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Sedimentation Counting and Morphology of *Mycoplasma*

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ABSTRACT

CLARK, HAROLD W. (The George Washington University School of Medicine, Washington, D.C.). Sedimentation counting and morphology of *Mycoplasma*. J. Bacteriol. **90**:1373-1386. 1965.—The sedimentation technique for counting viral particles was applied to the quantitation and morphological identification of *Mycoplasma* in broth cultures. *Mycoplasma*, apparently in their native form, firmly adhered to the surface, when sedimented on glass cover slips or onto electron microscope grids. The sedimented cover slip preparations stained with crystal violet could be readily counted in the light microscope. The cultures sedimented onto electron microscope grids were readily counted at low magnification and provided excellent preparations for morphological examination at higher magnifications. It was found that air-dried *Mycoplasma* particles were enlarged considerably because of excessive flattening. Fixation of sedimented *Mycoplasma* particles in diluted OsO_4 prior to air drying yielded a more realistic morphology, with various sizes and shapes in the stages of the growth cycle exhibited. A new technique of differentially staining *Mycoplasma* colonies on agar plates was developed to facilitate the quantitation of viable colony-forming units for comparison with total counts. The use of plastic or Parafilm gaskets for dry mounting was developed to facilitate the handling and examination of the stained cover slip preparations. The results of this investigation indicated that the growth cycle of some *Mycoplasma* species includes a stage of hexadic fission with the cleavage of minimal reproductive units (less than 100 mμ) containing a limited deoxyribonucleic acid genetic coding molecule (approximately 4×10^6).

A need exists for the standardization of *Mycoplasma* cultures to provide comparison of results and to improve interpretation of analytical data. It has become apparent in evaluating the chemical and immunological differences within a species and among different species that a more direct and accurate method of quantitating *Mycoplasma* is necessary. The methods currently used for the quantitation of *Mycoplasma* growth, i.e., colony counts, turbidimetric analysis, end point dilutions, dry weight, total protein, or total nitrogen, are indirect means of estimating the number or amount of *Mycoplasma* present. The analytical results obtained by these methods are influenced by a variety of factors, including viability, growth conditions and age of culture, species variations, residual and adsorbed media impurities, range of particle size, and aggregation.

Before any viral particle-counting technique can be applied to *Mycoplasma* broth cultures, it is first necessary to establish standard conditions and a countable unit. The hot-water fixation technique (Clark et al., 1961) is a rapid and accurate method for identifying *Mycoplasma* colonies. However, no satisfactory method exists for the identification of the naturally occurring *Mycoplasma* particles because of their pliable nature and resultant heterogenic size and shape. Consequently, a technique previously established for the enumeration and quantitation of virus particles was investigated. The sedimentation counting method developed by Sharp (1958) was modified and applied to the study of *Mycoplasma* cultures. When properly controlled, this method yields preparations suitable for both light and electron microscopy. The technique, originally developed to readily quantitate *Mycoplasma* in liquid culture, is also an acceptable method for the identification of *Mycoplasma* morphology.

In addition, correlation of *Mycoplasma* particle count with viable colony-forming units (CFU) was greatly simplified by the developmet of a rapid differential colony-staining technique. Such a technique facilitated the determination of CFU yield in a series of filtration studies made to compare the number, size, and shape of *Mycoplasma* particles with those obtained by sedimentation.

MATERIALS AND METHODS

Mycoplasma strains. M. hominis, type 1, strain JJ and *M. hominis*, type 2, strain CH used in this study were isolated from the human genital tract.

The avian strain 5969, *M. gallisepticum*, was obtained from M. E. Tourtellotte at the University of Connecticut. Other types of *Mycoplasma* were examined but are not included in this report.

Cultures. Mycoplasma strains were cultured in a freshly prepared broth of pancreatin-digested (PD) beef heart enriched with 10% heated bovine serum. The serum and broth were mixed just prior to use and passed through a large 220-mμ Millipore filter at 10 psi to minimize media sediment in the *Mycoplasma* preparations. The inocula, unless otherwise designated, were from 24-hr broth cultures and were added in 1% volumes.

Particle sedimentation. A Sorvall refrigerated centrifuge, RC-2, with a type SU particle-counting rotor was used (Ivan Sorvall, Inc., Norwalk, Conn.). The method of procedure as described by Sharp (1958) was used with few modifications noted below.

Preparations for the electron microscope were obtained by sedimentation of diluted *Mycoplasma* cultures onto nitrocellulose-covered stainless-steel grids at 27,000 × *g* for 20 min. The grids were held in place by a magnet mounted in Lucite at the base of the counting cell chamber (Fig. 1). The counting cells were filled with 1.0 ml of suitably diluted *Mycoplasma* cultures or suspensions. Supernatant fluids from the centrifuged cultures were drained off, and the cell sections containing the grid were immersed in a 0.4% OsO_4 solution of 0.8% NaCl and 5% acetic acid for 2 min. The cells were then rinsed with distilled water and allowed to air dry before the grids were removed. In the OsO_4 control preparation, the cell was rinsed with distilled water only and was fixed by air-drying. Shadow casting of specimens was done with Pallatium-palladium alloy at approximately 30°. When standard latex particles were sedimented along with *Mycoplasma* for verification of size and number, a 0.5% gelatin-coated nitrocellulose film was used as recommended by Sharp (1958).

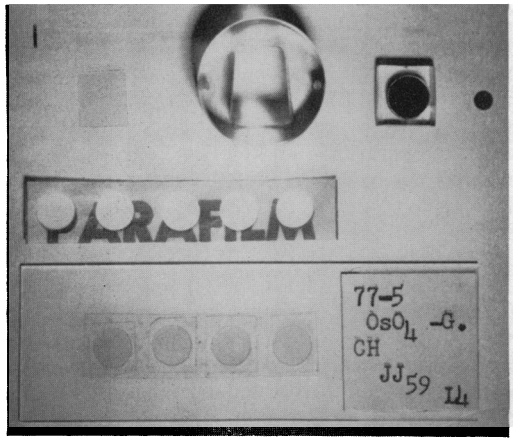

FIG. 1. *Center section of the particle-counting cell with the magnet and electron microscope grid (right) and the glass cover slip showing the mounting tape of Parafilm gaskets and the final stained and mounted cover slips.*

Preparation for light microscopy. Cover slips (8 × 10 mm) were cut from optical grade no. 1 glass and were acid-cleaned. A slip was mounted on the base (cm^2) of each counting cell with a trace amount of petrolatum. The counting cells were filled with 1.25 ml of a suitable dilution of *Mycoplasma* broth cultures (10^{-2}) and centrifuged at 27,000 × *g* for 20 min. The cells were drained and then immersed immediately in a 0.4% OsO_4 solution of NaCl and acetic acid for 2 min. The cells were drained again, and the cover slips were gently rinsed with water and were allowed to drain dry. The OsO_4 control preparations without fixation were also rinsed with water. After air drying, the cover slips were transferred to a microscope slide and were held in place with the residual petrolatum. One drop of 1.0% aqueous crystal violet was placed on each cover slip, and the slide was placed in a moist chamber for 1 hr. The stained cover slips were rinsed with distilled water and allowed to air dry.

Plastic mounted cover slips. Tapes of plastic mounting gaskets were prepared by cutting 1-cm wide strips from clear four-gauge plastic (Flex-O-glass, Warp Bros., Chicago, Ill.). These strips were mounted on centimeter graph paper and perforated with 6-mm holes at 1-cm intervals. A strip of four gaskets cut from the (plastic) mounting tape was centered on a clean microscope slide, and four of the stained cover slips were placed face down (inverted) over the center of each gasket hole. The microscope slide was placed on a low-temperature hot plate (approximately 100 C) for a few seconds until the plastic started to melt. The slide was removed, and the cover slips were gently pressed in place so as to exclude trapped air bubbles. After cleaning the residual petrolatum from the backs of the cover slips with soap and water, the slide was ready for microscopic examination and particle counting. Similar mounting gaskets were also prepared from Parafilm. Because of their organic solvent solubility, it is recommended that the plastic- or Parafilm-mounted cover slips be cleaned with soap and water. Figure 1 shows the center section of the counting cell which holds the cover slip or the magnet and grid. A portion of the Parafilm-mounting tape gasket and a final slide preparation are also demonstrated. Larger cover slip preparations of hot water-fixed *Mycoplasma* colonies were also permanently mounted with larger plastic gaskets (22 mm square) onto microscope slides.

Colony counts. Broth cultures or similar suspensions of washed *Mycoplasma* were usually diluted 10^{-2} or 10^{-3} for particle sedimentation counting. Higher dilutions, 10^{-4}, 10^{-6}, and 10^{-8}, were made for colony counts, and 0.05 ml of each dilution was spread over the surface of 60-mm agar plates. The plates were sealed with Parafilm and incubated at 37 C for 2 to 3 days. The colonies

were differentially stained by flooding the surface of the plate with approximately 2 ml of a filtered 0.1% Giemsa stain in chloroform (w/v) and gently rotating for 60 sec. The staining time varied somewhat depending on the agar hardness. The best results were obtained after evaporation of excessive moisture and condensation from the sealed plates. Plastic petri plates can be used because the chloroform solvent exposure is brief. The stained colonies were easily seen and rapidly counted with a stereomicroscope or hand lens with the whole plate in view. The stained colonies on a heavily inoculated plate (Fig. 2) were readily visible and were counted by placing it on a white sheet of paper containing centimeter square grid, thus eliminating the need for ruled plates. The stained *M. hominis* colonies selected for illustration in Fig. 2 required no magnification; they were exceptionally large because of culture and dilution phenomena. The smaller stained colonies readily counted at low magnification were also examined at higher magnification to observe colony morphology and to insure accuracy of count.

RESULTS

One of the most striking observations made in this investigation was the extremely pliable nature and size variation of the *Mycoplasma* particles. Although this morphological property has been described by Borrell et al. (1910) and later by Turner (1935) and many other investigators, it undoubtedly has also been the source of repeated misinterpretation. With only a membrane to confine their cellular contents, it would seem logical to expect multiple sizes and shapes dependent upon their physical-chemical environmental exposure.

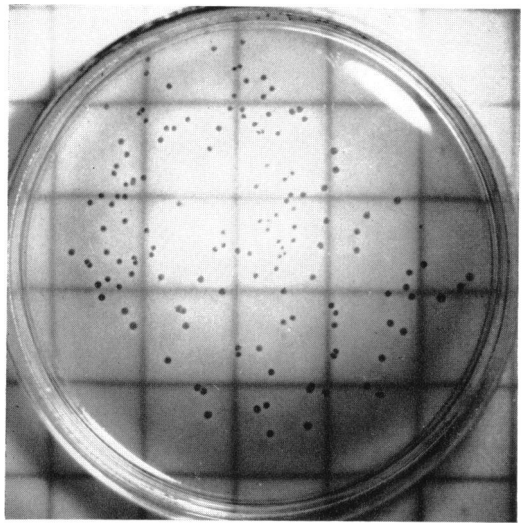

FIG. 2. *Mycoplasma hominis, type 2, colonies differentially stained intact with Giemsa for easier examination and counting.*

A look at the basic unit of *Mycoplasma* identification, the colony, that has been physically disrupted just prior to being fixed on a glass slide with hot water, will reveal several subunits. The dark-field photomicrograph of the *M. hominis* strain shown in Fig. 3 illustrates the variable large body composition of the colony as often described by Dienes (1945). It further illustrates that the membrane envelope holding the large bodies together is also pliable enough to stretch into filaments that hold the smallest particulate components in chains. Although the cotton fiber technique, used by Orskov (1927) and Freundt (1958) to demonstrate filaments in colonies, was not used in this preparation, it does seem possible that a more filamentous preparation could have been obtained under the right conditions. Another human *Mycoplasma* colony shown in Fig. 3b illustrates the chains of coccoidal particles branching out from the colony edge. The problem remains: What particle entity can be counted and what is the self-reproducing unit? The smallest separate particles seen in the colony chains are less than 300 mμ in diameter and cannot be used to measure *Mycoplasma* growth, especially in broth cultures.

An investigation of the effect of various diluents, rinses, and fixatives on *Mycoplasma* suspensions was made to determine optimal conditions for obtaining maximal yield, uniform-sized particles, and nonaggregated preparations. The effect of decreased osmolarity on *Mycoplasma* has been variously interpreted by different investigators to cause complete lysis and bactericidal or no apparent effect. Marked differences were observed in the centrifuged pellets of *M. hominis* type 2 (CH) washed with distilled water or hypotonic NaCl (less than 0.9%) and the pellets washed with hypertonic NaCl (2 to 8%). The hypotonic solution produces a diffuse and translucent pellet, whereas the hypertonic solution produces a smaller and white opaque pellet. However, no significant difference could be detected in the amount of protein released from an 18-hr culture washed three times with distilled water or with NaCl solutions of increasing concentrations (0.5 to 8%) or a fourth wash of 10% ethyl alcohol. The similar analysis of the water and NaCl washings was confirmed by the almost identical amounts of protein found in the washed *Mycoplasma* pellets obtained by centrifugation at 27,000 × *g* for 20 min. To avoid misinterpretation, it must be emphasized that these results obtained with an 18-hr culture do not

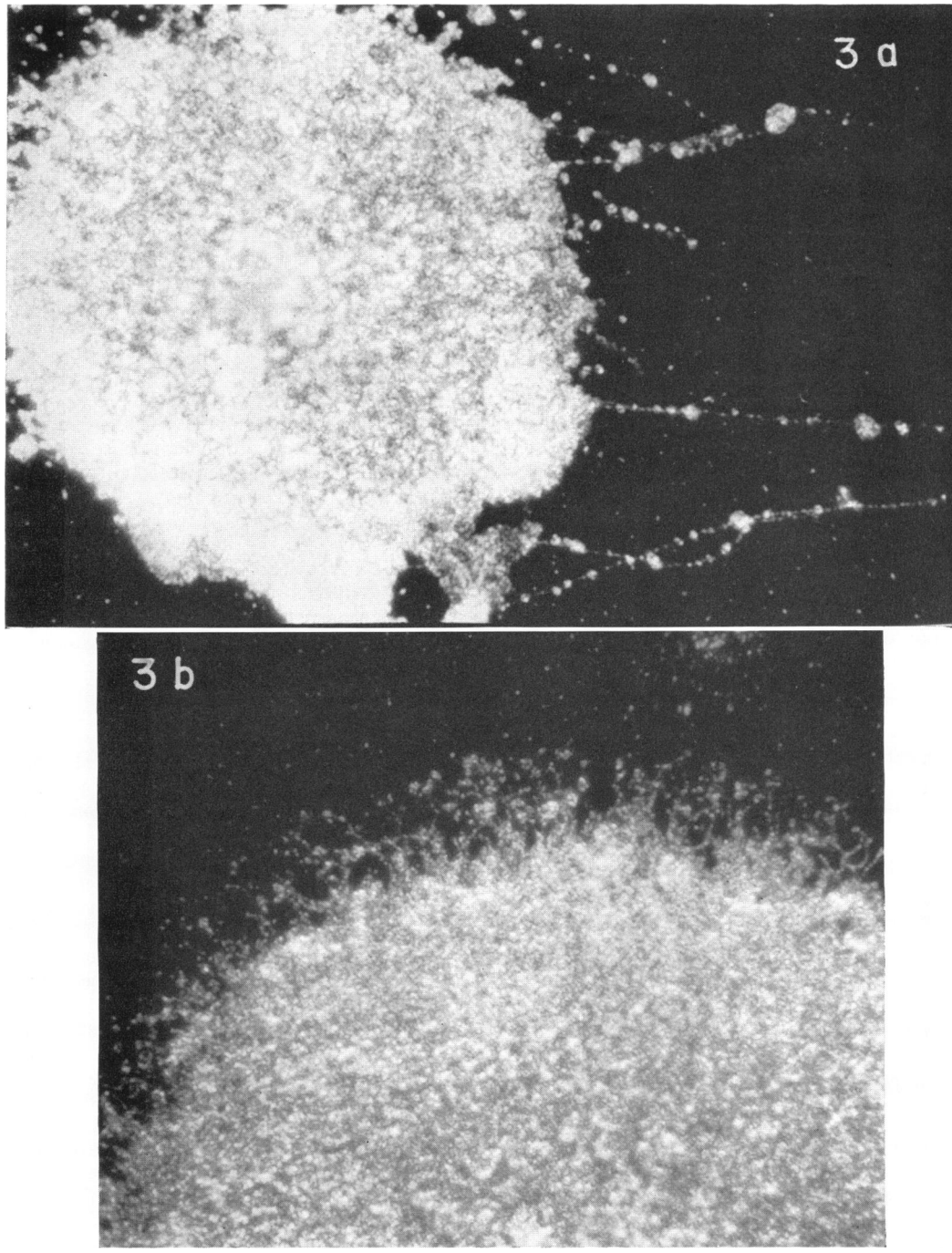

FIG. 3. *Dark-field photomicrographs of hot water-fixed Mycoplasma hominis, type 1, colonies,* × *450. a) Variable large body and particulate chainlike filaments disrupted from colony. b) Chains of particles branching out from edge of colony.*

necessarily apply to older cultures and other strains. Earlier experiments on chemical composition of *Mycoplasma* revealed marked instability of older cultures and aged preparations with the release of soluble components. Unless properly controlled, this variable would introduce considerable influence in both qualitative and quantitative analysis.

These results on osmolarity were confirmed by microscopic analysis of particles sedimented from *Mycoplasma* cultures diluted in various NaCl concentrations. There was a definite tendency for the *Mycoplasma* particles from the high salt concentrations to be smaller and more aggregated and usually to give slightly higher counts. However, the particles sedimented from water were less aggregated and slightly larger, which made these preparations (stained) easier to count. Some of these results on osmolarity effect are illustrated in Fig. 4a to 4d, with photomicrographs of the sedimented *Mycoplasma* particles diluted in various solutions and washed with water or 10% ethyl alcohol.

To simplify the counting procedures, photomagnification of particles sedimented on cover slips was kept constant at × 1,700. It was thus possible to use a 1.7-cm square (equivalent to 10 μ) overlay of the photos (Fig. 4d) to represent 10^{-6} of the centimeter square cell base upon which the 1.25 ml of *Mycoplasma* suspension was sedimented. The number of particles (n) counted per square, times 10^6, times the culture dilution (10^2), and divided by 1.25-ml volume gives a fairly accurate estimate of *Mycoplasma* particles per milliliter of culture. An area of average particle density was selected by use of lower magnification, and the average number of particles counted in five or more squares was used. Counts were also made direct from the projection of the microscope field on a ground glass plate when immediate analysis was required.

The appropriate dilution and condition of *Mycoplasma* suspensions for electron microscope analysis was pretested on cover slips. The electron microscope grids with sedimented *Mycoplasma* were pre-examined in the light microscope and photographed to assure a suitable specimen and to confirm the final results. Other than the variable size, shape, and particle distribution, the most striking and characteristic feature noted in the unfixed air-dried specimens was the marked flattening and stretching of particles. Similar results have been shown and variously interpreted by other investigators in earlier electron microscope photographs.

Actual comparison of pre- and postcentrifugation specimens indicated there was no apparent effect of the 27,000 × *g* sedimentation on the flatness because of size similarities. The flattening of *Mycoplasma* particles is primarily caused by surface drying, because fixation of *Mycoplasma* with OsO_4 prior to drying yielded more spherical and thicker (longer shadow) particles. The effect of OsO_4 fixation on *Mycoplasma* prior to drying is shown in the cover slip preparations (Fig. 5a and 5b) and the electron microscope photomicrographs (Fig. 6a and 6b). An additional advantage of OsO_4 fixation, other than producing a firm membrane, seems to be a differential cytological reaction resulting in the demonstration of electron-dense inclusion particles in the unshadowed preparations. The OsO_4-fixed preparations seemed to have maintained their consistent morphological growth-stage characteristics and were not reduced to flattened globs by air-drying nor altered to various pleomorphic forms.

Analysis of the number of colony-forming units (CFU) in a 24-hr broth culture of *M. hominis*, type 2, indicated a 66% viability in the total particle count. The per cent viability was dependent upon the size and viability of the inoculum. The accuracy of both CFU and total particle counts improved with minimal aggregation. Estimation of aggregation and CFU size by passage through a series of Millipore filters (450, 300, 220, and 100 mμ) indicated considerable variation. The CFU recovered from passage of a 48-hr *M. hominis* culture (CH) through a graded series of 25-mm diameter Millipore filters under pressures of 2 to 15 psi are shown in Table 1. These results showed that 99% of the viable CFU particles in the 48-hr culture were greater than 300 mμ and that one in 10 million was smaller than 100 mμ. Particle counts and examination of the filtrates below 300 mμ were difficult because of the low yields. No evidence of filaments or extruded forms was observed.

Standardization of *Mycoplasma* cultures in terms of CFU counts, particle counts, and washed sedimented protein or dry weight provide more specific basis for describing and relating metabolic activity, chemical composition, and antigenic activity. Table 2 illustrates the quantitative analysis of *Mycoplasma* growth in a 24-hr broth culture by means of colony counts (light and electron microscopy) and total protein. The average protein mass of a 24-hr *M. hominis* viable particle is less than 11×10^{-9} μg, and, correcting for nonviable particles, the average protein mass is 7×10^{-9} μg. This range of values is in approximate agreement with the dry weight value of 6.4×10^{-9} μg per *M. gallisepticum* (5969) particle calculated by Morowitz et al. (1962). One could approximate that the few

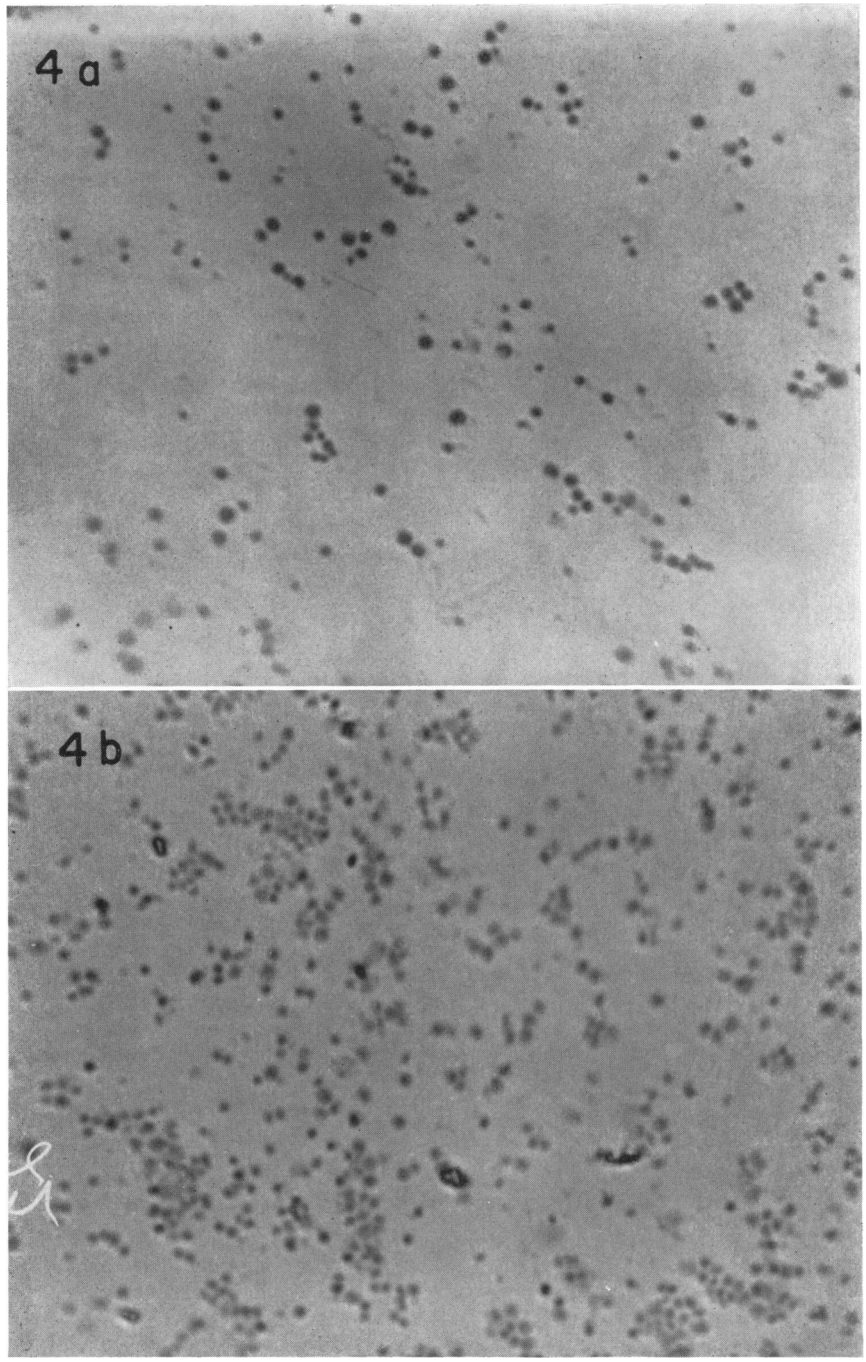

FIG. 4. *Diluted Mycoplasma broth cultures sedimented on cover slips, air-dried, and stained with crystal violet. (a) M. hominis, type 2, diluted with 0.9% NaCl and rinsed with 10% ethyl alcohol. (b) M. hominis, type 2, diluted with 4% dextrose and rinsed with water. (c) M. gallisepticum (5969) diluted in water and rinsed in 10% ethyl alcohol. (d) M. gallisepticum (5969) diluted in water and rinsed in water with the equivalent 10 μ square overlay to facilitate counting. Original magnification, × 1,700.*

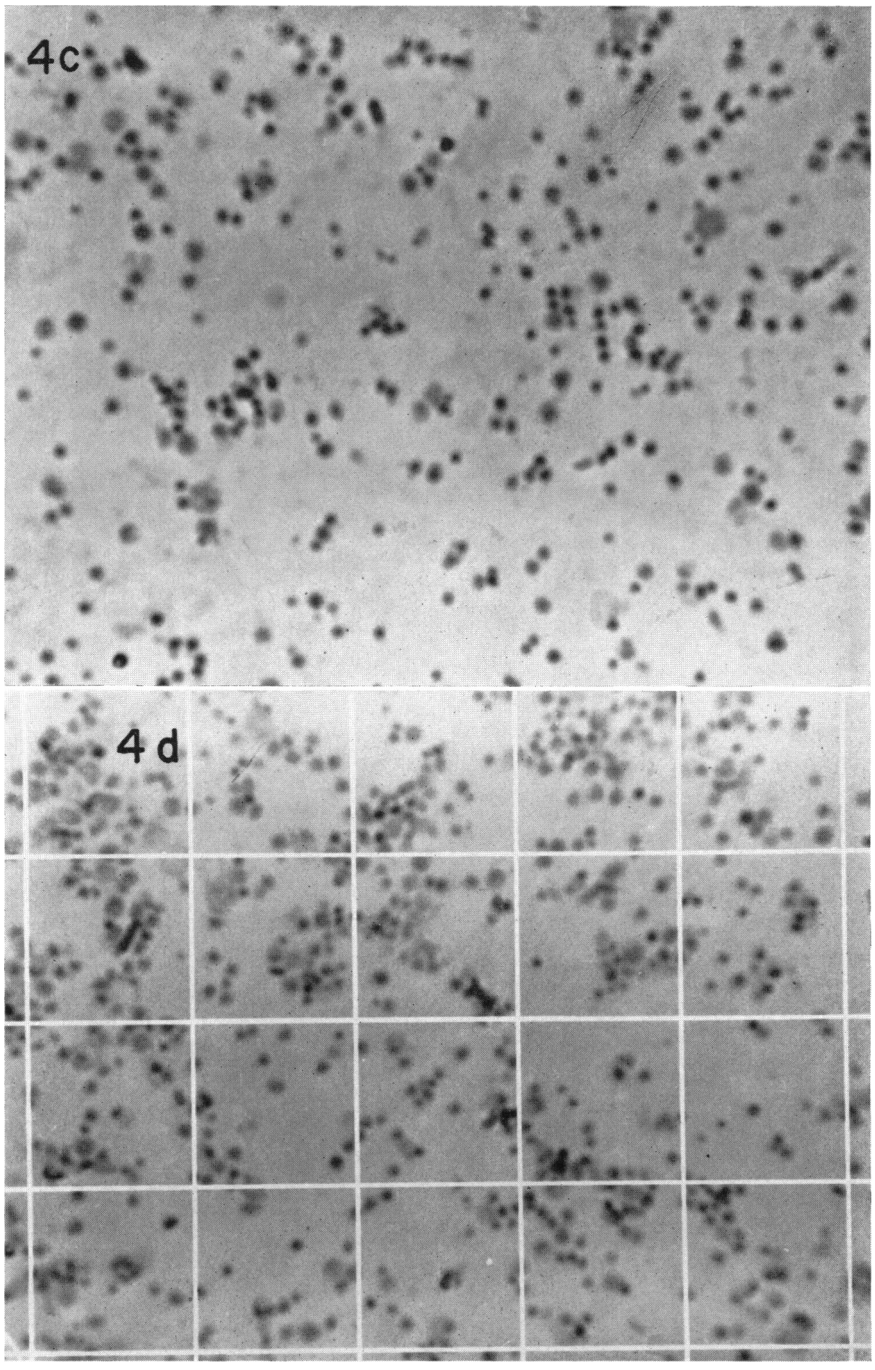

FIG. 4—*Continued*

CFU passing the 100-mμ filter are about r/4 or volume/64 with a mass of 1.2×10^{-10} μg, assuming the small *Mycoplasma* and large (400 mμ) were both spherical and had the same density. The average deoxyribonucleic acid (DNA) content of these preparations was about 5% by the diphenylamine test of hot trichloroacetic acid extracts. According to this, the amount of

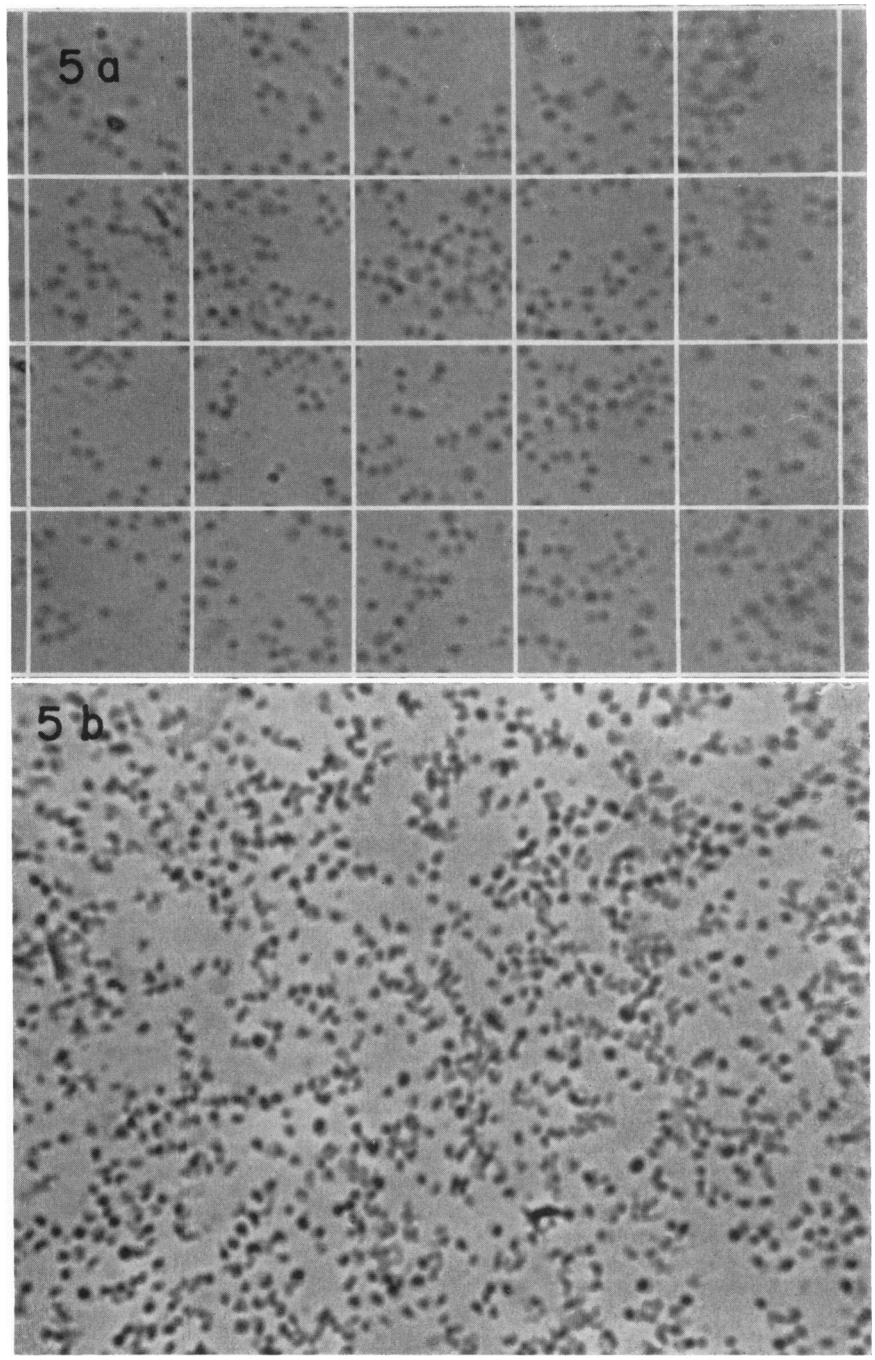

FIG. 5. *Mycoplasma hominis, type 2, broth cultures sedimented on cover slips. (a) Air-dried prior to staining shown with equivalent 10 μ square overlay. (b) Fixed with OsO_4 prior to air-drying to yield a greater number of irregular shaped and sized particles (same suspension used in Fig. 8b at higher magnification).*

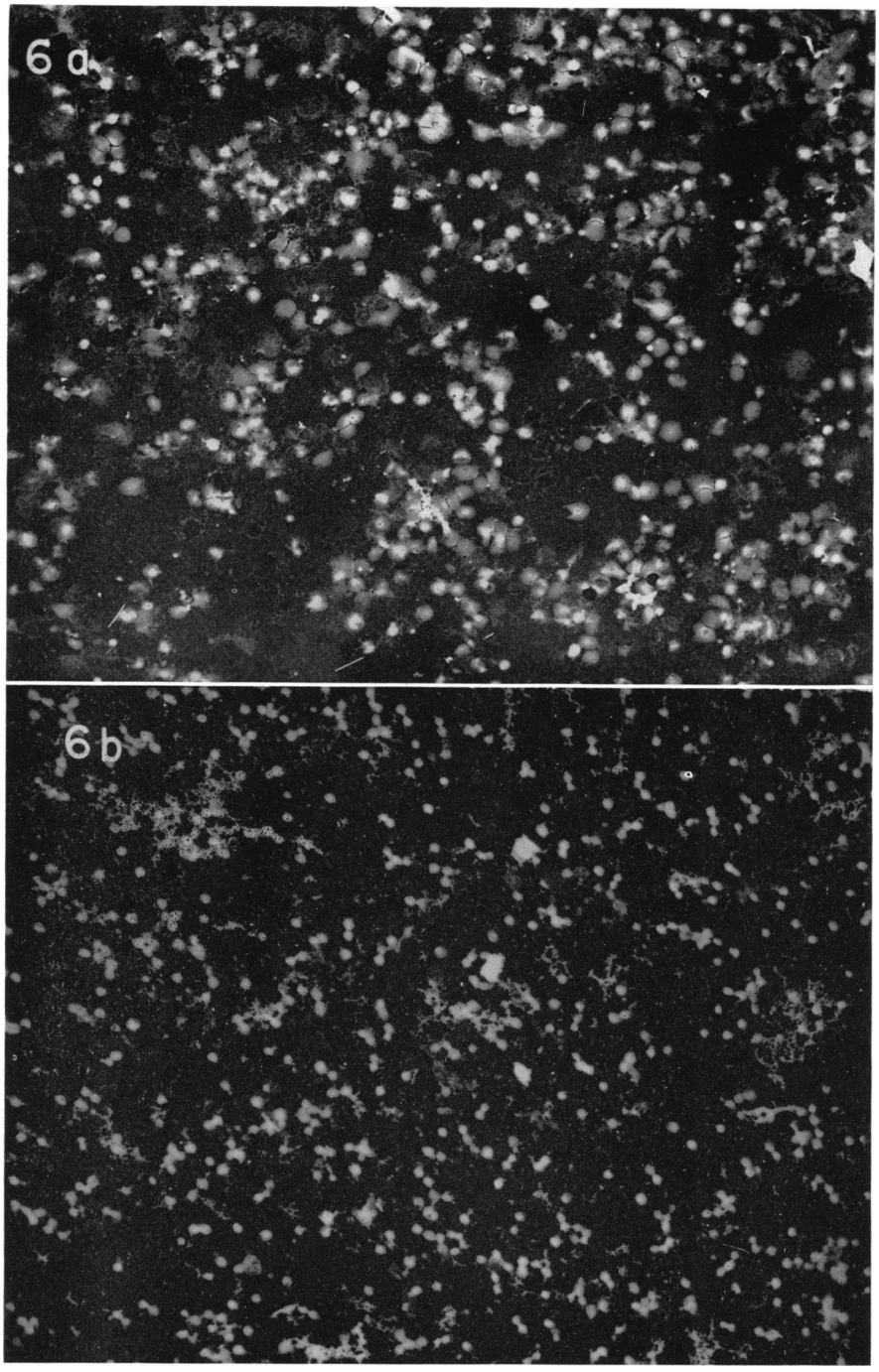

FIG. 6. *Mycoplasma hominis, type 2, diluted broth cultures sedimented on electron microscope grids and shadowed, × 2900 magnification. (a) Air-dried preparation producing large flattened particles (compare Fig. 5a cover slip). (b) Preparation fixed with OsO_4 yields spherical particles of more natural size. Unwashed medium fixed in the background does not affect particle counting but does affect chemical and immunological analysis.*

TABLE 1. *Recovery of Mycoplasma hominis, type 2, CFU through various-sized filters*

Filtrate tested	Dilution	CFU/plate*	Recovery fraction
Whole culture	10^{-6}	156	1
300 mμ	10^{-4}	104	10^{-2}
220 mμ	10^{-1}	10	10^{-6}
100 mμ	F S†	8	10^{-7}

* Average colony count from 0.05 ml of filtrate dilution.

† Full strength.

TABLE 2. *Quantitative analysis of Mycoplasma in broth culture*

Analysis	Total count /ml	Protein*/particle
		μg
CFU	26×10^8	11×10^{-9}
Electron microscope grid	23×10^8	12×10^{-9}†
Cover slip	40×10^8	7×10^{-9}
Estimated minimal CFU mass (volume/64)		1×10^{-10}

* Protein analysis of sedimented and washed *Mycoplasma* culture by use of a modified Folin-Ciocalteau method (28 μg/ml).

† Not applicable—low yield in this test condition.

genetic-coding DNA in the smallest reproductive units would be approximately 4×10^6 molecular weight per CFU, and suggests a limited synthetic capability.

Morphology. The accuracy of the average mass per *Mycoplasma* particle calculation is greatly limited, because of the wide range in size and shape during stages of development and growth. The uniform size and easily countable particles in a 24-hr culture of *M. hominis*, type 2 (CH), is shown in Fig. 6b along with the fixed but easily differentiated media fragments. Examination of other cultures or strains having different growth rates often yielded preparations exhibiting the various stages in development as shown by the 24-hr *M. gallisepticum* (5969) culture in Fig. 7a and 7b. It appears as though a single coccoidal particle develops from 100 mμ into a large body, 500 mμ, whereupon it concentrates at the periphery and segments into six minimal reproducing units that usually outgrow the restricting membrane. The number of segments seems to be dependent on the strain and culture conditions. Consequently, in a fluid state, it would be possible to obtain distortions of the growth stages resulting in an endless number of shapes. Generation time studies are further complicated by the cleavage failure after segmentation (Fig. 7a). These aggregates of segmented particles undoubtedly form the basis of colony formation on a stationary agar surface.

The results of this study are similar to some of the original morphological studies on *M. mycoides* by Borrel et al. (1910) and to the dark-field preparations of contagious bovine pleuropneumonia obtained by Turner (1935), and agree in general with some of the ideas on *Mycoplasma* growth and morphology presented by Klieneberger-Nobel (1962). Most of the pleomorphic sizes and shapes of *Mycoplasma* particles (rods, filaments, elipsoids, etc.) observed are apparently the result of the artificial chemical and physical manipulations employed during preparation of the specimen (Liebermeister, 1960; Weibul and Lundin, 1962). The flat, irregular disc-shaped *Mycoplasma* frequently observed in solid media growth were also observed in liquid media and were found to be the result of surface drying (Fig. 6a).

The size of colony growth on solid media is primarily dependent on such conditions as agar fluidity or hardness, chemical composition, and the inoculum concentration, which would influence the disruption and distribution of the mycoplasma particles. Although the absolute minimal reproductive unit size is difficult to determine, the few CFU that pass through the 100-mμ Millipore filters are certainly indicative of the minimal size range of particles in the *M. hominis* cultures. Chains and clusters of particles approximately 100 mμ can be seen in Fig. 7 and 8, evidently just prior to their cleavage from the other five segments. The shadowed electron photomicrographs of the avian and human strains (Fig. 8a and 8b) illustrate their similar stages of growth as well as their fragility with the resultant pleomorphic forms. With suitable precautions, the results of this technique seem to provide a new dimension in the visualization of *Mycoplasma* physical structure during stages of development.

DISCUSSION

The results of this investigation indicate that the modified sedimentation technique is a suitable method for standardizing and identifying *Mycoplasma* in broth cultures. The development and application of this method was based on the recognition and enumeration of the individual *Mycoplasma* particles. In previous studies, Clark et al.

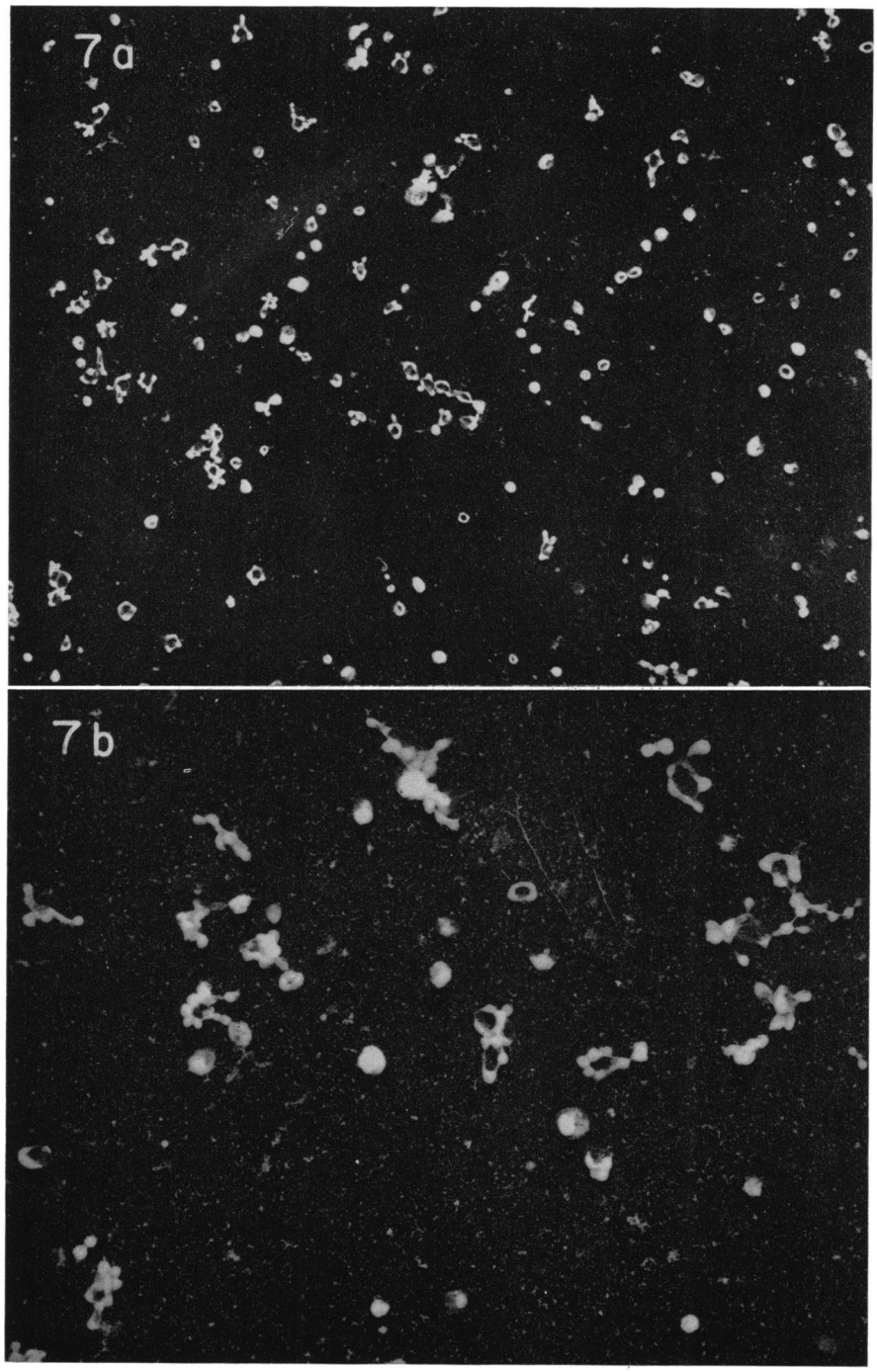

FIG. 7. *Mycoplasma gallisepticum (5969) broth culture sedimented on electron microscope grids, fixed with OsO_4, and shadowed as described. (a) Variable sizes and shapes in the growth cycle stages. (b) Another preparation at × 6,000 magnification showing the stages of growth and the segmented particles.*

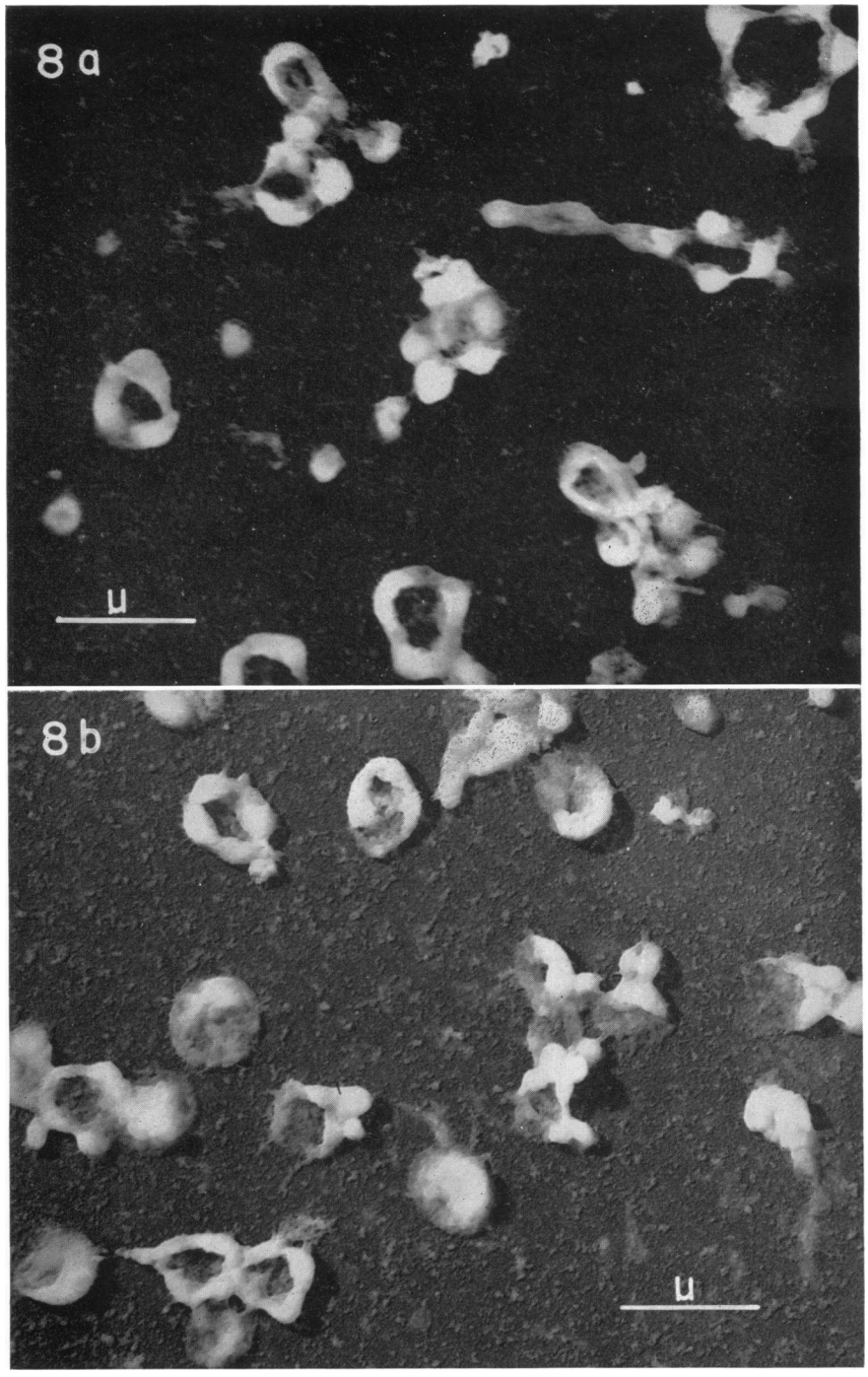

FIG. 8. *Diluted Mycoplasma broth cultures sedimented on electron microscope grids, fixed with OsO_4, and shadowed. (a) M. gallisepticum (5969) 24-hr culture showing three basic stages of growth. (b) M. hominis, type 2, 72-hr culture showing similar stages of growth along with the distorted shapes evidently produced in preparation.*

(1961) developed a new technique for the qualitative transfer and identification of *Mycoplasma* colonies growing on agar. The development of a rapid and simplified method for analysis of the individual *Mycoplasma* particle now makes it possible for other laboratories to compare and confirm *Mycoplasma* strain differences and similarities.

Although only 99.9% of the viable *Mycoplasma* particles are sedimented in 20 min at 27,000 $\times$ *g*, it is sufficiently complete within the limits of the fixation and counting procedures to be acceptable. Counting *Mycoplasma* particles by the sedimentation technique has the advantage of providing a suitable specimen for both morphological and quantitative analysis by using unaltered specimens and direct procedures. Under the conditions specified, the yield of sedimented *Mycoplasma* CFU is greater than 99.9%, which is as good or better than can be obtained from many physical and chemical comparative analyses. These results agree with the graded filtration studies which indicate that all of the 220 mμ and larger-sized CFU particles are sedimented, as are only a few of the smaller, minimal-sized CFU. Another possible source of error would come from particles with low density and the resultant flotation. Such might be the case if the missing "filamentous" forms were fragments of the lipoprotein membrane. However, the insoluble membrane fraction has been found to sediment readily under these conditions. Because of the many sources of variables and possible experimental errors, this direct method of quantitating *Mycoplasma* is undoubtedly less accurate than the indirect methods. The extent of *Mycoplasma* aggregation in broth cultures and its influence on CFU and particle counts can be estimated from the electron photomicrographs. The apparent influence of aggregation could be attributed to the colonylike growth of segmented particles with failure to undergo cleavage (Fig. 7a). Brief exposure of some cultures to sonic oscillation results in the release of additional CFU, whereas brief exposures in distilled water causes marked loss of viability with no appreciable change in general morphology.

The spray technique of particle counting (Backus and Williams, 1950) would probably yield more accurate quantitative analysis. However, this method was not attempted because it would have the disadvantages of being more complicated and yielding morphologically altered preparations by the dry contact fixation.

The *Mycoplasma* lipoprotein membrane has stretched our imaginations with about every conceivable pleomorphic form. It now seems advantageous to gently and rapidly stop the stretch and fix a uniformly distributed preparation with OsO_4. The primary advantage of the sedimentation technique is based on the *Mycoplasma* characteristic of strong adhesion to various surfaces while still in its original form and prior to complete fixation. Human, avian, and rat strains of mycoplasma have been examined by this method. The surprising and yet encouraging results obtained indicate that these strains are morphologically similar to the first identified strain, *M. mycoides*, characterized by Borrel et al. (1910) and Turner (1935).

If filaments are a characteristic morphological stage in growth development of *Mycoplasma*, as reported by some investigators (Freundt, 1958), they apparently are not produced in the culture media used in this laboratory. A few filaments have been observed occasionally in duplicate specimens; however, they apparently were artificial (Fig. 3a). On one occasion, filamentous forms were found contaminating the distilled water reservoir. As yet, no naturally occurring filaments have been observed in the sedimented cultures fixed with OsO_4, nor in the *Mycoplasma* colonies fixed with hot water after adhesion on a glass slide. Consequently, it is difficult to account for an intermediate or terminal filamentous stage of endomycelial development with the definite stages of coccoidal growth observed in the broth cultures.

The conclusions drawn from this study essentially confirm the basic stages of growth reported by Klieneberger-Nobel (1962). In addition, our studies indicate that this unique biological growth system is the orderly development of a constant number of segmented particles, primarily six, in the final stages of growth. It is anticipated that future investigations will indicate whether the segmentation number is a species characteristic (*M. laidlawii*), dependent upon culture conditions, or is due to the geometric physical resistance of the membrane. Any explanation of the conditions directing *Mycoplasma* reproduction should also include possible mechanisms of the hexadic fission with the production of minimal reproductive units of 1.2×10^{-10} μg mass and a small genetic coding DNA molecule (approximately 4×10^6).

This study again raises the old question whether Mycoplasmatales has filamentous or coccoidal morphology. If their fundamental morphology is coccoidal, as this study indicates, the appropriatness of the generic term Mycoplasmatales is certainly challenged.

ACKNOWLEDGMENTS

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