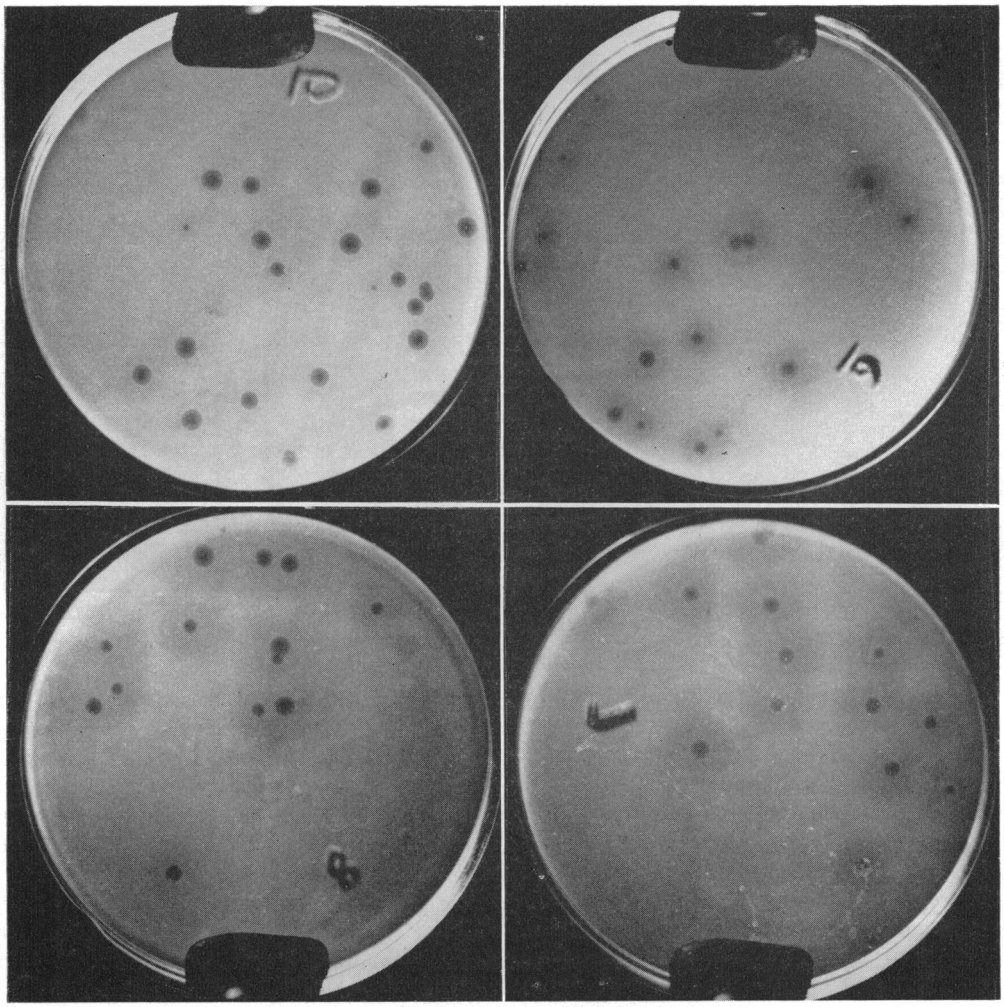

Figure 2. The morphology of T_3 plaques under various conditions (less than 15 hours incubation before treatment). Upper left: Untreated—same as 1 per cent mercuric chloride, 0.1 per cent chlorine, and 5 per cent phenol. Upper right: Chloroform-treated. Lower left: Ethyl acetate-treated. Lower right: Toluene-treated.

COLONIAL GROWTH OF LEPTOSPIRAE

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Although leptospirae have been grown in various fluid and semi-solid media (Wolff, *The Laboratory Diagnosis of Leptospirosis,* Charles C Thomas, 1954), their growth as isolated colonies has not been reported. Woratz (Z. Hyg. Infektionskrankh., **134,** 78, 1952) described the growth of these organisms on agar slants, although considering the inocula and description of growth, it seems

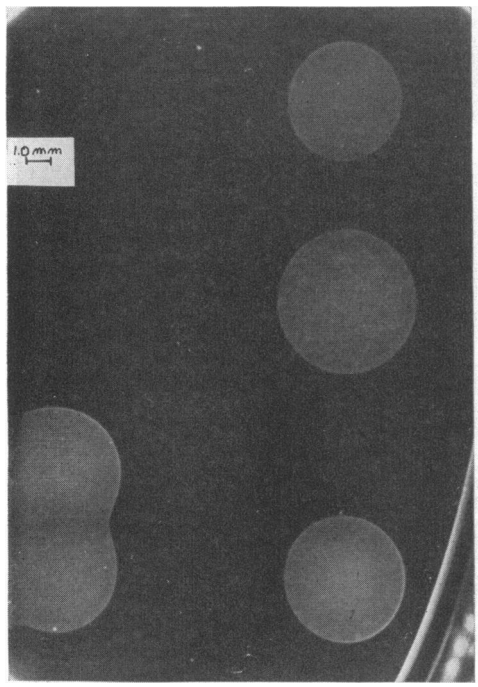

Figure 1. Type 2 colonies of *Leptospira biflexa*, 11 days old.

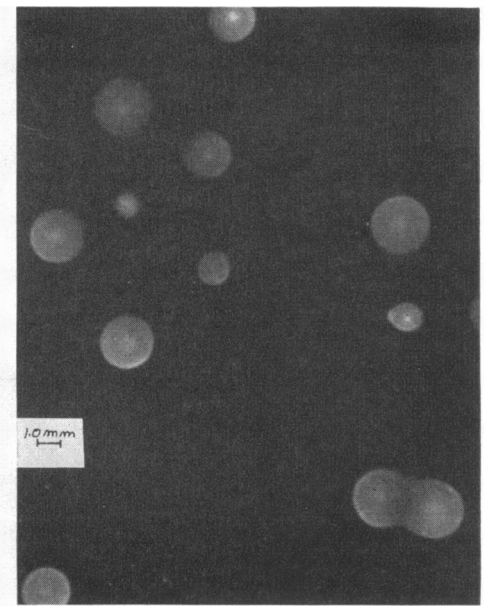

Figure 2. Types 1 and 2 colonies of *Leptospira icterohemorrhagiae*, 14 days old.

extremely doubtful that growth occurred as isolated colonies. The purpose of this note is to describe successful growth of leptospirae as isolated colonies on a solid medium, which is similar to an easily prepared fluid medium recently described from this laboratory (Cox, Proc. Soc. Exptl. Biol. Med., **90,** 610, 1955).

Each 100 ml of medium was prepared as follows: 0.2 g of tryptose-phosphate broth (Difco) and 1.0 g of agar (Difco) were dissolved in 90 ml of distilled water, the pH was adjusted to 7.5 and the medium sterilized in the autoclave. After cooling to 50 C, 10 ml of sterile rabbit serum and 1 ml of hemoglobin preparation was aseptically added. This completed medium was then heated in the 56 C water-bath for 30 min, after which plates were poured. Only rabbit serum which lacked leptospiral antibodies was used. The hemoglobin preparation was prepared by lysing washed and packed sheep erythrocytes in 20 vol cold distilled water, followed by removing stroma by centrifugation and sterilizing by Seitz filtration.

Plates have been inoculated by streaking in the usual manner for isolation as well as by spreading 0.1 ml vol decimal dilutions of fluid cultures evenly over the surface of plates. Plates were incubated at 30 C. All departmental cultures, consisting of *Leptospira biflexa, L. icterohemorrhagiae, L. pomona, L. bataviae, L. autumnalis, L. canicola, L. pyrogenes, L. ballum, L. grippotyphosa*, and *L. hyos*, have been grown as isolated surface colonies (figures 1 and 2) which, on the basis of preliminary evidence, we believe arise from single cells. *L. biflexa* colonies usually become visible on the fourth to sixth day and become 1–4 mm in diameter in 7 to 10 days. Similar colonies of pathogenic leptospirae require 3 to 5 days longer incubation.

Generally 2 types of colonies have been observed, the first (type 1) being smaller in diameter and more opaque, and the second (type 2) being quite translucent, of greater diameter, and veil-like in appearance. The margins of the latter give the impression of extending down into the medium. Darkfield examinations of material removed from both types of colonies reveal typical leptospirae both free and imbedded in bits of agar. Darkfield examinations of medium from areas between colonies have failed to reveal leptospirae. Type 1 colonies upon prolonged incubation often develop spreading peripheries and eventually resemble type 2 colonies with opaque centers. The usual form of both types of colonies is round and smooth with discrete, entire, well-

defined margins; however, some strains have developed colonies with irregular margins resembling rough variants. It has also been generally observed that zones of inhibition or less growth often form between closely adjacent colonies (figures 1 and 2). Successful transfers by needle and loop from isolated colonies to liquid media have been routinely accomplished.

Tryptose-phosphate broth (Difco) has been successfully replaced with similar quantities of tryptose, neopeptone, and proteose peptone No. 3 (Difco), but yeast extract and peptone (Difco) were more erratic in producing heavy colonial growth. Incorporation of hemoglobin preparation is not always mandatory, although colonial growth seems to be more abundant and observed earlier in its presence. Concentration of agar has proved to be fairly critical; with concentrations increasing above 1 per cent, colony size decreases and colonies eventually fail to develop.

These findings would seem to afford opportunity for further classical microbial studies on the leptospirae, some of which are now in progress in this laboratory.

TECHNIQUES FOR THE ISOLATION OF *LABYRINTHULA* AND *THRAUSTOCHYTRIUM* IN PURE CULTURE[1]

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The genus *Labyrinthula*, generally regarded as a slime mold but differing from the Acrasieae, the Myxomyceteae, and the Plasmodiophoreae (Alexopoulos, *Introductory Mycology*, John Wiley and Sons, New York, 1952), was described by Cienkowski (Arch. mikroskop. Anat., **3,** 274, 1867) almost a century ago. Detailed studies were made by Dangeard (Le Botaniste, **24,** 217, 1932) and Zopf (Beitr. Physiol. Morphol. niederer Org., **2,** 36, 1892), and more recently by Renn (Biol. Bull., **70,** 148, 1936) and Young (Am. J. Botany, **30,** 586, 1943). Many uncertainties still exist concerning the morphology, gliding motility, taxonomy, and ecology of these unusual organisms, owing largely to the fact that earlier investigators studied only crude enrichment cultures. We have devised a technique for growing these organisms in pure, reproducible laboratory culture to clarify these uncertainties and to reinvestigate Renn's contention that *Labyrinthula* was the epiphytotic agent in the devastating "wasting disease" of eelgrass (*Zostera marina*), which formerly flourished in the shallow waters along the Atlantic seacoast.

Labyrinthula was first obtained by us in 1949 from material collected in the surface waters of Puget Sound near Friday Harbor, Washington. Drops of plankton were spread over a solid agar medium composed of 0.9 per cent agar in sea water, and the seeded plates were incubated for 2 weeks at 18 C. Subcultures were made fortnightly by transferring actively growing cells of *Labyrinthula*. Profuse growth first occurred on a sea water medium supplemented with 5 per cent human blood or 10 per cent beef serum. After about 10 transfers a pure culture of *Labyrinthula* was obtained.

An improved isolation technique was subsequently developed (Watson, M.S. thesis, University of Washington, 1951). Source material was placed on a solid medium containing 0.9 per cent agar, 10 per cent beef or horse serum, 200 units per ml of penicillin and 300 μg per ml of streptomycin. Growth of *Labyrinthula* was observed usually within 24 to 48 hr after inoculation. Following the second serial transfer a pure culture of *Labyrinthula* was obtained, and its continued cultivation did not necessitate the use of antibiotics. Pure cultures have been successfully maintained for 6 years by monthly transfer and recultivation of the slime mold on this medium at the natural pH of sea water and at an optimum temperature of 15 C.

More recently, Vishniac and Watson (J. Gen. Microbiol., **8,** 248, 1953) reported the formula-

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