THE DEVELOPMENT OF METHODS FOR THE ISOLATION OF ENTEROCOCCI FROM WATER AND SEWAGE

By

Warren Litsky

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

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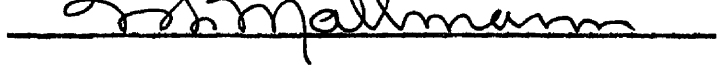
Approved 

Ethyl purple azide broth, a new medium, was developed. The formula for this medium is as follows:

Ingredient	Grams per liter
Tryptose	20
Dextrose	15
Sodium chloride	5
K_2HPO_4	2.7
KH_2PO_4	2.7
Sodium azide	0.4
Ethyl purple	0.00125

pH - 7.0

Sterilized at 121° C. for 15 minutes

When tested with laboratory strains of bacteria, it was found that the enterococci were the only group that would grow in this medium. These organisms also showed a characteristic growth of a purple compact button on the bottom of the tube of medium after 48 hours of incubation at 37° C.

The specificity of this medium has been demonstrated also with samples from river water, sewage, and soil.

A new test for pollution of river water, sewage, and soil has been advanced. This test employs dextrose azide broth as a presumptive medium and ethyl purple azide broth for confirmation.

A comparison of methods for the detection of enterococci was made and it was demonstrated that the new dextrose azide-ethyl purple azide broth test was the best and easiest of those in use today.

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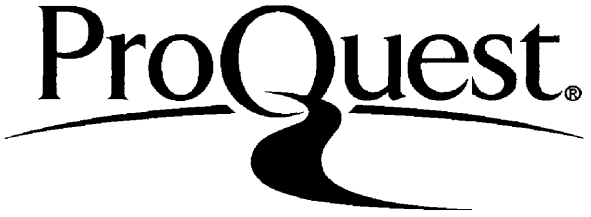

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INTRODUCTION

Workers throughout the years have investigated the problem of polluted waters and are all in agreement regarding the seriousness of the situation. Recently Dunlop, Twedt and Wang (15) have reported that 23 out of 113 samples of water used for irrigating vegetables in Colorado were positive for Salmonella and one sample yielded Salmonella typhosa. This organism was also isolated from East Lansing sewage and from the Red Cedar River. Although only a few epidemics have been charged to contaminated vegetables, it is possible that unrecognized endemic enteric infections or sporadic cases may be caused by eating vegetables grown on contaminated soil.

Up to now the test organisms employed for pollution are members of the coliform group, because these bacteria are supposedly indicative of sewage contamination and can be easily isolated and confirmed by a simple bacteriological test. It was felt, however, that the coliform organisms do not give a true indication of pollution because: (1) these organisms are found in uncontaminated soil and may be of non-fecal origin, (2) these organisms may persist in the soil and water for long periods of time and might not indicate a recent pollution, and (3)

fecal strains of coliform bacteria cannot be distinguished from non-fecal strains.

Streptococci are used as an indicator of pollution on the same grounds as Escherichia coli because (1) they are present in feces and sewage and are found in known polluted waters, (2) they are not found in pure waters, virgin soil, and sites out of contact with animal and human life, and (3) they do not multiply outside the animal body (except in such media as milk, etc.).

With respect to their numbers in feces and sewage, streptococci are subject to great variation. A review of literature reveals that while at times they may be almost as numerous as E. coli, at other times they may be considerably less numerous and absent. Mallmann and Litsky (41) showed that in soil that was treated with sewage the most probable numbers of streptococci was approximately that of the coliform organisms. It was also demonstrated that the streptococci disappeared from the soil rapidly while the coliform group persisted for long periods of time. Tested against the longevity of the typhoid bacillus, it was found that the virulent typhoid organisms died out much more rapidly than did the streptococci. This indicated that the fecal streptococci are a much more indicative organism of recent fecal pollution than is the coliform group.

A new medium, dextrose azide broth, Difco, was reported by Mallmann and Seligmann (42) to be an excellent

enrichment medium for the detection of the enterococci and other streptococci. This medium, however, must be confirmed for streptococci by microscopic examination.

With the above data indicating a new and more practical test organism for recent fecal pollution, as well as an excellent enrichment medium, work was started to devise a simple test for the confirmation of the enterococci after the presumptive test-growth in dextrose azide broth.

LITERATURE REVIEW

Ever since Escherich (16) in 1886 recognized the streptococci as normal inhabitants of the bowel of infants and described their morphology in detail, numerous workers have investigated these organisms but because of the lack of a well defined system of classification, much confusion still exists in the literature. Even today there is still some doubt as to what constitutes a fecal streptococcus, an enterococcus, or whether Streptococcus faecalis is a single species or a group of species.

The first important work in the classification of these organisms was carried out by Gordon in 1903-11 (19), who introduced the series of biochemical tests associated with his name. By means of seven chemical tests, Gordon distinguished 48 varieties among 300 streptococci from normal saliva. Houston (29), working along this line, examined and determined the main features of fecal streptococci. Andrews and Horder (3), utilizing the results of Gordon and Houston, applied an extended series of tests to a large number of streptococci isolated from disease conditions and undertook a wide statistical study of t he genus. As a result of this study, they were able to arrange the entire series of organisms into seven large groups, each

centering in a definite type demarcated by its biological activity, and connected with the other type by a graded series of intermediates. It was these authors who first described Streptococcus faecalis.

The streptococcus-like organisms found in feces have been the subject of numerous descriptions, and a host of loosely described types encumber bacteriological literature. It is evident that many of these should be entirely disregarded in the light of more modern investigations, since the distinctions made use of in defining them are now recognized as insufficient. There appears, however, to be certain constantly recurring and outstanding types, all considered more or less distinct by those who have worked with them. A survey of a mass of confused literature suggested the following three groups:

1. Described by European workers
   (a) The Enterococcus of Thiercelin (Micrococcus ovalis of Escherich).
   (b) The Streptococcus enteritis (Hirsh and Libman 1897).
2. Described by English workers
   (a) The Streptococcus faecalis of Andrews and Horder.

The enterococci, which were described by Thiercelin (59) in 1899 before the introduction of more recent methods and alleged by him to be the causative agent in certain types of diarrhea, biliary infections and appendicitis, have received little attention in the literature prior to

1925, whereas the Str. faecalis is well represented. The enterococci were not recognized, as such, in the majority of the English text-books, and the descriptions of this group in French and American works differed in some of the most important details from those of the Str. faecalis, as well as from such descriptions of the enterococci as appeared in more recent literature.

Mace (35) considered the enterococcus as identical with the Str. enteritis of Escherich, which appeared to be the chain-forming type described under the same name by Hirsh and Libman (24). He stressed the pleomorphic morphology of the organism and further stated that it was able to grow at 46° C.; it never liquefied gelatin, inconstantly coagulated milk. It was killed by an exposure of 15 to 20 minutes at a temperature of 60° C., and did not act upon any of the sugars.

Houston and McCloy (31) described an organism isolated from trench fever patients and from suppurating wounds. Among other characteristics, they laid much stress on the ability of the streptococcus to withstand heat and found it capable of surviving an exposure of one and one-half hours to a temperature of 55° C. This may be comparable to an earlier observation of Logan (34) who found a coccus in the feces of infants capable of surviving pasteurization for ten minutes at 80° C.

Wright (68) isolated a diplococcus from a wound which

grew more luxuriantly than Str. pyogenes and was able to grow at room temperature. This non-hemolytic coccus was identified as an enterococcus and also as Str. faecalis, although Gordon's tests did not appear to have been employed. This was the first time a connection between the two groups was made.

Donaldson (14) in 1917 gave a brief summary of the characters of the enterococci. He noted that they generally grew in the form of pneumococcus-like diplococci, did not produce hemolysis, and produced acid from glucose, lactose, maltose, saccharose, raffinose, glycerol, mannitol and inositol.

Weissenbach (61) in 1918 devised a differential test for distinguishing Str. faecalis from Str. pyogenes by employing a liquid medium containing 10 per cent bile. This medium supports the growth of the enterococci but not other streptococci. Bagger (4) utilized this observation and advocated the use of ox-bile with one per cent of peptone for the classification of the enterococci.

Dible (13) in 1921 established the relationship and connection between the enterococci and the Str. faecalis. In a classical piece of work he also reported that the power of withstanding exposure to heat was not a property of all intestinal streptococci. By its use, these are divisible into two classes: one of which largely consisted of organisms having fermentative reactions corresponding to

those of Str. faecalis. The other group consisted of types which frequently occur in saliva and included those raffinose fermentors of the feces which are not thermo-resistant and may be regarded as survivors of the salivary organisms.

Alston (1) confirmed the work of Dible and showed that there is a clearly defined group of organisms sufficiently differentiated to be classified together as enterococci. The primary attributes of this group are: (1) cocci tending to be oval in shape and occurring in pairs or short chains, (2) heat resistant up to 60° C. for 10 minutes, and (3) non-hemolytic and capable of fermenting mannitol, as secondary and not invariable characters. Among the 51 strains of streptococci isolated from the alimentary tract of man, dog, and rat, 16 or 31 per cent conformed to the description of enterococci suggested by Dible.

Holman (25) based a form of classification on the ability of hemolysin production and the ability to ferment lactose, mannitol and salicin. The streptococci derived by this method from feces were found almost entirely in five of the 16 types, viz., Str. faecalis, Str. equinus, Str. mitis, Str. pyogenes and Str. infrequens. Almost half were in the Str. faecalis group.

Welch (62) in 1929 indicated that there were six strains of streptococci common to human stools. Those

fermenting (a) all sugars used: glucose, lactose, sucrose, salicin, maltose, mannitol and galactose; (b) all but sucrose; (c) all but sucrose and mannitol; (d) all but mannitol; (e) all but mannitol and salicin; and (f) all but lactose.

Sherman, Mauer and Stark (56), in an exhaustive study of the enterococci, stated that because fermentation tests are extremely variable with Str. faecalis, there is considerable confusion concerning the boundaries of this group and whether or not one or more species are involved. Of 434 cultures identified as Str. faecalis all grew at 10° C. and 45° C. Other members of the enterococcus group, as defined by Bergy et al. (5) grow at these temperatures also (Str. zymogenes, Str. liquefaciens and Str. durans). In a later paper Sherman (55) also shows that only members of the enterococci grow in the presence of 6.5 per cent NaCl. The fermentation tests are diverse within the species and these characteristics are regarded by Sherman as of minor importance. He stresses the above two tests, the ability to grow at 45° C. and in the presence of 6.5 per cent NaCl as major tests in the classification of these bacteria.

Excellent reviews of the enterococci group, as well as its individual members, have been published by Sherman (55,56).

In 1894 Laws and Andrews (32) reported for the first time that streptococci could be isolated from sewage-

polluted water. This observation was not emphasized until six years later when Houston (27, 28) stressed the fact that streptococci, as well as staphylococci, were characteristic of sewage waste. The former being a more specific indicator of sewage pollution since they are "readily demonstrated in waters recently polluted and seemingly altogether absent from waters above suspicion of contamination". Streptococci were found in 0.1 to 0.001 ml of water from six rivers that were extensively polluted. On the other hand, eight rivers showed no streptococci in 0.1 ml, although ordinary chemical and bacteriological tests gave results which would condemn the waters. Horrocks (26) in 1901 found these organisms in great abundance in sewage and waters which were known to be sewage-polluted, but which contained no trace of Escherichia coli.

Winslow and Hunnewell (63, 64) were the first to study this organism in America in 1902 and later reported the isolation of streptococci from 25 out of 50 samples of polluted water. Prescott and Baker (49) found these organisms present in each of 50 samples of polluted water. On the other hand, Winslow and Nibecker (65) found streptococci by the direct plating method in only one of 259 presumably unpolluted water samples. Clemesha (9) in 1912 reported that in India, streptococci were present in 0.001 to 0.00001 gram of feces, but were rare in waters that were not grossly polluted.

Ostrolenk and Hunter (46), in a study on the distri-

bution of enteric streptococci, examined feces from the human, cat, mouse, guinea pig, dog, rabbit, chicken, flies, monkey and soil. Using Perry's S.F. broth, 51 fecal and two soil samples were examined. The two soil samples were negative for both the enterococci and E. coli. Forty-nine specimens representing 10 animals contained enterococci while E. coli was present in only 46.

Mallmann (36) reported that the streptococci were constant indicators of intestinal pollution and the number found in the swimming pool were parallel to the amount of pollution as indicated by the number of swimmers. It was also reported that the E. coli tended to multiply in the swimming pool while the streptococci did not. In a later paper, Mallmann and Sypien (43) compared the colon and streptococci indices of samples taken five feet from the shore of a bathing beach. It was found that while the colon indices and total plate count did not always respond to changes in the bathing load, the streptococci indices always did. The latter were not found at points free from the bathing pollution while the colon bacteria were. It was also reported that the streptococci disappeared overnight while the colon organisms and the total count sometimes showed an increase, although they were generally lower.

Ritter and Treece (52) isolated 79 strains of streptococci from swimming pools. Fifty-two or 65.8 per cent

were classified as Str. faecalis; these were confirmed by the Lancefield technique and were further classified as Type D.

Winter and Sandholzer (66) confirmed the work of Mallmann and Sypien in that they reported that coliform organisms persisted for a great distance from the source of pollution in water and the streptococci did not.

Horrocks (26) found by experiment that E. coli gradually disappeared from specimens of sewage kept in the dark at the temperature of an outside veranda whereas the streptococci and staphylococci persisted. Prescott (47) showed that the streptococci often overgrew E. coli in a few hours when inoculated into glucose broth. This was contradicted by Prescott and Baker (49) in a later paper. Clemesha (9) found that streptococci disappeared very rapidly in water, within two or three days at the most, when stored in bottles in the laboratory or in an artificially polluted outdoor tank.

Savage and Wood (53), in their study of the viability of streptococci in water, found that they died out in parallel with the coliforms, although a trifle faster.

Prescott (48) in 1906 reported streptococci occurring on hay and grain. Moore (44) reported as early as 1893 that streptococci were frequently isolated from garden soils. The gardens, however, had been heavily and frequently manured. Andrews (2) in 1906 stated that the streptococci cannot grow and multiply for any length of

time apart from the human body. Eighty-four samples of hay, grass, and leaves from country roadsides, pastures, park, and garden paths were examined. Only two samples showed streptococci. Soil and water from wood edges, moist railroad banks, brooks, woodland humus, etc., were examined. Only one of these eighteen samples, from a country roadside overflow, yielded a short-chained coccus organism.

Broadhurst (6) in 1915 was of the opinion that streptococci occurred less commonly in soils and water than most of the literature of that time implied. Sherman in 1937 reported, "Unpublished investigations have shown enterococci of the Str. faecalis and Str. liquefaciens types to occur rather commonly on plants. This may mean of course that these organisms were merely surviving, rather than growing, under these conditions".

Winter and Sandholzer (64) reported that while streptococci were present in all samples of human and animal feces tested, these organisms were never found in virgin soils or in soils from wooded areas.

Mallmann and Litsky (41), using dextrose azide broth as an enrichment medium, could not isolate enterococci from soils which were not treated with sewage. They also stated that other than the coliform organisms, the enterococci were the only organisms found in sewage that could be used as indicators of fecal pollution. While the coliform organisms were found to persist in sewage-treated

soil, the enterococci were found to die out rapidly but not as rapidly as virulent typhoid bacilli. It was also noted that the longevity of these three organisms in sewage-treated soils was prolonged with an increase of the organic content of the soil.

Both solid and liquid media have been used to estimate the number of streptococci in water and soil. Prescott, Winslow and McCrady (50) suggest a litmus lactose agar on which the streptococci colonies are generally distinguished from the other acid formers by their size, structure, and the formation of a permanent deep red color. These colonies, however, may be overlooked due to their slow growth or if they are present only in small numbers. Representative colonies must be examined microscopically.

In 1906 Prescott and Baker (49) reported that when E. coli and streptococci were grown in mixed cultures, E. coli reached a maximum growth before the streptococci but were gradually displaced by the latter 20 to 60 hours after the start of the experiment. From this time on, the streptococci predominated, on some occasions eliminating E. coli completely.

Since similar succession of growth occurs in lactose broth, Mallmann and Gelpi (40) suggested the ordinary lactose broth enrichment tube be employed in a similar manner. After the usual coliform confirmation tests were made, the tubes are reincubated for 48 hours, centrifuged and the sediment examined for streptococci by microscopical

examination. Another method suggested by these authors is to allow the tube to stand at room temperature for one to three days after the initial incubation to permit sedimentation. It was reported that a heavy sediment in the bottom of the tube, similar to that of the deposition in a macroscopic agglutination test, is an indication of the presence of streptococci, but this should be confirmed by microscopic examination. Mallmann and Cary (38) in 1933 recommended holding the incubated tube to the light and looking for a granular precipitate, flocs of which may adhere to the wall of the outer tube, or to the surfaces of the inverted vial. They stated that this indication is not always confirmed microscopically but that confirmation is not necessary if the granular precipitate is absent.

Houston (30), using the presumptive lactose tubes also, removed one ml to a nine ml blank, incubated this dilution in a 60° C. water bath for 15 to 20 minutes, and subcultured on MacConkey agar. After 48 hours incubation at 37° C. the pin-point red colonies were transferred into lactose broth, from this to the condensation water of a nitrate agar slant, and then streaked on the same slant. Acid production without gas from lactose, with a milky precipitate, usually signifies the presence of streptococci. Short-chain cocci in the condensation water of the nitrate agar slant, and the absence of nitrate reduction confirm the presence of streptococci. This method is still recommended by the Committee of the British Ministry

of Health (10).

In 1918 Weissenbach (61) was one of the first to describe a selective medium for enterococci in contrast to hemolytic and non-hemolytic streptococci. Sterile filtered ox-bile was used for the inhibitory agent. Beggar (4) also used sterile ox-bile with one per cent peptone to grow fecal streptococci. As confirmation he suggested the heat resistance test.

Fleming (17) in 1932 reported that fecal streptococci will grow in a concentration of 1:15,000 potassium tellurite which is inhibitory to coliform bacteria as well as most other gram-negative bacteria. It was applied to water analysis by Harold (21) in 1936 when he incorporated this chemical in a solid tellurite agar. Fecal streptococci appear as bluish-black colonies, about one mm. in diameter, with a peripheral opalescence. These were studied and confirmed by the method described by Houston. In 1937 Harold (22) compared this medium to those used at that time and found that from a series of over 250 positive MacConkey broths, 13.9 per cent contained fecal streptococci by Houston's heating technique, 30.6 per cent by the direct tellurite method and 44.0 per cent by the enrichment tellurite method.

Hartman (23) in 1937 was the first to use sodium azide to suppress the growth of gram-negative bacteria while permitting the streptococci to grow. Since this discovery many investigators have used this chemical in

media for the isolation of fecal streptococci.

Mallmann (37) reported a medium containing sodium azide, which was found useful in estimating the number of streptococci in sewage as it was found to support the growth of these bacteria while inhibiting the coliform group. Hajna and Perry (20) published another selective streptococci medium which was almost an exact duplicate of the medium suggested by Mallmann (37), but the former workers used an incubation temperature of 45° C. Growth and the production of acid in this medium were stated to be almost complete evidence of the presence of Str. faecalis.

Winter and Sandholzer (67) in 1946 published a procedure for detecting the presence of enterococci which is used by the U.S.P.H.S. at the present time. This method consists of a sodium azide - presumptive broth and a penicillin-methylene blue-NaCl broth-slant confirmation medium. This method, however, must be confirmed by a microscopic examination and a catalase test.

A study of the inhibition of gram-negative bacteria by sodium azide has been reported by Snyder and Lichstein (57).

Folpmers (18) in 1940 recommended two media for the detection of fecal streptococci. The first contained litmus as the effective agent and should be employed in deep columns. Microscopical examination of the sediment must be made.

The second medium contains one per cent caffeine.

The author stated that coliform bacteria do not interfere, but aerobic spore formers are sometimes troublesome.

Chapman (7) in 1944 published formulae of two media for the isolation of streptococci in mixed cultures. Tellurite streptococcus medium contains crystal violet, trypan blue and sodium tellurite as active agents. On this medium, Str. salivarius produces pale blue opaque colonies from 2 to 5 mm in diameter, Str. mitis produces blue colonies about 0.2 mm in diameter, while the enterococci give a dark brown or smooth black slightly-raised colony from 0.5 to 1.5 mm in diameter. Ninety-seven per cent of the transplants from feces were pure streptococci; the remainder were staphylococci.

Azide violet blood agar medium contains S. T. 37, sodium azide and crystal violet as inhibitory agents. In this medium Str. mitis grows in colonies producing large halos, enterococci produce large blue colonies with halos in some, while Str. salivarius produces no halo. It was reported that with the increase of sodium azide in this medium, more coliform bacteria were inhibited but the size and number of streptococci colonies were also reduced.

In 1946 Chapman (8) again perfected a medium for the isolation of fecal streptococci. This was mitis-salivarius agar, a modification of his former medium. Enterococci produces a dark blue or black slightly-raised colony about 1 mm in diameter, as contrasted to a blue "gum-drop" colony of Str. salivarius or the minute colony of Str. mitis.

Schuman and Farrell (54) in 1941 published a synthetic medium for the cultivation of Str. faecalis. This medium, however, is employed in vitamin and amino acid determinations.

Mallmann and Seligmann (42), in a comparative study of media for the determination of streptococci in water and sewage, after a screening procedure, studied the following media: lactose broth, azide broth (Mallmann), S. F. broth (Hajna and Perry) and azide dextrose broth (Roth). The results of this study can be summarized by the following:

Average streptococci indices of river water were as follows:

Lactose broth	930
Azide broth (Mallmann)	3700
S. F. broth (incubated at 45° C.)	600
Azide dextrose broth (Roth)	9200

The authors stated that the positive azide dextrose tubes should be checked microscopically because some of the gram-positive rods may show turbidity as well as the streptococci. It was concluded that azide dextrose broth offers a new means of testing for and measuring streptococci in water, sewage, and shellfish as well as other materials suspected of sewage pollution.

EXPERIMENTAL

In order to formulate a confirmation medium for enterococci, a base medium first had to be perfected that would not only support the growth of a minimal inoculum of these organisms, but would also permit them to demonstrate a very short lag phase. Many investigators are of the opinion that the lag phase is the most critical stage in the bacterial growth cycle. The following experiments were carried out on the assumption that the shorter the lag phase of a bacterial growth curve, the better the medium is for the particular species. This point can be further demonstrated by the fact that, under optimum conditions, bacteria multiply in a geometric progression, and that the more bacteria which are able to survive the critical lag phase, the better the chance for these organisms to begin multiplying, and the sooner the logarithmic growth phase will be reached.

Using E. coli as the test organism, Mallmann and Darby (39) noted that brilliant green lactose base medium gave a more rapid growth rate when one or two per cent tryptose was added to the base medium.

In order to ascertain the affect of various concentrations of the ingredients in the selective media on the

growth rate of Str. faecalis in small inocula, the culture media listed in Table 1, were prepared. Percentage composition is given for each ingredient. Azide dextrose broth (Medium 7) was the only dehydrated medium used; the rest were made in the laboratory from the listed formulae.

The procedure for determining the growth rate of the Str. faecalis is the same method which was used by Darby and Mallmann (12) and is as follows: A 24 hour culture, which was transferred each day for four days in brain-heart infusion broth, was diluted so that between 20 and 100 organisms per ml were present. This was seeded into flasks that contained 100 ml of the respective media. Initial plate counts were made immediately after the original inoculation and every three hours thereafter, up to and through the ninth hour. These flasks were incubated at 37° C. and were shaken for two minutes prior to plating. Azide dextrose agar, without the sodium azide, was used for the plating medium. All plates were incubated for 24 hours at 37° C. before counting.

This procedure was repeated five times in order to determine the medium that would best support the growth of the test organism. Each trial yielded similar results and a typical set of data is shown in Table 2. A close examination of these data revealed three outstanding facts: the toxicity of a large concentration of salt, which will be discussed later in the paper; the toxicity of the dehy-

TABLE 1

COMPOSITION OF BASE CULTURE MEDIA FOR THE DETECTION OF STR. FAECALIS

Ingredient	Per cent composition						
	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6	Medium 7
Tryptose	1.5	3.0	1.5	2.0	2.0	2.0	1.5
Beef Extract	0.45	0.45	0.45				0.45
Dextrose	1.5	1.5	1.5		1.5	1.5	1.5
Lactose				0.5			
Sodium Chloride	1.5	1.5	3.0	0.5	0.5	1.5	1.5
K_2HPO_4				0.27	0.27	0.27	
KH_2PO_4				0.27	0.27	0.27	
Sodium Azide	0.02	0.02	0.02	0.02	0.02	0.02	0.02

TABLE 2

PLATE COUNTS OF STR. FAECALIS ON THE VARIOUS EXPERIMENTAL BASE MEDIA

Incubation time	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6	Medium 7
0	17	17	13	15	15	16	17
3	75	46	23	75	84	45	68
6	2,900	2,400	700	5,050	4,150	1,950	1,850
9	177,000	135,000	20,000	319,000	380,000	61,000	54,000

drated medium; and the advantage of buffered media.

Medium 3 contained three per cent sodium chloride and no buffering agents. The inhibition of growth was quite evident after three hours of incubation when compared to the other media. Medium 6 contained one and one-half per cent sodium chloride and buffering agents. It did not support the growth of the test organism as well as Medium 5 which was of the same composition but only contained one-half per cent sodium chloride.

It was observed that Medium 7 made from prepared dehydrated material, showed the lowest count other than Medium 3 after six hours of incubation. It should be added that Medium 1 is of the same composition as Medium 7 but was made up from the ingredients in the laboratory. This medium was not as inhibitory as was its dehydrated counterpart. As is demonstrated in Table 2, Medium 1 showed a count slightly less than double that of Medium 7 after six hours. After nine hours of incubation Medium 1 contained 177,000 organisms per ml while Medium 7 contained only 54,000 per ml.

Of the three media which contained buffering agents, Media 4 and 5 were of the same composition, with the exception that the former contained one-half per cent lactose while the latter contained one and one-half per cent dextrose. Upon examination of the data, it was noted that these two media were the best of the experimental

base media for the support of Str. faecalis growth. Upon further repeated investigations, Medium 6 containing dextrose, was judged the better of the two. It is this medium that was employed as a base for the further experimentation for a selective confirmation medium for the enterococci.

In an attempt to increase the inhibitory action of the medium for the gram-negative bacteria, as well as to determine the limiting concentration for the enterococci, five media were prepared with a concentration of sodium azide ranging from 0.00 to 0.10 per cent.

An actively growing culture of Str. faecalis was seeded into the above media in minimal inocula and growth curves were plotted as previously outlined.

The results of this investigation, listed in Table 3, indicated that a concentration of 0.05 per cent and above demonstrated marked inhibition. It would seem that sodium azide increased the lag phase so that at the end of 24 hours no visible growth was observed in the medium with a concentration of sodium azide as low as 0.02 per cent. The control, base medium minus sodium azide, demonstrated normal growth and turbidity at the end of 18 hours of incubation.

The above results indicated the necessity of extending the incubation period to 48 hours. Media were made with the concentration of sodium azide ranging from 0.00 to 0.05 per cent. Again these media were tested by growth

TABLE 3

PLATE COUNTS OF STR. FAECALIS GROWN IN A BASE MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF SODIUM AZIDE

Hours of incubation	Bacteria per ml				
	Concentration of sodium azide in per cent				
	0	.02	.05	.07	.10
0	309	292	290	324	340
3	2,500	500	510	320	370
6	129,000	3,000	2,000	1,000	
9	3,300,000	20,000			
12	50,000,000		100,000		
18	930,000,000	300,000		1,000,000	1,000,000

curves as previously described.

The results of this investigation, shown in Table 4, indicated again that the growth peak for the control, Medium 1, was 18 hours. The growth peaks for media containing 0.02 to 0.03 per cent sodium azide was 42 hours, while for those containing 0.04 to 0.05 per cent, it was 48 hours or more. The medium with 0.05 per cent was the only one in this group that did not demonstrate visible growth after 48 hours of incubation.

It must be stated here that 0.02 per cent sodium azide was found to inhibit the coliform bacteria and this same concentration is used in dextrose azide broth as an inhibitory agent. It was felt that a greater concentration of sodium azide, which would allow Str. faecalis to grow and yet inhibit the gram-negative organisms, should be used in a confirmatory medium. Therefore, the medium containing 0.04 per cent sodium azide was chosen as a base medium for further investigations. It can be argued that 0.02 and 0.03 per cent sodium azide could be used but the results indicated that after 36 hours there is no appreciable difference in growth of the test organism using the two concentrations.

It was found that the above medium, as is the case with dextrose azide broth, demonstrated inhibition of the gram-negative bacteria but allowed some spore-formers, such as Bacillus subtilis, to grow. For this reason the

TABLE 4

PLATE COUNTS OF STR. FAECALIS GROWN IN A BASE MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF SODIUM AZIDE

Hours of incubation	Bacteria per ml				
	Concentration of sodium azide in per cent				
	0	.02	.03	.04	.05
0	255	295	326	275	320
6	7,000,000	5,700	950	800	950
12	133,000,000	120,000	3,900	1,000	450
18	780,000,000	3,260,000	15,000	2,200	400
24	360,000,000	148,000,000	185,000	7,300	900
36	550,000,000	435,000,000	148,000,000	7,400,000	490,000
42	368,000,000	2,270,000,000	230,000,000	115,000,000	10,000,000
48	300,000,000	461,000,000	180,000,000	155,000,000	63,000,000

addition of another inhibitory agent was necessary before the medium could be used for confirmatory work.

The following dyes: methyl violet, brilliant green, ethyl purple, malachite green, and crystal violet, were incorporated into the base medium in various concentrations in order to test their inhibitory powers towards the gram-positive bacteria. This also was carried out to find the concentration critical for the streptococci. The base broth, however, contained no sodium azide so that whatever inhibition occurred could be attributed to the bacteriostatic action of the dyes.

Ten ml of the base broth containing various concentrations of the dyes were seeded with 0.1 ml of a 24 hour culture of Str. faecalis, which had been transferred each day for three days in brain-heart infusion broth. The seeded tubes were then incubated at 37° C. and were observed at 24 and 48 hours.

The data as shown in Table 5 indicated that the following dyes and their concentrations could be used without inhibiting the test organism for more than 48 hours: methyl violet, 1:500,000; ethyl purple, 1:800,000; brilliant green, 1:2,000,000; malachite green, 1:1,800,000; and crystal violet, 1:800,000.

Next an attempt was made to determine whether these dyes would inhibit the common gram-positive organisms found in soil and water at concentrations below those which in-

TABLE 5

GROWTH OF STR. FAECALIS IN MEDIA CONTAINING VARIOUS CONCENTRATIONS OF DYES

Dilution	Methyl Violet		Ethyl Purple		Brilliant Green		Malachite Green		Crystal Violet	
	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.
500 T	≠	≠	-	-	-	-	-	-	-	-
600 T	≠	≠	-	-	-	-	-	-	-	-
700 T	≠	≠	-	-	-	-	-	-	-	-
800 T	≠	≠	-	≠	-	-	-	-	-	≠
900 T	≠	≠	-	≠	-	-	-	-	-	≠
1 M	≠	≠	-	≠	-	-	-	-	-	≠
1.2 M	≠	≠	-	≠	-	-	≠	≠	≠	≠
1.4 M	≠	≠	-	≠	-	-	-	-	≠	≠
1.8 M	≠	≠	≠	≠	-	-	-	≠	≠	≠
2 M	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠

hibit the enterococcus used as a test organism. To carry out this experiment B. subtilis, Staph. aureus, and Str. faecalis were used. Twenty-four hour broth cultures of the above-listed bacteria were mixed and 0.1 ml of the resulting mixture was inoculated into 10 ml of base broth. Incorporated into this base broth were various concentrations of dyes as shown in Table 6. These were incubated at 37° C. for 24 hours and examined microscopically. The following represent the highest concentration of the dyes which supported the growth of Str. faecalis (but not B. subtilis or Staph. aureus): crystal violet, 1:700,000; ethyl purple, 1:800,000; brilliant green, 1:1,500,000; malachite green, 1:1,000,000; and methyl violet, 1:10,000.

Various concentrations of the dyes were incorporated into a base medium containing 1.5 per cent agar. Plates were made and divided into sections, in which were streaked a loopful of the above cultures. These plates were then incubated at 37° C. for 48 hours. Growth on the plates was recorded as positive. The results of this investigation are shown in Table 7.

From the above table certain facts are evident. B. subtilis is not completely inhibited at a 1:600,000 concentration of crystal violet, while the same dye inhibited Staph. aureus at 1:1,000,000 concentration. Str. faecalis exhibited growth at 1:600,000.

Ethyl purple inhibited both B. subtilis and Staph. aureus at a concentration of 1:800,000 while no effect

TABLE 6

TWENTY-FOUR HOUR MICROSCOPIC EXAMINATION OF BASE MEDIA CONTAINING DYE INOCULATED WITH A MIXED CULTURE

Dye and dilution		Str. faecalis	B. subtilis	Staph. aureus
Crystal violet	600T	-	-	-
	700T	≠	-	-
	800T	≠	-	-
	1M	≠	-	-
	1.2M	≠	-	-
Ethyl purple	800T	≠	-	-
	.1M	≠	-	-
	1.2M	≠	-	-
	1.5M	≠	-	-
	2M	≠	-	-
Brilliant green	1.5M	≠	-	-
	2M	≠	-	-
	2.5M	≠	≠	-
	3M	≠	≠	-
	4M	≠	≠	-
Malachite green	1M	≠	-	-
	1.2M	≠	-	-
	1.5M	≠	-	-
	1.7M	≠	≠	-
	2M	≠	≠	-
Methyl violet	10T	≠	-	-
	25T	≠	-	-
	50T	≠	≠	-
	100T	≠	≠	-

TABLE 7

FORTY-EIGHT HOUR PLATE ANALYSIS OF GROWTH ON MEDIA CONTAINING DYES

Dye and concentration		Str. faecalis	B. subtilis	Staph. aureus
Crystal violet	600T	≠	≤	-
	700T	≠	≤	-
	800T	≠	≠	-
	1M	≠	≠	≠
	1.2M	≠	≠	≠
Ethyl purple	800T	≠	-	-
	1M	≠	≤	≠
	1.2M	≠	≠	≠
	1.5M	≠	≠	≠
	2M	≠	≠	≠
Brilliant green	1.5M	≠	-	≤
	2M	≠	≠	≠
	2.5M	≠	≠	≠
	3M	≠	≠	≠
	4M	≠	≠	≠
Malachite green	1M	≠	≠	-
	1.2M	≠	≠	≤
	1.5M	≠	≠	≠
	1.7M	≠	≠	≠
	2M	≠	≠	≠
Methyl violet	10T	-	≠	≤
	25T	≠	≠	-
	50T	≠	≠	≠
	100T	≠	≠	≠

was demonstrated on Str. faecalis.

Brilliant green inhibited B. subtilis at 1:1,500,000 but did not have a complete inhibitory effect on Staph. aureus at this concentration. Str. faecalis was not inhibited at the above concentration.

Malachite green, on the other hand, inhibited the growth of Staph. aureus at a concentration of 1:1,000,000 but allowed B. subtilis as well as Str. faecalis to grow.

Methyl violet inhibited the Str. faecalis at a concentration of 1:10,000 but did not inhibit B. subtilis or Staph. aureus. It must be added that the above experiments were used only as rapid screening methods. There was a possibility that some of the light growth, reported as plus-minus, could have been due to the carry-over of media on which the organisms were cultured. All things considered, the results of these experiments indicated that a 1:800,000 dilution of either crystal violet or ethyl purple could be used to inhibit the growth of the bacilli or staphylococci without effecting the growth of the streptococci considerably.

The investigation thus far suggested that the base medium with 0.04 per cent sodium azide and a 1:800,000 of either crystal violet or ethyl purple might be used as a selective medium for Str. faecalis. To investigate this possibility further and also to determine which is the better dye, the above media were prepared; one containing

crystal violet, the other ethyl purple.

Raw sewage, polluted water from the Red Cedar river, and soil were collected and the streptococci indices were determined using dextrose azide broth in triplicate for each dilution. Readings were made after 48 hours incubation at 37° C. and confirmed by microscopic examination. The following most probable numbers of streptococci were obtained from the dextrose azide broth presumptive tests:

Source	Visible MPN	Microscopic MPN
Raw Sewage	9,200,000	9,200,000
River Water	430,000	43,000
Soil	27,000	0

Comparing the visible MPN with the microscopic confirmation, it was noticed here, as well as by Mallmann and Seligmann (42), that dextrose azide broth supports the growth of organisms other than streptococci, especially in soil samples. In order to test the above two media as confirmatory media, three loopfuls of broth from each dextrose azide broth tube were seeded into tubes of the above media. After 48 hours incubation at 37° C. these were read visibly and confirmed by microscopic examination. Results of the visible growth are as follows:

Source	Ethyl Purple	Crystal Violet
Raw Sewage	9,200,000	4,300,000
River Water	43,000	43,000
Soil	0	0

It can be seen that the confirmation in ethyl purple azide broth was exactly the same as the microscopic readings on the dextrose azide presumptive test. The crystal violet broth displayed some toxicity in the confirmation of the raw sewage sample but was similar to the other medium usually. A compact purple button formed by the sediment seemed to be specific in all cases of positive growth. This was repeated and similar data were obtained.

In order to test the specificity of these two media the following experiment was performed. Cultures of various micrococci, isolated by Mr. Edward Seligmann and Miss Lisa Neu of this department, were seeded into the confirmation media. After 48 hours incubation at 37° C. observations were made for growth. The results of this experiment, as shown in Table 8, demonstrated the specificity of the ethyl purple azide broth, in that it only supported growth of the Str. faecalis (Nos. 1, 8, 9, 10, 11, 12, and 40) while the crystal violet azide broth supported the growth of these organisms plus three strains of Staph. aureus and possibly Str. mitis (Nos. 5, 6, 13, and 17). Since most of these cultures were only partially identified the certainty of the species is dubious. Such strains are indicated in Table 8 by a question mark. Cultures 23-36 were not identified but believed to be members of the buccal group of streptococci. Cultures 1-4 were obtained from the American Type Culture Collection.

TABLE 8

GROWTH OF VARIOUS ORGANISMS IN CONFIRMATION MEDIA

(48 Hrs.)

No.	Organism	Isolation	Ethyl Purple	Crystal Violet
1	Str. faecalis	ATCC	+++	+++
2	Str. salivarius	ATCC	-	-
3	Staph. aureus	ATCC	-	-
4	Str. mitis		-	-
5	Staph. aureus		-	+
6	Str. mitis ?		-	+
7	Staph. aureus		-	-
8	Str. faecalis	River	+++	+++
9	Str. faecalis ?	"	+++	+++
10	Str. faecalis ?	"	+++	+++
11	Str. faecalis	"	+++	+++
12	Str. faecalis	"	+++	+++
13	Staph. aureus or Str. faecalis	"	-	+
15	Str. mitis ?	Pool	-	-
16	Staph. aureus ?	"	-	+
17	Staph. aureus	"	-	-
18	Staph. aureus or Str. faecalis	"	-	-
20	Str. salivarius	"	-	-
21		"	-	-
22	Staph. aureus ?	"	-	-
23		"	-	-
24		"	-	-
25		"	-	-
26		"	-	-
27		"	-	-
28		"	-	-
29		"	-	-
30		"	-	-
31		"	-	-
32		"	-	-
33		"	-	-
34		"	-	+
35		"	-	-
36		"	-	-
37	Staph. citreus	Miss Neu	-	-
38	Staph. epidericus	"	-	-
39	Staph. aureus	"	-	-
40	Str. faecalis	"	+++	+++
41	Proteus vulgaris	"	-	-

On the basis of the results obtained from the preceding preliminary investigations, ethyl purple was found to be a better selective agent, for the gram-positive bacteria than was crystal violet. The latter dye was also found to be more toxic when used in a confirmation medium than was ethyl purple. On the basis of these findings, a more complete investigation of ethyl purple azide broth was carried out in order to prove the merits and discover its limitations. The formula for this medium, which will be used in the following experiments is as follows:

Ingredient	Grams per liter
Tryptose	20
Dextrose	15
Sodium chloride	5
K_2HPO_4	2.7
KH_2PO_4	2.7
Sodium azide	0.4
Ethyl purple	0.00125

pH - 7.0

Sterilized at 121° C. for 15 minutes

To investigate the specificity of ethyl purple azide broth more thoroughly, actively growing cultures of the following organisms were seeded into this medium: Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus aurantica, Staphylococcus albus, Sarcina citreus, Sarcina lutea, Serratia marcescens, Proteus vulgaris, Escherichia

coli, Escherichia communior, Salmonella typhimurium, Salmonella typhosa, Shigella alkaliscum, Micrococcus agilis, Bacillus subtilis, Bacillus cereus, Alkaligenes faecalis, Chromobacter violaceum, and Streptococcus faecalis. After incubation at 37° C. for 48 hours, these tubes were examined for visible growth. Str. faecalis was the only species which showed visible growth.

From the previous data it was quite obvious that ethyl purple azide broth supports the growth of streptococci but further information was needed to find its specificity limitation within the streptococci group. For this, actively growing cultures of streptococci, acquired from Cornell University and Iowa State College, were seeded into the test medium. Observations for visible growth were made after 48 hours of incubation at 37° C. It was found as shown in Table 9, that only the enterococci, Str. faecalis, Str. durans, Str. liquefaciens, and Str. zymogenes, demonstrated growth at 48 hours. Str. bovis grew out after five days and the rest did not show any visible sign of growth after a week of incubation.

This set of data was repeated with similar results. It showed that ethyl purple azide broth was not only specific for the growth of the streptococci, but it was specific to the extent of supporting the growth of only the enterococci. Again it was noted that all the enterococci demonstrated a compact purple button on the bottom of the tube after 48 hours.

TABLE 9

GROWTH OF VARIOUS STRAINS OF STREPTOCOCCI IN ETHYL PURPLE AZIDE BROTH

Species	Source	Growth after 48 hours incubation
Str. bovis	Bovine mouth	-
Str. faecalis	Intestine	≠
Str. durans	Intestine	≠
Str. liquefaciens	Intestine	≠
Str. mitis	Mouth	-
Str. zymogenes	Intestine	≠
Str. equisimilis	Pig heart valve	-
Str. lactis	Milk	-
Str. thermophilis	Milk	-
Str. salivarius	Milk	-
Str. agalactiae	Milk	-
Str. dyagalactiae	Milk	-
Str. uberis	Milk	-
Str. canis	Dog	-
Str. equi		-
MLB	Porcine abscess	-
419-9	Pig liver	-

Unidentified streptococci cultures were classified as to whether they were enterococci by their ability to grow at 45° C. and in the presence of 6.5 per cent sodium chloride. Tryptose phosphate broth was used as the base medium in both cases. These cultures were also seeded into ethyl purple broth, Perry's S. F. broth, Winter and Sandholzer presumptive medium and then positive tubes were confirmed in Sandholzer's confirmation broth-slant. Perry's S. F. broth and Winter and Sandholzer's presumptive broth were incubated at 45° C. while ethyl purple broth and Winter and Sandholzer's confirmation medium were incubated at 37° C. All tubes were incubated for 48 hours with the exception of those containing Winter and Sandholzer's presumptive medium (directions call for 8-12 hours of incubation) which were incubated only 24 hours.

The results of this test indicate, as shown in Table 10, that those strains having the characteristics of fecal streptococci, growth at 45° C. and in the presence of 6.5 per cent sodium chloride, grew in ethyl purple azide broth, with the exception of cultures 17, 18, and possibly 33. None of the enterococci showed acid production or visible growth in Perry's S. F. broth, while only Culture No. 25 was confirmed by the Sandholzer method. All cultures grew in dextrose azide broth. These data indicate the selectivity of ethyl purple azide broth for the enterococci as compared to other methods. The toxicity of these latter

TABLE 10

COMPARISON OF GROWTH OF VARIOUS STRAINS OF STREPTOCOCCI IN VARIOUS CONFIRMATION MEDIA

Culture No.	Growth at 45° C.	Growth in 6.5% NaCl	Ethyl Purple	Perry's S. F.	Sandholzer's Presump.	Sandholzer's Confirm.
1	++++	-	-	-	-	-
4	++	++	+	-	-	-
4	++	+++	++	-	-	-
9	-	++	-	-	-	-
10	++	++	++	-	-	-
12	-	+++	-	-	-	-
15	-	-	-	-	-	-
17	+	+++	+	-	-	-
18	++	++++	-	-	-	-
19	+++	++++	+	-	-	-
20	++++	+++	+	-	-	-
21	+	+++	+	-	-	-
22	-	+++	-	-	-	-
25	++++	++	++++	-	+	+
27	-	+++	-	-	-	-
28	-	-	-	-	-	-
29	-	+++	-	-	-	-
30	++++	+++	++++	-	-	-
31	++	++	++++	-	-	-
32	-	-	-	-	-	-
33	+	+++	?	-	-	-
34	-	-	-	-	-	-
39	-	-	-	-	-	-
40	-	-	-	-	-	-
41	++++	+++	+++	-	-	-
42	++++	+++	++++	-	-	-
43	++++	+++	++++	-	-	-

media is clearly demonstrated.

In order to test the effectiveness of ethyl purple azide broth as a confirmatory medium for the enterococci the following investigation was carried out. Samples of water were taken along the Red Cedar River at bridges and points at which sewage entered the river. Raw sewage samples were also collected. The river water (undiluted in ten-fold dilutions up to 1:1,000,000) and sewage samples (diluted in ten-fold dilutions up to 1:10,000,000) were seeded in dextrose azide broth, incubated at 37° C. for 48 hours. Microscopic examinations were made after 48 hours and used as a control for the positive streptococcus growth. Three loopfuls of the dextrose azide broth were then seeded into the ethyl purple azide broth, incubated at 37° C. for 48 hours. Microscopic examinations were also made of the 48 hour confirmation tubes. One hundred and sixty-four samples of river water were examined by this method.

A representative sample of the data is shown in Table 11. While this table does not include all of the results obtained, those not included tend to show the same trend. Ethyl purple azide broth confirmed the microscopic positive tubes of dextrose azide broth. It was also found that all positive confirmation tubes only contained streptococci when examined microscopically. Again it was noticed that the growth after 48 hours in ethyl purple azide broth

TABLE 11

RESULTS OF USING ETHYL PURPLE AZIDE BROTH AS A CONFIRMATION MEDIUM FOR STREPTOCOCCI IN TERMS OF M.P.N.

Site of Sampling	Sample	Dextrose Azide Presumptive		Ethyl Purple Azide Confirmation
		Visible	Microscopic	Visible
Railroad Bridge	C.	4,500	4,500	4,500
	J.	9,500	9,500	9,500
	P.	4,500	950	9,500
	V.	45,000	45,000	95,000
	3.	45,000	20,000	110,000
	9.	45,000	25,000	20,000
Women's Gym Bridge	D.	4,500	4,500	4,500
	E.	9,500	4,500	2,500
	K.	4,500	4,500	140,000
	Q.	950	950	45,000
	X.	25,000	2,000	2,000
	4.	2,500	2,500	2,500
Hotel Bridge	F.	2,500	4,500	4,500
	L.	25,000	95,000	25,000
	R.	45,000	45,000	95,000
	Y.	140,000	14,000	20,000
	5.	2,500	2,500	250
	12.	2,500	300	1,150
Kalamazoo Bridge	I.	4,500	9,500	4,500
	O.	140,000	140,000	140,000
	U.	45,000	45,000	95,000
	Z.	25,000	9,500	25,000
	8.	150,000	25,000	35,000

appeared as a compact purple button on the bottom of the tube.

Since some streptococci were found microscopically in the 48 hour dextrose azide tubes, but were not confirmed in the confirmation medium, an investigation was carried out to determine whether these were of fecal or non-fecal origin. Tubes which were not confirmed were diluted by loop dilution, and plated on brain-heart infusion agar. After incubation at 37° C. for 24 hours, colonies were fished and purified in tryptose phosphate broth. After purity was established these cultures were grown at 45° C. and also in a 6.5 per cent sodium chloride broth. The results indicated that of the 82 cultures checked only 7 were classified as fecal streptococci by Sherman's method of classification. Of these 7 cultures, five showed typical enterococci growth when reinoculated in the confirmation broth. These false negatives in ethyl purple azide broth may have been due to faulty laboratory technique when seeding from the presumptive to the confirmatory medium.

These results indicated that ethyl purple azide medium inhibited not only bacilli but also non-fecal streptococci while allowing the enterococci to grow readily. To further prove this, tubes which did show typical growth were picked at random and seeded in tryptose phosphate agar by loop dilution. Cultures were isolated, purified and tested as above. Of the 116 cultures tested by this method only

three typed out as non-fecal streptococci. One culture grew in the presence of 6.5 per cent sodium chloride and not at 45° C., while the other two did not grow in either test. It must be reported, however, that these cultures were isolated from plates that demonstrated fecal streptococci. Also, these three cultures did not produce a typical purple button in the confirmatory medium but showed very scanty growth upon reseeding.

To compare the dextrose azide-ethyl purple azide broth method, for the detection and confirmation of enterococci with present-day methods, samples of river water and sewage were seeded into these media and confirmation procedures carried out as previously described. The results of these studies, as shown in Table 12, proved that the ethyl purple azide confirmation method was far better than Perry's or Winter and Sandholzer's methods. In every case, with the exception of sample 21, ethyl purple azide detected and confirmed from 100 to 1000 as many enterococci as did the above methods. Samples 6 to 12 were taken from a relatively unpolluted stream. The results of both Perry's and Winter and Sandholzer's methods indicate no fecal streptococci in any of the six samples when the ethyl purple azide method detected two samples, 11 and 12, with streptococci indices of 73. Samples 1 to 6 and 36 show higher indices in Perry's than in Winter and Sandholzer's method but in the case of the remaining 35 samples Winter and

TABLE 12

THE M.P.N. OF ENTEROCOCCI WHICH WERE DETECTED AND CONFIRMED BY VARIOUS METHODS

Sample Number	Perry's S. F. Method	Winter and Sandholzer's Method	Dextrose Azide and E.P.A. Broth Method
1	91	0	920
2	91	0	430
3	36	36	430
4	36	0	740
5	240	0	920
6	91	0	150
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	0	0	73
12	0	0	73
13	0	0	480
14	0	0	240
15	0	0	920
16	0	36	430
17	0	0	9,200
18	0	0	2,500
19	0	73	140
20	0	91	920
21	0	240	240
22	91	150	2,500
23	240	1,470	4,300
24	7.3	36	91
25	0	90	1,500
26	40	150	700
27	91	210	4,500
28	0	70	4,500
29	0	450	2,000
30	40	450	25,000
31	240	1,500	20,000
32	91	450	25,000
33	0	3,000	110,000
34	0	40	2,500
35	0	0	15,250
36	91	40	15,000
37	0	40	35,000
38	40	40	20,000

Sandholzer's method detected as much or more enterococci than did Perry's medium.

It must be noted here that microscopic readings of the dextrose azide presumptive broth invariably resulted in higher indices than did the readings of the other two presumptive broths. Positive confirmation in the ethyl purple azide broth was noted by the compact purple button in the bottom of the tube. This was easily noticed after 48 hours of incubation. The tubes must not be shaken before this characteristic growth is noted.

DISCUSSION

Many investigators, especially those in England, have demonstrated that the enterococci can be used as indicators of fecal pollution. Mallmann and Litsky (41) have shown that these organisms can be used to test recent pollution in soils where the coliform bacteria are normally found, as well as in water. However, in the light of these reports, results have been published to the effect that the enterococci are not good test organisms for pollution because so few could be isolated as compared to the coliform organisms. This belief has been dominating the investigations for the past fifty years and consequently the enterococci were never given their proper role as test organisms.

The primary reason for not using the enterococci for testing for pollution was that there never has been a satisfactory medium that could be used for the isolation of these organisms. If and when these organisms were detected a complicated identification procedure had to be employed for their confirmation. It was not until 1937 that Sherman proposed the two test methods for the identification of enterococci.

From 1930 on, numerous media were reported in the literature for the detection of fecal streptococci. These,

however, were too toxic for the detection and demonstration of the actual number of streptococci in a sample of water, especially if the water was not grossly polluted. Consequently, those that did confirm were so few in number that the test was not practical. These media had to be confirmed by microscopic examination, which only tended to complicate the method as well as to add more work.

In 1950, Mallmann and Seligmann published a paper in which they showed that azide dextrose broth was the best test medium for the quantitative determination of streptococci as compared to the media used at that time. This medium had to be confirmed by microscopic examination also. It was felt that a microscopic determination defeated the purpose of this test because it required a trained technician and valuable time to prepare the stained slides and to examine them. The average water analysis laboratory unfortunately is poorly equipped and cannot employ a trained bacteriologist for routine analysis. It can also be stated that these workers have little time to examine confirmation slides.

Another objection to microscopical confirmation is that streptococci may take any coccus-like form including that of micrococci, diplococci, sarcina, staphylococci, etc. and conversely. It also must be added that it is very easy to call a smear negative for streptococci and find them on further examination. It can be stated further

that under the microscope all streptococci look alike and that fecal streptococci can not be differentiated from non-fecal streptococci. Because of these objections, a medium was sought to confirm the dextrose azide positive tubes without the use of the microscopic examination.

The preceding work in the development of ethyl purple azide broth is self-explanatory. It should, however, be noted that when 0.04 per cent of sodium azide was used in the base medium, a 48 hour incubation period was necessary for the visible growth determination. When the ethyl purple dye was incorporated into the medium, most of the positive tubes were observed in 24 hours or less. This suggests that the dye is in some way decreasing the toxicity of the sodium azide. The same phenomenon was reported in brilliant green bile medium, where the bile decreases the toxicity of the brilliant green dye concentration used.

To test the effect of dyes from various sources on the selectivity and toxicity of the medium in which they are employed, three batches of ethyl purple azide broth were made, each having the same ingredients with the exception of the dye source. When toxicity tests were carried out, it was found that media made from the dyes from National Aniline, Difco Laboratories, and Haricco showed different toxic effects. The media made with the dye received from Difco Laboratories supported the growth of Str. faecalis as well as dextrose azide broth, which was

run as the control. All five tubes supported growth when one ml of the culture containing approximately 1.56 organisms was seeded in the broth tubes and three out of five tubes showed growth when they were seeded with one-tenth of this number of organisms. The dyes from National Aniline and Haricco demonstrated more toxicity than the above. National Aniline dye, which was used in the development of ethyl purple azide broth, showed four of the five tubes positive when one ml of the dilution (containing approximately 1.56 test organisms) was seeded, and only two positives out of five when one-tenth of the above dilution was used. The medium made up with Haricco dye showed four out of five positive tubes in the first dilution and none in the second. All showed five out of five positive tubes when dilutions were used containing 15.6 organisms per ml or more.

Litsky, Mallmann and Fifield (33) showed that these dyes differ in actual dye content very greatly. Using spectophotometric analysis, they showed that the dye prepared by Haricco contained the greatest amount of active dye content of those examined. When these results were compared with the biological toxicity results it was discovered that there is a correlation between the toxicity of the ethyl purple azide media and the actual dye content of the dye. Haricco dye, with the largest content of active dye, was found the most toxic while the dye obtained from

Difco Laboratories containing the least amount of active dye was found the least toxic. Until further investigations are carried out as to the dye content, and until this dye is certified by the Certified Dye Commission, a concentration of 1:800,000 or 0.00125 gram per liter of ethyl purple dye produced by the National Aniline Company should be used in this confirmation medium.

The above results also show that very few organisms are required in the initial inoculum for growth in the medium. This indicates that although there are two inhibitory agents in the medium, together they demonstrate very little effect on the growth of enterococci.

A word of caution as to sterilization of this medium should be emphasized at this time. It was noticed that when the medium was sterilized for more than 15 minutes at a temperature of 121° C. browning took place and the longer the medium was sterilized the more the browning. Toxicity tests, in the same manner as described above, were made using a lot of medium that was sterilized for 15 minutes as a control. It was found that the longer the sterilization process was prolonged the more toxic the medium proved to be. It must be stressed that this medium should be sterilized only for 15 minutes at 121° C. for its greatest efficiency.

As was reported in the preceding sections, ethyl purple azide broth supports the growth of the enterococci and in-

hibits all the other bacteria that were tested. The specificity of this medium for the fecal streptococci can be put to much use in the field of medical bacteriology. With the use of this medium an enterococcus can be distinguished very rapidly and easily from that of an hemolytic or pyogenic streptococcus without employing a long series of media and reagents necessary for the separation at present.

Streptococcus faecalis has been named by Dack (11) as the causative agent in many food-poisoning outbreaks. Ethyl purple azide broth may find a major role in this field, thereby, making it possible for the investigator to trace this causative organism very rapidly and without too much effort.

It has been philosophized for many years that the fecal streptococci have a common characteristic which separates them from the rest of the group. Sherman found that these bacteria are the only streptococci which will grow at 45° C. and also in the presence of 6.5 per cent sodium chloride. The results of these investigations indicate another characteristic common among the enterococci, the fact that they will grow in the confirmatory medium while the other streptococci will not. This investigation was not made to determine whether this ability is due to tolerance or an enzyme system. It does, however, suggest very strongly, a common characteristic among the enterococci, and the

reason for this may be found in a further investigation of this medium.

A new method for the detection and confirmation of fecal streptococci in water, sewage and soil has been suggested. This method employs dextrose azide broth as a presumptive medium and ethyl purple azide broth for confirmation. Using the microscopical examination data as the control, it was demonstrated in the preceding sections that ethyl purple azide broth satisfactorily confirms the positive presumptive tubes that contain enterococci. In fact it may confirm a larger number of those than are called positive by the presumptive microscopical examination as can be seen in Table 11. This may be due to mistakes in the smear examination, in that the streptococci are confused with other forms or that they were so few in number that they were not detected. It also may be due to the original condition of the organism when first isolated. Weak strains may take 48 hours in dextrose azide broth to be revived. These few organisms may not be detected by the microscope. When they are transferred to the confirmation medium it is possible that they may be revived to the extent that they multiply more rapidly and consequently show visible growth at the end of an additional 48 hour period.

Dextrose azide broth presumptive tubes, showing streptococci by microscopical examination, but not growth in the ethyl purple azide broth, were plated and cultures isolated and classified. Of these only seven out of 82

cultures which were studied were classified as fecal streptococci. Five of these seven demonstrated typical growth on subsequent inoculation into the confirmation medium. The absence of growth in the primary confirmation medium might be attributed to faulty laboratory technique in the transfer. Of those not confirming, the majority were classified as micrococci; although staphylococci, diplococci and tetrads were frequently encountered.

The above method was compared with the former methods used for the detection of enterococci and it was found that it was superior to the Hajna and Perry method and also "The Winter-Sandholzer Enterococcus Test" in that it detected indices from 100 to 1000 times as great. One reason for the toxicity of these established tests might be due to either the inhibiting agent in the primary enrichment medium or the 45° C. incubation temperature. It is logical to surmise that the more optimum the growth conditions are, the more organisms will survive the critical lag phase of the growth cycle. The primary medium should be employed only to enhance the growth of the test organism and not to identify it. When growth occurs and a large number of these organisms are present, then and then only should confirmation be attempted. Both Hajna and Perry's S. F. broth and Winter and Sandholzer's presumptive broth are incubated at 45° C. It is true that the enterococci grow at 45° C. but this temperature is not the best temperature to grow a very small number of bacteria as often occur in

water and soil samples. Dextrose azide, on the other hand, is incubated at 37° C. and results in a larger index. Winter and Sandholzer's confirmation medium was compounded with the idea that if the enterococci are able to survive 6.5 per cent sodium chloride, 0.1 per cent methylene blue and penicillin, then these organisms should be able to grow in a medium containing all three. This is not the case. As the experimental data show, this medium is far too toxic to grow fecal streptococci when seeded in pure cultures, let alone enterococci found in water. Ethyl purple azide broth, on the other hand, showed visible growth when approximately 1.56 organisms were seeded into it. In ordinary routine analysis this is not important because dextrose azide broth increases the number of organisms and a very large inoculum is transferred for confirmation.

The following paragraphs were taken from a preliminary report of a "Tri-State Survey of Lake Michigan Waters" by the United States Public Health Service (60) and demonstrates the attitude at present concerning enterococci as test organisms for pollution:

> "The Enterococcus Test of Winters and Sandholzer was made simultaneously with the coliform examination of each sample. Without evaluation at this time of its usefulness as a pollution indicator, the following summary describes the results.
>
> "1. The enterococcus group was isolated from most sample points at one time or another during the period of the survey, including areas where the sanitary survey showed no evidence of immediate sewage pollution.

"2. In areas where the sanitary survey showed no evidence of immediate sewage pollution, the density of the enterococcus was extremely low. Generally the results were negative to the largest portion examined, in frequent instances the most probable number of organisms exceeded 3.6, but rarely did they exceed 43. . . .

"3. The enterococcus density was much higher in areas where fresh sewage or treated sewage effluent entered the waters of the area. The most probable number of organisms exceeded 3.6 more than 50 per cent of the time, frequently exceeded 43, and in some instances exceeded 240.

"4. The highest enterococcus densities occurred at the outlets of Pettibone and Sunderland Creeks in Lake County, Illinois. At these two points the density exceeded 240 per 100 ml in 60.4 per cent and 33.3 per cent of the samples examined.

"8. No characteristic enterococcus-coliform ratio existed for the entire section of Lake Michigan waters studied. However, it is apparent from the results secured that the enterococcus determination is a less sensitive measure of bacterial densities of waters than is the coliform determination."

Compared to the method advocated in this paper, the above results derived by the Winter-Sandholzer enterococcus test are very low and result in erroneous conclusions. Using the dextrose azide-ethyl purple azide broth method, as is shown in Table 11, samples from areas where no evidence of immediate sewage pollution could be detected (Women's Gym Bridge) showed enterococcus indices from 2,000 to 140,000, whereas the above report indicated that they rarely exceeded 43. Where sewage and treated effluent entered the waters the above report recorded indices more than 3.6 and frequently exceeding 43. Samples from the

Kalamazoo bridge, a quarter of a mile downstream from the East Lansing Sewage Treatment Plant, yielded indices from 25,000 to 140,000 per 100 ml of water. It is obvious that the figures of the U. S. P. H. S. would have been higher and more significant if a more efficient method for detecting enterococci were employed.

In conclusion it might be repeated that the method developed and described in this paper is the most accurate and easiest of all the methods used for the detection of fecal streptococci. It eliminates the microscopic examination and only two tubes of media are required. This test takes little time to carry out and can be employed in the smallest of water laboratories. It does not require any additional tests such as the catalase test of the Winter and Sandholzer method. It can be incubated in the ordinary 37° C. incubator and does not require 45° C. incubation.

SUMMARY

It was demonstrated that a concentration of 0.04 per cent sodium azide inhibited the gram-negative organisms but not the streptococci.

Data indicated that the following dye concentrations did not inhibit Str. faecalis for more than 48 hours: methyl violet, 1:500,000; ethyl purple, 1:800,000; brilliant green, 1:2,000,000; malachite green, 1:1,800,000; and crystal violet, 1:800,000.

Ethyl purple azide broth, a new medium, was developed. When tested with laboratory strains of bacteria, it was found that the enterococci were the only group that would grow in this medium. These organisms also showed a characteristic growth of a purple compact button on the bottom of the tube of medium after 48 hours of incubation at 37° C.

The specificity of this medium has been demonstrated also with samples from river water, sewage, and soil.

A new test for pollution of river water, sewage, and soil has been advanced. This test employs dextrose azide broth as a presumptive medium and ethyl purple azide broth for confirmation.

A comparison of methods for the detection of enterococci was made and it was demonstrated that the new dextrose

azide-ethyl purple azide broth test was the best and easiest of those in use today.

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