NOTES

A SIMPLE MEDIUM FOR THE CULTIVATION OF LEPTOSPIRA

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There are several different types of media used for the cultivation of *Leptospira* species (Stuart, J. Pathol. Bacteriol., **58,** 343, 1946; Chang, J. Infectious Diseases, **81,** 28, 1947; Van Thiel, *The Leptospiroses*, Universitaire pers Leiden, 42, 1948). Some of these are difficult to prepare while others do not maintain good growth over extended periods of time. The medium described in this note is easily made and has proven very satisfactory in maintaining stock cultures and in the preparation of antigens.

This medium is a modification of Gardner's medium (Topley and Wilson, *Principles of Bacteriology and Immunity*, The Williams & Wilkins Co., Vol. I, 920, 1946) and is prepared in the following manner. One capsule of Parstains Buffer Salt Mixture[1] (pH 7.2) is dissolved in 180 ml glass-distilled water. About 15-20 ml of this solution are drawn through a sterile Seitz filter and discarded. Twenty-four ml of fresh rabbit serum[2] (previously tested for antileptospiral qualities) are added to the remaining buffered water together with enough fresh rabbit hemoglobin to give the medium a distinct pink color. The medium is sterilized then by filtering through the prepared Seitz filter and tubed in 5 ml quantities in large screw-capped culture tubes. Immediately after preparation it should be placed in the ordinary type "deep freeze" for storage. Meanwhile about 10 of the tubes may be incubated for sterility.

Seitz filter pads can change the pH of solutions passing through them. It was noted that by filtering a small amount of buffer solution through the pad before using this effect could be reduced until there was no appreciable pH change.

Stock cultures have been maintained for periods up to 3 months without transfer and probably would remain viable for longer periods. This medium will support the growth of small numbers of organisms and has been used as the primary isolation medium when culturing strains of *Leptospira* from blood.

[1] Parstains Buffer Salt Mixture is obtainable directly from Hartman-Leddon Co., Inc., Philadelphia, Pa., and indirectly from scientific supply houses.

[2] To reduce formation of precipitate, each lot should be made with serum from one animal rather than with pooled sera.

A USEFUL BACTERIAL CELL WALL STAIN[1]

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Various techniques for staining the bacterial cell wall have been developed, of which the tannic acid-crystal violet method (Gutstein, Centr. Bakteriol. Parasitenk., **93,** 393, 1924) is one of the most widely used. This technique, which requires a very dilute solution of crystal violet, results in the cell wall and the cross walls of many bacteria being stained rather faintly. The tannic acid-crystal violet technique also has the disadvantage of being prepared as temporary water mounts.

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Bisset (Lab. Practice, **2,** 115, 1953) presents a phosomolybdic acid-methyl green cell wall stain which promises to be applicable to some bacteria not effectively stained by the tannic acid-crystal violet method. This stain also must be prepared as a water mount and does not give uniform results. A new principle in bacterial cell wall staining (Chance, Stain Technol., **28,** 205, 1953) involves the use of a basic dye, such as new fuchsin or crystal violet, and decolorization of the cell contents through the use of congo red, an acid dye. The Chance cell wall stain appears to be effective on most of the bacteria and on other forms considered difficult to stain, such as *Nocardia* and *Streptomyces*. This technique, however, has the disadvantage of only faintly staining cross septa.

An effective technique for staining the bacterial cell wall has been developed which utilizes procedures of both the tannic acid-crystal violet and Chance's fuchsin-congo red methods. By using tannic acid as a mordant and congo red as a selective decolorizing agent, a 0.5 per cent to 1.0 per cent solution of crystal violet can be used without leaving the cytoplasmic area of the cell colored. This method stains the outer wall and cross walls very sharply and has the added advantage of yielding permanent dry mounts. This technique appears to have wide application and should prove especially useful since cross septa are shown clearly. The procedure has been satisfactorily used to stain several genera of bacteria with uniformly good results. Figures 1 to 4 show the results obtained with the staining technique when applied to *Corynebacterium pseudodiphtheriticum*, *Micrococcus aureus*, *Nocardia corallina*, and an unknown bacillus and coccus.

The staining procedure is as follows: Bacterial smears are prepared and air dried in the usual manner and should be left unfixed. The slide is placed in 5 per cent tannic acid for ½ to 1 hour, removed and washed, but not dried. A 0.5 per cent aqueous solution of crystal violet is added to the smear and allowed to remain 1½ to 2 minutes. The slide is washed again and is flooded with a 0.5 per cent aqueous solution of congo red for 2 to 3 minutes, washed, and blotted dry. The slide is ready now to be examined. Somewhat better results may be obtained with the addition of a thin film of congo red to the smear after the slide has been warmed to 50 C on a slide warmer. The film of congo red is effective in further removing stain remaining in the protoplasm and slightly darkening the background which improves definition of the cell wall. The length of mordant time and congo red decolorization varies some with various bacterial species. However, the times are not critical and may be determined easily by trial and error. This technique is not superior to the tannic acid-crystal violet stain in all uses, but it is equally good and gives uniform results in cells not readily stained by other methods.

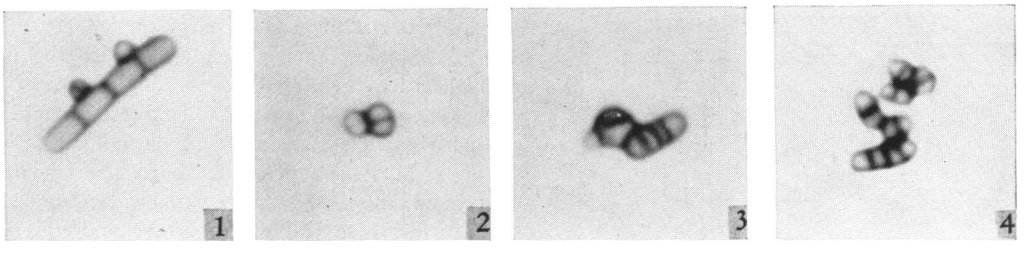

PLATE 1. Cell wall stain of various microorganisms (3,250 × mag)

Figure 1. Unidentified mixed culture.
Figure 2. Micrococcus aureus.
Figure 3. Nocardia corallina.
Figure 4. Corynebacterium pseudodiphtheriticum.