

THE MORPHOLOGY OF BACTERIUM TULARENSE

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Divergent views exist concerning the morphology of *Bacterium tularense*. McCoy and Chapin's (1912) original description depicted an apparently immotile, small, questionably encapsulated, pleomorphic organism occasionally presenting enlarged, irregular, and apparent involution forms; sometimes with predominant large globular forms. Ohara, Kobayashi, and Kudo (1935) claimed to have demonstrated flagella on both Japanese and American strains, stated that motility was observed repeatedly, and described clubbed, comma-shaped, dumbbell, and triangular forms. They further stated, "The more virulent is the bacterium, the greater is the pleomorphism"; also, "Virulence, pleomorphism, and motion are closely related and vary together." Galli-Valerio (1938), after working with cultures isolated by Drbohlav in Czechoslovakia, stated that both coccoid and bacillary forms were absolutely immotile and that no flagella were demonstrable by the Casares-Gil stain. He also failed to find any enlarged, elongated, filamentous, or involution forms. Most European language reports and textbooks omit all reference to flagella, state that the bacterium is non-motile, present inadequate descriptions of the extraordinary pleomorphism, give scant mention to encapsulation except as an occasional finding in tissues or in tissue smears, and classify the organism among either *Pasteurella* or *Brucella*.

Since the most extensive studies on morphology were conducted by Ohara and his associates, we reviewed thoroughly the Japanese literature. Ota (1936) states that Kudo and Kobayashi, working in Ohara's laboratory, first demonstrated a single polar flagellum in 1934, using the silver deposition method of Nishigawa and Sugahara (apparently the same as the Saisawa-Sugawara method mentioned by Ohara). They also observed capsules. Ota confirmed this work. He demonstrated flagella also with Victoria blue (4R), Burri's India ink method, and by his own modification of Benian's Congo red method. In his experience the methods of Loeffler, Benian, Zettnow, Inouye, Yokota, and Uyeno either failed entirely or showed few poorly stained flagella. Under dark-field illumination, "Refractile flagella were demonstrated." With regard to motility, "I found some actively motile, definitely changing their position." Capsules were well demonstrated by Ota's modification of Benian's method, mercurochrome negative staining, and by Gin's India-ink carbol-thionine method. The methods of Johnne, Wadsworth, Hiss, Welch, and Friedlander were said to stain them poorly or not at all. Ota stained *Bacterium tularense* and Yato-byo bacteria, also their flagella, in tissues with the Levaditi method. Successful preparations were made from human lymph node and skin, guinea pig spleen, and rabbit liver.

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Ohara, Kobayashi, and Kudo used the Saisawa-Sugawara silver deposition method. They described the typical *Bacterium tularensis* as a coccus or bacillus with a single polar flagellum. Yato-byo bacteria were said to have longer flagella than American strains of *Bacterium tularensis*. They noted bacterial forms connected by "flagella," also apparent multiple flagella, and instances of three cocci united by "flagella," but assumed that these images were preparational artifacts. Their American strains were 38, Hen, Va, and Col, all received originally from E. Francis, and sent to Kudo by us.

With regard to motility these workers stated that the bacterium "has a certain active movement," and that the degree of motility was proportionally related to virulence. They described its motility as "slower than the movement of cholera vibrio and much quicker than that of typhoid bacillus." Addition of a drop of mercuric chloride solution to the bacterial suspension was said to cause motility to disappear slowly, leaving only Brownian movement.

Ohara (1940) further mentioned the "specific active movement" and stated that the organism is monotrichate with a polar flagellum, also a capsule. A privately printed reprint of this complete paper, containing excellent photomicrographs, was presented to members of the Third International Congress of Microbiology. Ohara's statement that the marked difference in severity between tularemia and yato-byo is not due to inherent differences in virulence between Japanese and American strains, but rather due to the constitution of the Japanese people, should be modified in accordance with the experience related by Ota, who describes in detail the clinical course of disease in his laboratory technician infected by the American strain, Hen. The patient developed pleurisy and pneumonia with bloody sputum during the first week. During the second week he was irrational, with fever from 40.4 to 41.9 C (107.4 F), and had anuria for 7 days. Seven thoracenteses were performed. He was febrile for 64 days and disabled for more than 3 months. Agglutinin titers ascended from zero in the first week to 1:640 in the third week, and 1:2,560 in the sixth week of disease. The infecting strain was recovered from the pleural fluid.

We wish to record results of a systematic study of the morphology of this microorganism. Forty-three strains were examined, 21 from our collection and 22 others supplied by Dr. Edward Francis from the National Institute of Health. The histories of these strains are shown in table 1. Attention is directed to the wide range in geographic origin, to the diversity of pathologic sources, to an almost complete series of annual original isolations from 1920 to 1942, and to the periods of cultivation on artificial media which extended from 22 years to 2 days. The virulence range extended from maximal—killing all mice, guinea pigs, and rabbits within 3 to 5 days after dermal inoculation or after parenteral injection of 0.5 ml of 48- to 72-hour cultures in saline dilution of $T-500 \times 10^{-9}$ —to absolute nonvirulence—no illness in any of 12 mice and 12 guinea pigs after injection of approximately 12 billions of bacteria from a 48-hour culture into each animal.

METHODS OF STUDY

Motility. Motility was studied in salt-solution hanging drops and in vaseline-paraffin-luted cover slip preparations, by both direct illumination and dark-field

TABLE 1
Histories of the strains examined

DESIGNATION OF CULTURE	YEAR OF ORIGINAL ISOLATION	PATROLOGIC SOURCE	GEOGRAPHIC SOURCE	INTERVAL BETWEEN LAST ANIMAL ISOLATION AND OUR EXAMINATIONS
38	1920	Human Ly. node	Utah	22 years
26	1921	Human blood	Utah	21 "
SF	1922	Ground squirrel	Calif.	20 "
T	1923	Wood tick	Montana	19 "
V	1924	Human spleen	D. C.	11 "
Sn	1925	Wild hare	Montana	17 "
Jap	1926	Human Ly. node	Japan	16 "
LR	1927	Human ascitic Fl.	Arkansas	15 "
Max	1928	Human Ly. node	Russia	14 "
Russ	1928	" " "	"	14 "
Can	1930	Wild hare	Canada	12 "
Ohara	1931	Unspecified	Japan	11 "
Ri	1932	Human pus	Virginia	10 "
Fox I	1933	Gray fox spleen	Minnesota	9 "
Col	1933	Human pleural Fl.	So. Car.	8 "
Ll	1934	Human pus	Canada	8 "
Tol	1934	Human blood	Ohio	7 "
T-418	1934	Human blood	Ohio	6 "
L.C.	1935	Human ulcer	Virginia	7 "
H.D.	1935	Human eye	Austria	7 "
Md	1936	Human pleura	Maryland	6 "
PF	1936	Human lung	Ohio	2 "
Chr	1937	Human pus	Ohio	12 days
Sto	1937	Human blood	Ohio	5 years
Chil	1937	Human pus	Ohio	4 "
Trot	1937	Human lung	Ohio	8 months
Die	1938	Human ulcer	Calif.	2 years
Memp	1938	Human Ly. node	Tenn.	10 days
De P	1938	" " "	Ohio	2 years
Pi	1938	Human blood	Ohio	8 days
Pack	1939	Human sputum	D. C.	2 years
V.F.	1940	Human pleural Fl.	Tenn.	2 "
Hugh	1940	Human ulcer	Ohio	2 "
Clem	1940	Human blood	Ohio	2 "
Fox	1940	" " "	Ohio	1 "
Broo	1941	Human spleen	New York	1 "
Fish	1941	Human ulcer	Ohio	10 months
Well	1941	Human pus	Ohio	9 "
Fran	1941	Human sputum	Ohio	5 "
Gib	1941	Human ulcer	Ohio	3 "
Schu	1941	" " "	Ohio	3 days
Chur	1941	Human lung	Ohio	2 "
Bish	1942	Human pleural Fl.	Tenn.	4 months

examination. Our associates, Tamura and Gibby (1943), provided semisynthetic and synthetic liquid media in which heavy growth develops from inocula of approximately 15 organisms to 5 ml of media. Observations on morphology and motility were also made of cultures in these media. Some strains had been

propagated in liquid media for months; others were sown into liquid and observation preparations were made daily for 8 days, the average period of multiplication.

Fixed and stained smears. The methods used to stain for flagella were those of Gray, Fontana-Tribondeau, Saisawa-Sugawara, Casares-Gil, Von Ermengem, Leifson, Inouye, and Weiss. For capsule staining we used the methods of Churchman, Welch, Anthony, Wherry, and Hiss.

Preparation of bacterial suspensions. Most cultures were grown on solid media for 24 to 72 hours. Physiologic salt solution proved better than distilled water as a suspending medium. Satisfactory suspensions were made in either of two ways:

1. A small amount of surface growth was removed with a loop and very gently shaken into a tube of salt solution. The tube was incubated at 37 C for 1 to 2 hours until a uniform suspension resulted. Occasional gentle rotation accelerated this process, but shaking broke off most "flagella." The cohesive nature of the growth made it difficult to dislodge it from the loop.

2. One or 2 ml of salt solution were pipetted into the end of a culture slant, the tube was incubated until there was visible turbidity, then some of this suspension was pipetted to another tube of salt solution. This was the better method.

These suspensions were good for "flagella" demonstration for at least 3 days, but "capsules" were best shown during the first 6 hours. Preparations were also made from growth in liquid media.

Thin air-dried films were stained on both slides and cover slips. Preheating slides in a gas flame gave preparations with the clearest backgrounds. Although the Gray stain must be mixed for each day, we found it left less background deposit if it was allowed to stand 1 hour before using. Beyond this, the technique was simply that always necessary for success with special staining methods—scrupulously clean glassware, experience with the methods, and patience.

Supravital staining of living organisms. Each strain was also studied by supravital staining, either suspended in saline from growth on solid medium or directly in gelatin-hydrolyzate liquid medium. More than 30 dyes were used, but only Nile blue sulphate, Hofmann's violet (Dr. G. Grüber & Co.), Janus green B, malachite green, safranin O, gentian violet, and Bismarck brown Y proved useful. These were used chiefly as saturated solutions in saline, adding 1 small loopful of freshly prepared and filtered dye to an equivalent amount of suspension or culture on a slide, mixing, covering with cover slip, and sealing. Malachite green was used in 2, 3, and 5 per cent aqueous solutions. Examinations were made both by direct illumination and by dark field. Victoria blue (4R) also would probably have been satisfactory, judging from Ota's experience with fixed smears. We were unable to obtain it.

Since most of our photomicrographs of living organisms required exposures in excess of 15 seconds, we had difficulty with Brownian movement. Cover slips could not be rolled down close without producing numerous artifacts. Our most

successful method to avoid blurred images was to melt a drop or two of ordinary 14 per cent gelatin medium by gentle heat in the center of the slides, heating until the gelatin flowed into a thin, even layer. After solidification of the gelatin, preparations were made and sealed as usual. Organisms entrapped in the lower viscous layer were motionless and not deformed.

Examination of blood from infected rodents. Heart blood drawn from moribund or dead experimentally infected mice and guinea pigs was examined by direct illumination and by dark field in sealed cover slips immediately as drawn, diluted in sterile distilled water, in sterile saline, and in gelatin hydrolyzate medium; all both unstained and supravivally stained.

Control of methods of study by application to other bacteria. Since formation of artifacts by technical procedures is an ever-present hazard in studies on morphology, especially when new or infrequently used methods are employed, we subjected one or more strains of other bacteria to the special staining methods listed above. Strains of *Escherichia coli*, *Eberthella typhosa*, *Pseudomonas aeruginosa*, *Brucella melitensis*, *Pasteurella pestis*, and *Pasteurella pseudotuberculosis-rodentium* failed to reveal any new or unusual morphologic features when examined by these methods.

RESULTS OF OBSERVATIONS OF STAINED SMEARS

Examination of fixed smears stained by customary dyes, or even by special stains, is at best a poor method to reveal the morphology of this organism. No other method used produced as many deceptive images or as many artifacts except the examination of heart blood from infected rodents. Nevertheless the following observations are recorded, and illustrated in plates I and II, to provide comparison and correlation between previous studies and the results of more suitable methods of examination that follow.

Demonstration of structures that simulated capsules. Structures that simulated capsules were best demonstrated by either the Gray or the Saisawa-Sugawara flagella stains; less well by the Fontana-Tribondeau and Casares-Gil stains. We failed to demonstrate them with the Churchman, Welch, Wherry, Anthony, or Hiss capsule stains, though modifications of each were tried.

These structures were demonstrated on every strain. Although they looked like capsules to casual inspection, critical observation showed that they were not extracellular structures but actually cell walls, a feature better demonstrated by other methods of study that follow. Here it will suffice to note that the coccoid form of the organism, whether of small, medium, or large size, usually showed the cell wall. It was rarely seen on bacillary forms. When coccoid and bacillary forms appeared in chain formation it was usual to find cell walls only on the former. The very large coccoid forms often showed thick cell walls, and frequently two or three minute, circular, clear areas, or, sometimes, indentations were noted within such cell walls, usually almost exactly 120° of arc apart. Whenever such forms possessed "flagella," these structures seemed to arise from these circular areas.

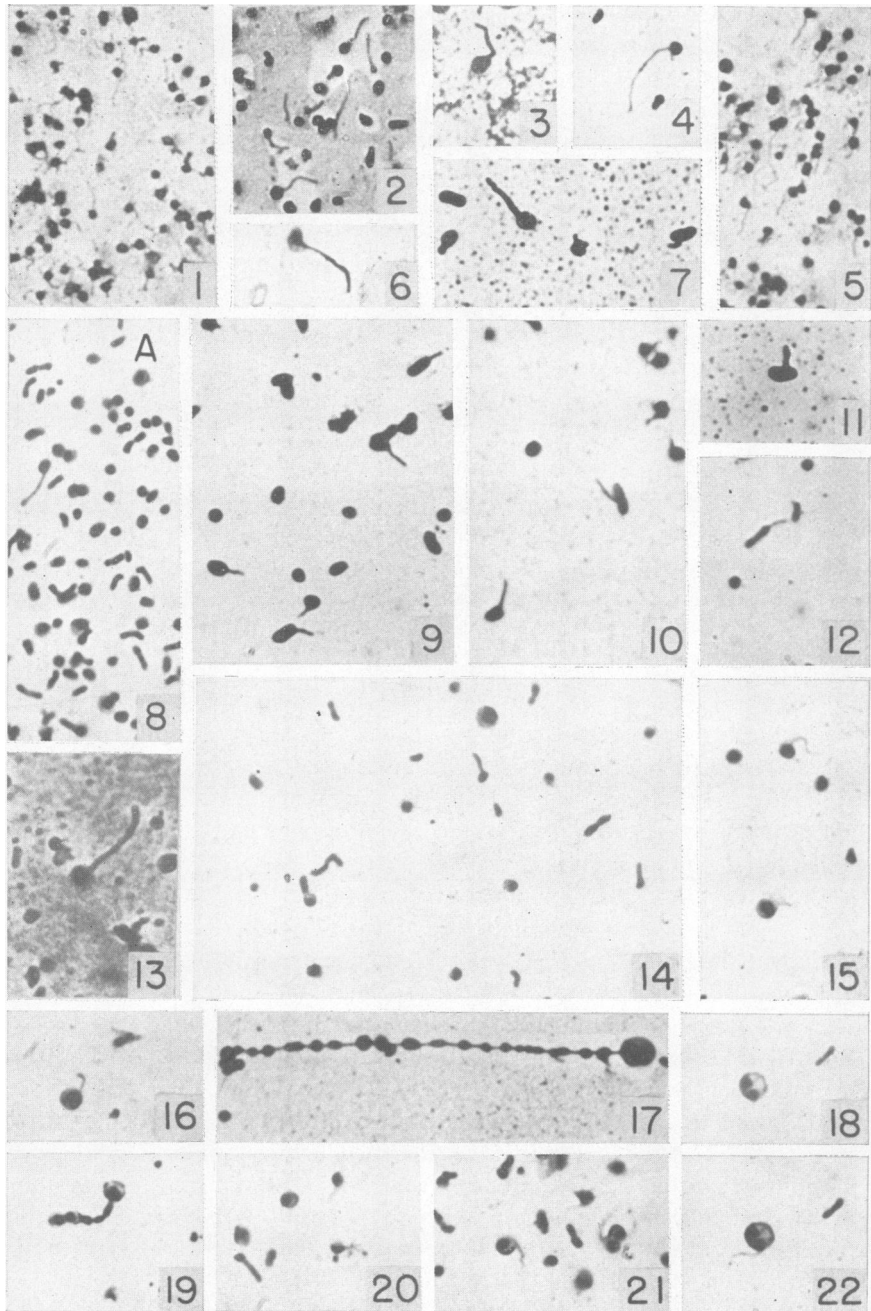


PLATE I

Photomicrographs of stained smears. Reproduced with slight reduction from initial magnifications of 2,250 diameters.

1. Chri strain, virulent. Saisawa-Sugawara silver stain. Usual coccoid forms. Note how closely the filaments resemble flagella; also the small budding form near top center. These coccoid forms average 0.45μ in diameter.

Cell walls were formed by both avirulent and virulent strains; by recently isolated cultures as well as by old cultures propagated only on media for more than 20 years.

Involution forms. Classical involution forms were observed in stained smears in about half of the strains. Although typical examples were seen and photographed in an avirulent strain propagated only on media for 22 years, they occurred more frequently in virulent strains, and most frequently in recently isolated cultures. Dumbbell, bean-shaped, spermatozoonlike, and many bizarre forms were seen, as well as L-shaped and irregularly knobby globoid forms. Long filamentous forms like those produced by the plague bacillus were not observed, but many shorter and bizarre filamented forms were seen, some of which are reproduced. It can be said here that none of these apparently typical forms were true involution forms, and that their real nature will be demonstrated in succeeding paragraphs.

The appearance of flagella in stained preparations. Very fine filamentous

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2. Col strain, virulent. S-S stain. Slide prepared by Kobayashi, in Fukushima, in 1935; our photomicrograph. Note budding, which the Japanese overlooked, also the minimal reproductive units on filaments near lower left center.
 3. 38 strain, avirulent. Gray stain. No animal passage since 1920.
 4. Schu strain, virulent. Gray stain. Note two M.R.U. near middle of filament. Note minute second filament, also budding of the lower coccoid form.
 5. H.D. strain, virulent. Casares-Gil stain. Note similarity of this Austrian strain to Ohio strain of figure 1. Filamented coccoid forms.
 6. 38 strain, avirulent. S-S stain. Note filament extending through cell wall.
 7. Schu strain. Gray stain. Short and long filaments. Budding at left. Heavily mordanted preparation.
 8. Same. Long filament connects usual coccoid form with a M.R.U. Cell walls on many "cocci." Drumstick form at bottom. At "A" is shown a heavily stained cell wall. Note evidence of budding, also suggestion of division by fission.
 - 9, 10, 11. Same. Filamented coccoid and bacillary forms. Attachments of filaments in various locations.
 12. Schu strain. Casares-Gil stain. Bacillary form with terminal filament.
 13. Schu strain. Gray stain, heavily mordanted. Thick filament on coccoid form.
 14. Same. Lightly mordanted. Delicate cell walls on large and small "cocci." Drumstick forms.
 15. Same. False "flagella" produced by protoplasmic streamers from ruptured cells. At bottom, part of a filament is seen within the cell wall, opposite the site of rupture.
 16. Same. Globule showing cell wall and a filament with a M.R.U. at tip. Coccoid form is about $1\ \mu$ in diameter.
 17. Max strain. Casares-Gil stain. Long beaded chain with filamentous linkage, probably formed by budding from the globus.
 18. Schu strain. Gray stain. Focused to show a segmented globus containing 2 coccoid forms. The filament of the upper "coccus" is within the cell wall and shows a bulbous tip.
 19. Same. Sessile budding producing nonfilamented chains. Note that coccoid forms show cell walls and that bacillary forms do not. This is usual.
 20. Same. Small globule with well-stained cell wall. Drumstick form at lower left.
 21. Same. Traumatized coccoid forms with ruptured cell walls (cf. 15), showing filaments partially uncoiled from original enclosed position.
 22. Same globus as in figure 18. Focused deeper to show external protrusion of filament of lower small coccoid form.

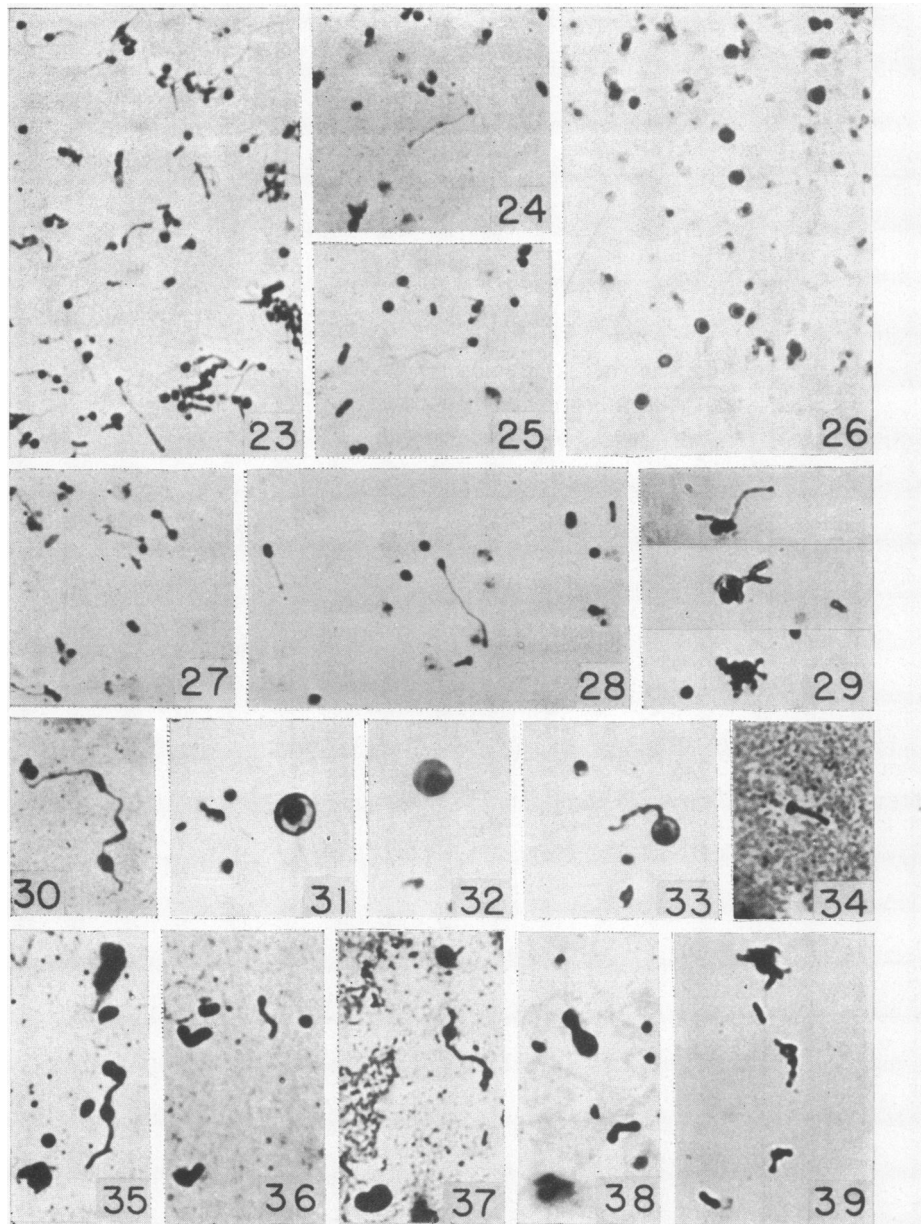


PLATE II

Photomicrographs of stained smears. Reproduced with slight reduction from initial magnification of 2,250 diameters.

23. Die strain. S-S stain. Note frequent filamentous linkage of various forms. A filamented triangular zooglealike mass at lower left. Many M.R.U. can be seen arising within filaments.
24. Ri strain. S-S stain. Filamentous connection (production?) of M.R.U.
25. Same. Note length of filament.

structures, identical with those the Japanese students call "flagella," were observed on every strain. They were best demonstrated by the Gray, Casares-Gil, and Saisawa-Sugawara staining methods. Excellent preparations were made by each method, and occasional good ones by the Fontana-Tribondeau stain. We were unable to demonstrate these structures with the staining methods of Von Ermengem, Leifson, Inouye, or Weiss. Prolonged staining with Wright's stain, Giemsa stain, or hematoxylin occasionally demonstrated them, but poorly. The pale colors, and the minute size owing to absence of previous mordanting, made such preparations poor subjects for photography. One plate was obtained from which measurements could be made.

In the unmordanted state a coccoid form of the usual size has a diameter of about 0.45μ . The form early noted by us as the "minute coccus" was not seen in unmordanted preparations. After heavy mordanting these forms have a diameter not greater than 0.2μ , and cannot be distinguished from precipitated stain unless they possess a "flagellum." Giant "cocci," and some so-called involution forms, have diameters as large as 3.0μ . Length of "flagellum" varies greatly, from 0.5μ to 8.0μ or longer. A few specimens stained without previous mordanting showed "flagella" of uniform thickness, but really so thin that they were discernible with difficulty under critical illumination. They appeared to be not greater than 0.05μ in thickness.

Although Ohara stated that the longer "flagellum" of Yato-byo bacteria differentiates them from *Bacterium tularense*, we were unable to find significant differences in length among our 43 strains.

These flagellalike structures were seen most frequently on coccoid forms, regardless of size; less frequently on bacillary forms. Many showed bulbous tips

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26. 38 strain. S-S stain. Note well-stained cell walls on this old laboratory strain.
 27. Ri strain. S-S stain. Filamentous linkage between various forms.
 28. Same. Illustrates filamentous development of coccoid and bacillary forms. Note M.R.U. at end of filament, at left.
 29. Memp strain. Fontana-Tribondeau stain.
Upper. Pullulation of filaments containing M.R.U.
Middle. Globus containing 2 coccoid bodies, the whole attached to a branched mycelium. Strongly suggestive of a sporangiophore, sporangium, and 2 sporangiospores.
Lower. Radial filamentation at right and below; continuous sessile budding at left upper.
 30. Max strain. Casares-Gil stain. Filamentous development of coccoid and bacillary forms, and M.R.U.
 31. Schu strain. Gray stain. A 2.5μ globus with thick cell wall. Note eccentric chromatin, and indentation in cell wall that denotes a potential budding site.
 32. Same slide. Globus with extremely delicate cell wall.
 33. Schu strain. Gray stain. Globule with a chain of small coccoid and bacillary forms produced by sessile budding. Note additional potential budding site on right edge of globule, marked by tiny ring in cell wall.
 34. Max strain. Casares-Gil stain, heavily mordanted. Drumstick form.
 35. Schu strain. Gray stain. Filamented chain.
 36. 38 strain. S-S stain. So-called involution forms, spermatozoon form.
 37. Same. Globoid "involution" forms, and filamented chain of coccoid forms.
 38. Same. More so-called "involution" forms.
 39. Memp strain. Fontana-Tribondeau stain. Sessile and filamentous budding, branching.

at the free end. Others frequently showed another coccoid or bacillary form at this end, giving the appearance of two bacteria united by a fine filament. Other fine filamentous structures united 3, 4, 5, or more than 20 bodies of either coccoid or bacillary shapes. The appearance of multiple flagella was also noted. At first we thought all such images were preparational artifacts and, indeed, by judicious variations in methods of preparation, especially in depth of mordanting and staining, most such images from a given suspension could be resolved into apparently monotrichate bacteria lying very near free "flagella," the free end of another "flagellum," and so forth. Although light mordanting revealed many such images as artifacts, it did not dispose of the matter of various bacterial forms united by fine filaments. Every strain continued to show united forms under critically controlled variations in technique.

Differentiation between flagella-sized filaments and protoplasmic streamers due to traumatic rupture of cell walls was easily made. Filaments stained evenly and deeply, with sides parallel throughout their length. Protoplasmic streamers stained lightly, often unevenly, and tapered from a broad base near the soma to fine single or multiple distal tips.

When the so-called "flagella" appeared on bacteria with cell walls, they sometimes seemed to arise from the soma and penetrate through the cell wall. Others seemed to arise from the cell wall. Similar appearances in relation to the "capsules" of *Bacillus subtilis* were noted and discussed by Churchman (1933). With regard to *Bacterium tularense*, we believe this disparateness is due to variations in staining technique. From a single suspension we made preparations that showed first one appearance, then the other. We occasionally saw both forms on the same slide, the "flagellum" not visible through the cell wall in lightly stained areas, but well shown within it in more heavily stained areas.

In some coccoid forms of *Bacterium tularense* the "flagellum" was very clearly seen coiled within the intact cell wall. This is shown in some of the photomicrographs. In the largest forms the internal location of the filament was exceptionally well shown, where they commonly heavily accentuated about one third of the internal perimeter of the cell wall. If cell walls had been broken by trauma, these filamentous structures could frequently be seen partially uncoiled from the original position and easily differentiated from the associated protoplasmic streamers. One photomicrograph shows this arrangement very clearly.

The coccoid form was the one most frequently seen, and most "flagella" and most cell walls were seen on this form. It was the only form in which "flagella" were observed within cell walls. One might argue that, if the filaments observed were true flagella, the almost universal failure of skilled observers to see motility of *Bacterium tularense* might result in part from the frequent internal location of flagella, especially since more than 3 hours are required for cell walls to disintegrate in salt solution. This hypothesis implies that proper or suitable conditions for observation of motility might never have been secured, an hypothesis which was readily made untenable by our study of living cultures in extremely favorable liquid media by dark-field illumination and by supravital staining.

OBSERVATIONS ON LIVING CULTURES UNDER DARK-FIELD ILLUMINATION

All forms previously seen in stained preparations were readily recognized under the dark field. The "minute coccoid forms" were seen singly, in groups, in zoogleealike masses, and at either or both ends, or along the courses of extremely delicate nonmotile filaments. They varied in size from just smaller than the usual coccoid forms to such minute bodies that neither size nor shape was discernible. Many control preparations showed that these bodies were not present in our saline solution or in uninoculated liquid media. Coccoid forms of usual 0.45 to 0.5 μ size were not spherical but rather crescentically obovoid, shaped somewhat like a segment of an orange, with one end larger and rounder than the other. The refractile chromatin body was seldom central; usually peripherally located within a scarcely discernible, delicate cell wall. A moderate number of coccoid forms of this size revealed small budding spherules. Some were sessile; others pedunculated or attached by extremely fine filaments. Coccoid forms of 1.0 to 1.5 μ in diameter were more spherical, usually contained one or more highly refractile chromatin particles eccentrically located, and frequently exhibited both sessile and filamentous buds. The bipolar forms of the stained preparations were seen to be diploforms of the 0.5 μ size, shaped like a figure 8 in the dark field view, with a chromatin particle in each end, or bacillary forms with polar chromatin particles. The "giant cocci" were actually globular forms 2.0 and 3.5 μ in diameter. They were occasionally twinned, sharing the largest chromatin particle at the junction of the rounded free surfaces with the plane interface surface. In addition, many globi of this size presented moderate to numerous peripherally located, smaller, refractile particles, often arranged in meridional or equatorial belts. It was these globular forms, especially when actively budding, together with certain bacillarylike forms to be noted presently, that appeared in stained smears as the so-called "involution forms."

Bacillary forms were present in many sizes and shapes. In general, they were not strictly bacillary in shape with the short diameters of approximately equal size. They were taeniform, or ribbon-shaped, with one short diameter very much shorter than the other. In addition to the common flattened "bacilli" we noted a less frequent long form which was almost round, or quite round, in transverse diameter. These appeared like true filamentous forms of bacillary dimensions. They differed in being more highly refractile throughout their length, often tapered to a smaller diameter at one end. These also showed frequent budding.

All sizes of "cocci" and "bacilli" appeared singly, in diploform, in short or long chains, and sometimes in chain formation connected by filaments of various lengths. The predominant form was almost always the 0.45 μ "coccus." All 43 strains showed the same range of morphologic diversity.

The "flagella" described and photographed by Ohara and his associates were readily seen. Their appearance in dark-field preparations was identical with that in fixed preparations stained by the Japanese, in similar preparations made by us, and in the photomicrographs published by Ohara as well as in our own.

However, all such "flagella" were absolutely nonmotile, whether observed in water, salt solution, or liquid media. We watched many living preparations all day for many days, making a fresh one for a single culture every day throughout its period of multiplication. We saw thousands of these flagella-sized filamentous structures attached to all observed forms of the organism, but they were invariably nonmotile. The bacteria multiplied well in the liquid medium, and there was seldom doubt concerning viability of cells observed. Suspended dead cells, with or without "flagella," were mere shadow outlines of living ones but all, living or dead, showed only Brownian movement. The easiest way to locate a cell with a long "flagellum" was to look for one that, for its diameter, had *less* Brownian movement than would be expected. The longer the filament the more it restricted Brownian movement in rate and amplitude. If there was also an organism at the other end of the filament it behaved as a drag anchor, permitting almost no Brownian movement. Multiple "flagellated" living forms were seen, confirming our findings in stained preparations.

In living cultures filaments of flagellar size were seldom attached to only one organism. In stained preparations this appearance was very frequent, simulating true flagella, but it seems almost certain that this was an artifact caused by trauma incident to the preparation of dried films. In cultures the great majority of fine filaments connected two or more organisms of any of the forms noted.

We can offer a possible explanation for the "peculiar motion" so frequently mentioned by Ohara and his co-workers. Some bacillary forms, and all refractile rounded filamentous forms, exhibited active flexional movements. These squirmings rarely accomplished any motion of translation, but occasionally an especially vigorous bend effected a change in position of a micron or two. We frequently left such forms located in a field for an hour, to return and find the "bacillus" or "filament" not more than one-half field diameter from the point at which we left it. Ohara writes of thin and thick flagella. From our observations we believe all "thick flagella" are slender bacillary forms or the round refractile filaments. When a long bacillary form or filament had a coccoid form at one end, noted as the "drumstick" forms in our photomicrographs, the flexional movements of the rod-shaped section gave the appearance of a thick flagellum. We watched many such forms, and the greatest rate of movement of translation recorded was of the order of 15 μ per hour. We confirm the constant presence of flagella-sized filamentous structures, true filaments of bacillary transverse diameter, small, medium, and large globules, and extraordinary pleomorphism, but not motility or encapsulation.

Figure 1 shows drawings from dark-field observations of living cultures of some strains at various stages of growth. It must not be inferred that the forms shown were present in every field. At least one example of every form drawn was observed in every strain, but the filamented and other-bizarre forms represented only a small fraction of the total populations. The most numerous forms seen during any of the first 8 days of cultivation were always the coccoid and bacillary forms. Many of these were not filamented.

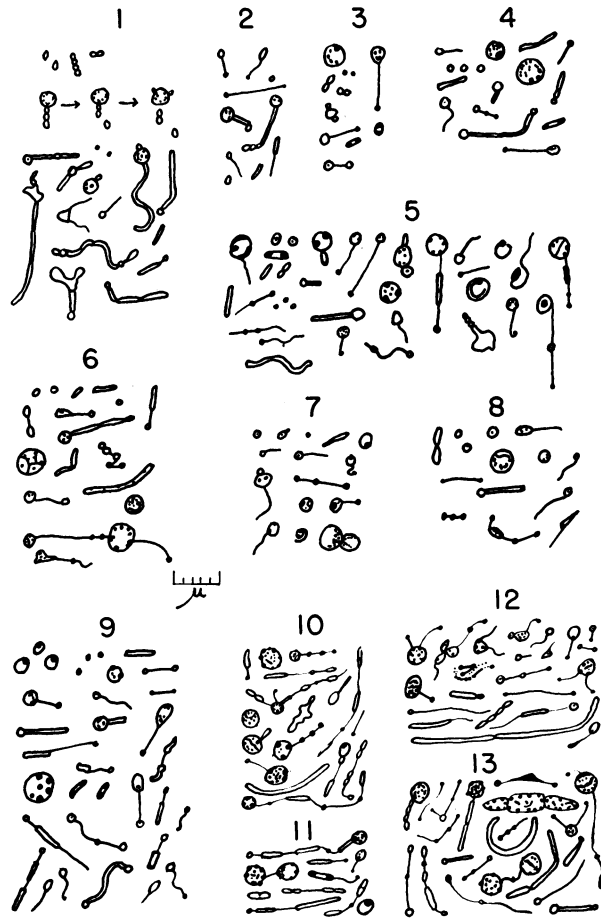


FIG. 1. SELECTED MORPHOLOGIC UNITS OF SEVERAL STRAINS OF BACTERIUM TULARENSE

Drawn from observations of living cultures in gelatin hydrolyzate media under dark-field illumination. The preparation from which no. 9 was drawn was made by suspending in saline solution a small amount of growth removed from a blood cystine agar slant; it is included to show that the same forms are produced by cultivation on the standard solid medium.

1. Strain Memp at 19 hours of incubation. Thirty to 40 minutes elapsed between the successive stages by the arrows. Note reproduction by budding.

2. Strain Memp, 44 hours.

3. Strain Memp, 90 hours.

4. Strain Memp, 5 days.

5. Strain Memp, 6 days. Note filament coiled within cell wall of one globule.

6. Strain Jap, 25 hours. Note filamentoed zooglee-like mass at bottom.

7. Strain Ohara, 48 hours.

8. Strain Ohara, 72 hours. At bottom note filaments derived from an ill-defined zooglee-like mass.

9. Strain Schu in saline solution; 72-hour culture. Signet ring forms, at upper left, were very common in all strains.

10. Strain Pack, 48 hours. Note evidence of reproduction by pullulation of filaments.

11. Strain Pack, 72 hours. Some forms shown near bottom suggested division by fission but this was never verified.

12. Strain Ri, 48 hours. Note multiple filamentation at upper left. Just below is a zooglee-like mass showing apparent fragmentation of filaments.

13. Strain Russ, 48 hours. The largest form, near top center, was not duplicated in entirety by any other strain but forms consisting of 1 or 2 such segments were noted in all strains.

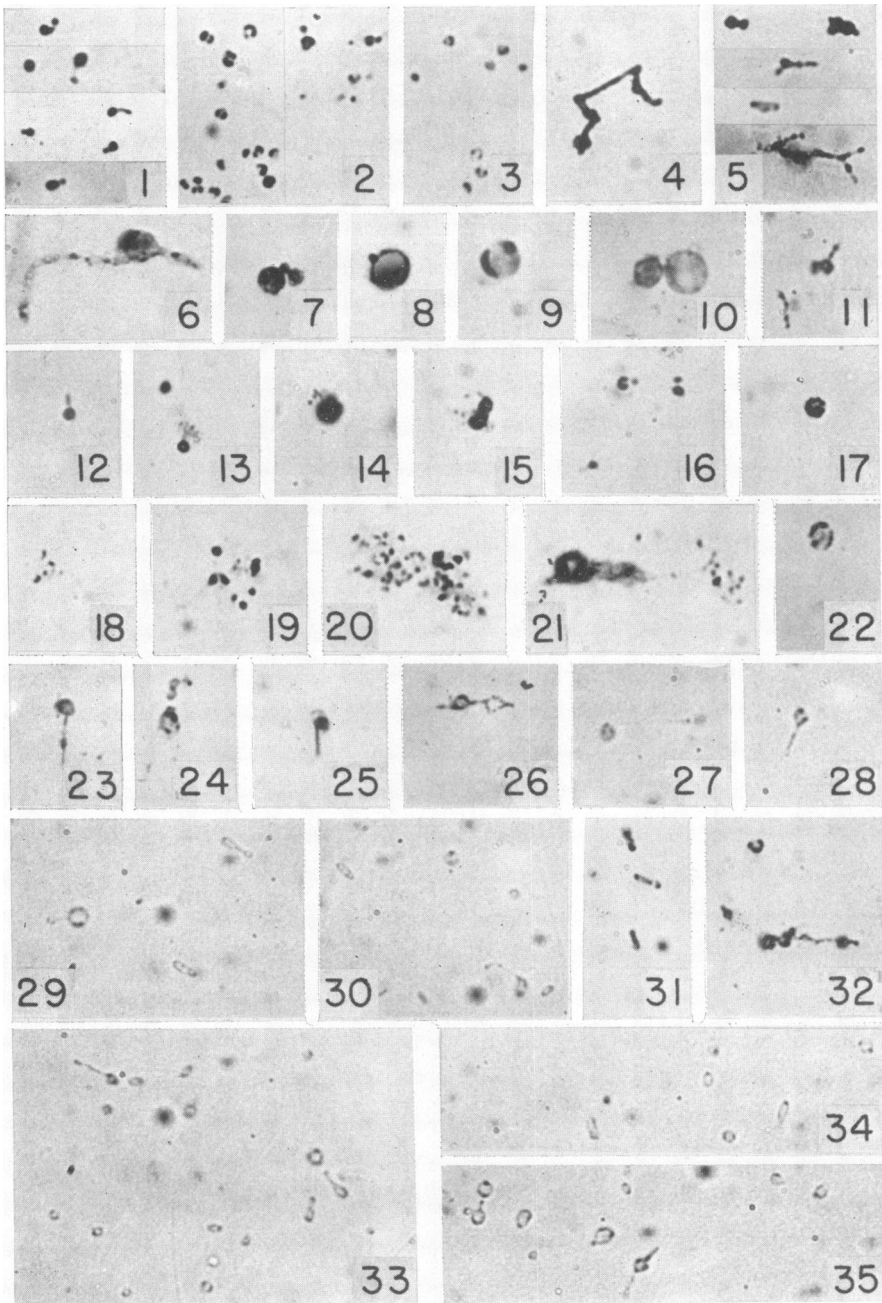


PLATE III

Photomicrographs of supravitaly stained organisms. Reproduced with slight reduction from original magnifications of 2,250 diameters.

1. Chri and Memp strains. Hofmann's violet and aqueous gentian violet stains. Sessile and filamentous budding. In gelatin hydrolyzate medium (Gel. HOH).

2. Left: Russ strain. Hofmann's violet in saline suspension from solid medium. Coccoid forms.
Right: Memp strain. Hofmann's violet, in Gel. HOH medium. Coccoid forms; filamented and sessile buds.
3. Memp strain. Hofmann's violet, in Gel. HOH. Coccoid forms.
4. 38 strain. Hofmann's violet, in saline suspension. Bizarre chain due to budding.
5. 1. Russ strain. Hofmann's violet, in saline suspension. Multiple budding.
2. Memp strain. Safranin O, in Gel. HOH. Budding producing branching.
3. Same. Bipolar staining bacillary form.
4. 38 strain. Bismarck brown Y, in saline suspension. Branched filament.
6. Memp strain. Bismarck brown Y, in Gel. HOH. Globus attached to a chain of coccoid and bacillary forms. Which gave origin to the other is not known. Globus shows heavy mass of chromatin at part opposite attachment site. Also within are 2 bodies of usual coccoid size. Suggests sporangium formation with 2 sporangiospores as usual number. (Cf. 29 in plate II, also 18 in plate I. See also 10 below.)
7. Russ strain. Hofmann's violet in saline suspension. Primary and secondary budding from a smaller globule. The globule also contains 2 bodies of usual coccoid dimensions. Note cell wall of secondary bud.
8. 38 strain. Bismarck brown Y, in saline suspension. Large globule with sessile bud.
9. 38 strain. Hofmann's violet, in saline suspension. Large globule. Note characteristic peripheral distribution of chromatin.
10. Schu strain. Bismarck brown Y, in saline suspension. Globus with globular bud on a short filament. Note 2 additional potential budding sites in perimeter of globus. The daughter globule contains 2 bodies of coccoid dimensions, also additional peripherally located chromatin.
11. 38 strain. Bismarck brown Y, in saline suspension. Twinned coccoid forms, one filamented. Note M.R.U. in filament.
12. Memp strain. Hofmann's violet in Gel. HOH medium. Coccoid form of usual size with very slender short filament containing 2 M.R.U.
13. Russ strain. Safranin O, in saline suspension. Small coccoid form with multi-branched filament. Note the M.R.U. at tips of fine branches.
14. Russ strain. Hofmann's violet, in saline suspension. A 2- μ globule showing part of a meridional budding fringe.
15. Same. Peripheral filamentous budding. Note the M.R.U. along the filaments.
16. Memp strain. Hofmann's violet, in Gel. HOH. Coccoid forms, one showing a filamented bud.
17. Schu strain. Hofmann's violet, in Gel. HOH. Globule with chromatin chiefly in granules at periphery.
18. Russ strain. Hofmann's violet, in saline suspension. Clump of filamentously connected M.R.U., some developing toward usual coccoid form size.
19. Same. Development of M.R.U. to larger forms. Note delicate filaments.
20. Memp strain. Hofmann's violet, in Gel. HOH. Clump of developing M.R.U.
21. Same. Ruptured globus. Parachutelike arrangement of delicate filaments, each connected to one or more minimal reproductive units.
22. Russ strain. Hofmann's violet, in saline suspension. Oval globule with peripherally distributed chromatin.
23. 38 strain. Bismarck brown Y, in saline suspension. Filamented coccoid form. Note peripherally distributed chromatin, and M.R.U. along course of filament.
24. Russ strain. Bismarck brown Y, in saline suspension. Globule with sessile bud. Also twinned coccoid forms, one filamented with a M.R.U. at tip.
25. Memp strain. Bismarck brown Y, in Gel. HOH. Filamented coccoid form showing delicate cell wall.
26. Russ strain. Bismarck brown Y, in saline suspension. Filamentous linkage between well-stained, living coccoid form and a lightly and unevenly stained, degenerating one.

OBSERVATIONS OF SUPRAVITALLY STAINED ORGANISMS

Supravital staining greatly enhanced visualization of all structural details and permitted better photographic registration than we could secure otherwise. Nuclear particles were stained within 10 minutes by most dyes and were seen to be typically peripheral in location and often multiple. Minute buds stained blue with Nile blue sulphate, and filaments stained faintly. Under the dark field the buds had a deep lilac-rose color. Hofmann's violet stained chromatin a deep violet color; minute buds and filaments a light violet. Under the dark field chromatin particles appeared red and yellow; the minute buds always a clear yellow. Malachite green also showed differential staining between nuclear chromatin and the minute buds, the former taking a clear green and the latter a terra cotta or brick red. Filaments stained poorly. Bismarck brown Y stained all structural parts including delicate cell walls and the finest filaments. Coccoid and bacillary forms, globules, globi, thick and thin filaments, and what appeared to be minimal reproductive units were readily visualized as demonstrated in plate III.

The chromatinlike particles within the coccoid forms were usually ring-shaped, discoidal, navicular, or crescentically obovoid, and almost always peripherally located, as noted previously. An extremely delicate, spheroidal, veil-like cell wall surrounded these forms, best seen when the diameters reached or exceeded 0.5μ .

In larger globoid forms nuclear chromatin was often dispersed into peripheral granules. The equatorial or meridional lines of granules around the circumferences of the larger globules sometimes gave origin to very short delicate filaments, each with a minute minimal reproductive unit as its free end, the entire rows undulating in harmonic wave motion and giving the appearance of budding fringes. Occasionally filaments were seen within globules; some forked or branched, others unbranched and coiled within the cell walls. Many of these internally located filaments were examined critically, and their cylindrical shape was repeatedly confirmed. However, it is quite possible that these structures

Two fine filaments spring from living unit, each with a M.R.U. at tip. Chromatin peripheral.

27. Memp strain. Bismarck brown Y, in Gel. HOH. Coccoid form with long slender filament with a M.R.U. at tip. Shows delicate cell wall and peripheral chromatin granules.
28. Same. Filamented coccoid form with M.R.U. in filament.
29. Same. Globular and bacillary forms. Note minute spherule at tip of filament.
30. Same. Coccoid and bacillary forms. Note M.R.U. in filament.
31. Schu strain. Hofmann's violet, in Gel. HOH. Bacillary forms with unevenly distributed chromatin.
32. Russ strain. Bismarck brown Y, in saline suspension. Filamentously connected coccoid forms with M.R.U. within filament.
33. Memp strain. Bismarck brown Y, in Gel. HOH. Coccoid and bacillary forms; some filamented with M.R.U. at tips of filaments.
34. Same. Coccoid and bacillary forms.
35. Same. Globules and dumbbell and coccoid forms, one filamented. Note peripherally distributed chromatin particles and minimal reproductive units within filaments.

may expand and eventually become globoid masses, thus accounting for the two sporangiosporelike bodies so often seen within globi.

Sessile and filamentous budding were observed. Chains of coccoid or bacillary forms, or chains composed of irregular mixtures of these forms, occasionally showed considerable branching from one or more nodal budding sites.

Minimal reproductive units were observed as suspended, discrete particles, at the termini of long or short filaments, and at various locations along the courses of filaments. Some small coccoid forms gave origin to a single short filament, which branched into a cluster of fine terminal filaments, each with a minute unit at its tip. These minute units were formed within or on filaments produced by coccoid, bacillary, or globular forms.

OBSERVATIONS OF INFECTED HEART BLOODS

Examination of infected heart bloods revealed with certainty only the small coccoid forms. The suspended minute particles in shed blood, the presence of degenerating blood cells, and filamentous structures derived from various cells made recognition of other microorganismal forms uncertain.

MODES OF REPRODUCTION OF BACTERIUM TULARENSE

Although we made frequent drawings of several strains at successive stages of growth, we could not consistently demonstrate a predominant morphologic phase at any time during the first 8 days. Even with very small inocula in gelatin hydrolyzate media it seemed that as soon as there were 15 to 20 organisms to the oil-immersion dark field a prolonged search would reveal at least one example of every form yet observed. Certain gross trends were noted. Large globi seldom developed before the third or fourth day unless the culture was incubated in an atmosphere of pure oxygen. Filaments, filamentous budding, and complex filamented chains seldom appeared before the third or fourth days. Early growth at room temperatures (24 to 26 C) was chiefly bacillary or mycelial in nature with frequent sessile budding. Filaments and filamentous budding appeared only after several days of growth, and were not frequent until the seventh day.

Figure 2 illustrates the trend of morphologic changes during the first week of cultivation in gelatin hydrolyzate medium of strain Memp. All tubes contained 5 ml of medium. The tubes for incubation at 37 C and at room temperature were each inoculated with 0.10 ml of a 24-hour liquid culture. The effect of an oxygen atmosphere was tested by inoculating similarly 7 other tubes of media, displacing air by pure filtered oxygen, pushing down the cotton plug, and sealing tube tops with parafilm. These tubes were incubated at 37 C. One tube was opened each day and swirled gently to resuspend any sediment; examinations were made of sealed cover slip preparations under dark-field illumination.

At no time during the two-year period of study could we demonstrate reproduction by binary fission. Since slide microcultures were seldom wholly satisfactory, possibly owing to obligate aerobic requirements of the organism, observations on modes of reproduction were necessarily limited to intermittent observations of cultures in gelatin hydrolyzate media.

Multiplication by budding was observed frequently, and this appeared to be the chief mode of reproduction. Both sessile and filamented buds were produced by bacillary forms, by refractile round filamentous forms, and by coccoid forms of all diameters from 0.45μ to the large globi of 3 to 3.5μ . Buds often appeared on the cell walls at the sites of the clear circular areas previously described, often at 120° of arc apart. They developed into coccoid forms of the usual size or, occasionally, into short or long bacillary forms. Chains of coccoid forms occasionally developed from a single budding site. Some chains were formed by

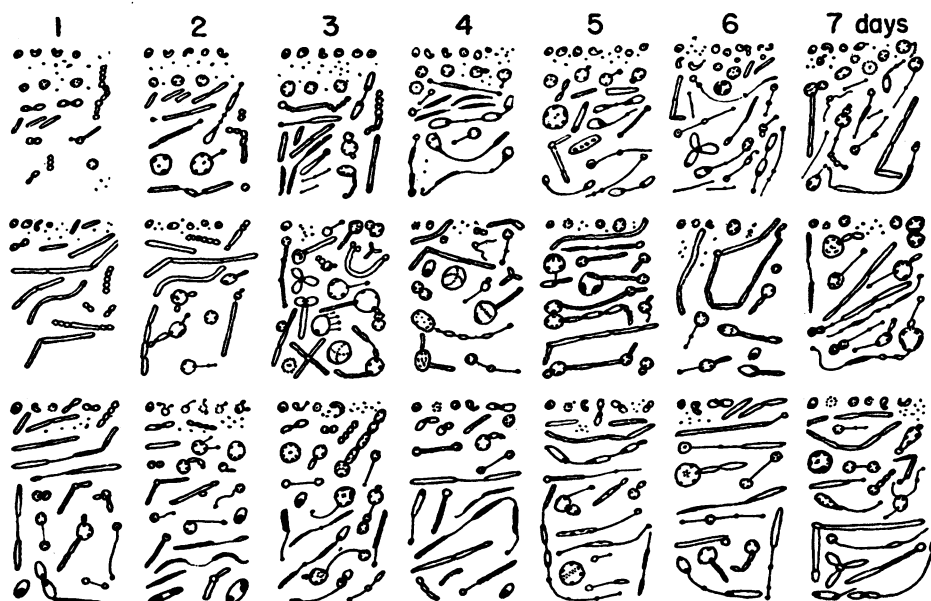


FIG. 2. Illustrates certain developmental trends of strain Memp in gelatin hydrolyzate medium during the first 7 days of cultivation. The upper row represents observations of a culture incubated at 37°C . Small coccoid forms outnumbered all others. Filaments, filamented forms, and globules were frequent only after the fourth day. The middle row is from a similar culture incubated at 24 to 26°C . Mycelial or bacillary forms predominated until the fourth day. Budding from these forms was frequent. Globules appeared on the third day. The lower row represents growth under an atmosphere of oxygen. Growth was heaviest in these tubes and predominantly the small coccoid form for 5 days. Thereafter bacillary forms and long filamented chains showed a marked increase. Sessile budding was frequent after the third day. Highly refractile bacillary forms capable of flexional movements are indicated by solid black.

continuous sessile budding; others developed with filamentous linkage between members. Chains seldom exceeded a total of 4 or 5 units unless they were of the filamented type. From a small globus we saw terminal nonfilamentously chained coccoid forms break off, singly and in pairs. Thereafter the same globus sprouted two more buds at new sites about 120° of arc apart and resumed formation of nonfilamented coccoid chains. There was considerable protoplasmic commotion within the cell wall at the budding site, and six or seven rapid, outpouching, and retracting movements were made through the cell wall before the bud finally remained outside. This process was repeated for each additional coccoid

form as it was added to the chain. Twenty to 30 minutes elapsed between delivery of each new bud. The protoplasm of the globus appeared to be consumed by the new forms, or converted entirely into them. After cessation of budding the residual globus consisted only of a delicate, lightly refractile, spheroidal, shrunken cell wall entirely devoid of refractile contents. We observed several examples of this type of budding from globoid forms of 1.5 to 3 μ in diameter.

One example of filamentous budding was observed. The globus was about 3 μ in diameter. It added a minute, solid, spherical, coccoid form, by extrusion, to a long filamented chain that already consisted of two 0.5 μ coccoid forms, a long bacillary form, and a long terminal filament. Here, again, it required 5 or 6 intermittent, vigorous, outpouching or extrusive movements through the cell wall at the site of junction with the fine filament, each outthrust apparently surrounding and enclosing the proximal end of the filament before the new spherule was delivered to a permanent place on the filamented chain.

Formation of fine filaments was not observed despite prolonged anticipatory search. Marked organisms without filaments were noted at later intervals to possess one or more of them, but the act of formation was not seen. This was true also for the development of minimal reproductive units. Filaments without them were noted later to have one or more minute condensed areas along their lengths or at their tips. Further observations demonstrated clearly that the filaments increased slowly in length, and that the minimal reproductive units enlarged to form spherules, coccoid forms, bacillary forms, and even large globi. This was true both for filaments that had and had not been broken off by Brownian movement from the generative form. It appeared almost certain that separation of coccoid or bacillary forms, due to forces exerted by Brownian motion or by manipulation of cultures, resulted in formation of filaments. Most long filaments are undoubtedly so formed, and it is possible that all are made in this manner. Both organisms and filaments adhere readily to glass surfaces.

Formation of minimal reproductive units was inferred from prolonged intermittent observations of living cultures. Though they lack the continuity that is necessary to establish certainty, we regard our serial drawings, collected in figure 3, as a reasonably good approximation of events. The figure also represents progressive development of minimal reproductive units into larger forms.

One variety of small spheroidal forms always had a dense, central, highly refractile body that stained solidly and deeply with the supravital dyes. Further development of this form always resulted in radial peripheral budding, as indicated in the upper and next to bottom rows of the right-hand column in figure 3. One is also shown at the bottom of 29 in plate II. The development of this uncommon, centrally nucleated form was observed in liquid media as well as in one series of slide microcultures stained at intervals with Bismarck brown Y.

The forms shown at 18 and 22 in plate I, the middle form of 29 in plate II, and 6 and 10 in plate III, all consist of globi containing 2 densely staining coccoid or ovoid bodies about 0.5 μ in diameter. Such globi may or may not possess additional chromatin material. The forms shown in plates II and III strongly

suggest the appearance of sporangia, each containing two sporangiospores. The one photographed and shown as 6 in plate III was studied for a long time, but it was not possible to be sure whether the globus had budded from the chain of coccoid forms or the chain had been produced by the globus. Nor were we able to determine this relation in other examples. The number of enclosed bodies was always two.

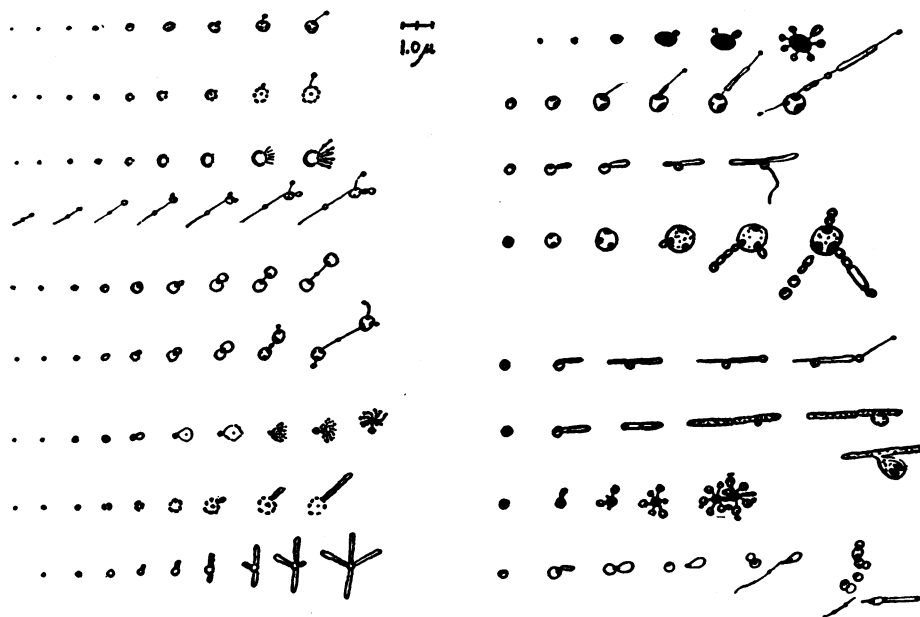


FIG. 3. The left-hand column illustrates progressive development of various forms from minimal reproductive units as inferred from many series of intermittent observations. It also indicates certain modes of production of these minute units. Development of additional forms from the predominant 0.45μ coccoid form is shown at the right. The suggested development of the sporangiumlike body, in the sixth row, is purely conjectural and possibly incorrect. All other drawings were based on actual observations. In all cultures it seemed that these various modes of reproduction were operative concurrently.

These preliminary studies indicate that *Bacterium tulareense* is "polygenethodic," possessing multiple modes of reproduction.

DISCUSSION

As knowledge of natural and experimental tularemia, and information about the causative agent, accumulated during recent decades it became increasingly apparent that the concept of *Bacterium tulareense* as an ordinary bacterium, classified usually as *Pasteurella tularensis*, was inadequate to account for the known facts and relations. The organism has obligate requirements for cystine and for an unidentified protein derivative and, until recently, was incapable of multiplication in any liquid medium. Filtration experiments with tested earthenware filters showed that about half of all filtrates were infective for rodents and that no exotoxin was formed. The agent passes the normal unbroken skin

or mucosa, and has a high degree of virulence or infectivity. Light suspensions of virulent strains, of usual agglutination antigen turbidity, will sometimes kill 20 to 25 per cent of mice or guinea pigs in dosage of 0.5 ml of a 10^{-12} dilution. The number of natural animal hosts is large, including many rodents, birds, reptiles, amphibia, felines, canines, and marsupials. The number of insect and arthropod hosts and vectors is large. Steinhaus (1942) lists 52 insects and arthropods associated in some manner with this organism. Three ticks are good biologic hosts, transmitting the organism via their eggs, larvae, and nymphs to adults of the next generation. It is further transmitted among adults by copulation. Within ticks the organism grows both in extra- and intracellular locations. In rodents intracellular multiplication also occurs; it is best seen in the hepatic cells of the mouse where large pseudocysts are eventually formed, but it is also noted in hepatic cells of the hamster and guinea pig, in pulmonary macrophages of several rodents, and in splenic vascular endothelium and lymph node reticular cells of mice. The disease is highly fatal for most rodents, but causes low mortality and prolonged morbidity in man, leaving recovered individuals with an amazingly solid immunity against subsequent exposures. Serum agglutinins acquired by infection persist for the remainder of life of the recovered patient. Practically all attempts to provoke active immunity in rodents with light and dense suspensions of bacteria killed by various means have failed to demonstrate any notable resistance to experimental infection against a single M.L.D. of a highly virulent strain. Similarly, hyperimmune sera produced in large animals by inoculation of killed or living cultures of titred high virulence have consistently failed to protect mice or guinea pigs against a single M.L.D. of a challenge strain of high virulence.

Knowledge of the morphology of the organism offers possible solutions to only some of the problems raised by the above statements. Nevertheless, the findings presented here, easy to confirm by simple methods of study, emphasize anew the necessity for reorientation of concepts concerning tularemia and the ecologic and biologic characters of its causative agent.

Since the morphologic units of *Bacterium tularense* consist of coccoid and cylindrical bacillary forms, flattened bacillary forms, globules and globi, filaments, and minimal reproductive units, and since the organism possesses several modes of reproduction and multiplies in cell-free media, but not in media devoid of protein or protein derivatives, this microorganism satisfies most of the criteria for classification in the pleuropneumonia group. Satisfactory evidence for filtrability of the minimal reproductive units, size of particles, and developmental forms of these units in animals and in culture media is not offered here. Some evidence that has been obtained will be presented separately. Examination of our photomicrographs and drawings will demonstrate that certain forms of *Bacterium tularense* bear striking resemblances to various organisms of the pleuropneumonia group as they are depicted in studies by Ledingham (1933), Klieneberger (1934), Turner (1935), and Sabin (1941). Our study of a recently isolated strain of *Streptobacillus moniliformis* by similar methods showed some forms which we could not differentiate morphologically from certain *Bacterium*

tularensis forms. The L₁ microorganism derived from this culture resembled *Bacterium tularensis* more closely. Similar resemblances are also apparent in the photomicrographs by Brown and Nunemaker (1942). The similarities are only partial, existing for only certain forms of these highly polymorphous microorganisms. Our plates also demonstrate morphologic features apparently unique for *Bacterium tularensis*.

Although these studies indicate that *Bacterium tularensis* should not be included in the *Pasteurella* or *Brucella* genera, we are not proposing a new generic name for it. We believe it is more closely related to the pleuropneumonia group than to any other group clearly defined at present, but that certain of its apparently unique morphologic features, herein described and photographed, set it apart from this group as it is now constituted. We think that creation of a new generic name now would be premature and based upon insufficient knowledge of this group and its relatives. We suggest postponement until these relations can be more clearly defined. Until then we believe that the more noncommittal name, *Bacterium tularensis*, is the one of choice and that *Pasteurella tularensis*, *Brucella tularensis*, and *Coccobacterium tularensis* should be declared invalid. We further suggest that when an appropriate generic name is adopted, the proper gender form of *tularensis* be retained as the specific name.

SUMMARY

Bacterium tularensis is an extremely polymorphous microorganism. It possesses neither capsules nor flagella, and is nonmotile. Its morphologic units include globi and globules, flat and cylindrical bacillary forms, coccoid forms, delicate filaments, and minimal reproductive units. Many forms, under certain conditions of staining, reveal prominent, thick cell walls. The cell wall is, of course, an integral structural unit with no relation to degree of invasiveness or virulence. All 43 strains were morphologically identical. We found no morphologic feature to differentiate a virulent from a nonvirulent strain. All strains seemed to possess multiple modes of reproduction, and budding was apparently the chief one. Division by binary fission was not observed, though it may occur. Although the organism seems to be related to the pleuropneumonia group, perhaps only distantly related, we suggest postponement of taxonomic efforts until the members and relations of this group are better defined. For the present we urge invalidation of the terms, *Pasteurella tularensis*, *Brucella tularensis*, and *Coccobacterium tularensis*, and retention of *Bacterium tularensis* as the most suitable temporary designation.

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