Selective Medium for Hydrogen Sulfide Production by Salmonellae

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Triple Sugar Iron Agar does not reveal hydrogen sulfide production by all Salmonella organisms nor does it permit clear-cut separation of those nonsalmonellae which produce H₂S. Numerous media with varied combinations of nutrients, inhibitors, selective agents, pH levels, and metal salts were tested for H₂S production of cultures of Salmonella, Citrobacter, Edwardsiella, Arizona, Proteus, Providencia, Klebsiella, and Enterobacter. An agar medium has been devised which promotes growth and H₂S production (generally within 6 hr) by Salmonella, Arizona, and Edwardsiella but which inhibits hydrogen sulfide production or growth of all other gram-negative organisms tested (including Citrobacter) or inhibits both. The use of this medium should facilitate the selection and identification of Salmonella.

As early as 1877, Gayon (5) referred to the production of hydrogen sulfide by microbes in spoiled chicken eggs and described the use of paper strips impregnated with lead acetate for detecting the presence of this gaseous by-product. Some years later, Beijerinck (1), Scharlenger (12), and Durham (3) described the production of H₂S by putrefactive organisms associated with soil and feces. The latter two laid the foundation for sanitary standards for water supplies and described the use of H₂S production for detection of Salmonella and other putrefactive organisms. During the same period, Orlowski (10) developed a medium containing metal salts to indicate H₂S production. In 1934, ZoBell and Feltham (20) presented data on the use of paper strips impregnated with lead compounds suspended over cultures to obviate the toxic effects of the heavy metal ions in the growth medium.

Through the years, there have been repeated attempts to couple H₂S production as measured by reaction with metallic salts to the inhibitory action of various metal ions as a means to differentiate selectively species of bacteria, particularly salmonellae. One of the most familiar examples of this approach is the bismuth sulfite medium of Wilson and Blair (19). Recent experimentation, using their work as a basis, has resulted in the development of a medium which selectively permits growth of the majority of Salmonella with concomitant H₂S production.

MATERIALS AND METHODS

Approximately 300 variations of media were screened for their ability to promote selectively the growth of Salmonella serotypes with H₂S production and give little or no evidence of this reaction with non-salmonellae. The media were prepared with various concentrations of several sources of nitrogen and carbohydrates as well as metal ions which produce distinctively colored sulfides. Numerous inhibitory compounds, pH indicators, and even supplements such as vitamins and amino acids were evaluated.

Organisms. The cultures employed (Salmonella, Citrobacter, Arizona, Edwardsiella, Proteus, Providencia, and Enterobacter) were from the stock collection of the Division of Microbiology, Bureau of Foods, Food and Drug Administration (FDA). Most of the serotypes of Salmonella had been identified as part of the regulatory activities of the FDA.

Nitrogen and sulfur sources. These included peptone (Difco), proteose peptone, proteose peptone no. 3, neopeptone, tryptone, tryptose, polypeptone, beef extract, yeast extract, calf serum, egg yolk, egg albumin (fresh and powdered), and the amino acids such as cystine and cysteine, all of which contain some sulfur which could contribute to the production of H₂S. However, an additional inorganic source of sulfur, usually as a sodium compound, was provided in most formulations at a level ranging from 0.01 to 0.4%. Some other sulfur salts were tested for their effect in H₂S production as well as for selective inhibition.

Carbohydrates. Nutrients expected to serve as energy sources were limited to glucose, dulcitol, and mannitol.

 Metals. Various salts of metals which form
distinctively colored sulfides were evaluated for use as indicators of \( H_2S \) production. They were ferrous sulfate, ferric sulfate, ferric chloride, ferric citrate, bismuth sulfite, bismuth ammonium citrate, and bismuth citrate. Tested concentrations ranged from 0.01 to 0.4%.

**Inhibitors.** A number of inhibitory compounds were evaluated. These were bile salts, sodium taurocholate, sodium deoxycholate, sodium azide, sodium chloride, Brilliant Green, ethyl violet, and various concentrations of manganese chloride, manganese nitrate, cobalt chloride, cobalt nitrate, and magnesium chloride.

**Supplements.** The amino acids cystine and tryptophan, reported as beneficial for flagellar formation and growth of *Salmonella*, were tested on a limited basis. The vitamins cobalamin and pantothentic acid were screened for their effect in eliciting the production of \( H_2S \).

**Hydrogen sulfide production.** All media for testing this response were prepared with 1.5% agar, steamed for 15 to 20 min at 100 °C for sterilization, and tempered to 45 °C. 2-ml portions were aseptically dispensed into 13 by 100 mm test tubes. The reaction tubes were incubated in a circulating water bath at 37 °C. Inocula were from Triple Sugar Iron (TSI) Agar slant cultures incubated at 37 °C for 18 to 24 hr. Controls consisted of known \( H_2S \)-producing strains of *Salmonella*.

**Screening procedures.** Each trial formulation was evaluated for \( H_2S \) production by a selected group of organisms consisting of *Salmonella senftenberg*, *E. coli*, *S. pullorum*, Arizona species, Klebsiella species, *Proteus mirabilis*, Providencia species, and *Citrobacter* species. If the proper pattern of \( H_2S \) response was obtained, the medium was subsequently tested with additional *Salmonella* isolates of various serotypes and several species of *Enterobacteriaceae*.

**Final formula for experimental agar medium.** Many tests were made to establish the level of most of the ingredients in this agar medium. The final formula is given in Table 1.

All of the ingredients except the \( MgCl_2 \cdot 6H_2O \) were dissolved in distilled water and brought to boiling for 1 min with frequent or continuous agitation. After slight cooling, the \( MgCl_2 \cdot 6H_2O \) was added. After solution of the \( MgCl_2 \cdot 6H_2O \) and additional cooling, the \( pH \) was adjusted to 7.5 to 7.6 with 1 N NaOH. The medium was then steamed 15 to 20 min in flowing steam in an Arnold’s sterilizer or in a conventional autoclave, tempered to 44 to 45 °C, and aseptically dispensed with continuous mild agitation to maintain suspension of the precipitate. The medium was dispensed in 2-ml portions into 13 by 100 mm tubes, which could be stored up to 6 months at 4 °C without adverse effect if properly sealed.

Whenever the effect of the absence or presence of a specific concentration of an ingredient was being established, the formula without that particular ingredient was called experimental agar base.

**RESULTS**

In the early stages of experimentation, inorganic sulfur compounds were examined for their efficacy in \( H_2S \) production. Tanner (14), Tilley (16), Tarr (15), and Clarke (2) have described the various sulfur-containing compounds metabolized by various bacteria. It was judged from these works that an inorganic source of sulfur would be best suited to yield detectable amounts of \( H_2S \). The presence of 0.01 to 0.05% of sodium thiosulfate in a medium generally resulted in abundant \( H_2S \) production. In fact, almost all of the test cultures of *Enterobacteriaceae* would produce \( H_2S \) in the presence of thiosulfate when it was used in a nutritionally adequate culture medium. Sodium sulfite was found to be more selectively used by *Salmonella* and fewer of the *Enterobacteriaceae*.

The data in Table 2 show that the experimental agar base without sodium sulfite does not exhibit \( H_2S \) production by any of the *Enterobacteriaceae* tested. However, the addition of 0.3% sodium sulfite appeared to be suitable for \( H_2S \) production by the *Salmonella*, Arizona, and *Edwardsiella*, but not for other gram-negative species tested.

The use of relatively high levels of \( MgCl_2 \), reported by Rappaport et al. (11) and Sperber and Deibel (13) to inhibit the growth of non-*salmonellae*, was investigated. Five percent \( MgCl_2 \cdot 6H_2O \) not only improved selectivity of the experimental medium by negating \( H_2S \) production by *Citrobacter*, *Proteus*, and *Providencia*, but enhanced the \( H_2S \) production of several of the test strains of *Salmonella*. A low concentration of proteose peptone no. 3 or polypeptone also improve the medium’s selectivity (Table 2).

### Table 1. Final formula for experimental agar medium

| Ingredient | Per cent | Amt (g/liter) |
|------------|----------|---------------|
| Proteose peptone no. 3 (Difco) or polypeptone (BBL) | 0.1 | 1.0 |
| Dulcitol (Difco) | 0.5 | 5.0 |
| Dextrose, anhydrous (Merck) | 0.15 | 1.5 |
| Dipotassium hydrogen phosphate (ACS) | 0.1 | 1.0 |
| Disodium hydrogen phosphate (ACS) | 0.15 | 1.5 |
| Sodium chloride (ACS) | 0.25 | 2.5 |
| Magnesium chloride ·6 water (ACS) | 5.0 | 50.0 |
| Sodium sulfite (ACS) | 0.3 | 3.0 |
| Bismuth citrate (USP) | 0.04 | 0.4 |
| Ammonium citrate (dibasic) (ACS) | 0.05 | 0.5 |
| Agar (bacteriological grade) | 1.5 | 15.0 |
| Distilled water | (QS) | 1,000 ml |
**TABLE 2. Effect of addition of sodium sulfite and magnesium chloride to experimental agar (EA) on \( H_2S \) production**

| Organism                                      | Effect of sodium sulfite on \( H_2S \) production in EA | Effect of \( MgCl_2 \cdot 6H_2O \) on \( H_2S \) production in EA |
|-----------------------------------------------|----------------------------------------------------------|------------------------------------------------------------------|
|                                               | EA base*                                                 | EA base with sodium sulfite                                       | EA base* (pH 7.5)                                               | EA base +5% \( MgCl_2 \cdot 6H_2O \) (pH 7.5) |
|                                               | 6 hr | 24 hr | 6 hr | 24 hr | 6 hr | 24 hr | 6 hr | 24 hr | 6 hr | 24 hr |
| *Salmonella senftenberg*                     | -    | -     | ++   | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. oranienburg*                             | -    | -     | ++   | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. typhimurium*                              | -    | -     | ++   | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. cubana*                                   | -    | -     | ++++ | ++++++| +++++ | ++++++| +++++ | ++++++| +++++ | ++++++|
| *S. berta*                                    | -    | -     | ++++ | ++++++| +++++ | ++++++| +++++ | ++++++| +++++ | ++++++|
| *S. tennessee*                                | -    | -     | +    | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. tennessee* (lactose +)                   | -    | -     | +    | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. newington* (lactose +)                   | -    | -     | +    | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. wassenaar*                                | -    | -     | +    | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. typhi*                                    | -    | -     | +    | ++++  | +    | ++++  | +    | ++++  | +    | ++++  |
| *S. paratyphi A*                              | -    | -     | +    | +     | -    | +     | (−+) | +     | (−+) | +     |
| *S. choleraesuis*                             | -    | -     | +    | +     | -    | +     | (−+) | +     | (−+) | +     |
| *Arizona*                                     | -    | -     | +    | +     | +    | +     | +    | +     | +    | +     |
| *Edwardsiella*                                | -    | -     | +    | +     | +    | +     | +    | +     | +    | +     |
| *Citrobacter*                                 | -    | -     | +    | +     | +    | +     | +    | +     | +    | +     |
| *Proteus mirabilis*                           | -    | -     | +    | +     | +    | +     | +    | +     | +    | +     |
| *Providencia*                                 | -    | -     | +    | +     | +    | +     | +    | +     | +    | +     |

* Symbols: (−) no reaction; (+) very slight reaction; (++) slight; (+++) moderate; (++++) strong; (++++) very strong. Indicates degree of development of black pigmentation around site of inoculation.

* EA base contains all ingredients of the medium except sodium sulfite.

* EA base contains all the ingredients of the medium except 5% \( MgCl_2 \cdot 6H_2O \).

The use of bismuth ammonium citrate as prepared by Wilson and Blair (17) was found to be extremely useful for selectively inhibiting most of the non-salmonellae. Metal salts, such as ferric chloride, nickel chloride, cobalt nitrate, manganese nitrate, and ferric ammonium citrate, were either too inhibitory or demonstrated undesirable selectivity. Extensive testing with various concentrations of bismuth sulfite, bismuth ammonium citrate, and finally a mixture of bismuth citrate, ammonium citrate; and sodium sulfite at several concentrations and various pH levels (Table 4) resulted in a medium which demonstrated reasonable selectivity for *Salmonella* growth with excellent \( H_2S \) production. However, this was evident only when a relatively high concentration of a carbohydrate, glucose, was present. As early as 1886, Hirschler (7) observed that carbohydrates were necessary for bacterial decomposition of proteins. This finding was confirmed by Heap and Cadness (6) and by Wilson (18). Consequently, dulcitol as a carbohydrate source was substituted in the formula for most of the glucose since nearly all salmonellae utilize dulcitol, whereas many of the other *Enterobacteriaceae* (4) do not. The carbohydrate requirement for \( H_2S \) production from experimental agar base is shown in Table 3. Although 0.5% dulcitol alone did not support \( H_2S \) production by all salmonellae, glucose alone (0.15%) did yield positive \( H_2S \) responses. However, the combination of glucose and dulcitol gave faster and stronger positive \( H_2S \) responses than either carbohydrate alone.

The importance of adjusting the medium to pH 7.5 is clearly indicated by those data presented in Table 4.

The experimental medium subjected to final testing and evaluation had the formulation described in Table 1. Table 5 compares the \( H_2S \) production obtained for a broad spectrum of *Enterobacteriaceae*, including 832 *Salmonella* isolates, on TSI Agar and the experimental medium. More positive \( H_2S \) responses for *Salmonella* serotypes and cultures were obtained with the experimental medium than with TSI Agar. This medium did not allow a positive \( H_2S \) response from *Citrobacter*, *Proteus vulgaris*, and *P. mirabilis*, whereas TSI Agar allowed these same organisms to give a response similar to that of *Salmonella*. Furthermore, the experimental agar readily yielded positive tests with lactose-positive *Salmonella* and the *Salmonella* of sub-genus.
IV [Kauffmann (8)], which are all KCN-positive.

The data from field trials show a high correlation of the positive H$_2$S response with the medium and the later diagnostic serotyping of a culture as *Salmonella*. These data are representative of the field trials of the experimental medium as reported by the FDA district laboratories. Other field trials of this medium with the methods described have been encouraging. Of the several hundred reported field tests, typical results were 2 false positives out of 351 positive with 349 confirmed *Salmonella*. This same study reported 23 false negatives (all were serotyped as *S. oranienburg* and all were from a single source material) out of 271 negatives with the experimental agar. Two other laboratories each reported over 200 tests with

### Table 3. Effect of carbohydrates in experimental agar (EA) on H$_2$S production

| Organism                        | EA base*       | EA base +0.5% dulcitol | EA base +0.15% glucose | EA base +0.5% dulcitol and 0.15% glucose |
|---------------------------------|----------------|------------------------|------------------------|----------------------------------------|
|                                 | 6 hr 24 hr     | 6 hr 24 hr             | 6 hr 24 hr             | 6 hr 24 hr                              |
| *Salmonella senftenberg*        | - -            | +++                    | +                       | +++                                    |
| *S. oranienburg*                | - -            | ++                     | +                       | +++                                    |
| *S. typhimurium*                | - -            | +                      | +                       | ++                                     |
| *S. cubana*                     | - -            | +                      | +                       | ++                                     |
| *S. berta*                      | - -            | ( - + )                | -                       | +                                      |
| *S. tennesse*                   | - -            | ++ ++ ++               | +                       | ++ ++ ++                               |
| *S. tennesse* (lactose +)       | - -            | + + +                  | +                       | ++ + +                                 |
| *S. newington* (lactose +)      | - -            | + + +                  | +                       | ++ + +                                 |
| *S. wassenar*                   | - -            | + + +                  | +                       | ++ + +                                 |
| *S. typhi*                      | - -            | + + +                  | +                       | ++ + +                                 |
| *S. paratyphi* A                | - -            | + + +                  | +                       | ++ + +                                 |
| *S. choleraesuis*               | - -            | + + +                  | +                       | ++ + +                                 |
| Arizona                          | - -            | + + +                  | +                       | ++ + +                                 |
| Edwardsiella                    | - -            | + + +                  | +                       | ++ + +                                 |
| Citrobacter                     | - -            | + + +                  | +                       | ++ + +                                 |
| Proteus mirabilis               | - -            | + + +                  | +                       | ++ + +                                 |
| Providencia                     | - -            | + + +                  | +                       | ++ + +                                 |

* EA base contains all ingredients of the medium except 0.15% glucose and 0.5% dulcitol.
* See Table 2, footnote a, for explanation of symbols.

### Table 4. Effect of pH of experimental agar (EA) on H$_2$S production

| Organism                        | EA (pH 6.0) | EA (pH 6.5) | EA (pH 7.1) | EA (pH 7.5) |
|---------------------------------|-------------|-------------|-------------|-------------|
|                                 | 6 hr 24 hr  | 6 hr 24 hr  | 6 hr 24 hr  | 6 hr 24 hr  |
| *Salmonella senftenberg*        | - -         | + -         | + -         | + -         |
| *S. oranienburg*                | - -         | + -         | + -         | + -         |
| *S. typhimurium*                | - -         | + -         | + -         | + -         |
| *S. cubana*                     | - -         | + -         | + -         | + -         |
| *S. berta*                      | - -         | + -         | + -         | + -         |
| *S. tennesse*                   | - -         | + -         | + -         | + -         |
| *S. tennesse* (lactose +)       | - -         | + -         | + -         | + -         |
| *S. newington* (lactose +)      | - -         | + -         | + -         | + -         |
| *S. wassenar*                   | - -         | + -         | + -         | + -         |
| *S. typhi*                      | - -         | + -         | + -         | + -         |
| *S. paratyphi* A                | - -         | + -         | + -         | + -         |
| *S. choleraesuis*               | - -         | + -         | + -         | + -         |
| Arizona                          | - -         | + -         | + -         | + -         |
| Edwardsiella                    | - -         | + -         | + -         | + -         |
| Citrobacter                     | - -         | + -         | + -         | + -         |
| Proteus mirabilis               | - -         | + -         | + -         | + -         |
| Providencia                     | - -         | + -         | + -         | + -         |

* See Table 2, footnote a, for explanation of symbols.
complete agreement except for one laboratory (Table 6) which reported one false negative which was later confirmed as S. oranienburg.

Additionally, since this medium appears to be highly selective and pure cultures are not mandatory, some other ways of using the experimental medium for presumptive Salmonella screening were tried with some success by the personnel in FDA district laboratories (Atlanta, Dallas, Minneapolis, New Orleans, and Philadelphia districts).

Inoculation of the experimental medium directly from a pre-enrichment broth frequently gave early indication of the presence of Salmonella in a product. When a loopful of lactose pre-enrichment broth (9) or enrichment me-

| Organism         | No. of cultures | H₂S production in Experimental agar | TSI Agar |
|------------------|-----------------|------------------------------------|----------|
| Salmonella isolations | 832 820 12 814 18 | + - + - |
| Salmonella serotypes | 128* 122 6* 117 11 | + - + - |
| Arizona          | 3 3 3 | + - + - |
| Edwardsiella sp.  | 2 2 2 | + - + - |
| Citrobacter sp.  | 17 17 17 | + - + - |
| Escherichia coli | 12 12 12 | + - + - |
| Proteus vulgaris | 6 6 6 | + - + - |
| P. mirebilis     | 20 20 20 | + - + - |
| P. morganii      | 8 8 8 | + - + - |
| P. reitgeri      | 5 5 5 | + - + - |
| Providencia      | 4 4 4 | + - + - |
| Shigella flexneri| 3 3 3 | + - + - |
| S. sonnei        | 3 3 3 | + - + - |
| S. dysenteriae   | 5 5 5 | + - + - |
| S. boydii        | 4 4 4 | + - + - |
| Klebsiella sp.   | 3 3 3 | + - + - |
| Enterobacter cloacae | 3 3 3 | + - + - |
| E. aerogenes     | 4 4 4 | + - + - |
| E. hafniae       | 2 2 2 | + - + - |
| E. liquefaciens  | 2 2 2 | + - + - |

*Total Salmonella serotypes. In this group, there are 18 Salmonella serotypes that belong to subgenus IV. All are positive in experimental agar and KCN-positive.

*Salmonella serotypes negative on experimental agar: S. abortus-equus, S. madelia, S. pullorum, S. gallinarum, S. havana, and S. rubislaw.

Value includes lactose-positive Salmonella which have given positive reactions on the experimental medium.

### Table 6. Comparison of Salmonella detection from 226 food samples by serological reaction versus experimental agar response

| Organism         | No. of cultures | Salmonella diagnostic serotyping | Experimental agar |
|------------------|-----------------|---------------------------------|-------------------|
| Salmonella       | 59              | 59 0 58 1*                      |
| Non-salmonellae  | 167             | 0 167 0 167                     |

* One isolation of S. oranienburg was agar-negative. Typical pure cultures of this serotype are positive with the experimental medium.

dium (17) was inoculated directly to the experimental medium, 66% of the confirmed positive samples gave positive reactions in 24 hr or less.

Direct inoculations were made with residues from the environment, equipment, machinery, etc., with subsequent incubation. If sufficient numbers of salmonellae were present in such materials to initiate growth in the experimental agar, positive reactions were obtained.

A positive test for Salmonella was obtained with any suitable nonrestrictive nutrient medium containing agar poured in a petri dish to a depth of approximately 2 mm, dried to remove surface moisture, streaked with an incubated pre-enrichment broth of residues from the environment, incubated for 4 to 5 hr to establish microcolonies, and overlaid with 5 to 6 mm of experimental agar followed by overnight incubation. The development of black colonies at the interface of the agars was presumptive evidence for the presence of Salmonella, Edwardsiella, or Arizona.

An early presumptive test can be obtained by using a recently patented device (Bacti-Lab, P.O. Box 1179, Mountain View, Calif. 94040) called a “probe disc” (Disposable Culture Assembly, U.S. Patent No. 3,632,478, Jan. 4, 1972). This probe disc is a small plastic plate with a handle on one side and tapered probe on the other side. It was placed, probe down, on a plate of the experimental medium which had been streaked from a pre-enrichment or selective culture. If the area around the probe or under the plate turned black, it was a presumptive positive test for Salmonella.

### DISCUSSION

The experimental medium when used for presumptive screening or in conjunction with
TSI Agar offered greater probability of early detection of the bulk of the Salmonella normally encountered to date in routine analyses. It will permit growth of Salmonella, Edwardsiella, and Arizona with concomitant H₂S production if Na₂SO₃ is present. No other Enterobacteriaceae tested have been observed to produce H₂S, exhibited as heavy black pigmentation, even though growth occurred.

The new medium is best used in conjunction with TSI Agar for early identification of the majority of Salmonella. Arizona and Edwardsiella are the only other groups of organisms that gave positive reactions with this medium. A few Salmonella species, such as S. gallinarum, S. pullorum, S. havana, S. madelia, S. abortus-equi, S. rubislaw, and some nontypable salmonellae with little or no flagellar antigen have given negative results with this medium, which indicates the need for dual testing with TSI.

The best results are obtained with heavy inoculation (e.g., 3-mm loopful) of the medium from fresh TSI culture followed by incubation in a water bath at 37 °C. Frequently, positive reactions can be seen within 3 hr. The culture should not be considered as negative for H₂S production before 24 hr of incubation has occurred.

Early presumptive results can be obtained by inoculating the medium from isolated colonies on selective media. One portion of the colony can be transferred to the experimental medium and the rest of the colony, to TSI Agar. To inoculate the agar, the center is stabbed and the needle is moved laterally to the wall of the tube, creating a slash-inoculation site.

Field trials of this medium in the methods described have been encouraging. Use of this medium as an adjunct to the usual biochemical characterization of Salmonella has frequently made early identification possible and resolved some of the problems that arose when lactose-positive or KCN-positive Salmonella were encountered.

As a presumptive screening technique, this experimental medium should be useful in facilitating the implementation of quality assurance programs for the detection of Salmonella.

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