose; ▲, S. bovis and soluble starch; △, S. faecalis and soluble starch.

much greater than the growth of S. bovis, a medium containing glucose would favor the growth of S. faecalis. However, the growth of S. faecalis with raffinose as the primary carbon source was very limited and was equal to the growth of S. faecalis in the control flask which contained no other carbon source other than that found in yeast extract. The maximum amount of growth of S. bovis on raffinose was equal to the growth of S. bovis on glucose. As the amount of growth of S. faecalis on raffinose was less than the amount of growth of S. bovis on the same sugar, a medium containing a trisaccharide such as raffinose would be somewhat selective for S. bovis. However, a medium containing raffinose would not inhibit all strains of S. faecalis because a S. faecalis with amylytic abilities similar to the amylytic abilities of S. bovis has been described (Geldreich and Kenner, 1969). The occurrence of this atypical S. faecalis strain in water has been slight. Figure 2 also indicates the ability of S. faecalis to grow as well on soluble starch as on glucose. Similar results were also true for S. bovis as growth of this microorganism was the same on both soluble starch and on glucose. The heavy growth exhibited by S. faecalis, a typical strain, on soluble starch was unexpected because of the lack of hydrolytic activity of S. faecalis on the trisaccharide, raffinose. This heavy growth was possibly due to partial hydrolysis of the soluble starch by the steam sterilization before use. The final analysis of the carbon sources indicates raffinose to be the selective carbon source for isolation of S. bovis.

Streptococcus bovis has the ability to utilize an inorganic nitrogen source (Wolin and Weinberg, 1960) as well as an organic

nitrogen source (Barnes, Seeley, and VanDenmark, 1961). Deibel (1964) reported the S. faecalis was unable to utilize NH_4Cl. If S. bovis can utilize NH_4Cl as well as it can utilize Bacto tryptose, NH_4Cl could be used to restrict the growth of S. faecalis without affecting the growth of S. bovis. Results obtained in this study indicate that NH_4Cl was inferior to Bacto tryptose for the growth of S. bovis (Table 1).

Table 1. Growth of Streptococcus bovis on various concentrations of Bacto tryptose and NH_4Cl in 10 hr.

Nitrogen source % (w/v)	Nitrogen mg/100 ml	Absorbance at 620 mμ
Bacto tryptose		
0.5	70.0	0.886
1.0	140.0	0.921
2.0	280.0	1.000
3.0	360.0	1.000
NH_4Cl		
0.005	1.3	0.469
0.010	2.6	0.469
0.020	3.2	0.495
0.100	16.0	0.377
0.200	32.0	0.222
Control		
0.0		0.387

Growth in the control flask was from nitrogen of the 0.2% yeast extract. Increasing the concentration of NH_4Cl did not increase the amount of growth of S. bovis. The total nitrogen of the Bacto tryptose was higher than that of the NH_4Cl used but concentrations of NH_4Cl above 0.02% showed toxicity of the chloride ion to S. bovis.

Under aerobic and anaerobic conditions the vitamin requirement for S. bovis was met by yeast extract. Biotin was the only vitamin required for growth of S. bovis and thiamine has been used as a growth stimulant for most strains of S. bovis (Deibel, 1964; Niven, Washburn, and White, 1948). Both vitamins were supplied in adequate amounts in this study by 0.2% yeast extract (Constituents of Bacteriological Culture Media, 1956).

Although many selective agents have been used in isolation media, sodium azide has been used most widely for the fecal streptococci (Kenner, Clark, and Kabler, 1961). In 1953 Litsky, Mallmann, and Fifield demonstrated the value of azide dextrose broth (0.02% sodium azide) for isolation of enterococci in conjunction with a confirmed test using ethyl violet azide broth (0.04% sodium azide). However, they also demonstrated that 0.04% sodium azide was inhibitive to S. bovis. Sodium azide concentrations of 0.02 to 0.03% were sufficient to inhibit coliform organisms. Reinbold, Swern, and Hussong (1953) demonstrated that 0.03% sodium azide was toxic to resting cells of fecal streptococci and levels from 0.01 to 0.025% were only slightly toxic. Streptococcus bovis was progressively inhibited as the concentration of sodium azide increased (Fig. 3). The data at 18 hr from Figure 3 were replotted to see if there was any relationship between sodium azide concentration and growth of S. bovis. In Figure 4 the inhibition of the growth of S. bovis by sodium azide is shown as a linear relationship. This relationship is probably due to the progressive inhibition of the oxidation processes of S. bovis by sodium azide.

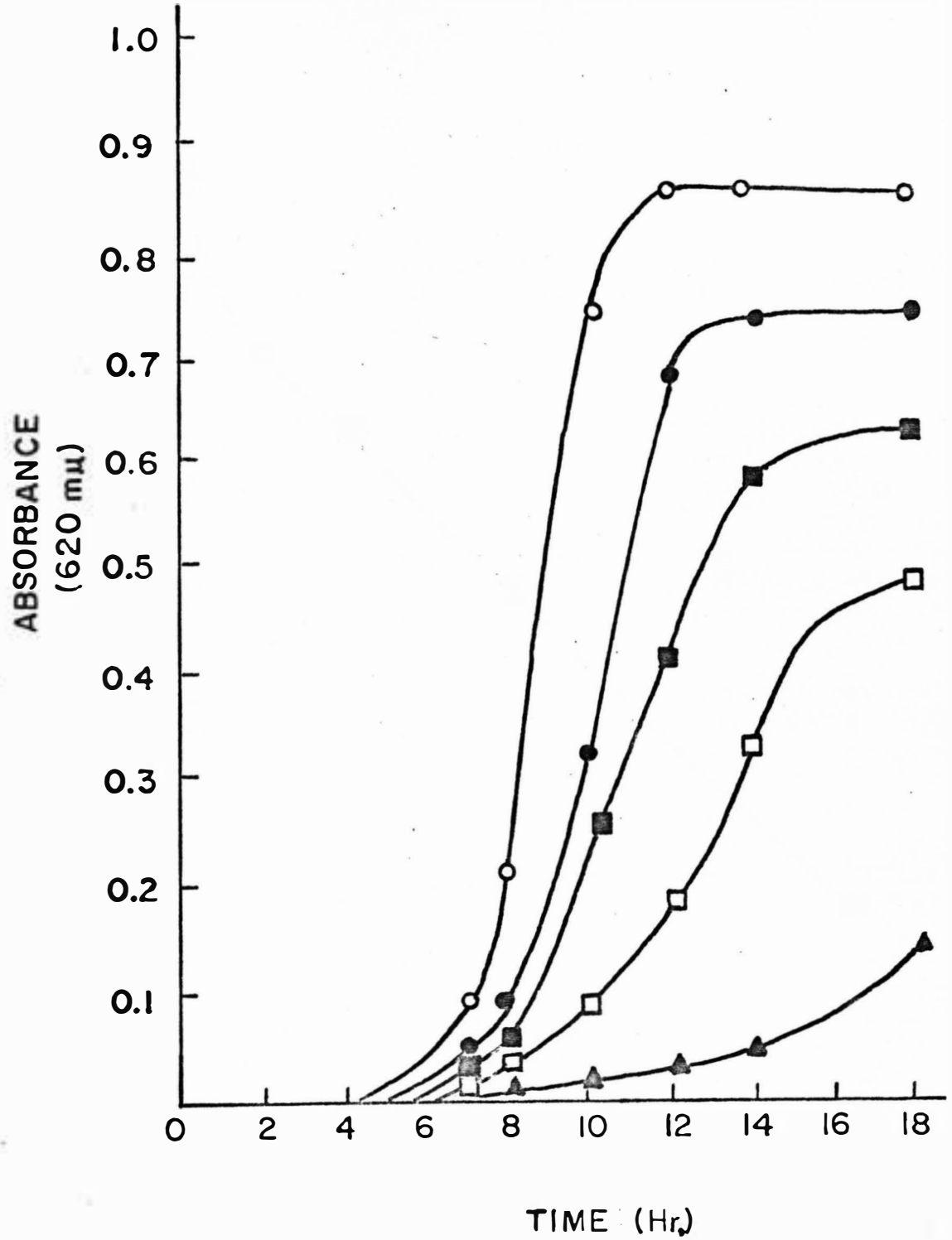

Fig. 3. Effects of various concentrations of sodium azide on the growth of Streptococcus bovis. Symbols: ○, 0.0%; ●, 0.0125%; ■, 0.025%; □, 0.05%; ▲, 0.1%.

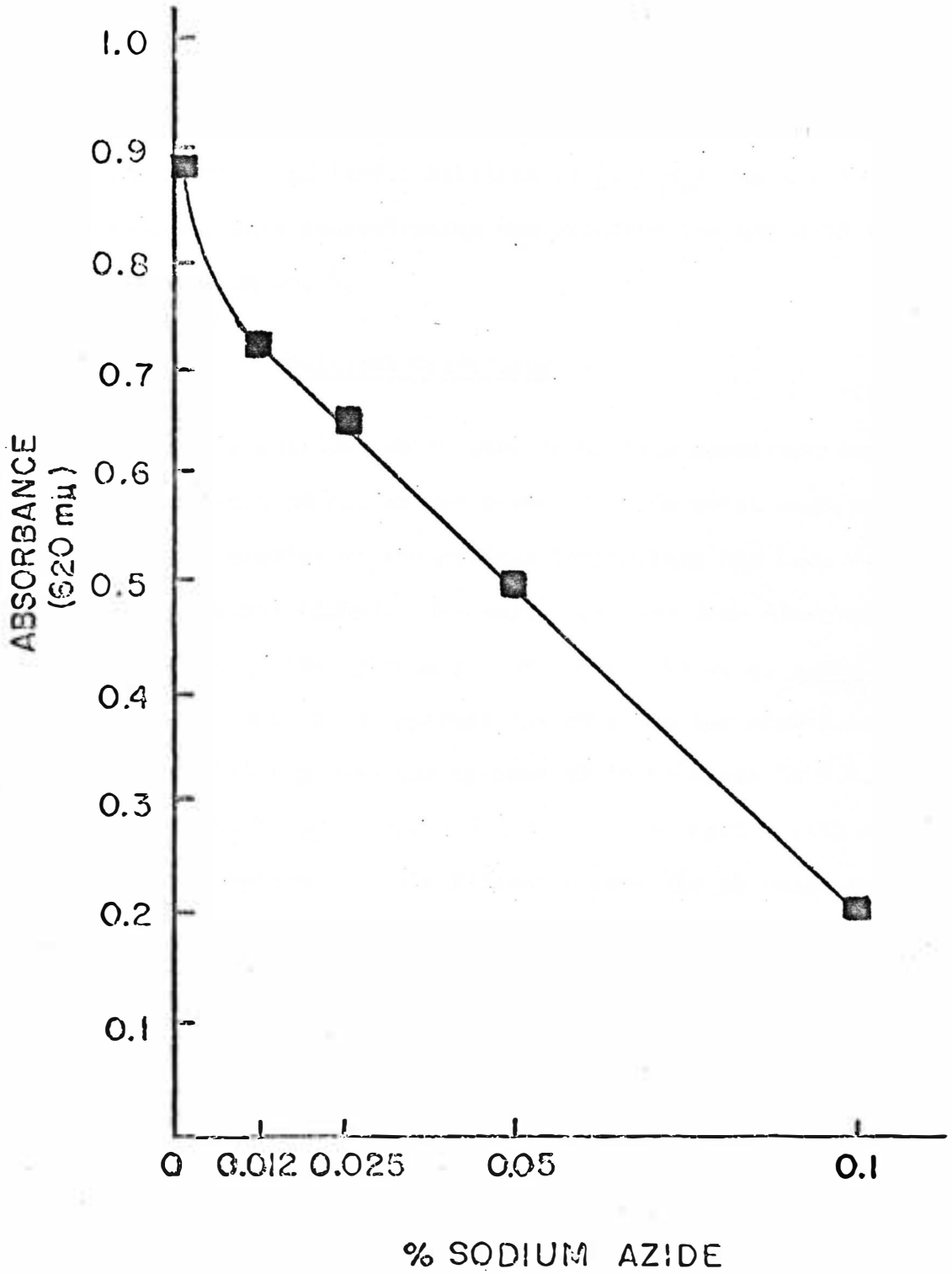

Fig. 4. Inhibition of the growth of Streptococcus bovis by sodium azide at 18 hr.

Coliform and spore-forming bacteria from river water were inhibited by concentrations of sodium azide as low as 0.02%. Since the data in Figure 3 indicated that inhibition of S. bovis was slight at 0.02% sodium azide, this concentration was selected for use with the final isolation medium No. 4.

Cultural Conditions

The cultural conditions which were taken into consideration in this paper were optimum pH and oxygen-carbon dioxide relationships. Temperature was not studied as the optimum temperature has been established as 37 C by Deibel (1964). However, there was some disagreement in the literature as to the optimum pH for the growth of S. bovis. Wolin and Weinberg (1960) have reported the pH range for growth to be 4 to 7. Deibel (1964) reported the optimum pH to be close to 7.4. If the optimum pH for S. bovis is below 7 and isolation medium with a pH above 7 will not be optimum. Table 2 demonstrates the pH range found in this study.

Table 2. Turbidimetric determination of the optimum pH of Streptococcus bovis in 12 hr of growth.

Initial pH	Final pH	Absorbance at 620 mμ
5.0	5.9	0.032
6.0	6.2	0.125
6.5	6.2	0.180
7.0	6.8	0.200
7.5	6.8	0.215
8.0	7.8	0.070
9.0	8.8	0.137

The optimum pH was indicated to be 7.0 or 7.5. The final pH of the medium was taken after 12 hr and found to be close to the initial pH.

Another requirement of the microorganism which has not been studied extensively for isolation is the requirement for carbon dioxide. Since the usual habitat of S. bovis before entry into water or soil is the rumen of animals, carbon dioxide may be essential for growth of S. bovis.

An increased concentration of carbon dioxide has been proven favorable to the growth of S. bovis (Prescott and Stutts, 1955: Prescott, Ragland, and Stutts, 1957). Wright (1960) suggested that carbon dioxide fixation was an essential part of the metabolism of S. bovis. Carbon dioxide fixation also explains the absolute requirement for the vitamin biotin. Preliminary studies with a turbidimetric growth curve have demonstrated an atmosphere of 75% nitrogen and 25% carbon dioxide to be the most favorable to the growth of S. bovis (Table 3).

Table 3. Turbidimetric determination of the growth of Streptococcus bovis in 24 hr with the described gaseous atmospheres.

Concentration of gas	Absorbance at 620 mμ	Final pH
100% air	0.347	7.4
100% CO_2	0.432	6.4
100% N_2	0.699	6.7
50% N_2 and 50% CO_2	0.456	6.5
75% N_2 and 25% CO_2	0.630	6.9

The carbon dioxide atmosphere altered the pH of the growth medium so that optimal conditions were not established and the buffer in the medium was not strong enough to hold a stable pH during growth of S. bovis. However, under increased carbon dioxide tension S. bovis will produce an insoluble dextran which may have affected the turbidimetric readings. It was for this reason that a plate count was used to determine a viable growth curve of S. bovis under aerobic and the selected anaerobic atmosphere. A difference was seen in the maximum growth by S. bovis in the 75% nitrogen and 25% carbon dioxide atmosphere (Fig. 5). This difference demonstrated the advantage of using carbon dioxide and anaerobic conditions when culturing S. bovis on isolation medium.

Isolation of Streptococcus bovis from Water and Feces

Enumeration of streptococci which hydrolyze starch was accomplished by flooding the starch agar layer on the membrane with a solution of 1% aqueous iodine. Zones of hydrolysis ranging in size from 3 to 5 mm in diameter allowed an accurate count of the numbers of starch hydrolyzing colonies (Fig. 6). However, approximately 1% of the starch positive colonies from the fecal samples were hard to distinguish because of their pinpoint size. Close observation of the zone at 70X magnification revealed a small white colony. Plates with 50 or more zones were difficult to count because of the confluence of the zones.

Even though heavily polluted water samples were filtered, no growth other than typical streptococcal colonies occurred on either

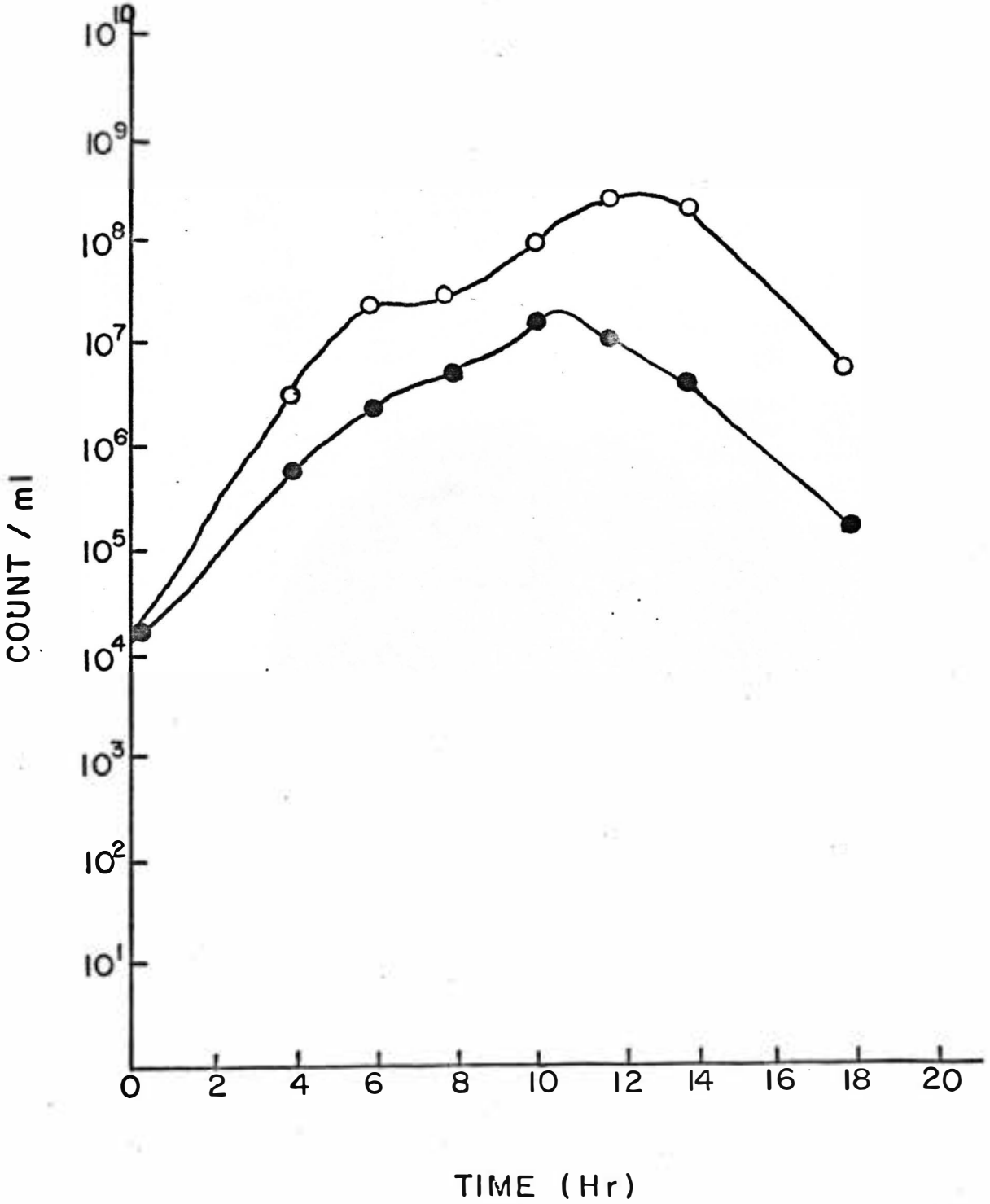

Fig. 5. Comparison of growth of Streptococcus bovis under anaerobic and aerobic conditions using a plate count technique. Symbols: O , 25% CO_2 and 75% N_2; ● , 100% air.

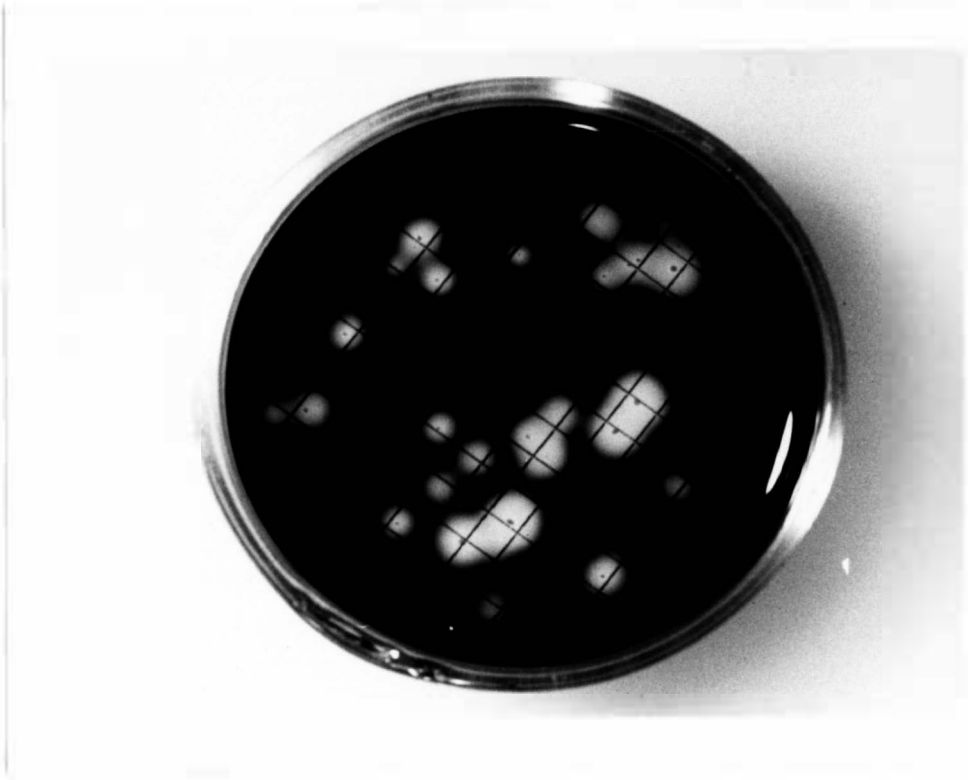

Fig. 6. Zones of starch hydrolysis produced by Streptococcus bovis on a membrane filter layered with 1% starch agar.

M-enterococcus medium or modified M-enterococcus medium. On M-enterococcus medium the pink to maroon entire colonies ranged from pinpoint size to 2 mm in diameter. On the modified M-enterococcus medium the pink to white entire colonies were of the same size range. The difference in colony color between the two media was due to the lower sodium azide concentration of modified M-enterococcus medium. Of the 124 isolations made, all were identified as fecal streptococci by biochemical tests.

Known cultures of S. bovis were filtered and grown on M-enterococcus medium and modified M-enterococcus medium for a comparison of the recovery of the organisms on 0.02% and 0.04% sodium azide. Duplicate counts showed that the recovery of S. bovis on modified M-enterococcus medium was higher than the recovery on M-enterococcus medium (Table 4).

Table 4. Comparison of M-enterococcus and modified M-enterococcus medium using known Streptococcus bovis cultures.

Culture	Count/ml x 10^5	
	Modified M-enterococcus	M-enterococcus
S. bovis ATCC 9809	3400	6.0
S. bovis ATCC 15351	1100	5.9
S. bovis	230	1.0

The membrane filter technique together with a 1% starch agar layer was used for counting purposes. The third known S. bovis culture used for the comparison in Table 4 was isolated by our laboratory from the Big Sioux River and was designated I-104.

Duplicate samples of polluted water and fecal material were filtered to demonstrate the ability of modified M-enterococcus medium to indicate more starch hydrolyzing streptococci than M-enterococcus medium (Table 5). In all samples counts of starch hydrolyzing streptococci were significantly higher on modified M-enterococcus medium than on M-enterococcus medium. Starch hydrolyzing streptococci in the cattle fecal samples averaged 63% of total fecal streptococci on modified M-enterococcus medium and 57% on M-enterococcus medium. Plate counts for the swine feces were 44% starch hydrolyzers for modified M-enterococcus medium and 8% for M-enterococcus medium. These data have good correlation with the 66 to 75% occurrence of S. bovis in cattle feces and 18 to 56% occurrence in swine feces reported in the literature (Croft, 1959; Kenner, Clark, and Kabler, 1960).

Of the 93 random isolations made from the starch hydrolyzing colonies found in all samples, 93% were identified to be S. bovis and 7% were identified as atypical S. faecalis (Mann, Masson, and Oxford, 1954). Isolations of atypical S. faecalis were made in cattle feces (4 isolations) and in fresh water (3 isolations).

The starch agar layer method used in conjunction with the membrane filter technique proved valuable in determining the number of

Table 5. Isolation of starch hydrolyzing streptococci from water and fecal material using the membrane filter and starch overlay technique[a]

Sample Source	Fecal coliform count/100 ml	Fecal streptococci count/100 ml	Starch hydrolyzing streptococci[b] count/100 ml		Ratio
	MPN	AD/EVA	MME	ME	FC/FS
Big Sioux River					
Site No.					
1	330	2,810	40	0	0.1
2	720	130	0	0	13.2
3	460	70	20	0	6.6
4	50	23	0	0	2.2
5	33	33	5	0	1.0
6	790	85	20	20	9.3
7	490	790	10	0	0.6
8	24,000	2,300	176	0	10.1
9	17,200	79,000	6,300	1,100	0.2
10	33,000	4,900	2,800	300	6.7
11	49,000	3,480	470	70	14.1
12	23,000	13,000	4,100	10	1.8

Skunk Creek					
Site No.					
1	2,300	790	210	0	2.9
2	330	330	150	100	1.0
Cattle Feces[c]					
Sample No.					
1	3,300,000	11,000,000	8,400,000	7,000,000	0.3
2		28,000,000	23,000,000	20,000,000	
3	790,000	27,000,000	10,000,000	15,000,000	0.03
4		2,900,000	1,400,000	100,000	
5	2,300,000	3,000,000	3,000,000	1,000,000	0.76
6	230,000	3,000,000	1,000,000	100,000	0.08
7	43,000,000	1,000,000	1,000,000	100,000	43.00
Swine feces					
Sample No.					
1	40,000	200,000	140,000	70,000	0.20
2	23,000	700,000	220,000	10,000	0.03
3	430,000	200,000	120,000	10,000	2.20

Table 5 (continued)

[a] Abbreviations: MPN, most probable number
AD/EVA, azide dextrose/ethyl violet azide media
MME, modified M-enterococcus medium
ME, M-enterococcus medium
FC/FS, fecal coliform/fecal streptococcus ratio

[b] Characteristic colonies producing a zone of hydrolysis. Random isolations (93) were made from the starch hydrolyzing colonies. Of these 86 were identified as Streptococcus bovis and 7 were identified as atypical Streptococcus faecalis.

[c] Taken with rectal swabs and diluted to 100 ml.

starch hydrolyzing streptococci in water. However, the presence of a zone of hydrolysis did not necessarily indicate that the organism responsible was S. bovis. Seeley and Dain (1960) demonstrated that S. bovis, S. equinus, and S. uberis are the principle starch hydrolyzers in the bovine rumen. Mann, Masson, and Oxford (1954) reported finding a starch hydrolyzing S. faecalis in the rumen of sheep. The possibility that water polluted from an animal source would contain a mixture of starch hydrolyzing streptococci cannot be ignored. In this study no isolations of S. uberis or S. equinus were made from the water samples or from the fecal samples because S. equinus and S. uberis are weak starch hydrolyzers which perhaps could not be detected easily. Atypical S. faecalis (capable of starch hydrolysis) was found in both feces and polluted water samples. The possibility exists that atypical S. faecalis can be normally found in the rumen, although atypical S. faecalis has also been reported in green bean waste discharge from a cannery (Geldreich and Kenner, 1969). Of the 93 random isolations made in this study from the zones of hydrolysis, the majority were proven to be S. bovis indicating that the streptococci isolated were of animal fecal origin.

The consistently higher counts of starch hydrolyzing streptococci were apparent on modified M-enterococcus medium. Apparently the 0.04% sodium azide found in M-enterococcus medium was sufficient to inhibit the growth of S. bovis. Decreasing the concentration of sodium azide to 0.02% did not reduce selectivity even though heavily polluted water and fecal material were filtered. The carbon dioxide and

anaerobic conditions may have played a role in the selectivity of the medium as well as enhancing the growth of S. bovis. Experimentation has proven that carbon dioxide and anaerobic conditions alone are inadequate to select for S. bovis. Lowering the sodium azide concentration to 0.02% created a problem by allowing the S. faecalis colonies to grow larger than normal after 24 hr. The larger size of the S. faecalis colonies made it more difficult to identify the smaller colonies present. The substitution of raffinose for glucose in modified M-enterococcus medium restricted the growth of S. faecalis without affecting the growth of S. bovis. The restriction of the growth of S. faecalis on raffinose is probably because S. faecalis is unable to ferment raffinose.

For each sample a fecal coliform/fecal streptococcus ratio (FC/FS) was calculated to give an indication of the relative origin of the pollution. A ratio of less than 0.6 indicated an animal pollution source while a ratio of 4.0 and above indicated a human pollution source (Croft, 1959; Geldreich, Clark, and Huff, 1964). The FC/FS ratio did not correlate well with numbers of starch hydrolyzing colonies counted in all samples. In the water samples where the starch hydrolyzing streptococci count consisted of 20 to 30% of the total fecal streptococci the FC/FS ratio indicated a human pollution source. The high percentage of starch hydrolyzing colonies would indicate a strong possibility of an animal pollution source. This may be because most of the water samples could have been of mixed animal and human pollution or that the FC/FS ratio does not correlate with the pollution source. It

is possible that the population levels of the fecal coliform and fecal streptococci have no relation to each other or to their source.

Persistency Study

Little is known about the survival of S. bovis after it is discharged from the animal into surface water. The persistency of S. bovis has been demonstrated to be very short in fresh water as well as salt water (Slanetz and Bartley, 1965; Bartley and Slanetz, 1960; Geldreich and Kenner, 1969). Geldreich and Kenner (1969) demonstrated the persistency of S. bovis to be less at 10 C than at 20 C but no explanation was given. In Figure 7 S. bovis showed a greater persistency at 10 C than any other temperature and which was contrary to the results reported in the literature. The other temperatures with the exception of 10 C gave greater persistency of S. bovis as the temperature rises. Duplicate results indicate that the temperature range from 15 to 20 C has little effect upon the persistency of S. bovis. The longer survival of S. bovis at 10 C can possibly be attributed to the relatively young age of the cells (18 hr) and to the fact that S. bovis will not grow at 10 C. Difficulty of storage of S. bovis at 10 C or lower was noted to be directly related to the stage of growth of the organism.

In the course of this study it was noticed that the persistency of S. bovis was greatest before it reached the maximum stationary phase of growth. After the maximum stationary phase was reached the persistency of S. bovis declined.

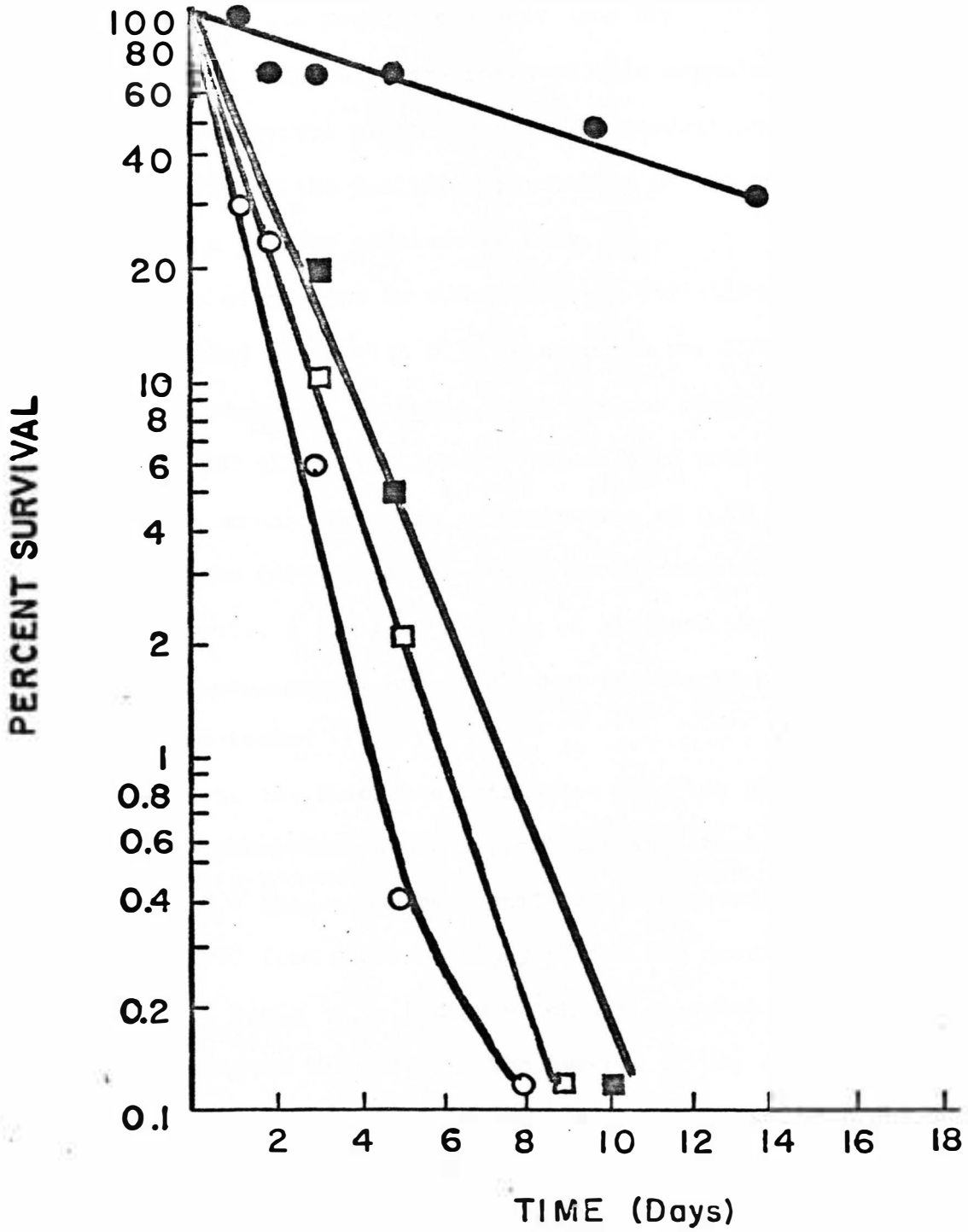

Fig. 7. The effects of temperature on the persistency of Streptococcus bovis in fresh water. Symbols: ● , 10 C; ○ , 15 C; □ , 20 C; ■ , 25 C.

Aeration had a favorable effect upon the survival of S. bovis in that aeration induced after-growth of this organism as indicated by the rise in survival after 3 days. The production of new cells probably stabilized the declining population of the aerobic flask to give S. bovis a greater persistence (Fig. 8).

The use of peptone to obtain organic variation of the water demonstrated that water with 0.72 mg nitrogen per 100 ml showed the greatest persistence of S. bovis while the raw sewage with 2.90 mg nitrogen per 100 ml had the lowest. These data would indicate that water with an organic nitrogen concentration of 0.72 mg per 100 ml would be optimum for some after-growth to increase the number of S. bovis present. A level of 2.90 mg of nitrogen was too high to support the microorganism and it allowed the lowest persistence of the concentrations tested (Fig. 9).

Combining the favorable attributes from the three conditions tested in the laboratory would support the idea that the persistency of S. bovis in a stream may be significant. However, results obtained by Zerfas (1970) from a stream study of the Big Sioux River showed the survival of S. bovis to be 3 days which was somewhat less than the results obtained in this study. The absence of any single stabilized condition in a stream would help explain the difference between the two studies. During the stream study, the temperature of the water deviated from 18 to 20 C and the nitrogen content of the stream was 0.28 to 0.30 mg organic nitrogen per 100 ml. These two conditions alone would not account for the difference in the two studies. Some

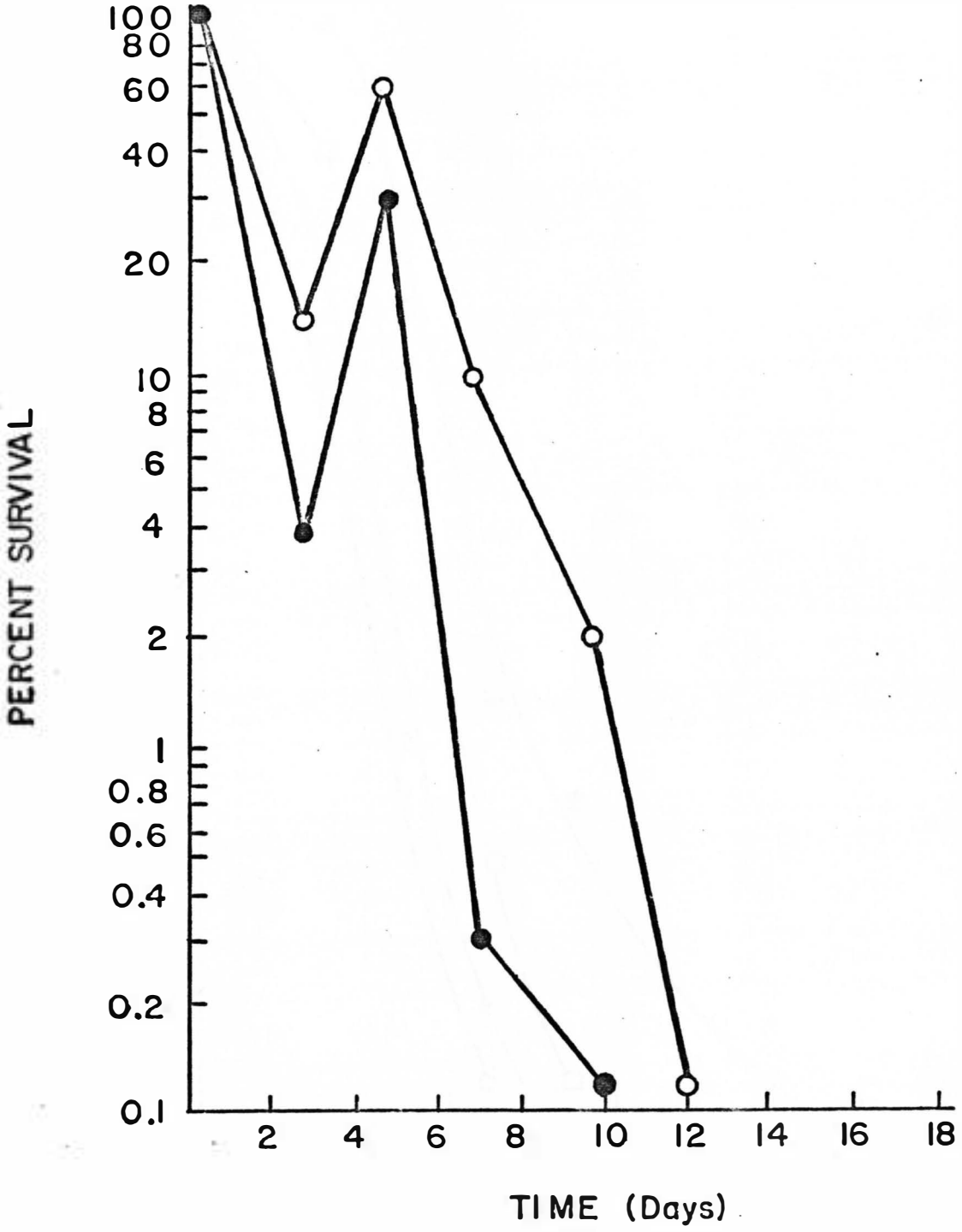

Fig. 8. The effects of aeration on the persistency of Streptococcus bovis in water at 20 C. Symbols: O , aerated; ● , static.

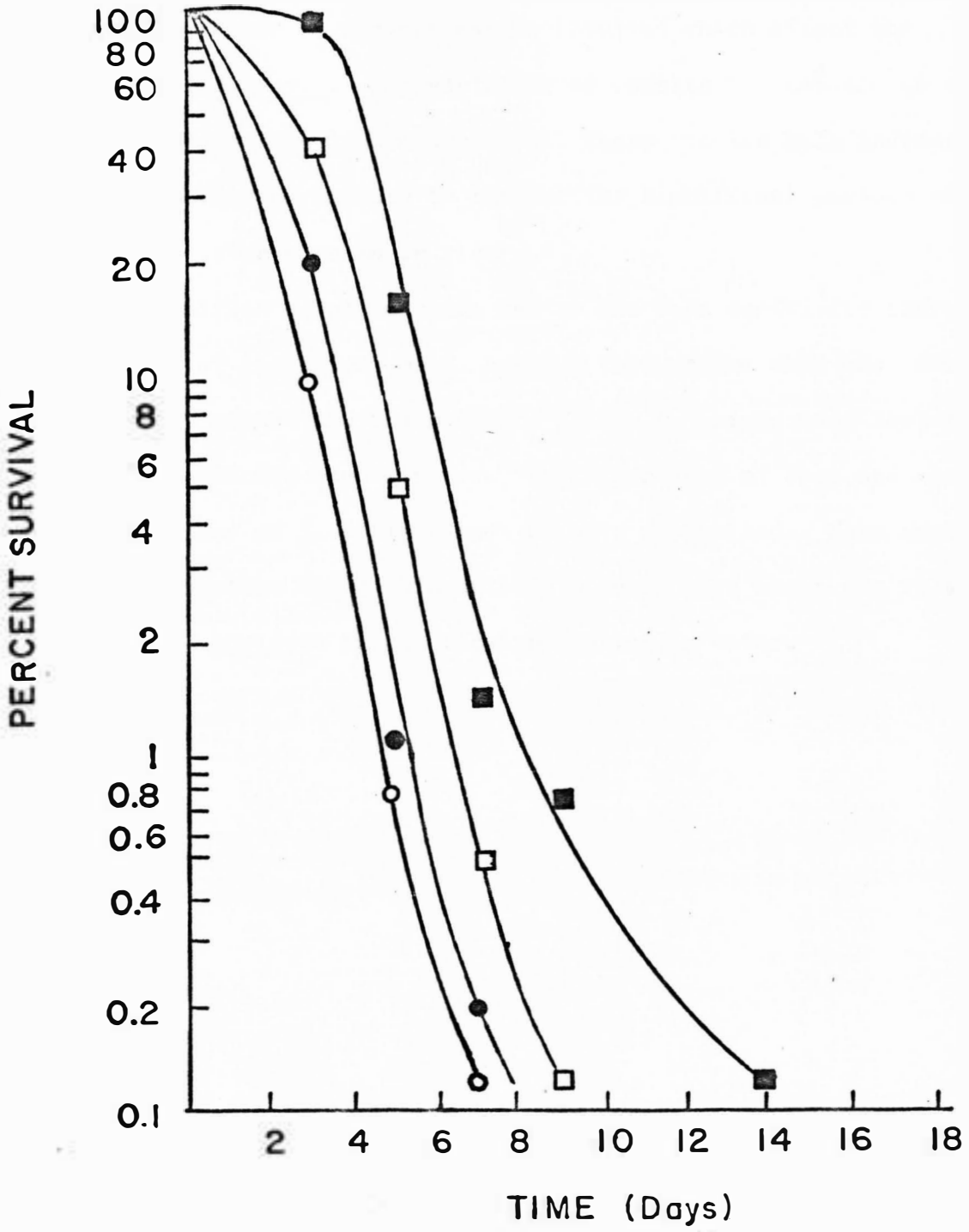

Fig. 9. Effects of organic nitrogen variation of fresh water on the persistency of Streptococcus bovis at 20 C. Symbols: ○ , 2.90 mg nitrogen/100 ml; □ , 1.45 mg nitrogen/100 ml; ■ , 0.72 mg nitrogen/100 ml; ● , 0.28 mg nitrogen/100 ml.

other unknown factor or factors may be involved which affect the persistency of S. bovis. The variation of results was not enough to warrant discarding the laboratory data. These studies help indicate that S. bovis has the ability to survive for significant periods of time in fresh water streams or rivers.

The modified M-enterococcus medium has been useful for increasing the frequency of isolations of S. bovis. This medium when used with the starch overlay method and the membrane filter technique made isolation of S. bovis rapid and quantitative. The importance of fast and quantitative isolations of S. bovis cannot be over emphasized. More work will have to be done using this method to learn more about the relationship of S. bovis to animal fecal pollution in water.

CONCLUSIONS

1. Substitution of raffinose for glucose serves to make the basal medium more selective for Streptococcus bovis.
2. Sodium azide at concentrations of 0.04% and greater inhibits the growth of S. bovis.
3. A sodium azide level of 0.02% was sufficient to inhibit the growth of coliform and spore-forming bacteria without inhibiting S. bovis.
4. A 25% carbon dioxide and 75% nitrogen atmosphere over the cultures enhances the growth of S. bovis while it maintains selectivity of a given medium.
5. Modified M-enterococcus medium was more suited for isolation of S. bovis than M-enterococcus medium when using the membrane filter technique.
6. The starch agar layer method when used in conjunction with the membrane filter and spread plate technique is an excellent method for rapid screening for S. bovis.
7. The majority of the starch hydrolyzing streptococci isolated from polluted water and animal feces was S. bovis (92%) with atypical S. faecalis representing 8%.
8. Streptococcus bovis exhibited the greatest persistence under the following conditions: in an organic concentration of 0.73 mg nitrogen per 100 ml as peptone, in an aerated state, and at a temperature of 10 C.

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