

## COLONIAL GROWTH OF ANAEROBIC SPIROCHETES ON SOLID MEDIA

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### ABSTRACT

HARDY, PAUL H., JR. (The Johns Hopkins University School of Medicine, Baltimore, Md.), YOUNG C. LEE, AND E. ELLEN NELL. Colonial growth of anaerobic spirochetes on solid media. *J. Bacteriol.* **86**:616-626. 1963.—A total of 14 strains of treponemes and one of *Borrelia vincentii* were cultivated as colonies on agar plates inoculated under ordinary atmospheric conditions, but incubated anaerobically. Every spirochete strain produced diffuse colonies with growth located primarily in the agar; in addition, eight treponeme strains also produced discrete colonies with surface growth. These represented stable colonial variants in several strains, and in one the spirochetes in the surface colonies showed permanently altered cellular morphology. Sufficient variation in colonial morphology was noted between strains to suggest that this may have some value in further classification of this group of microorganisms. Several conditions contributed to the successful growth of all strains as colonies, the most important of which was the use of a firm jelling agar preparation, Ionagar No. 2, that permitted preparation of plates with a final agar concentration of 0.7%. Agar concentrations of 0.9%, or above, in media inhibited treponeme growth.

Almost 60 years have elapsed since anaerobic spirochetes were first cultivated *in vitro* (Muhlen and Hartmann, 1906), yet they remain one of the most neglected and poorly classified groups of microorganisms indigenous to man and animals. Although much of the blame for the state of ignorance that surrounds these organisms can be attributed to a lack of interest, especially in recent years, the great difficulties that investigators have encountered in attempts to isolate new and pure strains undoubtedly represents the major obstacle to their study.

The strict anaerobic nature of spirochetes, together with their exacting growth requirements, make their initial cultivation difficult, but this

problem is minor in comparison with that of separating the organisms, once grown *in vitro*, from the many other bacteria that invariably contaminate initial cultures. To this end, most investigators have utilized one of two unique growth characteristics of spirochetes: (i) their ability to migrate in solid agar media and grow away from other microorganisms (Rosebury and Foley, 1941; Hampp, 1943), and (ii) their capacity to grow through filters that will retain most bacteria (Wichelhausen and Wichelhausen, 1942; Loesche, Socransky and Macdonald, 1962). Such procedures yield cultures free from other microorganisms but strain purity is not assured, since the spirochetes obtained by either method are almost certainly not the progeny of a single parent.

In an effort to acquire unquestionably pure strains, many attempts to grow these organisms as colonies have been made, but for the most part success has been achieved only in deep agar shake tubes, and colonial growth on surface-inoculated agar plates has been reported very infrequently. Gates (1923), who was the first to describe such growth, cultivated two strains of *Treponema pallidum* (nonpathogenic) and one each of *T. caligyrum* and *T. microdentium* on blood agar plates incubated in a Brown anaerobic jar, but was unable to subculture every strain. Fortner (1929), using the bacterial method of producing an anaerobic environment, also obtained colonies of several *T. pallidum* strains and one oral treponeme strain on blood agar, as did Aksjanzew-Malkin (1933) several years later. However, Wichelhausen and Wichelhausen (1942) were able to grow treponemes as colonies on streaked plates only three times in many attempts.

In recent years, Berger (1958) achieved colonial growth of some oral treponemes on both streaked and poured agar plates, but was less successful with others. Socransky, Macdonald, and Sawyer (1959), on the other hand, consistently grew these organisms as colonies on streaked plates, but only in cultures that were inoculated as well as incubated in an anaerobic environment, and not

in parallel cultures streaked under ordinary atmospheric conditions. On the basis of these findings, Socransky et al. (1959) concluded that exposure of treponemes to atmospheric oxygen for even the short time required to inoculate plates was detrimental to their subsequent growth as colonies.

In view of the limited and somewhat inconsistent findings of others, further investigation of the colonial growth of these organisms was considered worthwhile. The purpose of this paper is to report the results of such a study, in which all available spirochete strains were grown as colonies on streaked plates, and the colonies so obtained were subcultured without difficulty, thus assuring unequivocally pure strains. It was further observed that the colonial morphology of different strains was sufficiently distinct to suggest that this might be of possible value in the future classification of these organisms.

#### MATERIALS AND METHODS

Eight treponeme strains reputed to be in vitro cultivable variants of *T. pallidum* were studied. The Reiter treponeme was the same strain used in previous studies (Hardy and Nell, 1961). The Noguchi, Nichols, and Kazan (re-labeled Kazan A upon receipt of additional Kazan cultures) strains were obtained from E. G. Hampp of the National Institute of Dental Health, and were probably the same strains as those described by Eagle and Germuth (1948). Four additional Kazan strains (Kazan 2, 4, 5, and 8) were obtained from A. Nielsen of Copenhagen who had received them from the USSR in 1962. The history of these is at present somewhat obscure.

Six strains of small oral treponemes and one *Borrelia vincentii* strain (N-9) were also studied. The N-9 and FM (oral treponeme) strains were obtained from E. G. Hampp. The other five oral treponemes were isolated from personnel in the laboratory approximately 1 year prior to the initiation of the present study.

Each strain was maintained in an appropriate broth medium in screw-cap tubes filled to the neck. Solid media were prepared by the addition of Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.) to maintenance broth at a final concentration of 0.7%, except where otherwise specified. All cultures were incubated at 35 C.

Reiter, Kazan A, and the five oral treponemes

isolated in this laboratory were cultivated in USP alternate thioglycolate broth supplemented with 10% normal calf serum. Noguchi, Nichols, and the other Kazan strains were grown in a broth composed of Trypticase (BBL), 3%; yeast extract, 0.5%; glucose, 0.5%; NaCl, 0.25%; and cysteine hydrochloride, 0.2%; with pH adjusted to 7.2. Normal rabbit serum (10%) was added as a supplement to this medium, because calf serum was toxic for the Noguchi and Nichols treponemes. The FM and N-9 strains were maintained in a medium composed of three parts thioglycolate broth and one part Brain Heart Infusion, plus 10% normal rabbit serum.

Media for the study of hemolysis were prepared by adding 2.5% washed erythrocytes as well as serum supplement to melted agar. This was done to eliminate the factors inhibitory to treponeme growth that are sometimes found in unheated serum or plasma.

Plate cultures were incubated in sealed glass cylinders equipped with side arm for the evacuation of air. For the most part, the chromium sulfate-metallic zinc method described by Marshall (1960) was used for the production of anaerobiosis because of its efficiency and relative simplicity. Other methods that appeared to be satisfactory on the basis of limited studies were the Brewer anaerobe jar, when thoroughly flushed with illuminating gas prior to heating the catalyst, and the modified Fildes-McIntosh jar which employs a cold catalyst but necessitates the use of hydrogen gas.

#### RESULTS

*General cultural requirements.* In the early phase of this study, it was found that the ability of various treponeme strains to grow as colonies on streaked plates was markedly influenced by the nutritional properties of the media employed. It had previously been shown that no single broth medium would support the growth of all spirochetes equally well, and that several modifications to the basal medium were necessary to achieve luxuriant growth of some strains. Limited investigations with several different solid media revealed that colonies developed only under the most optimal growth conditions; for this reason, attempts to study all strains in a single medium were discontinued. Instead, each organism was grown on an agar jelled version of the broth me-

TABLE 1. *Effect of agar concentration upon colonial growth of the Reiter treponeme*

Broth culture inoculated per plate	Colonies per plate at various agar concn			No. of treponemes in inoculum*
	0.7%	0.9%	1.2%	
<i>ml</i>				
10 <sup>-6</sup>	>300	116	28	350
10 <sup>-7</sup>	41	11	3	35
10 <sup>-8</sup>	3	0	0	3.5

\* Determined by direct count on 10<sup>-1</sup> dilution.

dium previously found to promote growth most satisfactorily.

The agar used for the preparation of solid media was Ionagar No. 2. Because this was reputed to have a much higher jelling capacity than conventional agars, media were prepared with various Ionagar concentrations to determine the one that would give most satisfactory colonial growth of treponemes. It was found that the amount of agar in the medium exerted a pronounced effect upon the growth of these organisms, both as to the size of colonies that developed and the number of treponemes capable of colonial growth. In general, colony size was inversely related to agar concentration, but this was not invariably the case as will be described later. The effect of agar concentration upon the number of colonies to develop on plates was even more striking, and although this was apparent on streaked plates it was more clearly demonstrated by pour plate techniques (Table 1).

In this experiment, tenfold dilutions of a young broth culture of the Reiter treponeme were prepared, and samples of appropriate dilutions were inoculated into several series of pour plates that differed only in the amount of agar present. After incubation for 1 week, the number of colonies per plate was determined with the aid of a colony microscope. It will be noted from the figures in Table 1 that the number of colonies to develop on each plate with the same inoculum was inversely proportional to the agar concentration in the medium; it can be further observed that every treponeme in the inoculum, as determined by direct count, was capable of colonial growth only on the plates containing 0.7% agar. Where an agar concentration of 1.2% was present, approximately 90% of the organisms failed to grow. On the basis of these and similar findings with other treponemes, most subsequent studies were made with media containing 0.7% agar.

Socransky et al. (1959) reported that successful colonial growth of oral treponemes could be achieved only when all manipulations related to the inoculation of cultures were carried out in an anaerobic environment. This was not found to be necessary in the present study, and all plates were streaked under ordinary atmospheric conditions. However, the establishment of anaerobiosis within a short time after inoculation was shown to be essential, and colonies appeared on plates only when the procedure for removal of atmospheric oxygen was both rapid and efficient. It should be emphasized that the media used in this study, in contrast to that of Socransky et al. (1959), contained either cysteine or sodium thioglycolate, which undoubtedly accelerated the establishment of an adequately reduced state within the agar. Because of the presence of these sulfhydryl compounds, plates were not dried before use but were inoculated immediately after hardening. The water of condensation present on the agar surface at the time of inoculation was absorbed sufficiently early in the incubation period to have no effect on the development of well-isolated colonies.

The slow growth of anaerobic spirochetes, which have division times ranging from approximately 5 to 15 hr, necessitated long incubation of plates before colonies appeared. Incubation times varied from 6 days for some strains to 12 days or longer for others, and colonies sometimes required 3 to 4 weeks of growth before reaching maximal size.

*General characteristics of treponeme colonies.* Considerable variation was observed between the colonies produced by the different strains of treponemes studied, but in general they could be divided into two distinct types: flat, diffuse colonies (Fig. 1) and raised, discrete ones (Fig. 2). Every strain produced some variation of the diffuse colony type in which growth occurred entirely within the agar, as shown in Fig. 3 and 4. These colonies, therefore, appeared flat except for a few that had a small central nipple of elevated growth; this, however, was also imbedded in agar and not on the surface. Most frequently, these colonies appeared as homogenous hazes of spreading growth that thinned out toward the periphery with no discernible margin. In some, however, there was a central dense area surrounded by small clusters of various-sized microcolonies that gave a rather granular appearance to the colony as a whole. These two variations often occurred

together on the same plate and appeared to be quite interchangeable in some strains.

The discrete colonies, which were usually much smaller than the diffuse ones, were found only in strains that produced more than one colony type. These colonies, which could be further divided into rhizoid and round subtypes, were raised above the agar surface and had a well-defined margin. The rhizoid colonies were of a low conical shape but, in spite of this, growth occurred in a matrix of agar, and they could be picked only by removing the medium in which they were imbedded. The round colonies, on the other hand, were primarily surface colonies and could be picked with ease.

When more than one colony type was first observed with several of the treponeme strains, the possibility that the original culture contained more than one variety of spirochetes was considered. However, subsequent investigations indicated that this was not the case, and the occurrence of colonial variants of the same organism was established.

*Colony forms of the Reiter treponeme.* This organism gave visible colonial growth on plates after 6 to 7 days of incubation, and was of particular interest because three distinct colony types grew from the original culture. The two predominant colonies were the rhizoid and subsurface diffuse types described above. Both measured 0.3 to 0.6 mm in diameter after approximately 1 week of growth and increased somewhat upon further incubation, but rarely exceeded 1.0 mm in size. Microscopically, the rhizoid colonies were found to be composed of branching filaments of growth that radiated from the center of the colony and sometimes, but not always, fused (Fig. 5). Under shadowed light, it could be observed that the conical shape of these colonies was contoured with ridges and valleys corresponding to the growth filaments and the spaces between them (Fig. 6). As mentioned above, these colonies were imbedded in agar, and a loop pulled across the surface did not disturb them.

The diffuse colonies were always granular in appearance (Fig. 7), and were completely flat except for a small central elevation that was a rather consistent feature. As with the rhizoid type, subcultures of these produced colonies that were primarily of the same morphology as the parent, although there were almost always present a few

colonies of the other type, and occasionally colonies intermediate between the two.

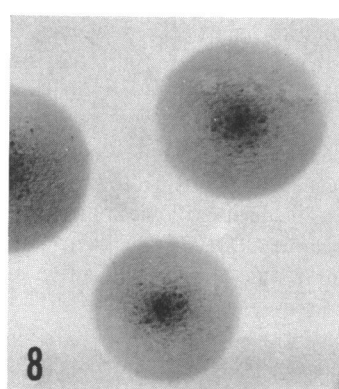
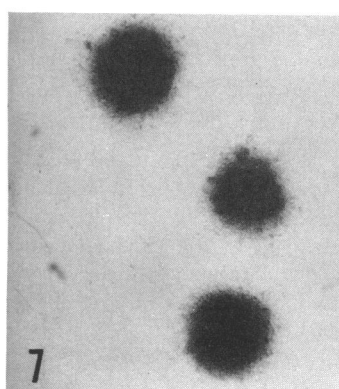
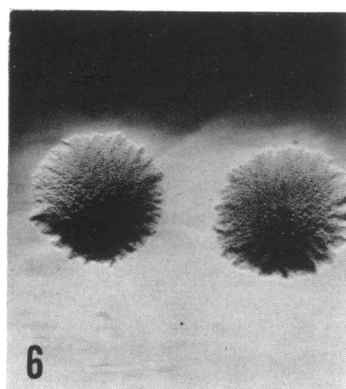
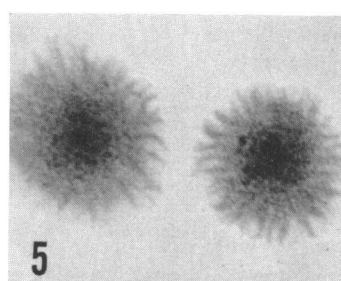
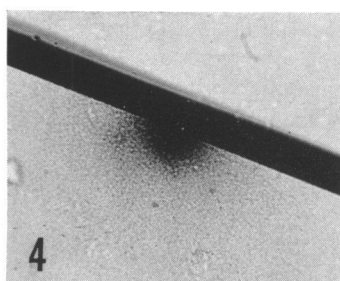
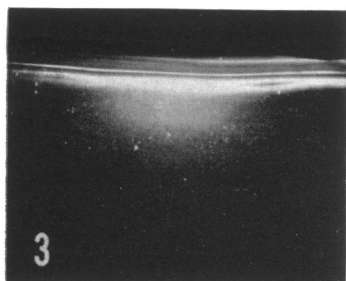
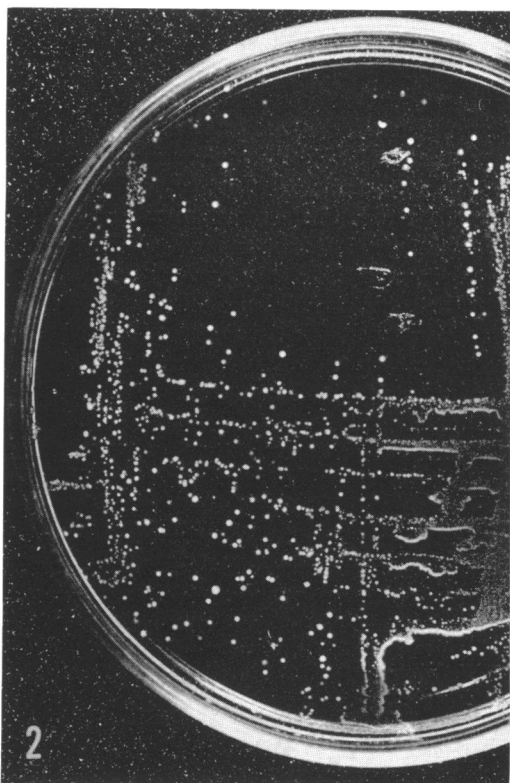
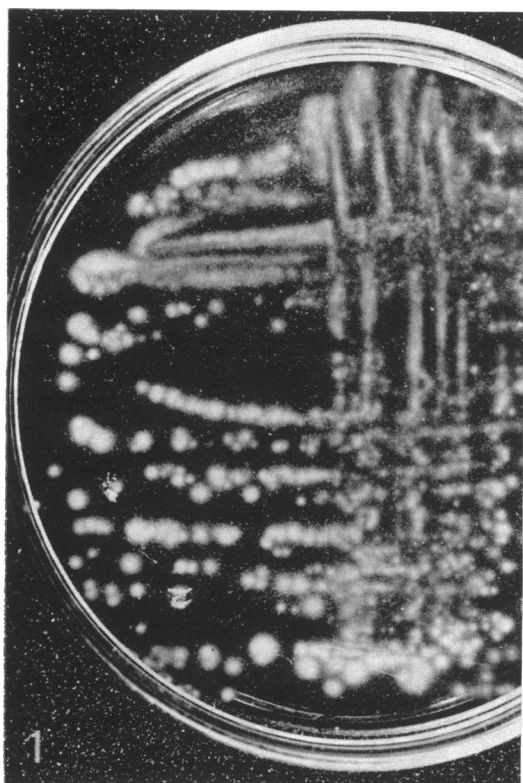
In addition, the original Reiter culture gave rise to a few small, round, convex colonies with a glistening, almost mucoid appearance. They also were 0.3 to 0.6 mm in diameter after 1 week of growth, but unlike the other types they increased very little in size upon further incubation. They had a grayish, opalescent color and the consistency of a very thin syrup, which permitted them to be rubbed off the plate with ease. When this was done, the outline of the colony frequently remained visible, suggesting a small amount of growth within the agar. When viewed microscopically, these colonies usually showed a few centrally placed dark granules, whereas the remainder of the colony appeared relatively homogeneous and only slightly more dense than the surrounding medium (Fig. 8).

The round colonies represented a very stable form of growth, and were transferred repeatedly from plate to plate, through a number of broth cultures, and then back to plates, without reverting to either of the other types. The only indication that the spirochetes in these colonies were related to those in the other colony types was the observation that plates streaked with organisms from rhizoid colonies very occasionally contained a few round colonies as well.

When the Reiter treponeme was grown on medium containing 1.0% agar, the round colonies, though fewer in number, remained essentially unchanged. The rhizoid colonies, on the other hand, were quite different in character. They were larger than those on softer agar and, with the exception of the colony center which remained in the agar, growth was entirely on the surface where it spread in a pseudopod-like fashion (Fig. 9) as if growing in a film of moisture. In this respect, these colonies behaved somewhat like those of mycoplasma where the agar surface was moist (Razin and Oliver, 1961).

None of the colony types produced hemolysin in the presence of human erythrocytes, but the rhizoid colonies were frequently surrounded by a narrow hemolytic zone on rabbit blood agar. This was not a consistent finding, however, and seemed to vary with different batches of rabbit blood, an observation previously made by Fortner (1929) for a *T. pallidum* strain that he had obtained from Reiter.

The microscopic appearance of individual



treponemes from the round surface colonies was remarkable in the extent of pleomorphism they exhibited. Only occasional organisms had a truly spiral shape, and even these were sometimes short, thick, and composed of only a couple of spirals so that they resembled spirilla more than treponemes. Many were straight or irregularly bent filamentous forms that varied greatly in both length and thickness. Some were doubly contoured, and none was motile. In addition, there were many of the spherical bodies that appear in treponeme cultures under certain environmental conditions. All of the cellular forms found in the round colonies could also be observed among the organisms from the other colony types. However, in young cultures of the latter, many organisms possessed a more regularly spiral shape, and some were noted to have a lazy flexing or rotatory movement, but vigorous motility was never observed. Subcultures from all three colony types into broth gave rise to spirochetes that were regularly coiled and actively motile.

*Colony forms of Kazan A.* Colonial growth of this organism could be observed first after 6 days of growth, but colonies increased in size upon further incubation and frequently reached a maximal diameter of 3 to 4 mm after 8 to 10 days. Only diffuse hazy colonies were observed, and they differed from similar colonies produced by other treponemes in that they were larger and seldom penetrated as deeply into the agar. Because of this, there was insufficient contrast between colony and surrounding medium to make them visible under a microscope except in cross sections examined with dark-field illumination (Fig. 3). The thin, spreading nature of these colonies was probably due to the marked motility of the organisms, which were the most vigorously motile of any studied, and were the only ones in which the motility observed in young colonies approached that found in broth cultures.

Kazan A grown on blood agar hemolyzed both

rabbit and human erythrocytes. The colonies appeared to be surrounded by a zone of partial hemolysis, but when compared with the colonies on other plates inoculated at the same time, and on the same medium without blood cells, it was observed that the colonies on plain agar were of the same diameter as the areas of hemolysis on the blood plates. Microscopy of material fished from the periphery of the hemolytic area revealed it to be rich in treponemes, which indicated that hemolysis occurred only in the immediate area of growth and was, therefore, probably not due to a soluble hemolysin diffusing through the medium. Another feature of Kazan A growth on blood agar plates was the presence of a depression in the agar surface over each colony. This began at the edge of the hemolytic zone and extended downward in a shallow saucer shape toward the center of the colony, which was not depressed and, therefore, stood out as a small nipple of growth. This phenomenon, which was also seen with the Noguchi strain, was observed previously by Wichelhausen and Wichelhausen (1942) on the one occasion when they successfully grew the Kazan A treponeme on blood agar.

*Colony forms of Noguchi and Nichols strains.* Attempts to grow these organisms as colonies were unsuccessful, until it was realized that an insufficient incubation time was allowed. Both strains required 14 days of growth before the appearance of visible colonies, some of which increased upon further incubation and required 3 to 4 weeks to reach maximal size.

Each of these strains produced both diffuse and round surface colonies. The diffuse types were more numerous and were identical in the two strains. They ranged from about 0.3 mm when first visible to almost 2 mm in diameter when fully developed. Frequently, they appeared as enlarged versions of the granular spreading colonies of the Reiter treponeme (Fig. 10), but more often growth was homogenous throughout (Fig. 11).

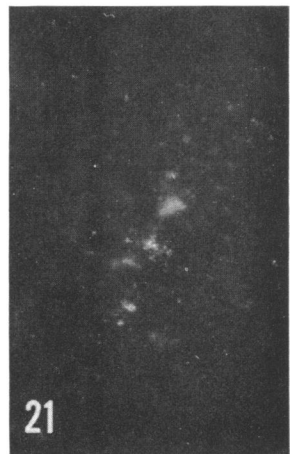
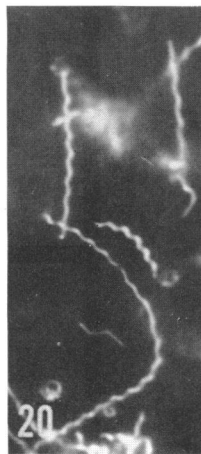
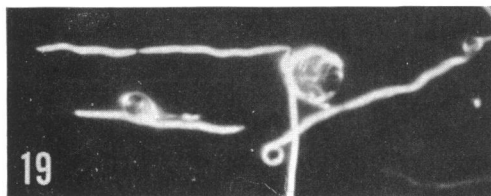
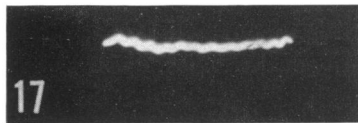
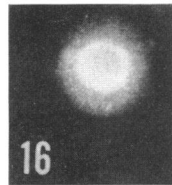
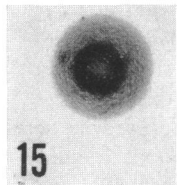
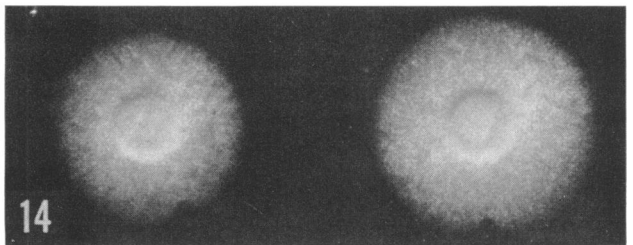
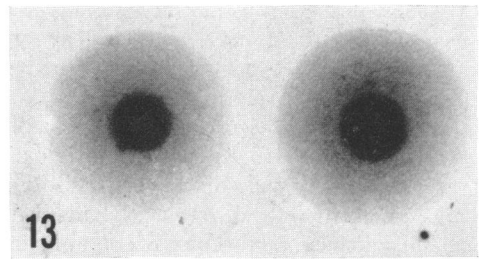
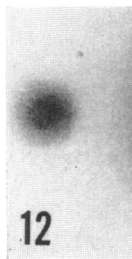
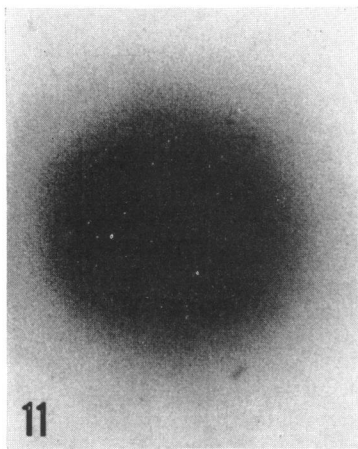
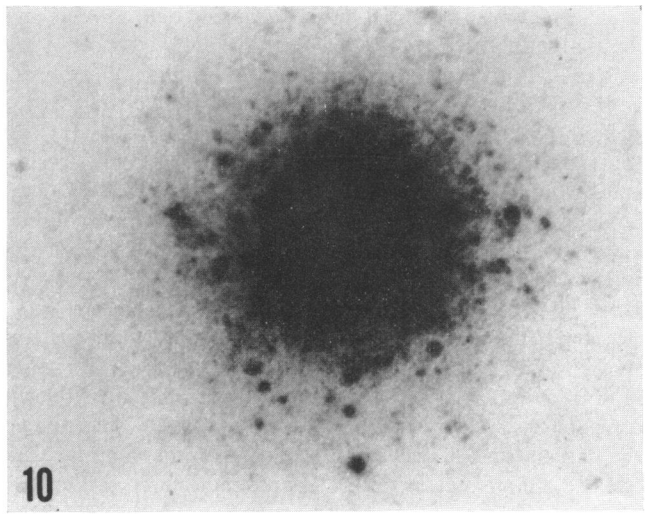
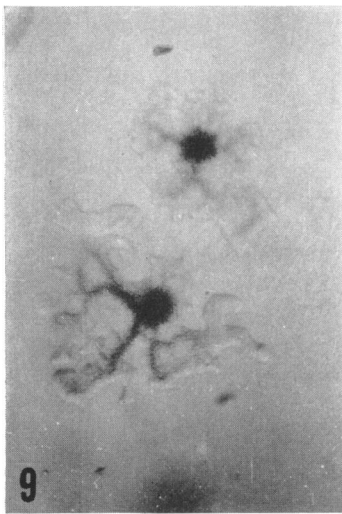
FIG. 1. *Kazan A strain of Treponema pallidum.* Diffuse colonies on 0.7% agar after 8 days of incubation.

FIG. 2. *Reiter strain of T. pallidum.* Rhizoid and round discrete colonies on 0.7% agar after 8 days of incubation.

FIG. 3. *Kazan A.* Cross section of 6-day colony under dark-field illumination ( $\times 30$ ).

FIG. 4. *Noguchi strain of T. pallidum.* Cross section of 17-day diffuse colony under transmitted light ( $\times 30$ ). Note extremely dense center with peripheral zone of spreading growth.

FIG. 5-8. *Reiter treponeme colonies* ( $\times 30$ ): (5) 7-day rhizoid colonies; (6) 6-day rhizoid colonies under shadowed light (note conical shape and contoured surface); (7) 6-day granular colonies; (8) 7-day round colonies.



The round colonies produced by these treponemes differed somewhat between the two strains. In cultures of the Noguchi treponeme, they were very few in number and appeared as minute, slightly raised colonies, which ranged from 0.1 to 0.3 mm in diameter and were most easily identified on blood agar plates. The central growth of these was in the agar, but the peripheral portion of the colony was on the surface and microscopically appeared somewhat lacy in character (Fig. 12). When subcultured, these colonies increased in size and showed a definite double zone of growth (Fig. 13). Under dark-field illumination, it could be observed that the dense central zone was depressed in relation to the peripheral surface growth so that the colonies possessed a characteristic dimpled shape (Fig. 14). These, like the round colonies of Reiter, represented a stable colonial form that did not change further upon subsequent subcultures.

The round colonies of the Nichols treponeme were initially slightly larger than those of Noguchi, and resembled more closely colonies of the Reiter treponeme. However, upon subculture they assumed characteristics of the Noguchi colonies but differed in that they were never as large, the dimpled center was less pronounced, and the central subsurface growth was never as uniformly dense (Fig. 15 and 16). Although these differences were minor, they were consistently present and were characteristic of this strain.

The diffuse colonies of the Noguchi and Nichols treponemes had a peripheral zone of hemolysis on both human and rabbit blood agar. In addition, the Noguchi strain frequently, but not always, showed the saucer-shaped depression of the surface above the colonies as previously noted with Kazan A. This feature was not observed with the Nichols strain. The round colonies of both organisms produced no hemolysin.

The individual spirochetes in the diffuse colonies of both strains were regularly coiled (Fig.

17 and 18) and motile, although their motility was less vigorous than that seen in broth cultures. In contrast, the organisms in the Noguchi round colonies had many bizarre shapes; some were loosely, and frequently irregularly, waved, but many more were straight and often long and filamentous as though they had failed to divide (Fig. 19). These organisms were unusual in that they were the only spirochetes which retained their atypical morphology upon repeated subcultures in broth. The organisms in the Nichols round colonies also differed somewhat from those in the spreading colonies, but the changes were neither as marked nor as permanent. They retained their spiral shape, but the periodicity of their spirals was consistently greater than that of the organisms in deep colonies; this can be noted in Fig. 20 when compared with the deep colony treponeme in Fig. 18. When subcultured in broth, these organisms usually retained their loose spirals for one or two transfers, but subsequently many reverted to normal morphology and gave rise to diffuse subsurface colonies.

*Colony forms of other cultivable strains of T. pallidum.* The Kazan strains 2, 4, 5, and 8 all produced visible colonies after 6 to 7 days of incubation, and colonial morphology was the same for all. Only diffuse, homogenous colonies appeared in cultures of these organisms, and they were indistinguishable from similar colonies of the Noguchi and Nichols strains. The organisms within the colonies were regularly coiled and motile. Studies with regard to hemolysis of erythrocytes were not made.

*Colony forms of small oral treponemes.* The five oral treponeme strains isolated in this laboratory readily grew as colonies which were visible after 6 to 7 days of growth. All five produced three colony forms, each of which was similar to one of the colony types of the Reiter treponeme. In these strains, the diffuse granular colonies were far more frequent than the rhizoid type, and

FIG. 9. Reiter treponeme colonies ( $\times 30$ ): 10-day rhizoid colonies on 1.0% agar. Compare with same type colonies on 0.7% agar in Fig. 5.

FIG. 10-14. Noguchi treponeme colonies ( $\times 30$ ): 20-day-old granular (10) and 16-day diffuse (11) colonies; discrete surface colonies as seen in 16-day-old initial culture (12), and in 20-day subcultures under transmitted (13) and dark-field (14) illumination.

FIG. 15 and 16. Nichols treponeme colonies ( $\times 30$ ): 14-day colonies under transmitted and dark-field illumination.

FIG. 17-20. Individual treponemes from colonies ( $\times 1500$ ). Noguchi (17) and Nichols (18) from diffuse colonies. Noguchi (19) and Nichols (20) from round, surface colonies.

FIG. 21. FM strain of small oral treponeme. Cluster of 14-day-old microcolonies ( $\times 30$ ).



around both colony types there was hemolysis on human as well as on rabbit blood agar. The individual organisms within the colonies had the same marked pleomorphism previously observed with the Reiter treponeme, but as in the latter they reverted to their original spiral shape upon subculture in broth.

Considerable difficulty was encountered in attempts to grow the FM strain of oral treponeme as colonies, because it possessed more rigid growth requirements than most other strains studied. When colonies were finally achieved, they required 14 days to become visible and then could be seen only by indirect (dark-field) illumination. Grossly, they appeared as small, diffuse colonies 0.3 to 0.5 mm in diameter which, upon microscopy, were found to be composed of clusters of microcolonies without a central area of dense growth (Fig. 21). They resembled minute versions of the "bits of cotton" colonies of *Leptospira pomona* described by Armstrong and Goldberg (1960), and probably represented colonial growth under adverse conditions. However, we have not succeeded to date in devising a medium that will give better colonies of this organism. The treponemes within the colonies were regularly coiled and motile.

*Colony forms of B. vincentii.* The N-9 strain of *B. vincentii* required the same medium as the FM strain for colonial growth. Colonies were visible after 2 weeks of incubation and ranged from 12 to 15 mm in diameter. They were smaller but otherwise resembled the colonies of Kazan A, in that growth occurred as a shallow homogenous haze insufficiently dense to be observed microscopically. The spirochetes were less motile but were otherwise the same as those in broth cultures; many were loosely and irregularly spiralled, but some were tightly coiled and could not be differentiated from treponemes.

#### DISCUSSION

The findings reported in this paper clearly show that many anaerobic spirochetes can be readily grown as colonies on streaked agar plates; on the basis of the limited number of strains studied, they further suggest that there may be sufficient colonial variation between strains to be of some value in the further classification of these organisms. However, it is recognized that many more spirochetes must be investigated before any significance can be assigned to their colonial

morphology. Whether or not this proves to be of value, the growth of these organisms as colonies makes possible the establishment of definitely pure strains, for there seems to be little doubt from the results recorded in Table 1 that colonies arose from individual spirochetes.

Several features of the colonial growth of spirochetes, as observed in this study, are of interest, especially the appearance of more than one colony type in several of the strains. Whether this variation of colonial morphology within a given strain corresponds to the smooth- and rough-colony variants of other microorganisms, or whether it simply represents differences in motility of various members of the population, is still to be determined. In general, the spirochetes in the diffuse, subsurface colonies were more regularly coiled and motile than those in surface colonies, and, as a rule, the more motile strains produced the larger colonies. This would suggest that colony morphology was largely a reflection of spirochetal motility. However, the stability of the round colonies produced by several strains indicated a permanent change in some characteristic of the organisms, and this would make them more like rough variants of other bacteria. Further evidence that the organisms were changed could be observed in the permanently altered morphology of Noguchi treponemes from round colonies.

The fact that anaerobic spirochete colonies grow within the agar, as well as on the surface, is of interest because it is sometimes stated that such growth is a unique feature of *Mycoplasma* and L forms. It is now apparent that *Leptospira* (Armstrong and Goldberg, 1960), in addition to the organisms studied here, also grow in this fashion.

The growth of anaerobic spirochetes as colonies on streaked plates was reported previously by a number of investigators, but, with the exception of Socransky et al. (1959), studies of this nature were only partially successful. Most investigators either failed to achieve colonial growth consistently (Wichelhausen and Wichelhausen, 1942), or were unable to subculture colonies of all strains (Gates, 1923). Rosebury (1962) suggested that only well-established laboratory strains could be grown as colonies, and indicated that this might be the result of adaptation to less rigorous anaerobic requirements. In support of this, he pointed to the results of Socransky et al. (1959)

who succeeded in growing spirochetes from mixed oral microflora as colonies when plates were inoculated in an anaerobic environment, but not when inoculations were made under ordinary atmospheric conditions. However, such a hypothesis does not explain the failure of Socransky et al. (1959) to grow (presumably adapted) stock treponeme strains, unless these also were transferred under anaerobic conditions. While there is no doubt that stock strains are easier to grow, and therefore produce colonies more readily, than fresh isolates, studies to be published later by us indicate that adaptation toward easier growth is almost certainly nutritional rather than environmental. We have found that new treponeme strains can be grown readily as colonies with the procedures employed in this study, provided that their nutritional requirements are adequately met.

Since the results presented in this paper are somewhat in contrast to those of previous investigators, it is perhaps worth considering possible reasons for this. Insofar as can be determined, almost all previous attempts to grow spirochetes as colonies were made on blood agar media with a meat infusion base. This method has several disadvantages. First, the opacity of such media makes the observation of nonhemolytic subsurface colonies difficult, and it is quite possible that colonial growth was achieved at times in the past but was not recognized. Even with clear media, as used in this study, some treponeme colonies could be seen only with the aid of indirect (dark-field) illumination. Second, the use of a single medium for the study of all strains was not successful in our hands, and the colonial growth of several strains was achieved only after their growth requirements had been more satisfactorily met. Third, Wichelhausen and Wichelhausen (1942) were the only previous investigators to specifically mention the addition of a sulfhydryl compound to media employed for colonial growth, as was done in the present study. Such compounds not only accelerate the production of a suitably reduced environment within the medium, but they are also specifically required for growth by some spirochetes as shown by Eagle and Steinman (1948).

The observation that the amount of agar in the medium affects the ability of treponemes to grow as colonies may have played a major role in the successful results reported here, and may explain

some of the difficulties encountered by others in the past with the use of agar concentrations two to four times those employed by us. It is still to be determined why increased concentrations of agar inhibit treponeme growth, but the fact that this occurs in poured as well as streaked plate cultures suggests that it is not due to the failure of spirochetes to migrate through the firmer medium in search of a more suitable environment for survival. Two other possibilities seem more likely. Agar, or some contaminant in agar preparations, may have a direct toxic action, which is dose-related, upon treponemes. This possibility cannot be excluded at the present time, but is somewhat difficult to reconcile with the findings of Socransky et al. (1959) who were able to grow treponemes on media containing 3% agar. Another, and perhaps more likely explanation, rests with the fact that the rate of diffusion of gases, including oxygen, from jelled media is dependent upon the concentration of agar present. Because of this, it seems reasonable to assume that anaerobiosis sufficient to permit survival of treponemes could be more rapidly established in a medium of low agar concentration than in one where the agar concentration was higher. This would explain the ability of Socransky et al. (1959) to grow colonies on a more firmly jelled medium, since these investigators incubated plates in an anaerobic environment overnight prior to inoculation.

In conclusion, it appears likely that the one factor most responsible for the successful growth of spirochete colonies reported here was probably the use of media with a low agar concentration. However, other conditions, such as different media for different strains, the presence of sulfhydryl compounds in media, and recognition of the need for long incubation periods, undoubtedly contributed to the success of this study.

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