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AN EVALUATION OF MEDIA FOR  
THE ISOLATION OF SALMONELLA  
FROM FECES

Thesis for the Degree of M. S.

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Sylvia Lela Laine

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This is to certify that the  
thesis entitled  
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**AN EVALUATION OF MEDIA FOR THE ISOLATION  
OF SALMONELLA FROM FECES**

**by**

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## Introduction

The isolation of pathogenic organisms from mixed bacteriological cultures has always been a difficult problem, primarily because the pathogenic bacteria are generally a small minority of the organisms present. Furthermore, the pathogens, as a rule, are fastidious organisms that demand special media, whereas, the non-pathogens frequently grow vigorously, particularly on the special media employed for the pathogens. To hold back the growth of the non-pathogenic bacteria, it has been necessary to employ inhibitory agents which are in general, more selective for the non-pathogenic group than they are for the desired pathogen. The isolation of the Salmonella group from feces is a good example of this problem. Numerous media and kinds and types of selective agents have been tried and recommended. Some have proved to be very useful and others which have been highly recommended have failed completely in practical use.

A review of the literature revealed considerable confusion as to what constituted the best method of isolating enteric organisms. It appeared advisable to make a detailed study of the various recommended media both with pure cultures and in practical application. This thesis presents such a study.

## Historical Review

No attempt will be made intentionally of citing the various researchers and their contributions, but rather the different media will be reviewed, to show the author's development of a medium and his reasons for recommending the medium.

## Solid Media

The Wilson bismuth sulfite agar (1) and Wilson and Blair (2) modification of this medium have been used as selective medium for enteric pathogens by almost all investigators. The principle upon which this medium is based includes the fact that the *Salmonella* are able to reduce sulfites to sulfides in medium containing a fermentable carbohydrate (glucose) and metallic salts (ferrous sulfate) and as a result of the reduction, the colonies of *Salmonella* and the medium surrounding the colonies are blackened. Wilson and Blair (2) found the glucose an indispensable source of energy and found further, that the acid produced from this sugar facilitated the characteristic changes by bringing the metallic salts into solution. A combination of bismuth and sodium sulfite causes a suppression of *Escherichia coli* without reparably suppressing the development of the *Salmonella* colonies. Sodium phosphate was used as a buffer to absorb excess acids produced by the fermentation of glucose, thus insuring the blackening of the *Salmonella* colonies. Brilliant green (3) aided in the inhibition of the colon group as well as promoted the blackening of the *Salmonella* colonies. Schmidt (4) in a

practical study of bismuth sulfite glucose medium confirmed the findings of Wilson and Blair. Gunther and Tuft (5) obtained many more positive results from bismuth sulfite agar medium in a comparison of the results from Endo's, eosin methylene blue agar, desoxycholate citrate agar and bismuth sulfite agar medium. Ruy (6) found that bismuth sulfite agar gave rather poor results for the isolation of *Salmonellas*. Hynes (7) concluded from his research that bismuth sulfite agar medium inhibited gram positive organisms completely and all *E. coli* and supported a good growth of *S. typhi*, and the *Salmonellas* in general. Gibbons and Moore (8) found bismuth sulfite medium too selective in their experiments with egg powder.

MacConkey's agar (9) is probably the most commonly used selective medium for the isolation of the typhoid-dysentary group. Most state health departments employ MacConkey's agar in combination with other solid media for the isolation of enteric pathogens. This medium is based upon the general principles that organisms of the intestinal flora may be isolated by utilizing lactose, bile salts and dyes. Bile inhibits the non-colon types and enhances the growth of intestinal bacteria. Lactose indicates the presence of colon forms by the production of acid and gas. Neutral red is used as the indicator of this oxidation and reduction phenomenon. Crystal violet inhibits the growth of gram positive organisms by its selective bacteriostatic action.

Hynes (7) suggested a variation of the usual formula; peptone, sodium chloride, sodium taurocholate, neutral red and 1 percent lactose. In the place of the lactose, he used 0.5 percent sucrose and 0.5 per

cent lactose. Typical red colonies developed and sucrose fermenters as well as lactose fermenters were easily recognized.

Knox, Gell and Pollack (10) found that though MacConkey's medium was usually reliable and supported a good growth of pathogens it appeared to favor the lactose fermenters at the expense of the non-lactose fermenters. Gibbons and Moore (8) found MacConkey's agar medium not selective enough for isolation of the *Salmonellas* from egg powder.

Leifson (11) presented a medium based on the principles that if a solution of neutral red or a similar dye and sodium desoxycholate are acidified, the neutral red goes out of the solution with the precipitate of the desoxycholic acid. The bile reduces the toxicity of the dye to bacteria, and iron salts (ferrous citrate) increase the inhibitory effect of the medium upon contaminants such as E. coli and other lactose fermenters, while they lessen the inhibitory effect on the pathogens. There are several modifications of Leifson's medium. Difco's modification S S agar was studied in this investigation. Difco adds brilliant green to the medium to inhibit gram positive and non-intestinal bacteria. Sodium thiosulfate indicates the sulfite reducers by production of hydrogen sulfide.

Gibbons and Moore (8) obtained good results with desoxycholate citrate medium. Hynes (7) modification of this medium gave good results but was difficult to prepare. Gunther and Tuft (5) found bismuth sulfite agar superior to desoxycholate citrate medium. Darby (12) recommended S S agar medium for the isolation of S. pullorum from chicks.

Harrie-Holt and Teague (13) recommended the use of eosin-methylene blue agar which utilized the two dyes in combination with lactose and sucrose, for the isolation by differentiation and inhibition, of enteric organisms. Teague and Clurman (14) devised an eosin-brilliant green medium which inhibited lactose fermenters and gram positive organisms and differentiated between those that grew and the pathogenic non-lactose fermenters. Knox, Gell and Pollack (10) suggested a medium utilizing the three dyes, eosin, methylene blue and brilliant green. They found that the gram positive organisms and lactose fermenters were suppressed by the brilliant green, the lactose fermenters that grew could be readily differentiated by the oxidation-reduction action of the methylene blue. The *Proteus* group was also inhibited.

Brilliant green liver-infusion agar medium was recommended as a selective medium for *S. pullorum* by Mallmann, Thorp and Semmes (15). Liver-infusion agar was suggested by Huddleson (16) as a base medium for fastidious organisms. The effectiveness of brilliant green as a selective inhibitory agent has been proved by Torrey (17) and confirmed by Rakieta and Rettger (18) and others.

Gruickshank (19) recommended Hoyle's brilliant green acid fuchsin agar which was found to be inhibitory to *Proteus*. This medium which is comparable to eosin-brilliant green agar (14) in formula, was simpler to prepare and more consistent results were obtained. The acid fuchsin did not have to be titrated for each lot of medium, as did the eosin. Better

colonial differentiation was obtained. This medium contains besides the base medium and the dyes, sodium taurocholate which is inhibitory to non-intestinal organisms and seems to stimulate the growth of enteric pathogens when present.

#### Enrichment Media

The literature revealed that many enrichment media have been used. Conradi (14) demonstrated the inhibitive action of brilliant green on E. coli at dilutions which allowed the typhoid organisms to grow. Torrey (17) tested the efficacy of brilliant green broth as a specific enrichment for the paratyphoid-dysentary group. Browning, Gilmour and Mackie (20) utilized brilliant green peptone water. They demonstrated the bacteriostatic action of the dye and the stimulation of the enteric pathogens. Rakieten and Rettger's methods were found to entail too much work by Orzechowski (21). Ray (6) suggested a medium for enrichment which she found useful. Hynes (7) recommended Ray's medium. He found that it suppressed *Proteus* and inhibited E. coli and gram positive organisms. This medium utilizes brilliant green dye which is inhibitory to the gram positive and lactose fermenters and picric acid which is inhibitory to gram positive organisms.

Sodium tetrathionate broth was first discovered to be useful as an enrichment broth by Mueller (21). The principles upon which this medium is based involved the regulation and inhibition of the physiological activities of contaminating organisms by sodium tetrathionate which is

formed by the reaction of sodium thiosulfate and potassium iodide. Iodine is inhibitory to gram positive organisms and the bile salts inhibit the non-intestinal types of organisms. Proteose-peptone is used in the Difco formula as a ready source of energy for bacteria and aids as a buffer against too great a change in the reaction of the medium. Khalil (23) found a high incidence of the Salmonella organisms in rats and mice and ascribed his success of isolation to the use of tetrathionate broth and eosin-brilliant green agar medium. Hoeden (24) obtained twice as many positive results from feces and obtained pure cultures of the pathogens directly after enrichment with tetrathionate broth by plating on Endo's medium.

## Experimental Studies

### I. Studies with Pure Cultures

#### A. Solid Media - Non-diagnostic

Comparative studies are useful and necessary in any scientific inquiry. To initiate this research, a series of tests was set up to demonstrate and compare typical characteristics and reactions of various bacteria of the intestinal flora on non-diagnostic media. Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus subtilis, Aerobacter aerogenes, Shigella dysenteriae, Salmonella typhi, Salmonella typhimurium, Salmonella pullorum, and Salmonella paratyphi were selected as representative organisms that might be isolated from fecal samples. Plain nutrient agar and tryptose agar were compared. Darby and Mallmann (25) compared Bacto-tryptose, a peptone used extensively for cultivation of fastidious microorganisms, with Bacto-peptone and showed that much more rapid growth occurred with the former peptone. The following procedure was set up.

A standard seeding of a 24 hour culture of each of the control organisms was suspended in sterile test tubes containing 10 cc of sterile saline, and thoroughly mixed. Each of these suspensions was streaked on plain nutrient agar and tryptose agar. The plates were incubated for 24 hours at 37° centigrade. At the end of this time the plates were examined. Table 1 shows the appearance of the colonies of the various organisms on plain and tryptose agar plates.

Table 1

APPEARANCE OF BACTERIAL COLONIES ON TRYPTOSE AND PLAIN NUTRIENT AGAR AND A COMPARISON OF THE RELATIVE SIZE AND AMOUNT OF GROWTH ON THE MEDIA AFTER 24 HOURS INCUBATION AT 37° C.\*

| Bacteria               | Description of Colonies and Appearance of Medium                                                                                                       | Plain Nutrient Agar                                                           | Tryptose Agar                                        |
|------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|------------------------------------------------------|
| <u>Staph. aureus</u>   | Colonies are round, smooth, glistening butyrous, entire, raised, yellowish to orange in color. Media not changed by growth.                            | Size 2-6 mm. Good growth                                                      | Size 2-8 mm. Good growth                             |
| <u>Ps. aeruginosa</u>  | No individual colonies, confluent growth surface of agar covered by moist grayish to yellowish glistening even layer. Medium bright green fluorescent. | Not measurable. Very luxurious growth.                                        | Not measurable. Very luxurious growth.               |
| <u>P. vulgaris</u>     | No individual colonies; very spreading growth, flat opaque, smooth, grayish. Distinct fishy odor. Media not changed.                                   | Variable colonial size. Not measurable as to spreading. Very abundant growth. | Size of colony not measurable. Very abundant growth. |
| <u>E. coli</u>         | Colonies white to grayish opaque, smooth, moist. Some colonies slightly brownish, entire to undulate media not changed.                                | Size 1-10 mm. Good growth.                                                    | 1-12 mm. Size. Abundant growth.                      |
| <u>B. subtilis</u>     | Colonies smooth, raised to convex grayish white, round to amoeboid crenate, margins, butyrous adherent media slightly grayed.                          | Size 4-6 mm. Growth abundant.                                                 | Size 4-10 mm. Growth abundant.                       |
| <u>A. aerogenes</u>    | See <u>E. coli</u> ; growth and colonies                                                                                                               | Similar to <u>E. coli</u>                                                     | Similar to <u>E. coli</u>                            |
| <u>Sh. dysenteriae</u> | Colonies small, discrete, slightly raised; grayish in color, smooth entire. slightly convex media not changed.                                         | Size 2-3 mm. Growth scant.                                                    | Size 2-4 mm. Moderate growth.                        |
| <u>S. typhi</u>        | Colonies small, entire, smooth, grayish, convex translucent. Media not changed.                                                                        | Size 2-5 mm. Growth Scant.                                                    | Size 2-5 mm. Good growth                             |
| <u>S. typhimurium</u>  | Same as <u>S. typhi</u>                                                                                                                                | Size 2-5 mm. Moderate growth                                                  | Size 2-5 mm. Good growth                             |
| <u>S. pullorum</u>     | Colonies very small - same as <u>S. typhi</u>                                                                                                          | 1-2.5 mm. Fair growth                                                         | 2-3 mm. Moderate growth                              |

\*Appearance similar in both media. However a variation of size and amount of growth on plain nutrient agar and tryptose agar plates was noted.

The colonies of Staph. aureus, Ps. aeruginosa, and B. subtilis were quite characteristic. P. vulgaris because of its spreading characteristic, could be readily identified. E. coli, A. aerogenes, Sh. dysenteriae and the Salmonellas were more difficult to identify because of marked colonial similarity. The need for selective media for the isolation of pathogens was quite obvious from this series of tests. The tryptose plates demonstrated better growth and larger colonies than did the plain nutrient agar plates, particularly of the pathogenic organisms. The formulae for plain nutrient and tryptose agar media will be found in the appendix.

#### B. Selective Solid Media

The following solid selective media were studied in the next series of tests: MacConkey's agar, Wilson and Blair's bismuth sulfite agar, S S Agar, a modification of desoxycholate citrate agar, eosine-brilliant green-methylene blue agar suggested by Knox, Gell and Pollack, brilliant green liver-infusion agar recommended by Mallmann, Thorp and Semmes, and Hoyle's brilliant green-acid fuchsin agar medium. Pure culture studies of the control organisms were made on each of the six selective media, employing the saline suspensions of 24 hour cultures as in the first series of tests. The colonies were studied at the end of 24 hour and 48 hours incubation at 37° C.

Pure culture studies of Wilson and Blair's bismuth sulfite agar indicated that this medium supported a good growth of the Salmonellas. The gram positive organisms were inhibited. Proteus grew, but had no

tendency to spread and was readily distinguished from the pathogens since it did not blacken on the medium as did the pathogens. E. coli survived on bismuth sulfite agar, appearing as droplets of moisture and after 48 hours very pale yellowish green, indicating that care had to be exercised in picking colonies from mixed cultures to avoid contamination.

Experiments with MacConkey's agar showed that this medium supported a good growth of the Shigellas and Salmonellas. Gram positive organisms were inhibited. Proteus grew well and spread over the entire plate by the end of 48 hours incubation, giving off a distinct and characteristic odor. Lactose fermenters grew with characteristic red to pinkish color and a precipitation of bile salts surrounding some of the colonies.

Brilliant green liver-infusion agar, when tested in pure culture studies, inhibited E. coli and the gram positive organisms completely. Proteus was not inhibited and was difficult to differentiate from the pathogens. The Salmonellas grew well. The preparation of the base medium was tedious and difficult.

The media studied up to this point, demonstrated more or less satisfactory results in eliminating or differentiating gram positive bacteria and the coliform and other lactose fermenters. However the Proteus group was the most obnoxious non-pathogen that grew on culture plates, for it not only spread over media, making isolation of individual colonies impossible, but was difficult to differentiate from the pathogenic organisms.

Knox, Gell and Pollack (10) found eosine-brilliant green-methylene blue agar medium inhibitory to the *Proteus*. In this investigation, tryptose agar was used as the basal medium instead of tryptic digest agar was suggested by the authors. The preparation of the medium was difficult as fresh dyes had to be prepared often and the techniques were time consuming. Pure culture studies showed excellent results. The lactose fermenters that grew were few in number and these readily differentiated by colonies which were large, mucoid and with deep indigo centers. *Proteus* was inhibited completely. The *Salmonellas* grew luxuriously. The *S. pullorum* colonies which are usually quite tiny on most media, grew to a much larger size, as did the colonies of all the *Salmonella* tested. Gram positive organisms were inhibited completely.

Cruickshank (19) recommended Hoyle's brilliant green-acid fuchsin agar which had been shown to be inhibitory to *Proteus*. With pure cultures, experimentally, the medium inhibited *Proteus* and gram positive organisms completely. *Salmonellas* grew well on the medium. *Shigellas* were inhibited. This medium was shown to be highly selective, was not difficult to prepare and kept well.

The comparative amounts of growth on each of the media studied are presented in Table 2. In Table 3 are presented the typical colonial characteristics of the control organisms on the various selective media. Formulae for the media are given in the appendix.

Table 2

The Growth of Organisms Representative of Intestinal  
Flora Compared on Selective Media

| Bacteria               | Mac. C. | Bi S. | S S | B. G.<br>L. I. | E.B.G.<br>M.B. | B. G.<br>A. F. |
|------------------------|---------|-------|-----|----------------|----------------|----------------|
| <u>Staph. aureus</u>   | -       | -     | +   | -              | -              | -              |
| <u>E. coli</u>         | +++     | +     | +   | -              | +              | +              |
| <u>B. subtilis</u>     | -       | -     | -   | -              | -              | -              |
| <u>P. vulgaris</u>     | +++     | +++   | +++ | ++             | +              | -              |
| <u>A. aerogenes</u>    | ++      | -     | ±   | -              | -              | -              |
| <u>S. typhi</u>        | +++     | +++   | +++ | +++            | ++++           | +++            |
| <u>S. typhimurium</u>  | +++     | +++   | +++ | +++            | ++++           | +++            |
| <u>S. pullorum</u>     | +++     | +++   | +++ | +++            | ++++           | +++            |
| <u>Sh. dysenteriae</u> | +++     | -     | +++ | +++            | +++            | -              |

Key: No Growth -  
Very scant growth +  
Moderate Growth ++  
Good growth +++  
Very good growth ++++

Table 3

## APPEARANCE OF COLONIES OF VARIOUS BACTERIA ON SELECTIVE AND DIFFERENTIAL MEDIA

| Bacteria              | Bismuth Sulfite Agar                                                                                                                                              | S.S Agar                                                                                                                 | Brilliant Green-Acid Fuchsin Agar                                                                                          | MacConkey's Agar                                                                                                                                 | Brilliant Green Liver infusion                                                      | Eosin-Brilliant Green-Methylene Blue                                                               |
|-----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|
| <u>Staph. aureus</u>  | No growth                                                                                                                                                         | No significant growth; very tiny pale pinkish brown.                                                                     | No growth                                                                                                                  | No growth                                                                                                                                        | No growth                                                                           | No growth                                                                                          |
| <u>E. coli</u>        | No significant growth, those present pale, opaque, yellowish to green without pitting medium, sometimes brownish.                                                 | Growth inhibited. If present large opaque, pink to red colored; mucoid; umbilicate. Some whitish edges.                  | Mostly inhibited. If present, large mucoid umbilicate, opaque greenish blue with violet zone. 1-5 mm.                      | Colonies 1-9 mm. opaque brick red to pinkish occasionally white or gray cream usually surrounded by zone of pptd. bile salts. Smooth glistening. | No growth                                                                           | Scant growth, or none. Deep indigo centers. Large mucoid, umbilicate or convex.                    |
| <u>B. subtilis</u>    | No growth                                                                                                                                                         | No significant growth                                                                                                    | No growth                                                                                                                  | No growth                                                                                                                                        | No growth                                                                           | No growth                                                                                          |
| <u>P. vulgaris</u>    | Colorless to pale bluish or yellow green. No pitting in medium. 1-2 mm. No tendency to spread.                                                                    | Resemble Salmonella though usually larger cloudier. Fishy odor.                                                          | No growth                                                                                                                  | Colorless, transparent, spreading colonies. 2-8 mm. in size. Flat to convex. Similar to Salmonellas but larger.                                  | Dirty gray mucoid or somewhat dry. Spreading flat to convex. Opaque distinct odor.  | No growth usually. Similar to Salmonella if they do not grow.                                      |
| <u>A. aerogenes</u>   | Usually inhibited. Similar to <u>E. coli</u>                                                                                                                      | See <u>E. coli</u>                                                                                                       | No growth                                                                                                                  | See <u>E. coli</u>                                                                                                                               | Grayish green, shiny raised centers, butyrous, opaque. Medium greened.              | No growth                                                                                          |
| <u>S. typhi</u>       | Round, about 1-3 mm. Jet black surrounded by blackish zone, with intense metallic sheen, young colonies, green with black centers. Soft with black pit in medium. | 1-5 mm. Colorless translucent to yellowish or tan; May have tan center Smooth butyrous dome shaped sometimes umbilicate. | Size 1-5 mm. Smooth transparent, colorless to slightly yellow. Flat to slight convex. Older colonies develop central peak. | Colonies colorless to bluish gray, convex. Size 1-3 mm. Glistening smooth butyrous. Clear area around colony. Transparent.                       | Lobate, dull grape-leaf like. Colorless, size 1-5 mm. Medium sometimes yellowed.    | Uniform pinkish purple large translucent lacerate edges. Umbilicate. Size 3-8 mm. Grape leaf like. |
| <u>S. typhimurium</u> | Colorless to bluish gray, convex, 1-5 mm. Smooth butyrous, transparent. Clear area surrounding colony.                                                            | Colorless to gray green slightly raised, lobate clear 1-5 mm. Size. No change in medium.                                 | Uniform pinkish purple. translucent with lacerate edges, umbilicate. Size 3-8 mm. Grape leaf like.                         | Dark green to dark brown lustrous, size 1-5 mm. Flat to raised colonies pitting of medium. Black centers with transmitted light.                 | Colonies similar to <u>S. typhi</u> , larger. Dark brown to black center. 1-7 mm.   | Similar to <u>S. typhi</u> .                                                                       |
| <u>S. Pullorum</u>    | Same as above (Sal) though much smaller                                                                                                                           | Same as <u>S. typhimurium</u> except much smaller and dark center                                                        | Same as <u>S. typhimurium</u> though somewhat smaller.                                                                     | Colorless to bluish gray. transparent, glistening. Smooth butyrous clear area surround colony tiny 0.5-2mm                                       | Tiny colorless to pale bluish green. Clear moist lobate dull granular. Size 1-2 mm. | Same as <u>S. typhimurium</u> or <u>S. typhi</u> . Colony size 1-6 mm.                             |
| <u>S. dysenteriae</u> | No significant growth                                                                                                                                             | Colonies same as <u>S. typhi</u>                                                                                         | No growth                                                                                                                  | Colorless to whitish small 1-6 mm. transparent to opaque, uneven edges. Flat to slightly convex.                                                 | Similar to <u>S. typhi</u>                                                          | Similar to <u>S. typhi</u> and Salmonella                                                          |

A recapitulation of the findings showed that bismuth sulfite agar was quite selective, and the pathogens could be readily identified. MacConkey's agar medium was not very selective and seemed to favor the growth of the lactose fermenters more than the non-lactose fermenters. S S agar, though selective, did not inhibit *Proteus* and these colonies were difficult to differentiate from the pathogens. Rosine-brilliant green-methylene blue agar was highly selective and very favorable to the *Salmonellas*. E. coli could be readily distinguished from the non-lactose fermenters when they grew, and P. vulgaris was inhibited completely. The medium was difficult to prepare and did not keep well. Brilliant green liver-infusion agar inhibited E. coli and gram positive organisms completely but allowed the *Proteus* to grow. Brilliant green-acid fuchsin agar medium inhibited gram positive organisms and P. vulgaris completely. The *Salmonellas* grew well. The medium was not difficult to prepare and kept well.

#### C. Enrichment Media

The next series of tests with pure cultures of the test organisms was made with the enrichment broths, Ruy's medium and sodium tetrathionate broth (Difco).

A standard seeding of each test organism was planted into Ruy's medium and sodium tetrathionate broth, the cultures mixed thoroughly and then incubated for 18 hours at 37° C. From each tube was streaked a tryptose agar plate and the plates incubated for 24 and 48 hours. At the end of each incubation period, the plates were examined for growth. The results showed that Ruy's medium was very highly selective and was toxic to the pathogens as well as to the non-pathogens.

Table 4

## The Effects of Enrichment on the Representative Bacteria\*

| Organism               | Saline | Tetrathionate<br>Broth | Roy's<br>Medium |
|------------------------|--------|------------------------|-----------------|
| <u>Staph. aureus</u>   | ++     | -                      | -               |
| <u>Ps. aeruginosa</u>  | ++     | -                      | -               |
| <u>Sh. dysenteriae</u> | ++     | +                      | -               |
| <u>P. vulgaris</u>     | ++++   | ++++                   | -               |
| <u>E. coli</u>         | ++     | -                      | -               |
| <u>B. subtilis</u>     | ++     | -                      | -               |
| <u>A. aerogenes</u>    | ++     | -                      | -               |
| <u>S. typhi</u>        | ++     | ++                     | +               |
| <u>S. typhimurium</u>  | ++     | ++                     | +               |

Key:      ++      Good Growth  
           ++++      Very Good Growth  
           +      Scant Growth  
           -      No Growth

\*Plated on tryptose agar after 18 hours incubation in enrichment media.

(1) Streaked directly on tryptose agar plates (control)

Sodium tetrathionate broth inhibited the lactose fermenters and the gram positive organisms completely. The pathogens grew well. However, *Proteus* grew even more luxuriously than the pathogens.

In Table 4 are shown the results of enrichment by Ruy's medium and sodium tetrathionate broth of pure cultures of the test organisms, when plated on non-diagnostic medium.

#### D. Enrichment with Sodium Tetrathionate Broth and Solid Media

The final series of tests to be run with pure cultures, utilized enrichment with sodium tetrathionate broth followed by plating on six selective media. A plate of each of the selective media, MacConkey's agar, bismuth sulfite agar, S S agar, brilliant green liver-infusion agar, eosine-brilliant green-methylene blue agar and brilliant green-acid fuchsin agar was streaked directly from saline suspensions as controls of growth. A standard seeding of each of the test organisms was planted into tubes of tetrathionate broth and incubated for 18 hours at 37° C., and then a plate of each of the selective media was streaked from the cultures. These plates were in turn incubated for 24 and 48 hours. At the end of the incubation period the plates were examined.

The lactose fermenters were suppressed on all plates after enrichment with sodium tetrathionate broth. Gram positive organisms were completely inhibited. The pathogens grew well but demonstrated little variation from the direct plating methods. The *Shigellas* which did not grow on bismuth sulfite agar or brilliant green acid fuchsin on direct plating did not grow on these media after enrichment. *Proteus* seemed

to be favored by the enrichment with sodium tetrathionate broth and grew even better on all the media except brilliant green-acid fuchsin agar which inhibited its growth with the direct plating method also.

The compared results of this series of experiments are presented in Table 5.

## II. Mixed Known cultures added to Fecal Material

As a preliminary procedure to the study of mixed cultures, five unknown chicken fecal samples were streaked on tryptose agar and MacConkey's agar plates. Colonies were picked from both sets of plates after incubation at 37° C. The organisms which predominated were from the genera *Escherichia* and *Proteus*. Practical application proved this to be true of most poultry fecal samples. *Proteus* appeared to predominate and to be the most obnoxious and confusing group of organisms found on the culture plates for the isolation of enteric pathogens from chickens in this investigation.

### A.--Non-Diagnostic Solid Media

Methods similar to those used by Mallmann, Ryff and Matthews (26) were employed, using pure cultures of *P. vulgaris*, *E. coli* and *S. typhimurium* to represent the genera of most significance to this investigation. Into each of five large sterile test tubes containing 10 cc of sterile saline was placed approximately 1 gram of sterile fecal material. Into the first three tubes, a standard seeding of 24 hour cultures of *P. vulgaris*, *E. coli* and *S. typhimurium* was introduced respectively. To the fourth tube, a standard seeding of a mixture of *E. coli* and *S. typhimurium* was added. To the fifth tube a mixture of *P. vulgaris* and *S. typhimurium* was introduced. The mixtures were carefully suspended and then allowed

Table 5

Comparative Growth of Bacteria on Selective Media From  
Direct Plating and After Enrichment in Sodium Tetrathionate Broth

| Solid Media →          | Mac C |      | Bi S |   | S S |   | BGL-I |   | EBG-MB |      | BG-AF |   |
|------------------------|-------|------|------|---|-----|---|-------|---|--------|------|-------|---|
| Organisms ↓            | D     | E*   | D    | E | D   | E | D     | E | D      | E    | D     | E |
| <u>S. aureus</u>       | -     | -    | -    | - | -   | - | -     | - | -      | -    | -     | - |
| <u>Pa. aeruginosa</u>  | s.g.  | -    | -    | - | -   | - | -     | - | -      | -    | -     | - |
| <u>Sh. dysenteriae</u> | +     | +    | -    | - | +   | + | +     | + | +      | +    | -     | - |
| <u>P. vulgaris</u>     | ++    | ++   | s.g. | M | M   | M | +     | + | -      | s.g. | -     | - |
| <u>E. coli</u>         | +     | s.g. | -    | - | -   | - | -     | - | s.g.   | -    | -     | - |
| <u>B. subtilis</u>     | s.g.  | -    | -    | - | -   | - | -     | - | -      | -    | -     | - |
| <u>A. aerogenes</u>    | s.g.  | -    | -    | - | -   | - | -     | - | -      | -    | -     | - |
| <u>S. typhi</u>        | +     | +    | +    | + | +   | + | +     | + | ++     | ++   | +     | + |
| <u>S. typhimurium</u>  | +     | +    | +    | + | +   | + | +     | + | ++     | ++   | +     | + |

\*Enrichment in sodium tetrathionate broth for 18 hours at  
37° C. prior to plating.

Key:

|        |   |                                            |    |   |                  |
|--------|---|--------------------------------------------|----|---|------------------|
| +      | = | Good Growth                                | ++ | = | Very good growth |
| -      | = | No Growth                                  |    |   |                  |
| s.g.   | = | slight growth                              |    |   |                  |
| M      | = | Moderate growth                            |    |   |                  |
| D      | = | Direct Plating                             |    |   |                  |
| E      | = | Enrichment                                 |    |   |                  |
| Mac C  | = | MacConkey's agar                           |    |   |                  |
| Bi S   | = | Bismuth Sulfite agar (Difco)               |    |   |                  |
| S S    | = | S S Agar (Difco)                           |    |   |                  |
| BGL-I  | = | Brilliant green liven infusion agar        |    |   |                  |
| EBG-MB | = | Eosine brilliant green methylene blue agar |    |   |                  |
| BG-AF  | = | Brilliant green acid fuchsin agar          |    |   |                  |

to settle. Each tube was streaked on plain nutrient agar and tryptose agar plates. The plates were incubated for 24 hours at 37° C., and then examined for growth.

The first three plates of tryptose agar showed luxurious growth of the individual organisms with which each had been streaked. The fourth plate, which had been inoculated with the mixture of E. coli and S. typhimurium also demonstrated abundant growth. However, identification of ten colonies picked at random, since there was no way of distinguishing the pathogen from the non-pathogen, revealed all ten colonies to be E. coli. The spreading growth of the P. vulgaris on the fifth plate obscured any individual colonies and made picking of isolated colonies impossible. The five plates of plain nutrient agar showed similar results, though the growth of the pathogen was not as abundant as that on the tryptose agar plates. It was again obvious that a selective medium was essential for isolation of pathogenic organisms. Table 6 shows the comparative results obtained from this series of tests.

### B - Enrichment Media and Mixed Cultures

To compare the growth of mixed cultures in feces and with enrichment broth, into each of five tubes of sterile saline, Ruy's medium (though it had proved too inhibitory in pure culture studies) and sodium tetrathionate broth was placed approximately 1 gram of sterile fecal material, and into the first three tubes of each medium, a standard seeding of S. typhimurium, E. coli and P. vulgaris. Into the fourth tube of each was placed a mixture of E. coli and S. typhimurium, and the fifth, a mixture of P. vulgaris and S. typhimurium. The suspensions were care-

Table 6

To Demonstrate the Growth of Organisms from Genera *Escherichia*, *Proteus*, and *Salmonella* in Mixed Cultures and the Necessity of Selective Media for Isolation of Pathogens

| Plate Number | Organisms                                   | Growth                                   | Colonies Isolated*                                              | Growth                  | Colonies Isolated                                                       |
|--------------|---------------------------------------------|------------------------------------------|-----------------------------------------------------------------|-------------------------|-------------------------------------------------------------------------|
| 1            | <u>S. typhimurium</u>                       | Moderate                                 | 10 <u>S. typhimurium</u>                                        | Good                    | 10 <u>S. typhimurium</u>                                                |
| 2            | <u>E. coli</u>                              | Abundant                                 | 10 <u>E. coli</u>                                               | Abundant                | 10 <u>E. coli</u>                                                       |
| 3            | <u>P. vulgaris</u>                          | Very abundant spreading                  | 10 <u>P. vulgaris</u>                                           | Very abundant spreading | 10 <u>P. vulgaris</u><br>(difficult to pick as not individual colonies) |
| 4            | <u>S. typhimurium</u><br>and <u>E. coli</u> | Abundant Growth                          | 10 <u>E. coli</u>                                               | Abundant Growth         | 10 <u>E. coli</u>                                                       |
| 5            | <u>S. typhimurium</u><br>and <u>E. coli</u> | No isolated colonies abundant spreading. | Impossible to pick individual colonies<br>10 <u>P. vulgaris</u> | Abundant Spreading      | Impossible to pick isolated colonies.<br>10 <u>P. vulgaris</u> .        |

\* 10 colonies picked from each plate.

Key: Very abundant - plate overgrown with colonies - spreading growth  
 Abundant - individual colonies - over 40 colonies per plate  
 Good - individual colonies - 25-40 colonies per plate.  
 Moderate - 20 - 30 colonies/plate.

fully mixed. The saline suspensions were streaked immediately on tryptose agar plates. Into the tetrathionate broth tubes, a sterile piece of cotton was pressed, to force the coarser particles of fecal material to the bottom of the tubes. The enrichment broth cultures and the tryptose agar plates were incubated for 24 hours at 37° C. At the end of this period, tryptose agar plates were streaked from each of the enrichment broths, and incubated for 24 and 48 hours. Colonies were examined and identified from the direct plating at this time. The results were similar to those obtained in the previous mixed culture experiments. E. coli and P. vulgaris far outgrew the pathogens.

The examination of the tryptose agar plates made from the enrichment medium showed that Buy's medium inhibited E. coli and P. vulgaris but was also toxic to the S. typhimurium. The sodium tetrathionate broth, though apparently favoring the P. vulgaris allowed the S. typhimurium to grow. This medium inhibited E. coli completely in this series of tests. Table 7 (a and b) show the evaluation of enrichment media with mixed cultures.

#### Q. Mixed cultures in sodium tetrathionate broth and Selective Solid Medium

To conclude the series of tests with mixed cultures, the three representative organisms were compared on the solid selective media with enrichment in sodium tetrathionate broth and from direct plating. The procedures used in the foregoing experiment were followed, except for plating on the six selective media instead of non-diagnostic media.

Table 7-A  
An Evaluation of Enrichment Media\*

| Plate No. | Organisms                                               | Direct Plating | Ruy's Medium | Sodium Tetrathionate |
|-----------|---------------------------------------------------------|----------------|--------------|----------------------|
| 1         | <u>S. typhimurium</u>                                   | +++            | +            | +++                  |
| 2         | <u>E. coli</u>                                          | +++            | -            | +                    |
| 3         | <u>P. vulgaris</u>                                      | ++++           | ±            | ++++                 |
| 4         | Mixture of <u>E. coli</u> and <u>S. typhimurium</u>     | +++            | ±            | ++                   |
| 5         | Mixture of <u>P. vulgaris</u> and <u>S. typhimurium</u> | ++++           | +            | ++++                 |

Key -

- = No Growth
- ± = Scant growth -less than 10 colonies per plate.
- ± = Fair growth -approximately 10-20 colonies per plate.
- ++ = Moderate growth
- +++ = Good growth
- ++++ = Very abundant spreading growth

\*After enrichment in broth plated on tryptose agar medium.

Table 7-B

Identification of Colonies Picked from Plates 4 and 5  
which were Streaked with Mixtures of Microorganisms\*

| Plate | Mixture                 | Direct<br>Plating | Ruy's<br>Medium | Sodium<br>Tetrathionate |
|-------|-------------------------|-------------------|-----------------|-------------------------|
| 4     | <u>S. typhimurium</u>   | 0%                | 100%            | 100%                    |
|       | +<br><u>E. coli</u>     | 100%              | 0%              | 0%                      |
| 5     | <u>S. typhimurium</u>   | 0%                | 70%             | 40%                     |
|       | +<br><u>P. vulgaris</u> | 100%              | 30%             | 60%                     |

\*Percentages based on 10 colonies picked.

After enrichment with tetrathionate broth, E. coli grew poorly on MacConkey's agar. P. vulgaris grew well as did the S. typhimurium. Direct plating allowed all three organisms to grow well. On the plate inoculated with the mixture of E. coli and S. typhimurium, after enrichment, E. coli grew lightly and could be readily identified. On the direct plate the growth of E. coli was much heavier though it could be identified as such. The plate containing the mixture of S. typhimurium and P. vulgaris indicated that this combination of media was not of much value for eliminating the obnoxious Proteus group.

On busmuth sulfite agar, after enrichment with sodium tetrathionate broth E. coli did not grow. P. vulgaris grew better after enrichment but could be recognized. S. typhimurium grew well with enrichment and on the directly plated medium. The mixtures of E. coli and S. typhimurium streaked on busmuth sulfite agar directly and after sodium tetrathionate enrichment showed no recognizable E. coli colonies. The plates streaked with the mixture of P. vulgaris and S. typhimurium, directly and after enrichment both allowed the P. vulgaris to grow. The colonies could be identified as such, and had no tendency to spread.

On S S agar E. coli was inhibited with and without enrichment. P. vulgaris showed little variation on direct plating and enrichment prior to plating. In the mixtures, E. coli was inhibited and S. typhimurium grew well. P. vulgaris seemed to be favored slightly when enrichment with sodium tetrathionate broth was used prior to plating.

On eosine-brilliant green-methylene blue agar, with direct plating, S. typhimurium grew luxuriously, E. coli grew scantily, and P. vulgaris grew poorly. After enrichment with sodium tetrathionate broth E. coli

did not grow, P. vulgaris showed a slight growth, and S. typhimurium good growth. On the plate containing the mixture of the pathogen and E. coli, S. typhimurium could be readily identified. P. vulgaris grew better after enrichment with sodium tetrathionate broth on this medium and the colonies were difficult to identify from S. typhimurium colonies in the mixed cultures.

Brilliant green liver infusion agar suppressed E. coli completely both with direct plating and sodium tetrathionate enrichment. P. vulgaris grew well with both methods.

Brilliant green-acid fuchsin agar was not affected by the utilization of sodium tetrathionate enrichment, the pathogens grew well on direct plating and after enrichment. The non-pathogens were suppressed with direct plating and enrichment methods.

The results of these tests are presented in Table 8 (a and b).

### III. Practical Applications

#### A. Selection of Media to use for Isolations

In selecting the media to use for practical application, the isolation of Salmonella organisms from feces, the following qualifications were utilized: (1) the medium should allow the Salmonella group to grow well, (2) it should be inhibitory to the non-pathogenic organisms without being toxic to the pathogens (3) the medium should show adequate differentiation of various bacteria, (4) it should be relatively stable and give uniform results and (5) it should be relatively easy to prepare.

Table 8 A

A Comparison of the Growth of *E. coli*, *P. vulgaris* and *S. typhimurium* on Various Solid Media with Direct Plating and After Enrichment

| Medium                                | <i>S. typhimurium</i> |             | <i>E. coli</i> |            | <i>P. vulgaris</i> |            |
|---------------------------------------|-----------------------|-------------|----------------|------------|--------------------|------------|
|                                       | Direct                | Enrichment* | Direct         | Enrichment | Direct             | Enrichment |
| MacConkey's Agar                      | +++                   | +++         | +++            | +          | +++                | +++        |
| Bismuth Sulfite Agar                  | +++                   | +++         | -              | -          | +                  | +          |
| S S Agar                              | +++                   | +++         | -              | -          | ++                 | ++         |
| Eosine-Brilliant green-methylene Blue | ++++                  | ++++        | +              | -          | +                  | +          |
| Brilliant green liver-infusion agar   | +++                   | +++         | -              | -          | +++                | +++        |
| Brilliant green and fuchsin agar      | +++                   | +++         | -              | -          | -                  | -          |

Key -

- = No Growth

+ = Scant Growth - readily identified from appearance of colonies

++ = Scant Growth but difficult to identify by appearance.

+++ = Moderate growth

++++ = Good Growth

+++++ = Very Abundant Growth

\* Enrichment in Sodium Tetrathionate Broth prior to plating.

Table 8-B

Identification of Colonies from Mixtures of Representating Organisms of the Genera  
Escherichia, Proteus and Salmonella on Various Selective Media

| Selective Medium                              | Mixture of Organisms                   |                                            |                                            |
|-----------------------------------------------|----------------------------------------|--------------------------------------------|--------------------------------------------|
|                                               | <i>S. typhimurium</i> + <i>E. coli</i> | <i>S. typhimurium</i> + <i>P. vulgaris</i> |                                            |
|                                               | Direct Plating*                        | Enrichment                                 | Direct Plating*                            |
| MacConkey's Agar                              | 10 Salmonella<br>(1)                   | 10 Salmonella<br>(2)                       | No Individual colonies -<br>Proteus picked |
| Bismuth Sulfite Agar                          | 10 Salmonella<br>(2)                   | 10 Salmonella<br>(2)                       | 10 Salmonella<br>(1)                       |
| S S Agar                                      | 10 Salmonella<br>(2)                   | 10 Salmonella<br>(2)                       | 5 Salmonella<br>9 Proteus<br>(3)           |
| Tosine-brilliant green<br>Methylene blue agar | 10 Salmonella<br>(2)                   | 10 Salmonella<br>(2)                       | 9 Salmonella<br>1 Proteus                  |
| Brilliant-Green Liver<br>infusion agar        | 10 Salmonella<br>(2)                   | 10 Salmonella<br>(2)                       | 9 Proteus<br>1 Salmonella<br>(3)           |
| Brilliant green-acid<br>fuchsin agar          | 10 Salmonella<br>(2)                   | 10 Salmonella<br>(2)                       | 10 Salmonella<br>(2)                       |
|                                               |                                        |                                            | Proteus picked from<br>Spreading Growth    |
|                                               |                                        |                                            | 10 Salmonella<br>(1)                       |
|                                               |                                        |                                            | 6 Proteus<br>4 Salmonella<br>(3)           |
|                                               |                                        |                                            | 5 Salmonella<br>5 Proteus<br>(3)           |
|                                               |                                        |                                            | 8 Proteus<br>2 Salmonella<br>(3)           |
|                                               |                                        |                                            | 10 Salmonella<br>(2)                       |

Key: (1) Pathogen and non-pathogenic colonies on plate, each showing colonial characteristics and readily identified.

(2) No recognisable non-pathogenic representative on plate.

(3) Colonies difficult to differentiate because of colonial similarity.

\*10 colonies picked from each plate for identification.

With these specifications in mind, a comparison was made of the various media studied. Of the enrichment media, Ruy's medium was disqualified because it was too toxic to the Salmonellas. Sodium tetrathionate broth was inhibitory to the gram positive organisms and the lactose fermenters and was easy to prepare. The medium was made in small lots for each group of specimens and was found to give better results when freshly prepared.

A review of the solid selective media indicated that in pure culture studies, MacConkey's agar, while it allowed the pathogens to grow, it was not selective enough and seemed to favor the lactose fermenters and the Proteus group to the desired pathogens.

Bismuth sulfite agar medium was highly selective and differentiated those non-pathogens that grew. It allowed the Salmonellas to grow well in pure culture studies. Difco's prepared medium was used.

S S Agar (Difco) showed good growth of the Salmonellas. It was inhibitory to the gram positive organisms for the most part, although during actual testing of specimens a few strains of Streptococci were recovered. Lactose fermenters were inhibited and if they grew were readily identified. The proteus was not inhibited in pure culture studies and were difficult to differentiate from the Salmonellas in most instances.

Brilliant green liver infusion agar was inhibitory to lactose fermenters and gram positive organisms. It allowed the Salmonellas to grow well. The preparation of the base medium, liver infusion agar was difficult and time consuming.

Eosine-brilliant green-methylene blue agar showed good results: It inhibited lactose fermenters, gram positive organisms and the Proteus in pure culture studies. It stimulated the growth of the Salmonella group. However, the medium was exceedingly difficult to prepare, because of the number of sterile techniques employed with the addition of each dye and the sugar. The dyes had to be freshly prepared often, as they were found to be relatively unstable.

Brilliant green acid fuchsin was found to be highly selective and allowed a good growth of the Salmonellas. This medium was easily prepared and kept well.

A comparison of these findings, led to the selection of the following media for the practical experimental phase of this investigation: bismuth sulfite agar, S S Agar, and brilliant green acid fuchsin agar medium in combination with direct plating methods and sodium tetrathionate broth enrichment. Later in the investigation direct plating was found to be of little value for the isolation of Salmonellas from chicken feces and was discontinued.

#### B. Source and description of fecal samples.

P. R. Edwards (27) stated in 1939 that "fowls are the greatest reservoir of paratyphoid in the United States". Since it was difficult to obtain an adequate number of specimens from chickens known to be suffering from Salmonella infections, the suggestion made by Mallmann, Ryff and Matthews (26) of the possibility of the normal chicken as a source of infection (Salmonella) to man and animals, led to the choice of normal chickens for investigation. With the cooperation of the

Michigan State College Poultry Department and the Regional Laboratory, U.S.D.A., fecal samples were readily available.

Fecal samples from 376 chickens were examined. Of these, 298 specimens were from droppings of live birds. These specimens were collected into sterile petri plates from the cages of the isolated chickens, numbered, dated and histories obtained for each chicken. Seventy-six specimens were from the intestines of killed birds. The intestines removed at autopsy were brought directly to the laboratory for examination, or were refrigerated until they could be examined. All the birds studied had normal life histories and gave no clinical manifestation of any disease. The chickens had had no known contact with diseased birds of any kind. Of particular interest was the fact that the chickens studied were from groups of birds which had been segregated from all other flocks throughout their lives.

The preparation of the fecal samples for examination varied only in the preliminary procedures because of physical differences. The intestines of the killed birds were divided into three parts, the large and small intestine and the caecum, each section split open and the contents removed to a separate sterile petri dish. The samples were labeled as to source, large, small intestine or caecum as well as the bird number. From this procedure, they were handled as individual specimens. Specimens of feces from the live chickens were obtained by collecting approximately 10 grams or more of the droppings from the tray under the cage of each bird. The trays had been lined with clean paper the night be-

fore the collection was made. Portions of each sample of feces were taken from various parts of the tray, and care taken to collect any suspicious material, such as that which contained any unusual amount of mucus, was especially liquid, or contained gross blood.

### C. Examination of Fecal Samples

The following procedures were employed in examining each fecal specimen. From each specimen, approximately one gram of feces was transferred to a test tube containing 10 cc of sterile saline, and one containing 10 cc of freshly prepared sodium tetrathionate broth. The tubes were mixed thoroughly. Into the tetrathionate broth tube was pressed a sterile piece of cotton to force the coarse material to the bottom of the tube. The saline suspension was allowed to settle during this procedure and then a plate of bismuth sulfite agar, S S agar and brilliant green acid fuchsin agar medium streaked from it. The sodium tetrathionate broth cultures and the directly plated agar plates were incubated at 37° C. After 18 hours incubation, S S agar, bismuth sulfite agar and brilliant green acid fuchsin agar plates were streaked with a fairly heavy amount of inocula from the sodium tetrathionate broth enrichment tube. These plates were incubated for 24 and 48 hours at 37° C.

At the end of 24 hours, all the plates were examined for typical colonies, and when possible at least five colonies picked for further identification. If at the end of 24 hours there was no growth or very light growth, the plates were reincubated for another 24 hours and reexamined. The suspicious colonies were transferred into lactose broth. From the lactose broth, non-lactose fermenters were transferred into tubes of semi-solid agar for stock cultures and motility tests and into tryptone broth for indole production test.

The tryptone broth culture was tested for indole at the end of 48 hours, and if indole was produced, a transfer was made from the stock culture into gelatin and Kligler's iron agar slant. A typical reaction in the Kligler's agar slant indicated the inoculation of the following carbohydrates: glucose, lactose, sucrose and salicin. Production of acid or acid and gas in glucose and negative reactions in the other three sugars led to further identification in maltose, mannitol and adonitol. Voges-Proskauer's test and a Gram's stain were run as supplementary examinations. If the V-P test was negative and the Gram's stain demonstrated a typical gram negative rod, fresh stock cultures were made and incubated for 24 hours at 37° C. These cultures were then sent to the Michigan State Health Department for antigenic identification.

The flow sheet which follows shows the general methods of procedure.

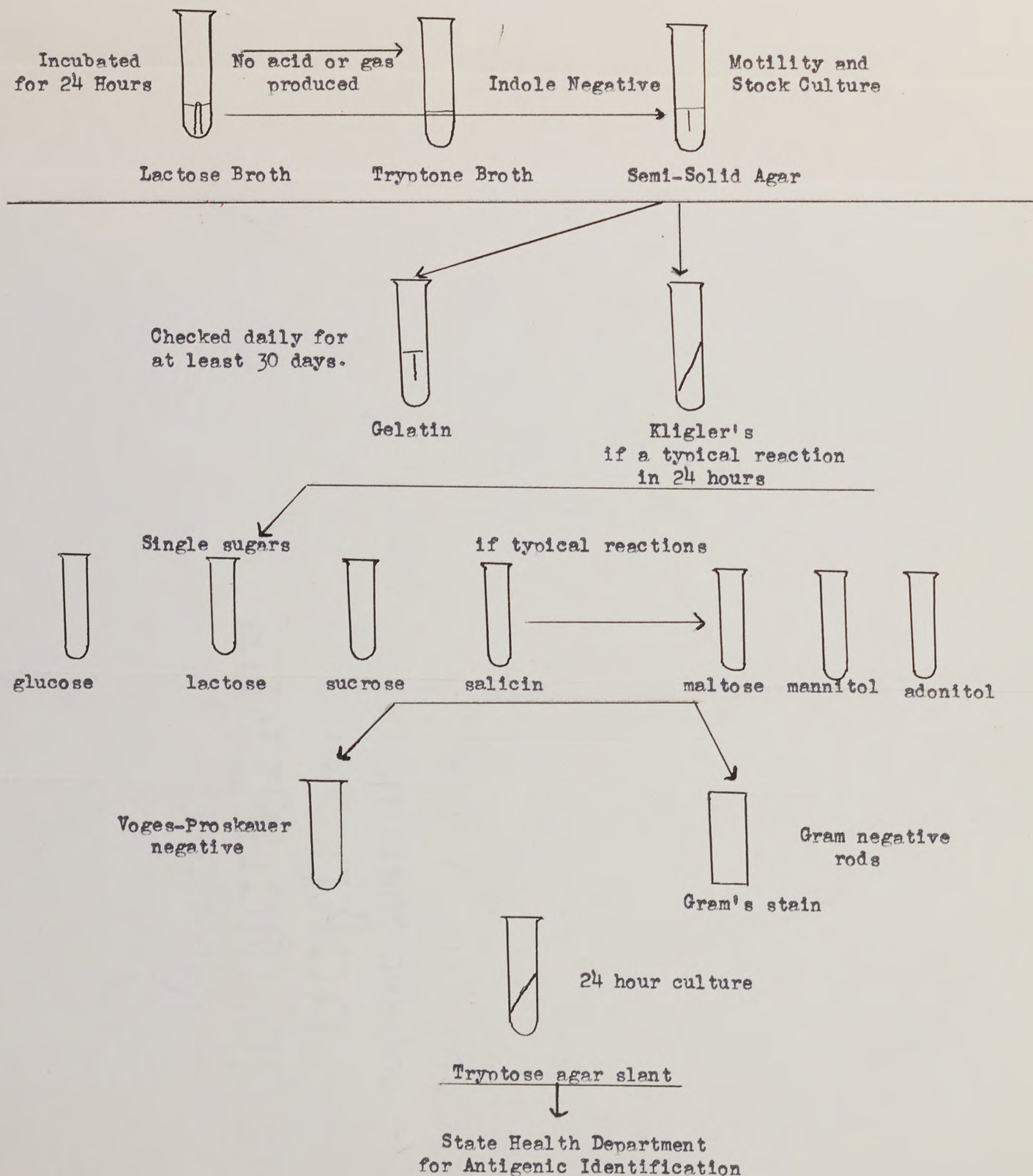
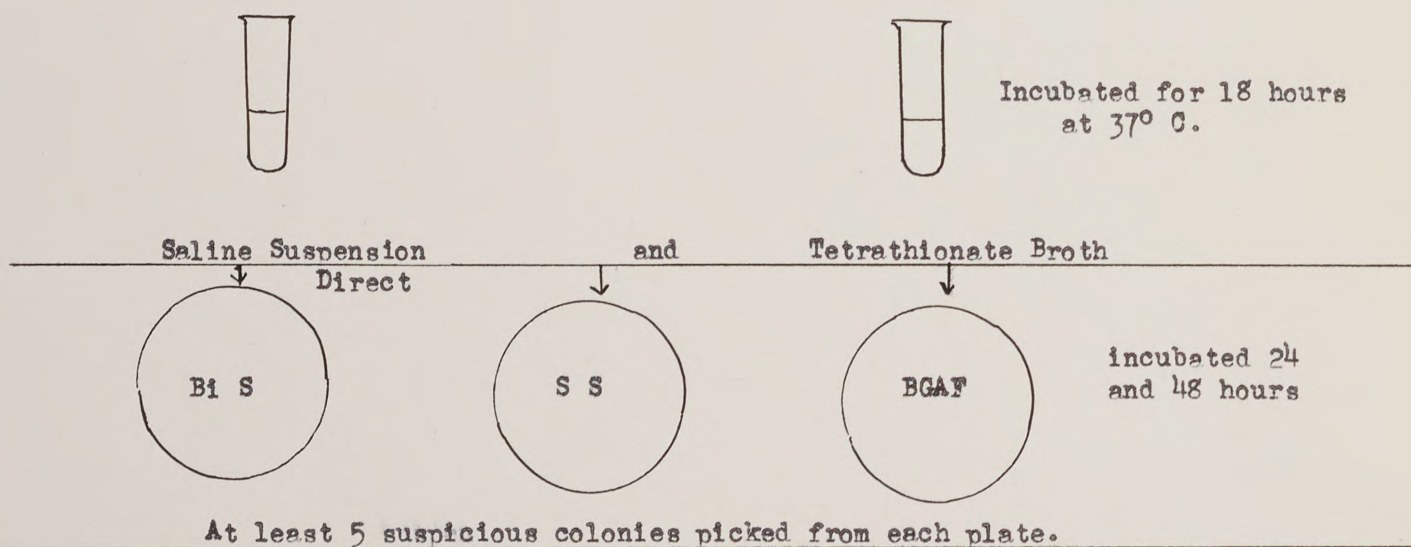
#### D. Results from practical experiments

The findings from the practical applications were twofold: the normal chicken was proved to be a carrier of Salmonellas and a practical evaluation of S S agar, bismuth sulfite agar and brilliant green acid fuchsin agar medium was made.

An examination of 376 normal chickens showed an incidence of 1.6 percent of carriers of Salmonellas which were identified as S. oranienberg by antigenic identification.

A study of the findings in relation to the media, showed that 21 colonies were isolated and tested serologically. Fourteen of these colonies proved to be Salmonellas, five were identified as numbers of the paracolon group, and two were rough colonies which could not be typed.

# GENERAL METHODS OF PROCEDURE



All of the positively identified *Salmonellas* had been subcultured in sodium tetrathionate broth prior to plating. Of the fourteen positive colonies, 9 were made from brilliant green acid fuchsin agar medium, and 5 from S S agar plates. Four of the colonies identified as paracolons were from S S agar and 1 from brilliant green acid fuchsin. The 2 rough colonies were isolated from S S agar. No isolations were made from bismuth sulfite agar medium.

A compendium of the practical findings showed that approximately 64 percent of the *Salmonellas* isolated were made from brilliant green acid fuchsin agar and approximately 36 percent from S S agar. No isolations were made from bismuth sulfite agar.

The results of this experiment are presented in Table 9.

**Table 9**

**Results from Practical Experiments in Relation to Media**

| Bird Number   | No. of Isolations | Isolations Made From: |          |          |          |          |          |          |          |          |
|---------------|-------------------|-----------------------|----------|----------|----------|----------|----------|----------|----------|----------|
|               |                   | S S agar              |          |          | BGAF     |          |          | Bis. S   |          |          |
|               |                   | S                     | P        | R        | S        | P        | R        | S        | P        | R        |
| 8605          | 4                 | 1                     | 1        | -        | 2        | -        | -        | -        | -        | -        |
| D712G         | 2                 | -                     | -        | -        | 2        | -        | -        | -        | -        | -        |
| D854E         | 7                 | 3                     | -        | 1        | 3        | -        | -        | -        | -        | -        |
| 2802          | 1                 | 1                     | -        | -        | -        | -        | -        | -        | -        | -        |
| 541           | 1                 | -                     | -        | -        | 1        | -        | -        | -        | -        | -        |
| 5503          | 2                 | -                     | -        | 1        | 1        | -        | -        | -        | -        | -        |
| 8270          | 2                 | -                     | 1        | -        | -        | 1        | -        | -        | -        | -        |
| D954S2        | 2                 | -                     | 2        | -        | -        | -        | -        | -        | -        | -        |
| <b>Totals</b> | <b>21</b>         | <b>5</b>              | <b>4</b> | <b>2</b> | <b>9</b> | <b>1</b> | <b>0</b> | <b>0</b> | <b>0</b> | <b>0</b> |

Total Salmonellas - 14

Total Paracolons - 5

Total Rough - 2

**Key:** S - Salmonella identified  
P - Paracolon group  
R - Rough colonies could not be typed.

S S - S S agar plates

BGAF - Brilliant green acid fuchsin agar plates.

Bis. S. - Bismuth sulfite agar plates.

## DISCUSSION

Numerous selective and enrichment media have been used and recommended for the isolation of the Salmonella group from fecal specimens. A detailed study was made of the various selective and enrichment media both with pure cultures and in practical applications. The necessity of a selective medium for the isolation of Salmonellas was substantiated with pure culture studies and in practical experiment.

Studies of a number of fecal sample cultures revealed that organisms from the genera Escherichia and Proteus were the contaminants most frequently found on media used for the isolation of intestinal pathogens from chickens. On all the media studied, gram positive organisms and lactose fermenters were in general inhibited or readily identified. The Proteus group proved to be the most prevalent and obnoxious organism to be dealt with, being difficult to inhibit or differentiate on most of the media.

An evaluation of MacConkey's agar, bismuth sulfite agar, S S agar brilliant green liver-infusion agar, eosine-brilliant green-methylene blue agar and brilliant green acid fuchsin agar medium was made using pure cultures.

MacConkey's agar medium though it allowed the enteric pathogens to grow, appeared to favor the lactose fermenters and was not at all inhibitory to Proteus in pure culture studies.

Bismuth Sulfite agar medium in pure culture studies supported a good growth of the Salmonellas and though it did not inhibit Proteus the colonies could be identified as such on the medium. Gram positive organisms and lactose fermenters were inhibited.

On S S agar medium the Salmonellas grew well, and gram positive and lactose fermenters were for the most part, inhibited and if not inhibited were readily differentiated. Proteus was not inhibited and the colonies appeared similar to the colonies of the Salmonellas.

Brilliant green liver infusion agar medium was found to be completely inhibitory to the lactose fermenters and gram positive organisms and supported a good growth of the Salmonellas. It was not of much value in eliminating the Proteus group.

Eosine-brilliant green-methylene blue agar medium gave good results; inhibiting non-pathogenic organisms including the Proteus and at the same time stimulating the growth of the pathogens.

Brilliant green acid fuchsin agar medium was found to be superior to the other selective media studied with pure cultures. It was highly selective and supported a good growth of the Salmonellas. It was found to be inhibitory to the Shigellas.

Two enrichment media were tested, Ruy's medium and sodium tetrathionate broth (Difco). Ruy's medium was found to be toxic to the enteric pathogens as well as to contaminants. Sodium tetrathionate broth did not appear to affect the growth of Salmonellas appreciably in pure culture studies. It was found to be inhibitory to lactose fermenters and gram positive organisms, but seemed to favor the Proteus group. Sodium tetrathionate broth combined with the selective media studied, showed somewhat superior results to direct plating though it had no inhibitory effect on the Proteus group and, in fact, seemed to stimulate its growth.

Bismuth sulfite agar, S S agar and brilliant green acid fuchsin agar medium were employed in combination with sodium tetrathionate enrichment broth and direct plating for examining fecal samples. Direct plating was found to be of little value in the isolation of Salmonellas from chicken fecal samples.

An examination of 376 chickens specimens was made. All the specimens were from birds having normal case histories and had at no time shown any manifestations of any kind of disease. Six of these birds, or an incidence of 1.6 percent, were shown to be carriers of Salmonella organisms. The organisms were identified serologically as S. oranienberg.

Twenty-one suspicious colonies were isolated and tested serologically. Fourteen of these proved to be Salmonellas, 5 were identified as members of the paracolon group, and 2 were rough colonies and could not be typed. Nine of the colonies that were identified as Salmonellas were isolated from brilliant green acid fuchsin agar plates and five from S S agar plates. Four of the colonies identified as paracolons were picked from S S agar, and 1 from brilliant green acid fuchsin agar. The 2 rough colonies were isolated from S S agar. All the isolations were made from specimens which had been subcultured in sodium tetrathionate enrichment broth prior to plating.

A compendium of the findings showed that 64 percent of the isolations were made from brilliant green acid fuchsin agar medium, and 36 percent from S S agar medium. No isolations were made from bismuth sulfite agar medium.

## CONCLUSIONS

A detailed study of six selective solid media, MacConkey's agar, bismuth sulfite agar, brilliant green liver-infusion agar, S S agar, eosine-brilliant green-methylene blue agar and brilliant green acid fuchsin agar medium, and two enrichment broth media, Ray's brilliant green broth and sodium tetrathionate broth, both with pure cultures and in practical applications is presented.

The necessity of a selective medium for isolation of *Salmonellas* from feces of chickens is substantiated by results from pure culture studies and practical applications. Organisms from the genera *Escherichia* and *Proteus* were found to be the contaminants most frequently encountered on media used for the isolation of enteric pathogens. Gram positive organisms and lactose fermenters were, in general, inhibited or readily identified on the media studied in this investigation. The *Proteus* group was the most difficult to inhibit or identify on selective media.

Methods utilizing combinations of sodium tetrathionate enrichment broth and three solid media, bismuth sulfite agar, S S agar, and brilliant green acid fuchsin agar medium for practical isolation of *Salmonellas* from feces of chickens is presented.

An examination of 376 fecal specimens from normal chickens, revealed an incidence of 1.6 percent carriers of *S. oranienberg*.

The specimens from which isolations were made had all been cultured in sodium tetrathionate broth prior to plating on solid media. Sixty-four percent of the isolations of *Salmonella* were made from brilliant green acid fuchsin agar medium. Thirty six percent of the isolations were from S S agar medium. No isolations were made from bismuth sulfite agar medium.

## APPENDIX

### 1. Tryptose Agar

|                                       |            |
|---------------------------------------|------------|
| Tryptose.....                         | 20.0 grams |
| Sodium Chloride.....                  | 5.0 grams  |
| K <sub>2</sub> HPO <sub>4</sub> ..... | 4.0 grams  |
| KH <sub>2</sub> PO <sub>4</sub> ..... | 1.5 grams  |
| Agar.....                             | 20.0 grams |
| Distilled Water.....                  | 1000.0 cc. |

Dissolve ingredients in steamer, adjust pH to 6.8 or 7.0. Autoclave at 15 pounds pressure for 20 minutes. Pour plates.

### 2. Liver Infusion

(For liver infusion agar plates)

|                                   |           |
|-----------------------------------|-----------|
| Liver (finely ground, fresh)..... | 1.0 pound |
| Water (tap or distilled).....     | 500.0 cc. |

To the ground liver in an agateware pail add the 500 cc water, mix thoroughly and allow to stand in a cool place (refrigerator) for not more than 16 to 24 hours. Strain meat infusion through clean cheese-cloth in a large funnel, thoroughly pressing out all the juice. 500 cc should be recovered. Sterilize in autoclave for 30 minutes at 15 pounds pressure. Ready for use in medium.

### 3. Liver Infusion Agar

|                      |            |
|----------------------|------------|
| Agar.....            | 20.0 grams |
| Liver Infusion.....  | 500.0 cc.  |
| Peptone.....         | 10.0 grams |
| Sodium Chloride..... | 5.0 grams  |
| Distilled Water..... | 500.0 cc.  |

Adjust pH to 7.0. Sterilize at 15 pound pressure for 30 minutes.

### 4. Brilliant Green Liver Infusion Agar

|                          |            |
|--------------------------|------------|
| Liver Infusion Agar..... | 1000.0 cc. |
| Brilliant Green.....     | .0015 gm.  |

Add Brilliant green dye to the melted agar base aseptically and pour plates aseptically. Use about 20 cc in each petri dish.

## 5. Ray's Enrichment Broth (6)

|                                        |            |
|----------------------------------------|------------|
| Peptone.....                           | 20.0 grams |
| Sodium Chloride.....                   | 5.0 grams  |
| Distilled Water.....                   | 1000.0 cc. |
| Brilliant green solution (1:1000)..... | 10.0 cc    |
| Esbach's reagent.....                  | 20.0 cc    |

(Esbach's reagent is prepared by dissolving 1.0 gram picric acid and 2 grams of citric acid in 100 cc. of distilled water.)

Dissolve all ingredients by steam and adjust pH to 7.2. Dispense in 10 cc amounts in test tubes. Sterilize at 15 pounds pressure for 20 minutes.

## 6. Tetrathionate Broth (Bacto)

### Broth base

|                                     |            |
|-------------------------------------|------------|
| Proteose-peptone No. 2 (Difco)..... | 5.0 grams  |
| Bacto-bile salts.....               | 1.0 grams  |
| Calcium carbonate.....              | 10.0 grams |
| Sodium thiosulfate.....             | 30.0 grams |
| Distilled water.....                | 1000.0 cc. |

### Iodine Solution

|                       |           |
|-----------------------|-----------|
| Iodine crystals.....  | 6.0 grams |
| Potassium iodide..... | 5.0 grams |
| Distilled water.....  | 20.0 cc.  |

To prepare 1000 cc of medium, to 1000 cc. of broth base which has been boiled and then cooled to below 45° C., add 20 cc. of the iodine solution. Shake well to mix and dispense in 10 cc. quantities in test tubes, taking care to obtain an even distribution of the insoluble material. The medium was found to give better results when freshly prepared and used on the same day.

## 7. Bismuth Sulfite Agar - (Bacto) (Difco-dehydrated)

|                                |             |
|--------------------------------|-------------|
| Peptone.....                   | 10.0 grams  |
| Beef extract.....              | 5.0 grams   |
| Dextrose.....                  | 5.0 grams   |
| Disodium Phosphate.....        | 4.0 grams   |
| Ferrous sulfate.....           | 0.3 grams   |
| Bismuth sulfite indicator..... | 8.0 grams   |
| Agar.....                      | 20.0 grams  |
| Brilliant green.....           | 0.025 grams |
| Distilled water.....           | 1000.0 cc.  |

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Suspend all dry ingredients in the distilled water and heat to boil as rapidly as possible; then allow to simmer for a minute. Into sterile petri plates pour 15 to 20 cc of the medium. This medium should not be autoclaved as prolonged heating destroys its selectivity. Final pH 7.6  $\pm$ .

### 8. MacConkey's Agar

|                          |            |
|--------------------------|------------|
| Peptone.....             | 2.0 grams  |
| Sodium chloride.....     | 0.5 grams  |
| Sodium taurocholate..... | 0.5 grams  |
| Distilled water.....     | 100.0 cc.  |
| Agar-agar.....           | 2.0 grams  |
| Lactose.....             | 1.0 gram   |
| Neutral red.....         | 0.001 gram |

The first four ingredients are steamed for an hour and filtered while hot. Agar, lactose and neutral red are added, the medium thoroughly mixed and then autoclaved for 20 minutes at 15 pounds pressure. Approximately 12 to 15 cc. of the medium is poured into sterile petri dishes.

### 9. MacConkey's Agar-Bacto (Dehydrated - Difco)

|                                    |            |
|------------------------------------|------------|
| Bacto-peptone.....                 | 17.0 grams |
| Proteose-peptone.....              | 3.0 grams  |
| Bacto-lactose.....                 | 10.0 grams |
| Bacto Bile salts No. 3.....        | 1.5 grams  |
| Sodium chloride.....               | 5.0 grams  |
| Bacto-agar.....                    | 17.0 grams |
| Bacto-neutral red.....             | 0.03 grams |
| Bacto-crystal violet (D.C.-2)..... | 0.001 gram |
| Distilled water.....               | 1000.0 cc. |

To prepare the medium 53.5 grams of the dehydrated medium are suspended in 1000 cc. distilled water. Boil for one or two minutes to dissolve the medium. Sterilize for 20 minutes at 15 pounds pressure.

### 10. Desoxycholate - citrate medium

|                             |               |
|-----------------------------|---------------|
| Agar.....                   | 22.5 grams    |
| Lab lemco.....              | 5.0 grams     |
| Difco proteose-peptone..... | 5.0 grams     |
| Lactose.....                | 10.0 grams    |
| Sodium citrate.....         | 8.5 grams     |
| Sodium thiosulfate.....     | 8.5 grams     |
| Ferric citrate.....         | 1.0 grams     |
| Sodium desoxycholate.....   | 5.0 grams     |
| Neutral red.....            | 0.025 grams   |
| Water.....                  | to 1000.0 cc. |

**11. Salmonella and Shigella Medium**  
(Difco - dehydrated)

|                         |             |
|-------------------------|-------------|
| Beef extract.....       | 5.0 grams   |
| Proteose-peptone.....   | 5.0 grams   |
| Lactose.....            | 10.0 grams  |
| Bile salts.....         | 8.5 grams   |
| Sodium citrate.....     | 8.5 grams   |
| Sodium thiosulfate..... | 8.5 grams   |
| Ferric citrate.....     | 1.0 grams   |
| Agar.....               | 13.5 grams  |
| Brilliant green.....    | 0.33 grams  |
| Neutral red.....        | 0.025 grams |
| Distilled water.....    | to 1000 cc. |

Dissolve ingredients in distilled water. Steam for 15 to 20 minutes or bring to the boiling point. Do not allow to boil or do not sterilize. Pour about 20 cc. medium in each sterile petri plate. Allow to dry with covers partially removed.

**12. Eosine-Brilliant green - Methylene blue agar (10)**

Base Medium - Tryptose agar (See Page 1 - Appendix)

|                                |          |
|--------------------------------|----------|
| Tryptose Agar base medium..... | 89.0 cc. |
| Eosine 1%.....                 | 3.5 cc.  |
| Brilliant green 0.1%.....      | 2.5 cc.  |
| Methylene blue 0.1%.....       | 2.5 cc.  |
| Lactose 20%.....               | 2.5 cc.  |

The base medium is melted by steaming and each dye and the lactose added using sterile techniques. Stock solutions of the dyes are made and diluted 1-10 immediately before use.

Stock solutions for E-B-G-M B Medium.

|                      |            |
|----------------------|------------|
| Lactose.....         | 10.0 grams |
| Distilled water..... | 100.0 cc.  |

Sterilize at 12 pounds for 15 minutes Store in dark bottle.

Eosine (10 percent)

|                      |            |
|----------------------|------------|
| Eosine 10%.....      | 10.0 grams |
| Water distilled..... | 100.0 cc.  |

Sterilize at 12 pounds for 15 minutes. Store in dark place.

**Brilliant Green (1 percent)**

|                      |           |
|----------------------|-----------|
| Brilliant green..... | 1.0 grams |
| Distilled water..... | 100.0 cc. |

**Methylene blue (1 percent)**

|                      |           |
|----------------------|-----------|
| Methylene blue.....  | 1.0 grams |
| Distilled water..... | 100.0 cc. |

Store in dark.

**13. Brilliant Green Acid Fuchsin Agar (19)**

**Base medium**

|                          |            |
|--------------------------|------------|
| Agar.....                | 20.0 grams |
| Peptone.....             | 20.0 grams |
| Sodium taurocholate..... | 5.0 grams  |
| Sodium chloride.....     | 5.0 grams  |
| Distilled water.....     | 1000.0 cc. |

Autoclave at 10 pounds pressure for 20 minutes, adjust pH to 7.4 and filter. Add 10 grams lactose, mix and distribute in 200 cc. quantities in flasks and sterilize at 12 pounds pressure for 15 minutes.

To prepare plates, to 200 cc. of base medium, melted and cooled to 50° C. add 2 cc. of Andrade's indicator and mix. Add 0.5 cc. of freshly prepared brilliant green (1 percent) solution. Mix and pour plates.

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