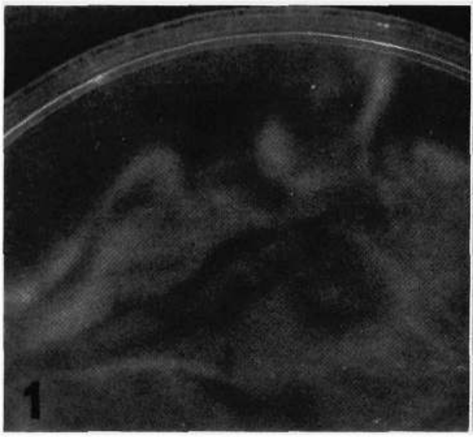


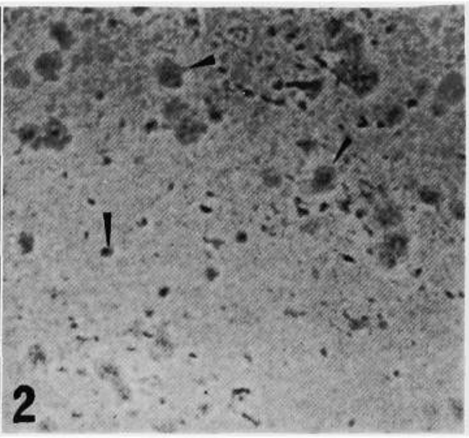


Fig. 1. Human oral treponeme strain G7201 grown for 11 days on 0.9% agar medium. Photographed under transmitted light.

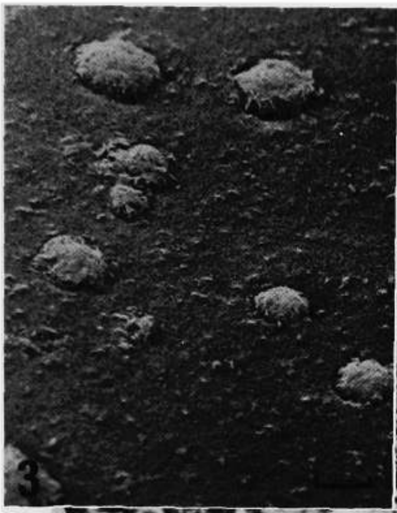
Fig. 2. Phase-contrast micrograph showing 11-day-old colonies of strain G7201 (arrows).

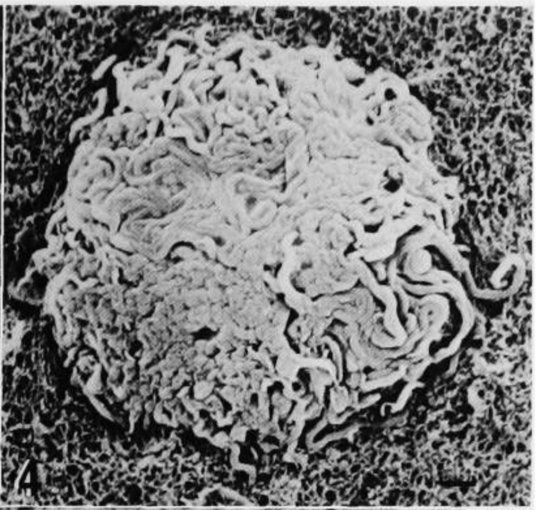
morphological differences among the colonies of three cultivable treponemes including the human oral treponeme.

MATERIALS AND METHODS

Organisms. The bacteria used were three strains belonging to the genus *Treponema*. *T. phagedenis* strains Reiter and Kazan were kindly given to us by Prof. Z. Yoshii of Yamaguchi University School of Medicine. A human oral treponeme, strain G7201, was isolated from a human oral cavity in our laboratory. These treponemes were subcultured in sterility test broth supplemented with 12.5% horse serum (16).

Formation of colonies. The culture medium used to determine the time of formation of colonies consisted of sterility test broth, 10% rabbit or horse serum, rifampin (100 μg/ml), and 0.9 to 3.1% agar (Special Noble Agar, Difco). About 0.3-ml quantities of a 1-wk culture of the treponemal strains were inoculated on the surface of agar plates preincubated for 2 days under anaerobic conditions and were then incubated at 37 C in an anaerobic chamber (Hirasawa Anaero-Box, SNB-1) maintained by the constant circulation of an atmosphere composed of 80% N_2, 10% CO_2, and 10% H_2. Subcultures of bacteria from one colony to freshly prepared solid agar plates were handled in the anaerobic chamber. The effect of agar concentration on the time required for formation of colonies of the three strains of treponemes was determined as follows: Concentrations of agar to be added to sterility test broth containing 12.5% horse serum were 0.9, 1.3, 1.8, 2.2, 2.6, and 3.1% (w/v). After one drop of suspension of actively growing treponemes (optical density 0.05–0.5 at 590 nm) was smeared onto three anaerobically preincubated agar plates for each agar concentration, the plates were examined every day for colonial growth. The time at which colonies became visible on two or three





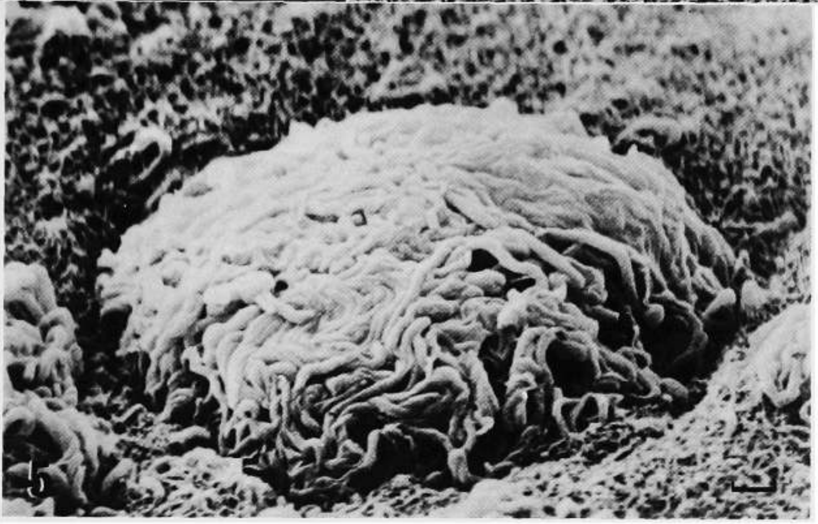


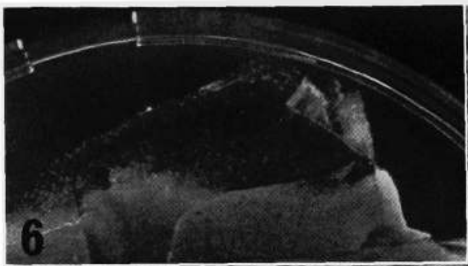
Fig. 3. Convex colonies of strain G7201 grown on a 0.9% agar plate for 11 days. Bar = 10 μm.

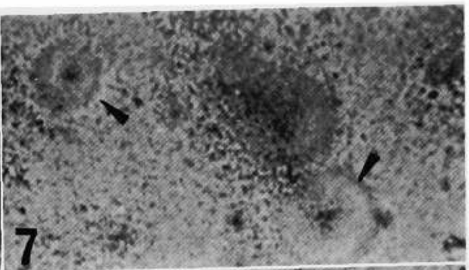
Fig. 4. A plane view of a colony of strain G7201 grown for 11 days. Scanning electron microscopy. Bar = 1 μm.

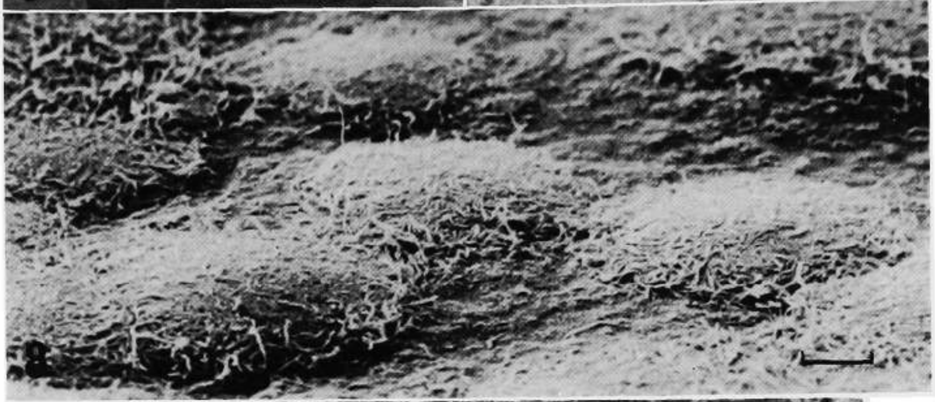
Fig. 5. An 11-day-old colony of strain G7201. Scanning electron microscopy. Bar = 1 μm.

plates was recorded as positive colonial growth.

Microscopy. Colonies that formed on the agar media were observed under a light or phase-contrast microscope. The colonies were also observed in detail under a scanning or transmission electron microscope. Agar blocks containing 11- or 25-day-old colonies, each about 5 mm square, were cut out and then fixed for 1 hr in 2% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2). After being washed once with 0.1 M cacodylate buffer solution, the agar blocks were postfixed with 1% osmium tetroxide in cacodylate buffer for 1.5 hr, and washed again with the same buffer. The specimens were then dehydrated in a graded







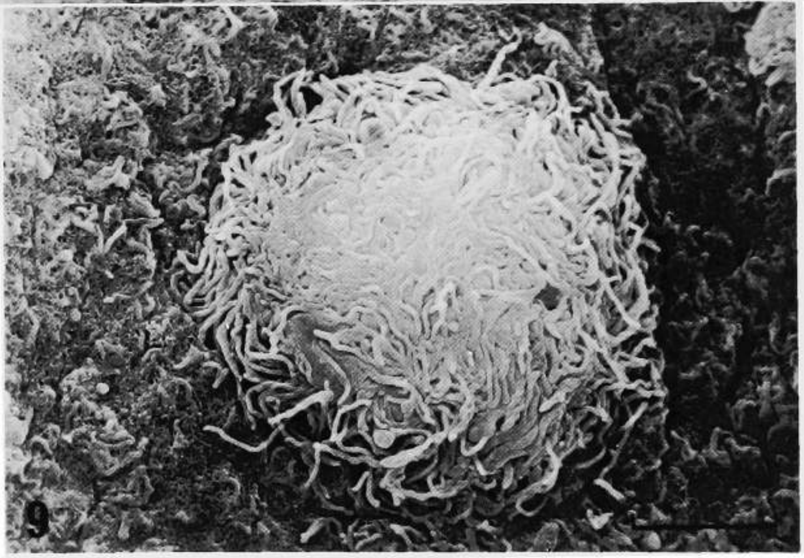
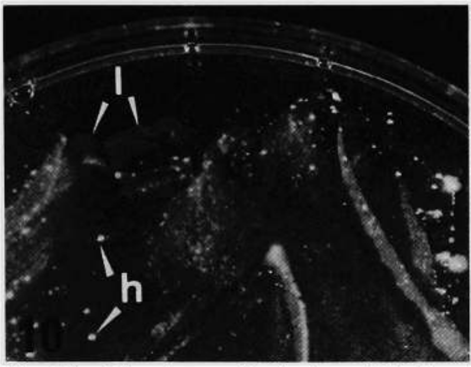


Fig. 6. Colonies of *T. phagedenis* Kazan grown on a 0.9% agar plate for 11 days. Photographed under transmitted light.

Fig. 7. Phase-contrast micrograph showing 11-day-old colonies of *T. phagedenis* Kazan (arrows).

Fig. 8. Eleven-day-old colonies of *T. phagedenis* Kazan. Note low convex colonies with a round periphery. Scanning electron microscopy. Bar = 10 μm.

Fig. 9. A plane view of a colony of *T. phagedenis* Kazan grown for 11 days. Note flat arrangement of spirochetal cells on the surface of the colony. Bar = 10 μm.



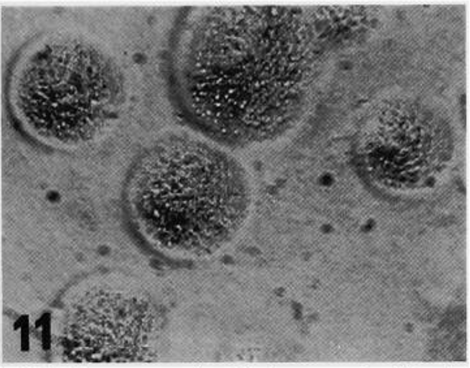


Fig. 10. Two types of colonies of *T. phagedenis* Reiter grown for 11 days on sterility test agar medium. Photographed under transmitted light. Letters l and h indicate low and high convex colonies, respectively.

Fig. 11. Phase-contrast micrograph showing 11-day-old colonies of *T. phagedenis* Reiter. Note the rough surface and round edge of the colonies.

ethanol series (30, 50, 70, 90, 100, and 100% for 20 min each) and dried by the critical point method (Hitachi Critical Point Dryer, HCP-2). To obtain vertical sections of the colonies the critical point-dried specimens were cut vertically to the agar plate with a razor blade. The specimens mounted on a flat specimen holder were coated with gold for 10 min in a vacuum evaporator (Fine Coat Ion Sputter JFC-1100) and observed under a scanning electron microscope (JSM-35C, JEOL) at 25 kV.

Thin sections of Reiter colonies were prepared from the dehydrated specimens as described previously (15).

RESULTS

The human oral treponeme, strain G7201, grew on the surface of solid medium containing 12.5% rabbit or horse serum. This strain grew as diffused zones on sterility test medium containing 0.9 to 3.1% agar (Fig. 1). The colonies were seen to have a somewhat irregular margin when observed with a phase-contrast microscope (Fig. 2). Scanning electron microscopy clearly showed their colonial characteristics (Figs. 3, 4, and 5). The 11-day-old colonies with irregular edges were comparatively small, 5 to 15 μm in diameter, regardless of inoculum size.

In contrast, *T. phagedenis* strain Kazan formed circular colonies 20 to 70 μm in diameter on solid medium containing rabbit serum (Figs. 6 and 7) and were elevated to produce a low convex form (Fig. 8). The surface of Kazan colonies was relatively smooth (Figs. 8 and 9).

T. phagedenis strain Reiter developed morphologically distinctive colonies. Light microscopy revealed two types of colonies, low and high convex colonies, with entire edges on solid medium containing rabbit serum (Figs. 10 and 11) or horse serum. Low convex colonies were smaller than high convex ones. Observation of 11-day-old Reiter colonies by scanning electron microscopy revealed clear margins

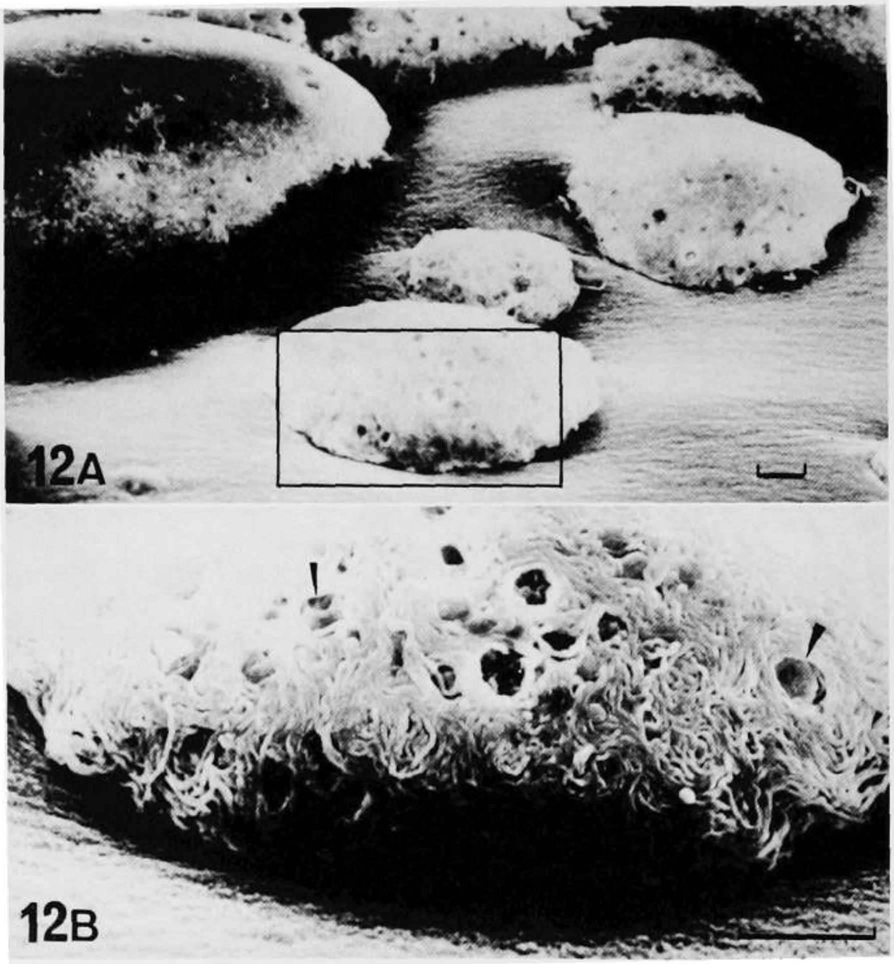


Fig. 12. Scanning electron micrographs showing high convex colonies of *T. phagedenis* Reiter grown for 11 days on solid medium. A: Many crater-like structures can be seen on the surface of high convex colonies. B: Higher magnification of the area enclosed in A showing the crater-like structures (arrows). Bar = 10 μm.

on both low and high convex colonies and also many oval or spherical crater-like forms up to 5 μm in diameter on the surface of both types of colonies (Figs. 12 and 13). Some of these spherical forms were partly exposed and others were almost buried in interlocking treponemes (Figs. 12 and 14). These structures were seen to have a surrounding membrane (Fig. 14B). Their random distribution and variation in size gave a rough appearance to the surface of Reiter colonies. Vertical sections of the Reiter colony were made to determine the morphology of these structures and their distribution within the colony. Oval or spherical cavities or spaces were present far from the surface of the colony (Fig. 15). These cavities or spaces seemed to consist of at least a surrounding membrane and a folded treponemal main body (Fig. 15B), although all these structural elements were not always

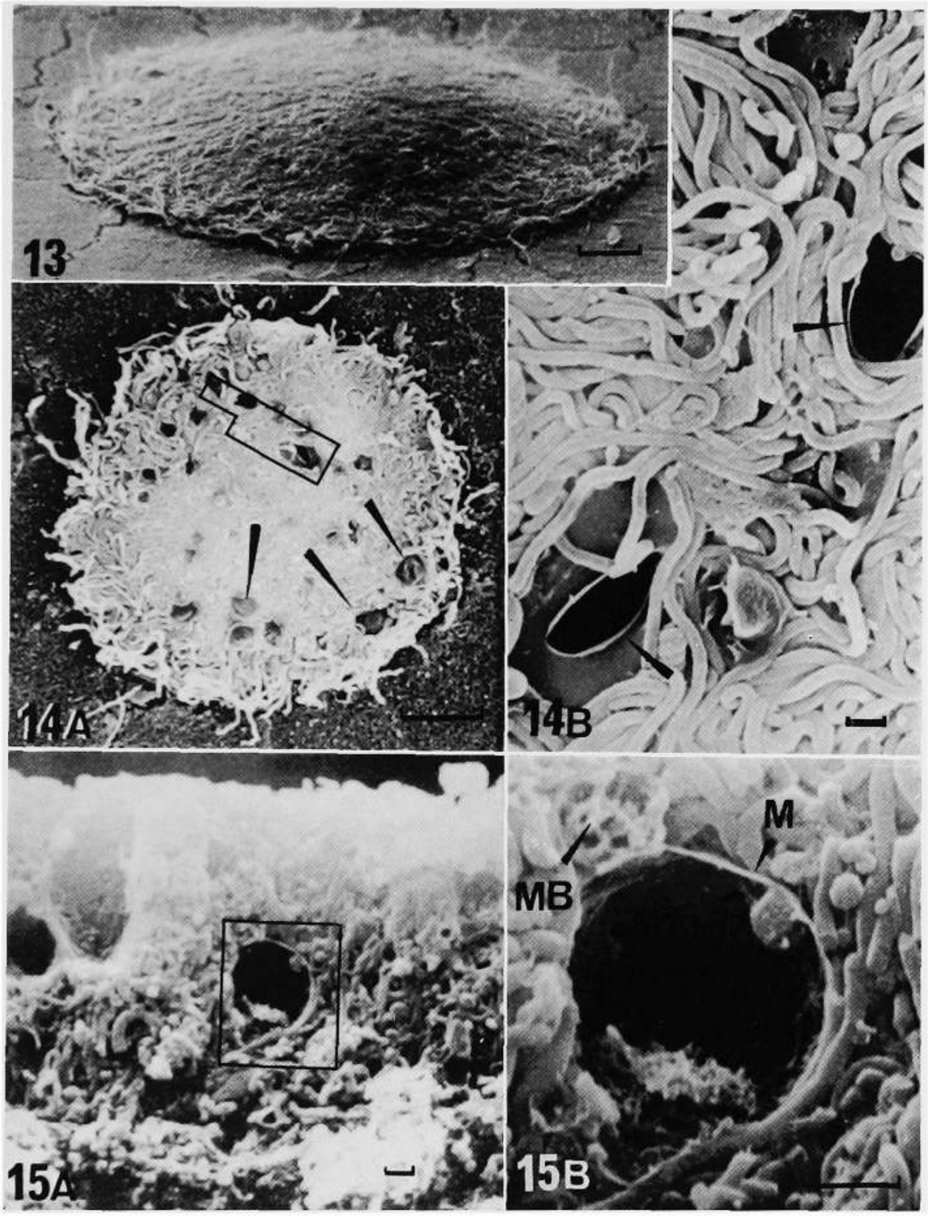


Fig. 13. Scanning electron micrograph showing a low convex colony of *T. phagedenis* Reiter grown for 11 days. Bar = 10 μm.

Fig. 14. A plane view of a low convex colony of *T. phagedenis* Reiter grown for 11 days. A: Spherical forms (arrows) each surrounded by a membrane. Bar = 10 μm. B: Higher magnification of the area enclosed in A. A partially broken membrane can be clearly seen (arrows). Bar = 1 μm. Scanning electron microscopy.

Fig. 15. Scanning electron micrographs showing vertical section of an 11-day-old colony of *T. phagedenis* Reiter. A: Oval or spherical spaces less than 5 μm in diameter within the colony. B: Higher magnification of the area enclosed in A. Note a membrane (M) and a folded treponemal main body (MB). Bar = 1 μm.

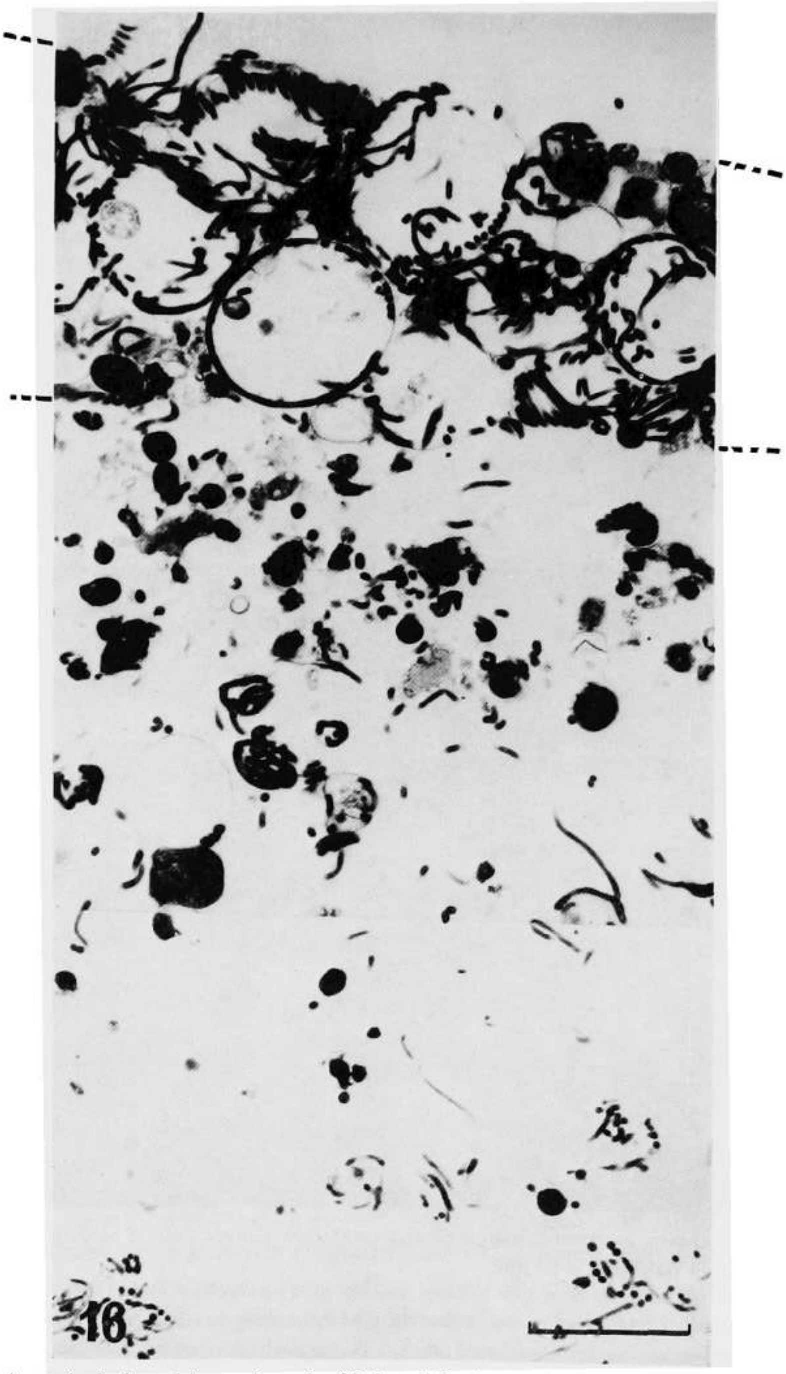
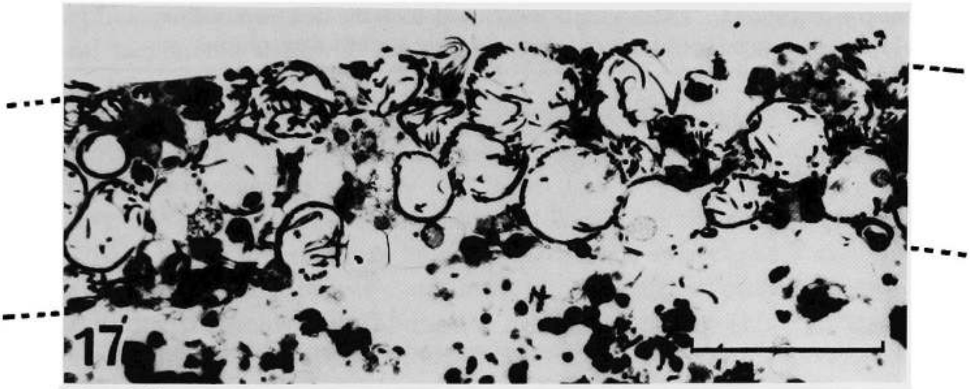


Fig. 16. A vertical ultra-thin section of a 25-day-old colony of *T. phagedenis* Reiter showing many spherical forms near the surface of the colony, but few in the agar. Bar $= 5$ μm.



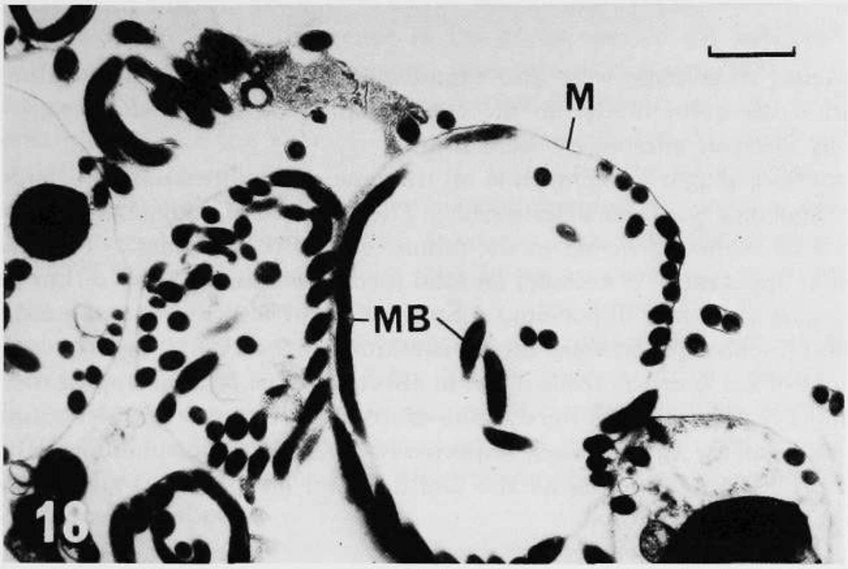


Fig. 17. A vertical ultra-thin section of a 25-day-old colony of *T. phagedenis* Reiter. Note the dense zone of the colony, approximately 10 μm thick packed with organisms including many spherical forms, and the coarse zone with few spherical forms in the agar. Bar = 10 μm.

Fig. 18. Thin section showing spherical forms quite near the surface of a 25-day-old colony of *T. phagedenis* Reiter. An outer membrane (M) and main bodies (MB) can be seen as elements of the spherical form. Bar = 1 μm.

observed in one cross-section of a spherical cavity or space.

Ultra-thin sections of the Reiter colony clearly revealed spherical forms up to 5 μm in diameter within a colony on an agar plate, but few in the agar except for morphologically varied organisms (Figs. 16 and 17). Each spherical form consisted of an outer membrane as a surrounding membrane which had a similar thickness to the outer envelope of normal treponemal cells, and protoplasmic cylinders or main bodies (Fig. 18). The spherical forms were mainly observed in colonies which were situated on the agar.

When the microorganisms from young colonies, for instance, 4-day-old colonies,