INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UNIVERSITY OF CINCINNATI

May 31, 1947

I hereby recommend that the thesis prepared under my supervision by Henry T. Eigelsbach

entitled "The Morphology of Bacterium Tularense as Revealed by Light and Electron Microscopy"

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

Lee Frobisher

Form 668—G.S. and Ed.—500—3-42

THE MORPHOLOGY OF BACTERIUM TULARENSE AS REVEALED BY LIGHT AND ELECTRON MICROSCOPY

A dissertation submitted to the

Graduate School
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1947

by

Henry T. Eigelsbach

B. S. Purdue University 1941

M. S. University of Kentucky 1943

TABLE OF CONTENTS

ACKNOWLEDGEMENT

The author desires to express his sincere appreciation for the direction, unfailing interest, and constructive criticism given him during the course of this work by Dr. Lee Foshay. He also wishes to express thanks to Dr. Leslie A. Chambers, whose continued interest, technical advice and cooperation made possible the section on electron microscopy, to Dr. Keith H. Lewis for aid in the preparation of the manuscript, and to all those who have given so freely of their time and counsel.

INTRODUCTION

A divergence of views concerning the complex morphology of Bacterium tularense has been emphasized by Hesselbrock and Foshay (1) as recently as 1945. In general, American investigators have depicted Bacterium tularense in pure culture as being absolutely nonmotile and possessing neither flagella nor capsule, while Japanese workers have reported that the organism is motile and possesses a monotrichate polar flagellum as well as a capsule.

The systematic study of the morphology of Bacterium tularense by means of supravital staining and dark field examination, as reported by Hesselbrock and Foshay (1), suggested that a study of the organism during its various growth phases, as well as an investigation made with the aid of the electron microscope, would be of value in the interpretation of its multiple morphological features. The great resolving power of the electron microscope and the utilization of special techniques for enhancing contrast in electron microscopic preparations permit precise observation of details of size, shape, and structure which are not discernible by ordinary microscopy.

Accordingly the present investigation was undertaken in an attempt to acquire a better understanding of the extremely complex morphology of Bacterium tularense.

HISTORICAL

A review of the literature has revealed that since McCoy and Chapin (2) in 1912 initially described Bacterium tularense as a small, nonmotile, questionably encapsulated, Gram-negative, pleomorphic organism occasionally presenting involution forms, relatively few investigations of the extremely complex morphology of this organism have been reported in either Europe or the United States. In Japan, however, Ohara (3) and Ota (4), who refer to endemic Japanese tularemia as Yato-Byo, have carried out rather extensive studies on the morphology of Japanese and American strains of Bacterium tularense.

In a recent article Hesselbrock and Foshay (1) presented an extensive review of the Japanese literature and emphasized that divergent views exist concerning the morphology of Bacterium tularense. These investigators stated, with regard to European literature,

> "Most European language reports and textbooks omit all reference to flagella, state that the bacterium is nonmotile, 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."

Ohara, Kobayashi, and Kudo (3) claimed to have demonstrated by the Saisawa-Sugawara silver deposition method that both Japanese and American strains possess a single polar flagellum and present clubbed, comma-shaped, dumbbell, and triangular forms. Concerning motility these workers

described a "certain active movement" of the organism. The intensity of movement or motility was said to be directly related to virulence. It was stated that, "The more virulent is the bacterium, the greater is the pleomorphism," as well as "Virulence, pleomorphism, and motion are closely related and vary together."

Galli-Valerio (5) after having observed cultures isolated by Drbohlav in Czechoslovakia reported that he was unable to find any enlarged, elongated, filamentous, or involution forms and stated that both coccoid and bacillary forms were nonmotile. He failed to demonstrate flagella by means of the Casares-Gil stain.

Ota (4) stated that in 1934 Kudo and Kobayashi while working with pure cultures under the direction of Ohara successfully demonstrated a single polar flagellum by means of the Nishigawa-Sugahara silver deposition method. A light staining mucoid capsule-like zone which surrounded the organism was also observed during the investigation. Ota further mentioned that in his experience the Nishigawa-Sugahara method was more complicated and less satisfactory for staining organisms in tissue than his own modification of Benian's Congo red method. After an extensive investigation of various staining techniques he reported that flagella were stained poorly or not at all by the direct

methods of Loeffler, Zettnow, Inouye, Yakota, Imai-Hidaka, and Uyeno. Satisfactory results were obtained by means of the methods of Nishigawa-Sugahara, Tsushima-Koike, and Ohara-Kobayashi. Of the negative staining methods investigated the India-ink technique was found to be unsatisfactory whereas flagella were well demonstrated with nigrosin, mercurochrome, and by means of Ota's modification of Benian's method, as well as the Ota-Hasegawa Congo red-aniline gentian violet method. In Ota's experience capsules were successfully demonstrated by Gin's India-ink carbol-thionine method, the Ota-Hasagawa method, and by mercurochrome while the methods of Johne, Wadsworth, Hiss, Welch, and Friedlander failed entirely or stained capsules poorly. Concerning the motility of Bacterium tularense Ota stated,

> "While studying the organisms suspended in a hanging drop, I found some actively motile, definitely changing their position. This motion gradually became more sluggish and finally ceased. When the organisms were suspended in 0.01 per cent mercuric chloride, they became motionless within a short time."

Ota does not concur with McCoy, Chapin, Francis, Foshay, and others who have reported that Bacterium tularense is a pleomorphic nonmotile organism and suggests that the pleomorphism observed by the forementioned investigators probably resulted from the variation in the degree of staining since only simple staining methods, which in his

experience did not reveal the complete outline of the organism, were employed.

Kobayashi, Kawakami, and Ota (6) failed to demonstrate flagella by Loeffler's method but demonstrated monotrichate flagella by the Nishigawa-Sugahara method and by means of Victoria blue (4R) with the aid of a mordant.

Ohara (7) in 1940 reported that Bacterium tularense is monotrichate with a polar flagellum, has a specific active movement, and possesses a capsule.

In 1945 Hesselbrock and Foshay (1) reported a systematic study of the morphology of Bacterium tularense. By means of various techniques, including vital staining and dark field examination, 43 strains varying from highly virulent to completely avirulent were examined. No morphological feature which would differentiate virulent from avirulent strains was found. All strains examined were found to be morphologically identical. Flagella-sized structures described by the Japanese as true flagella were readily observed but were found to be absolutely nonmotile. Capsules were not observed but the extreme pleomorphism of the organism was readily demonstrated. In addition to coccoid and bacillary forms dumbbell, bean-shaped, filamentous, and minute coccoidal forms were described and photomicrographic illustrations presented. Multiplication by budding appeared to be the chief mode of reproduction. The authors concluded that

Bacterium tularense is an extremely pleomorphic nonmotile microorganism possessing neither capsule nor flagella.

Foshay and Hesselbrock (7) in 1945 demonstrated that minute morphologic units of Bacterium tularense were able to pass the Elford 600 mu gradocol membrane but not the 500 mu membrane and hence were calculated to be in the range of 300 to 350 mu in diameter. These investigators demonstrated that the minute forms are capable of reproducing all other morphological forms of the organism and termed them minimal reproductive units, M.R.U. The authors stated that they were reasonably sure that minimal reproductive units of smaller size exist, and that accurate measurement will require better cultural conditions than those hitherto obtained.

In 1946 Eigelsbach, Chambers, and Coriell (8) reported an investigation of the morphology of Bacterium tularense by means of electron microscopy. This investigation was in agreement with the systematic study of the morphology of the organism by Hesselbrock and Foshay (1). Electron micrographs were presented which confirmed previous observations that Bacterium tularense possesses multiple morphological units including large and small coccoid and bacillary, oval, minute, filamented, bean-shaped, dumbbell, bizarre, and so-called "involution" forms. Minute morphological forms as

small as 110 mu were observed. The authors found that the typical cell possesses little opacity to the electron beam and presents a semitransparent nebulous appearance. Finely filamentous structures, thought by the Japanese investigators to be flagella, were observed. Critical examination for the presence of a cell wall revealed an extremely delicate structure of very low electronic density. Capsules were not demonstrated.

In the present study there is presented a discussion of the morphology of Bacterium tularense observed during its various growth phases. Numerous photomicrographs depicting the extremely complex morphology of the organism are presented. In addition electron microscopy of Bacterium tularense is reviewed and electron micrographs of material prepared according to the modifications of the replica technique recently described by Hillier and Baker (9) and the metal shadowing technique described by Williams and Wyckoff (10) are presented and discussed.

MATERIALS AND METHODS

Strains. Since Hesselbrock and Foshay (1) could not differentiate between 43 virulent and avirulent strains on the basis of morphology, only three strains were used in the present study. They are designated as Schu, Memp, and 38 and represent a portion of the strains examined by Hesselbrock and Foshay (1). Schu and Memp were virulent and strain 38 completely avirulent. Strain 38 had originally been sent to Dr. Lee Foshay by Dr. Edward Francis of the National Institute of Health. The histories of the strains used in this study are shown in table 1.

Media. Four varieties of liquid media, peptone broth (12), peptone broth plus 0.1 per cent cysteine hydrochloride (12), gelatin hydrolyzate (13), soybean hydrolyzate (14), and two solid media, dextrose cysteine blood agar (DCBA) (15) and dextrose cysteine blood infusion agar* were used for the cultivation of the organism.

Conditions of Growth. Liquid cultures were either continuously agitated by means of a shaking machine with an excursion of 3 inches and an oscillation rate of 100 times per minute or permitted to remain static. Both liquid and solid cultures were incubated at 37 C. The length of the

*Prepared as DCBA (15) except that infusion agar is used and beef extract omitted. The author found that slants prepared in this manner supported more luxuriant growth of Bacterium tularense.

TABLE 1

HISTORICAL DATA CONCERNING STRAINS OF BACTERIUM TULARENSE USED IN THIS INVESTIGATION

Data	Strain Designations		
	38	Memp	Schu
Year of original isolation	1920	1938	1941
Pathologic source	Human lymph node	Human lymph node	Human ulcer
Geographic source	Utah	Tennessee	Ohio
Present pathogenicity	Avirulent	Virulent	Virulent

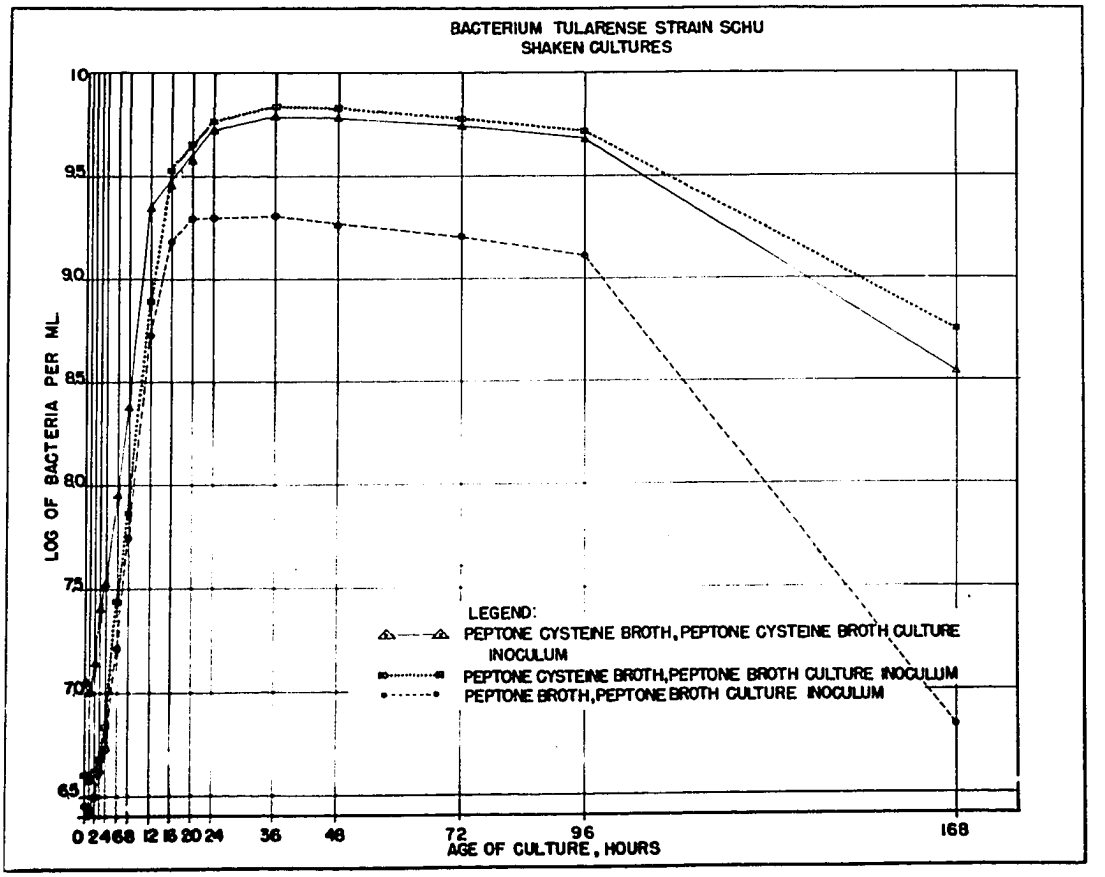

Figure V.

Viable Count Growth Curve.

incubation period depended upon the conditions of the experiments to be described subsequently.

Preparation of Material for Optical Miscroscopy. A small amount of surface growth on a slant culture was removed with a sterile wire and shaken into a tube containing 3 to 4 ml of either sterile distilled water or sterile physiological salt solution. The tube was gently rotated and a drop of the suspension carefully spread on the surface of a chemically clean glass slide. Preparations from broth cultures were made by spreading a drop of the culture on a similarly prepared glass slide. Smears were air dried, fixed in methyl alcohol for 5 minutes, and stained by Giemsa's method for 45 minutes. Periodically control staining methods were employed to determine the possibility of artifact formation. Unfixed smears were stained by Giemsa's method and suspensions were stained with Hofmann's violet according to the supravital staining technique described by Hesselbrock and Foshay (1).

Optical Microscopy. Stained preparations were examined with a B. and L. Type BD research microscope. Photomicrographs were made with the aid of a Wratten B filter. Negatives were developed with DK60a developer and prints made on No. 4 paper to insure contrast.

Preparation of Cultures for Electron Microscopy. Vari-

ous procedures were followed in preparing cultures for electron microscopic study. From solid media suitable suspensions were prepared by one of the following techniques: (A) A small amount of surface growth was removed and suspended in sterile physiological salt solution or in sterile distilled water. The suspension was usually incubated at 37 C for 5 hours. (B) Three to four ml of sterile physiological salt solution were pipetted onto the surface of a slant culture, which was then allowed to incubate for a few hours at 37 C. (C) A small amount of surface growth was removed with a sterile wire and passed three or four times through a wire loop containing a film of distilled water until a fairly uniform suspension resulted.

Suspensions from liquid media were used directly or diluted with one to two volumes of distilled water. A drop of the suspension prepared by the previously described techniques was placed upon a collodion or polyvinyl formal (formvar) electron microscope mount by means of a capillary pipette or wire loop and allowed to dry in air. Kirchner (16), Marton (17).

Preparation of Collodion and Polyvinyl Formal (Formvar) Electron Microscope Mounts. Collodion film mounts were prepared as follows: A drop of a 1 per cent solution of collodion in amyl acetate was placed on a clean distilled

water surface. When the water surface is touched the drop spreads over a relatively large area. The amyl acetate then readily evaporates and leaves the desired collodion film floating upon the surface of the water. A number of fine mesh stainless steel screen disks, 1/8 inch in diameter, constructed from screen possessing 200 meshes per square inch, were placed on the surface of the collodion. Each was picked up in a circular loop, the edges cut by running a sharp needle around the circumference of the loop, inverted, and placed on the small flat top of a metal cylinder. The cylinder was held by a pair of forceps and the loop pressed downward and removed. The cylinder was then inserted into a shallow hole in a wooden block and the forceps removed. Surplus water adhering to the collodion film was removed with a capillary pipette. The film was then air dried for at least ten minutes before mounting the specimen. Collodion films prepared in the previously described manner were very clean and apparently free from structure within the limit of resolution of the electron microscope.

Films of polyvinyl formal which have been reported to exceed collodion films in mechanical strength and durability Zworykin et al. (18) were prepared according to the method of Schaefer and Harker (19). A chemically clean glass

slide was dipped into a 0.4 per cent solution of polyvinyl formal in chloroform and withdrawn, permitting evaporation of the solvent. After evaporation of the solvent had taken place, the film was scored in approximately one-half inch squares with a sharp needle. The slide was slowly dipped at a 45 degree angle into a container of clean distilled water and the film segments floated on the water surface. These films were then processed in the same manner as the collodion films.

Collodion and polyvinyl formal films prepared by the above methods yielded structure-free films and were approximately 10 mu in thickness.

Replica Technique. The replica technique of Schaefer and Harker (19) as modified by Hillier and Baker (10) was used in preparing specimens for examination with the electron microscope. A 1 per cent solution of collodion in amyl acetate, or a 0.4 per cent solution of polyvinyl formal in chloroform, was allowed to flow over a colony or over the surface of confluent growth. After the film had dried it was floated off onto a clean distilled water surface and prepared in the usual way.

Replicas were also prepared in the following manner. A drop of culture suspended in distilled water was allowed to dry on a chemically clean glass slide. The slide was

then dipped into a solution of 0.4 per cent formvar in chloroform. After the slide was removed and the solvent had evaporated, the film was floated off onto a distilled water surface and prepared as previously described.

Metallic Shadow Casting Technique. Many specimens prepared in the conventional manner, and all those prepared by the replica technique, were shadowed with gold according to the procedure described by Williams and Wyckoff (11). This technique consisted in obliquely depositing in a vacuum a thin layer of gold onto the electron microscope mount. Approximately 28 cm of 3 mil gold wire (0.003 inches in diameter) were wrapped around a tungsten wire (evaporation point 3370-5900 C). One to five mounts were placed on a chemically clean slide which was located six inches from the base of the tungsten wire attachment poles and two inches below the center of the tungsten wire. The tungsten wire was electrically heated to incandescence and gradually to the evaporation temperature of gold, 1063-2600 C. Evaporation of the gold in the vacuum produced a continuous essentially grainless metallic film on the surface of the mount.

The R.C.A. Vacuum Type EMV-1 unit used in this investigation is shown in figure I.

Electron Microscopes Employed. The initial electron

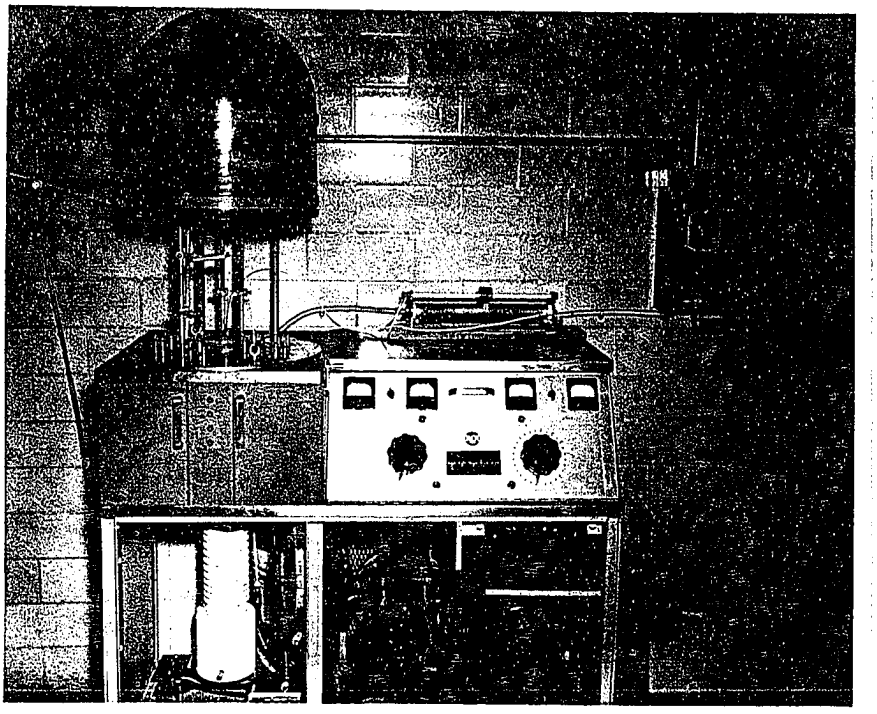

Figure I.

R.C.A. Vacuum Type EMV-1 Unit

for Metallic Shadow-Casting Technique.

microscopy investigation of the morphology of Bacterium tularense by the author and his associates (8) was made with the aid of the R.C.A. Type B electron microscope. This instrument was not equipped with an objective aperture, and was located at the Johnson Foundation, University of Pennsylvania.

A continuation of the electron microscopy investigation was made at Camp Detrick, Frederick, Md. with the aid of the R.C.A. Type E.M.U. electron microscope equipped with an objective aperture. This instrument is shown in figure II.

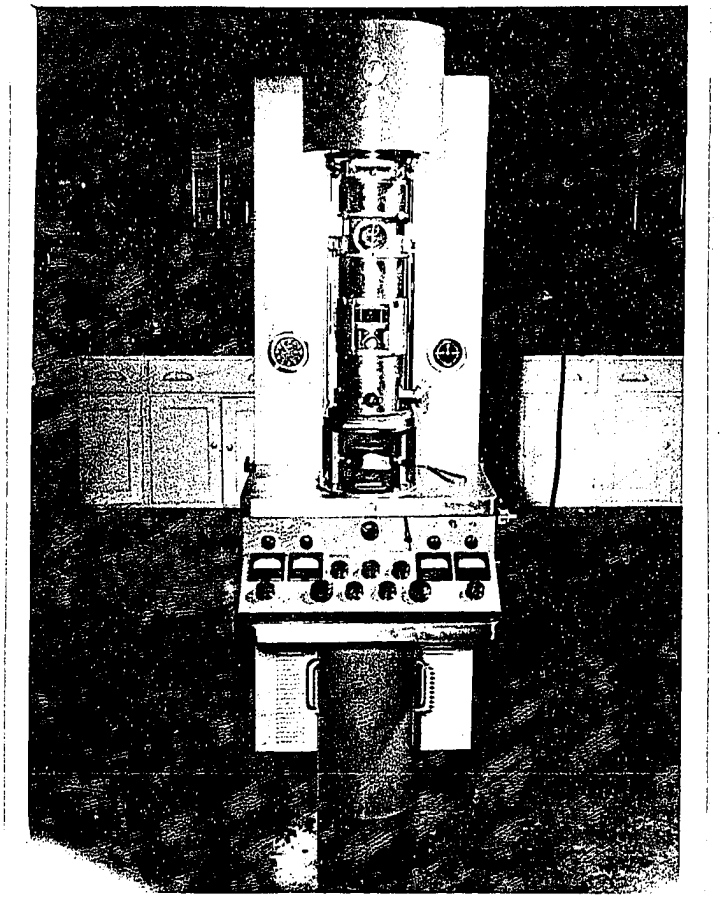

Figure II.

R.C.A. Type EMU Electron Microscope.

EXPERIMENTAL

A description of the morphology of a particular organism carries with it the implication, unless otherwise stated, that the description applies to young actively growing cells cultivated under optimal physical conditions on a medium favorable to the growth of the particular species. When a morphological study of an organism is reported, it is of utmost importance that exact conditions of cultivation of the organism be cited. Since Francis in 1942 (21) stated that no liquid medium was suited to the growth of Bacterium tularense, special effort was made to determine whether or not the cultural methods to be employed in the present investigation were particularly favorable to the growth of the organism.

Snyder et al. (12) recently reported that the minimum effective inoculum of strain Schu in a static peptone broth medium incubated at 37 C was between 4,000 and 40,000 organisms per ml of medium. Addition of 0.1 per cent cysteine hydrochloride to the medium decreased the minimum effective inoculum to approximately 1 organism per 100 ml of medium. Since shaken as well as static cultures in peptone broth, both with and without 0.1 per cent cysteine hydrochloride, were to be used in the subsequent morphologic investigation, the minimum effective inoculum of strain Schu was determined for shaken cultures in a manner

similar to that employed by Snyder et al. (12).

A saline suspension of strain Schu was prepared from a 24 hour dextrose cysteine blood infusion agar slant and adjusted to a transmission of 40 per cent (T-40), approximately 2 billion cells per ml, with a Coleman spectrophotometer using a wave length of 650 mu. Several tenfold dilutions of the adjusted suspension were made. DCBA plate counts (15) were made possible by pipetting 0.1 ml of appropriate dilutions, 10^{-5} to 10^{-7}, of the T-40 suspension onto the center of the agar, and by gently spreading the fluid over the surface of the agar with a sterile bent glass rod. Plates were incubated at 37 C for 72 hours and the colonies counted. Typical Bacterium tularense colonies on DCBA plates together with a glass spreader are shown in figure III.

One ml amounts of serial tenfold dilutions of the T-40 suspension were inoculated respectively into each of 10 Erlenmeyer flasks (250 ml) which contained 25 ml of peptone broth or peptone cysteine broth. The cultures were held at 37 C for 3 hours and then shaken at the same temperature until turbidity appeared or for the duration of the experiment. Gram stains of the turbid broth cultures were made to rule out the presence of contamination.

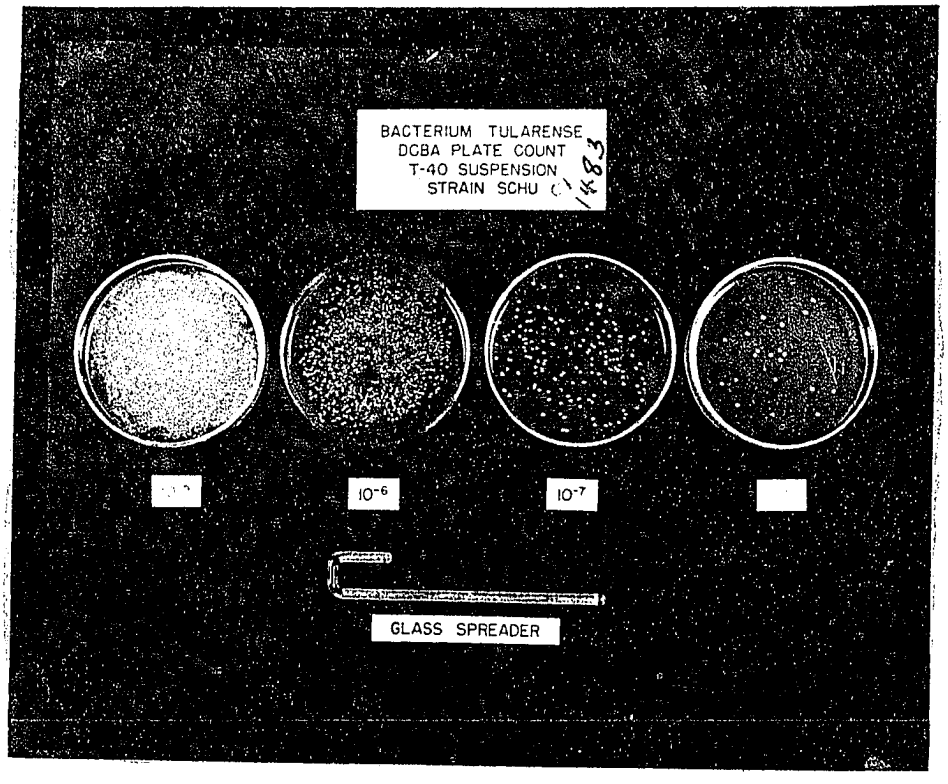

Figure III.

An average of 168 colonies were obtained from colony counts of 5 DCBA plates inoculated with 0.1 ml of the 10^{-6} dilution of the T-40 suspension. Therefore the number of viable organisms as determined by the DCBA plate method was 1.68 billion cells per ml of the original suspension.

It has been assumed (15) that one organism gives rise to a colony on DCBA and also constitutes a lethal dose for the mouse. In the author's experience enumeration of viable Bacterium tularense in a given suspension by means of the DCBA plate count method compares exceptionally well with the enumeration of viable cells by means of the mouse titration method when calculated according to the method of Stevens (20).

Table 2 shows that between 6.7 million and 67 million viable cells per ml of peptone broth are required to initiate growth under the conditions of the experiment. Table 3 shows that the addition of 0.1 per cent cysteine hydrochloride to peptone broth reduces the minimum inoculum requirement for growth to between 1 and 6.7 viable cells per ml of medium.

Thus it has been shown that both static and shaken peptone cysteine broths will initiate growth of Bacterium tularense, strain Schu, from approximately 1 viable cell

TABLE 2

EFFECT OF INOCULUM SIZE ON GROWTH OF THE SCHU STRAIN OF BACTERIUM TULARENSE IN SHAKEN PEPTONE BROTH

Dilution of T-40 suspension	Inoculum (no. of cells per 25 ml medium)	No. of flasks inoculated	No. of flasks becoming turbid after incubation (days) 1 2 3 4 5 6 7
Undiluted	1,680,000,000	10	10
10^{-1}	168,000,000	10	0 0 6 3
10^{-2}	16,800,000	10	0 0 0 0 0 0 0
10^{-3}	1,680,000	10	0 0 0 0 0 0 0
10^{-4}	168,000	10	0 0 0 0 0 0 0
10^{-5}	16,800	10	0 0 0 0 0 0 0

TABLE 3

EFFECT OF INOCULUM SIZE ON GROWTH OF THE SCHU STRAIN OF *BACTERIUM TULARENSE* IN SHAKEN PEPTONE CYSTEINE BROTH

Dilution of T-40 suspension	Inoculum (no. of cells per 25 ml medium)	No. of flasks inoculated	No. of flasks becoming turbid after incubation (days) 1 2 3 4 5 6 7
10^{-5}	16,800	10	0 10
10^{-6}	1,680	10	0 10
10^{-7}	168	10	0 10
10^{-8}	16.8	10	0 5 0 0 0 0 0
10^{-9}	1.68	10	0 0 0 0 0 0 0
10^{-10}	0.168	10	0 0 0 0 0 0 0
10^{-11}	0.0168	10	0 0 0 0 0 0 0

per ml of medium. A considerably greater inoculum was required to initiate growth in peptone broth under the same conditions.

Growth of Bacterium tularense, Strain Schu, in Static and Shaken Peptone and Peptone Cysteine Media. After data concerning the size of inoculum required to initiate growth of strain Schu in static and shaken peptone and peptone cysteine media had been obtained, a growth curve study, as well as a morphologic study, of the organism during its various growth phases in the above media was made. Five hundred ml Erlenmeyer flasks each containing 100 ml of either peptone or peptone cysteine broth were each inoculated with 0.5 ml of a 24 hour broth culture. The organism had been subcultured in both peptone and in peptone cysteine broths, respectively, for approximately 20 times since animal passage and subsequent isolation on solid medium. A cysteine broth culture was used to inoculate peptone cysteine broth whereas a peptone broth culture was used to inoculate both peptone and peptone cysteine broths. Flasks were divided into two identical groups. One group was shaken and the other permitted to remain at rest. All cultures were incubated at 37 C. Approximately 0.2 ml samples were withdrawn immediately after incubation (0 hours) and thereafter at 1, 2, 3, 4,

6, 8, 12, 16, 20, 24, 36, 48, 72, 96, and 168 hour intervals for DCBA plate counts and morphologic examination.

Static cultures. Peptone and peptone cysteine broth cultures which had been inoculated with a 24 hour peptone broth culture showed a short lag phase of 1 to 2 hours' duration. Peptone cysteine cultures which had been inoculated with a 24 hour peptone cysteine broth culture showed no detectable lag phase. For all cultures the logarithmic phase continued until approximately 12 hours after inoculation. The maximum viable count in both peptone cysteine broth cultures was recorded between 72 and 96 hours after inoculation and ranged between 2 and 3.1 billion organisms per ml. The phase of decline of these cultures began approximately 96 hours after inoculation. The peptone broth culture reached its maximum viable count, 900 million cells per ml, approximately 48 hours after inoculation. For this culture the phase of decline began 48 hours after inoculation. (See figure IV.)

Shaken cultures. One hour after inoculation, shaken cultures showed a slight drop in viable cells. After the drop no further lag phase was noted, and the logarithmic phase began and continued until 16 hours after inoculation. The maximum viable count in both peptone cysteine broth cultures was recorded between 36 and 48 hours after inocu-

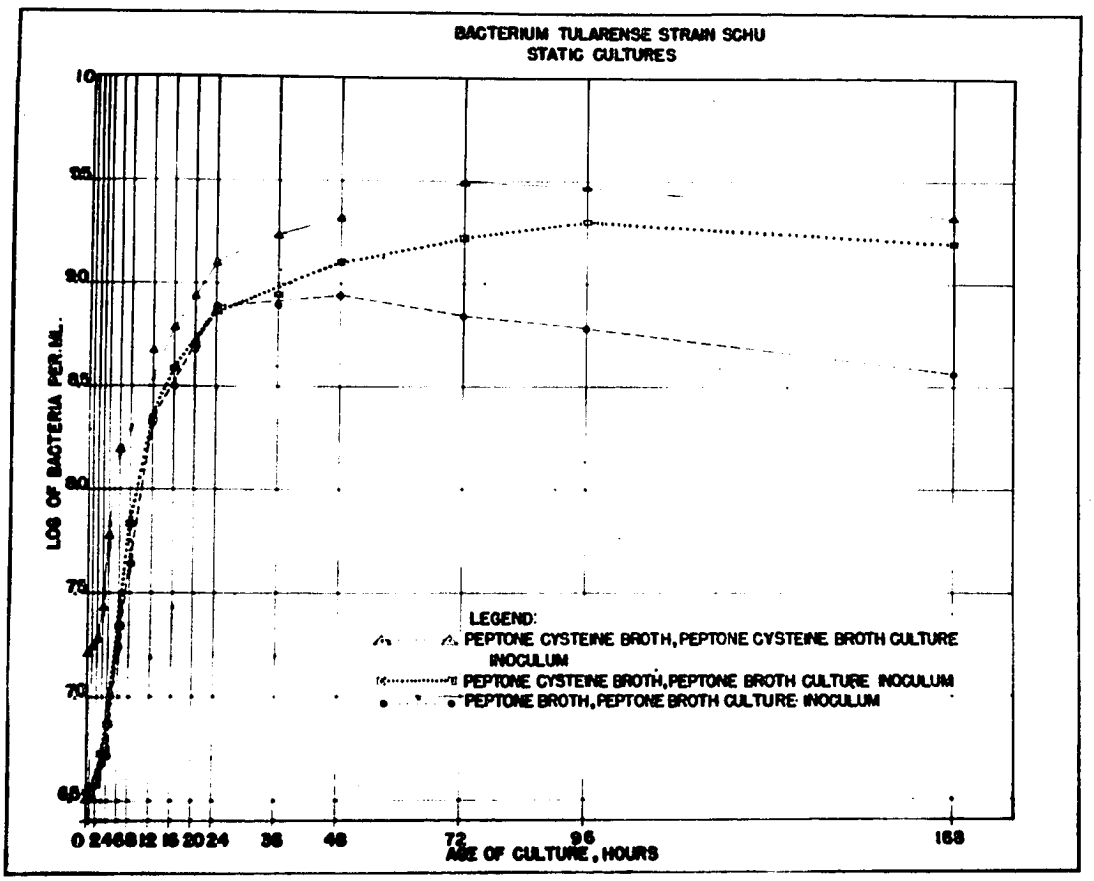

Figure IV.

Viable Count Growth Curve.

lation, and ranged between 6 and 6.7 billion organisms per ml. The phase of decline of these cultures began approximately 96 hours after inoculation.

The peptone broth culture reached its maximum viable count, 1.9 billion cells per ml, approximately 24 hours after inoculation. The phase of decline for this culture also began at 96 hours after inoculation but dropped much more sharply after that time than did that of either of the peptone cysteine broth cultures. (See figure V.)

In general, both static and shaken peptone cysteine broth cultures gave a higher maximum viable cell count, retained the number of viable cells at a higher level for a proportionally greater time, and exhibited a more delayed and less abrupt phase of decline than did the peptone broth cultures.

Thus, from the above and previously presented data, it can be concluded that active growth of Bacterium tularense, strain Schu, takes place in peptone and peptone cysteine broths and that peptone cysteine broth is particularly well adapted for growth of the organism.

Optical Microscopy. The morphological appearance of Bacterium tularense, as shown microscopically by visible light, was investigated in conjunction with the growth curve studies described in preceding sections. Strains

Schu and 38 were examined from both static and shaken cultures prepared in peptone broth with and without addition of cysteine hydrochloride. Organisms of strain Schu from dextrose cysteine blood infusion agar were also observed.

Strain 38 is more fastidious in its growth requirements, and it requires a larger inoculum to initiate growth on liquid and solid media than does strain Schu. Approximately 250 passages of strain 38 in peptone broth had been made prior to the present investigation. A 0.5 per cent inoculum (0.5 ml of a 24 hour peptone broth culture) was used, as had previously been done with strain Schu, to inoculate 500 ml Erlenmeyer flasks each containing 100 ml of broth.

Slides for microscopic study of all cultures were prepared from 24 hour inocula immediately before transfer to new media, and thereafter from the inoculated media at 4, 8, 12, 16, 20, 24, 36, 48, 72, 96, and 168 hour intervals.

Figures VI to XIV depict the morphology observed during the various phases of growth. The photomicrographs present the typical morphology observed during the various growth phases by careful study of each preparation. Measurement of organisms was not attempted and no particular significance was attached to the observation of isolated

unusual forms. Gross trends in morphology during the growth of the organism were observed and recorded. Table 4 will serve as a guide to the ages of the cultures as depicted by the number shown in the lower right corner in photomicrographs of figures VI to XIII. In figure XIV, however, culture ages in hours designated by numbers 1 to 9 are 0 (24 hour culture used for inoculation), 4, 8, 12, 16, 20, 24, 48, 96, and 168 respectively.

Control staining methods employed to detect artifact formation failed to reveal any significant variation in general morphology.

Although several minor morphological differences were noted between strains Schu and 38 during the various growth phases, no distinctive morphologic features were observed which would permit differentiation of the strains examined. In general, young cultures, 4 to 20 hours old, were composed principally of bacillary forms showing bipolar staining. As growth continued coccoid forms increased in number. Subsequently filamentous forms also became numerous. With increasing age large coccoid, irregular, and bizarre forms were observed. Strain Schu, when cultivated in static peptone broth, tended to produce a greater number of large irregular forms after 48 hours than when cultivated in peptone cysteine broth. In shaken peptone broth cultures

TABLE 4

CULTURE AGE DESIGNATED BY PHOTOMICROGRAPH NUMBER

(FIGURES VI TO XIII)

Photomicrograph Number	Culture Age (Hours)	Photomicrograph Number	Culture Age (Hours)
1	0(24)*	7	24
2	4	8	36
3	8	9	48
4	12	10	72
5	16	11	96
6	20	12	168

*Photomicrograph of 24 hour culture used for inoculation.

of this strain a predominance of coccoid forms was noted after 20 hours, whereas in similar cultures containing cysteine a predominance of coccoid forms was observed only after 72 hours of incubation. After either static or shaken peptone or peptone cysteine broth had been inoculated with a 24 hour shaken peptone broth culture, which consisted mainly of coccoid forms, a definite alteration in form occurred. In all cases a predominance of small bipolar bacillary forms was observed 4 hours after inoculation.

An increase in filamentous forms was noted as early as 36 hours after inoculation of shaken peptone broth with strain 38. In shaken peptone cysteine broth this feature was not observed until 96 hours after inoculation. With the above exception no significant difference in morphology of strain 38 was observed when cells were grown in either peptone or peptone cysteine broth.

A 24 hour dextrose cysteine blood infusion agar culture of strain Schu, consisting mainly of coccoid forms, showed a change to a predominance of large coccoid forms 4 hours after inoculation onto fresh medium of identical composition. Observation of the culture at 8 hours revealed that a dramatic change in morphology had occurred. Uniformly stained bacillary forms predominated. It was interesting to note that few bipolar staining forms were seen. With

the above exceptions, the morphology observed was similar to that of the organism in broth culture. Although bacillary forms seemed to predominate at some period during the early growth of the organism in all media, large and small coccoid, oval, dumbbell, bizarre, and even the so-called "involution" forms were also observed in practically every preparation. Indeed each culture examined seemed to contain all the forms which make up the complex morphology of Bacterium tularense. It is noteworthy that the above observations were made from preparations of cultures which had been grown under optimal physical conditions in media which have been shown to be well suited to the growth of the organism.

Bipolar staining bacillary forms best illustrated the extremely delicate cell wall of Bacterium tularense. In some instances it was extremely difficult to detect the cell wall which enclosed the deeply staining polar chromatin concentrations. The cell wall was much better demonstrated in original photomicrographs than in the reproductions subsequently presented. In these it is even more difficult or impossible to differentiate the limiting edge of the cell wall from the surrounding area. Capsules were not demonstrated.

Description of the Morphology of Bacterium tularense During the Growth Phases of the Organism.

Figure VI. Static peptone cysteine broth inoculated with shaken peptone cysteine broth culture strain Schu. The character of the cells used for inoculum is shown in the first photomicrograph. It will be observed that long and short bipolar staining bacillary forms predominated, and that relatively few large or small coccoid forms were present. As growth proceeded after inoculation of new medium, no significant change in the complex morphology, with the exception of an increase in the number of plump bipolar staining bacillary forms, was noted until 24 hours after inoculation. However, during relatively early growth of the organism, 4 to 24 hours, oval, bean-shaped, dumbbell, and bizarre forms were occasionally observed. After that time coccoid forms became increasingly more abundant. Seventy-two hours after inoculation coccoid and filamented forms were predominant. Diploid, bipolar staining, bacillary, comma-shaped, and bizarre forms were readily observed. Thereafter an even greater predominance of filamented forms was noted. All other forms previously described were present.

Figure VII. Static peptone cysteine broth inoculated with shaken peptone broth culture strain Schu. The cells used as inoculum consisted mainly of large and small coccoid and filamented coccoid forms. With the initiation of growth

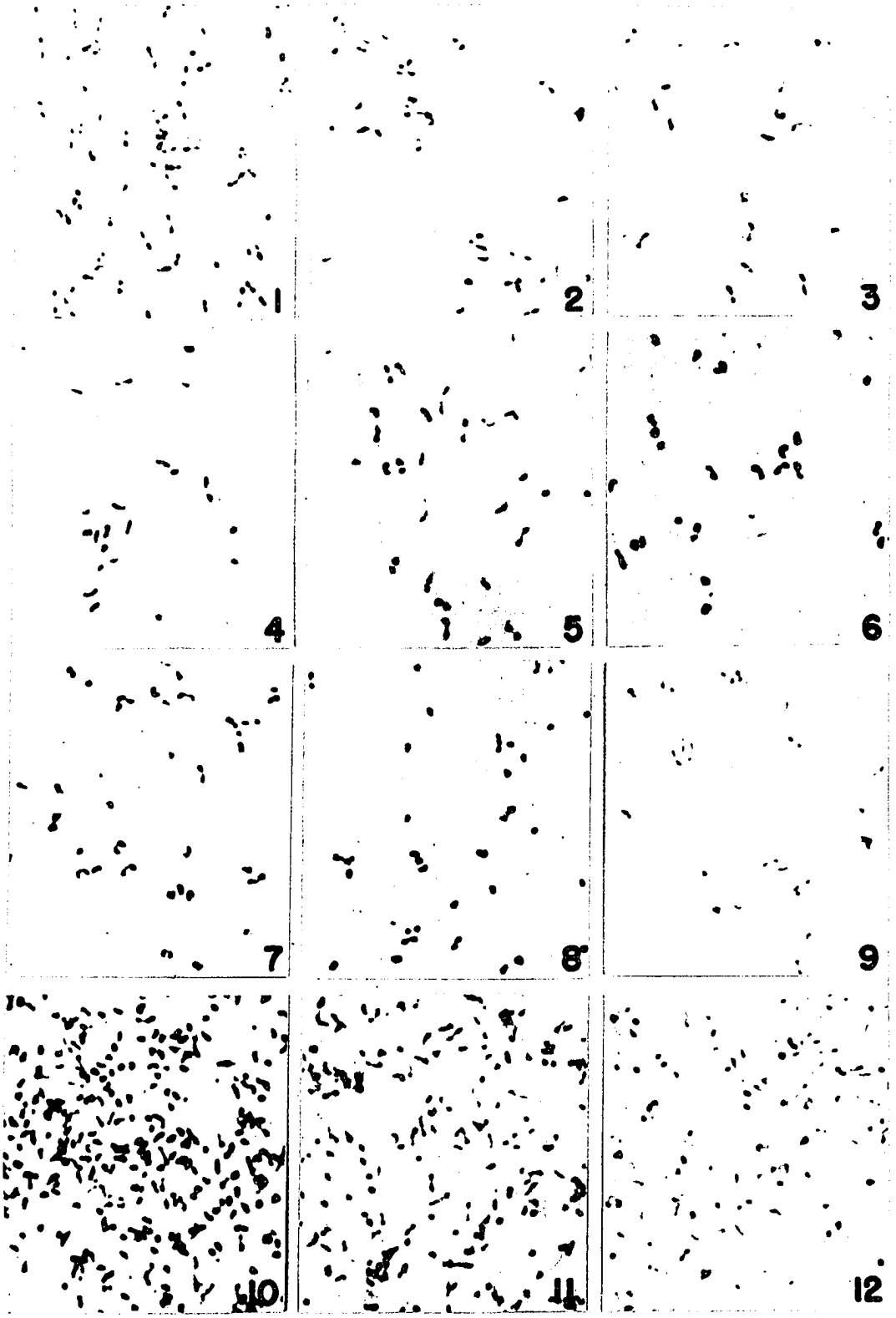

Figure VI.

Static Peptone Cysteine Broth Inoculated with Shaken Peptone Cysteine Broth Culture Strain Schu.

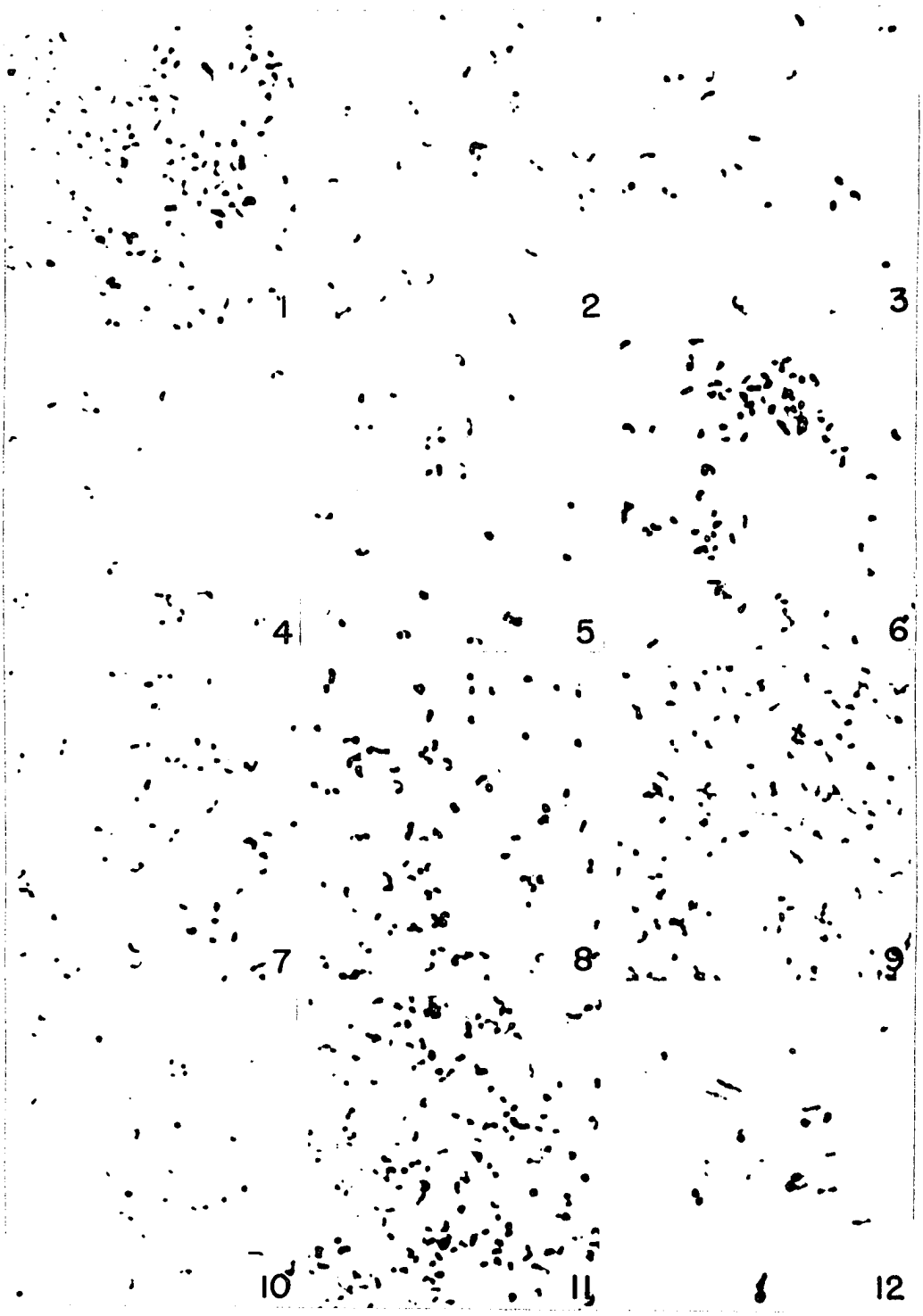

Figure VII.

Static Peptone Cysteine Broth Inoculated with Shaken Peptone Broth Culture Strain Schu.

following inoculation of new medium a definite alteration in form occurred, the majority of cells being of the bacillary type possessing bipolar staining characteristics. Thereafter the morphology of the organism did not differ significantly from that described for figure VI.

Figure VIII. Static peptone broth inoculated with shaken peptone broth culture strain Schu. As in figure VII, the cells used as inoculum consisted mainly of large and small coccoid and filamented coccoid forms. Observation of the morphology of the organism in a 4 hour culture revealed that a definite change in form had occurred. Bipolar staining bacillary forms predominated though occasional coccoid forms were observed. After 20 hours there appeared more and more large and small coccoid forms until these forms predominated at approximately 48 hours after inoculation. Thereafter irregular cell forms became more abundant and many of the so-called "involution" forms were observed.

Figure IX. Shaken peptone cysteine broth inoculated with shaken peptone cysteine broth culture strain Schu. The culture used as inoculum was the same as that described for figure VI. The majority of the organisms present were of the bipolar staining bacillary type. Four hours after inoculation a predominance of plump bacillary forms was

Figure VIII.

Static Peptone Broth Inoculated with Shaken Peptone Broth Culture Strain Schu.

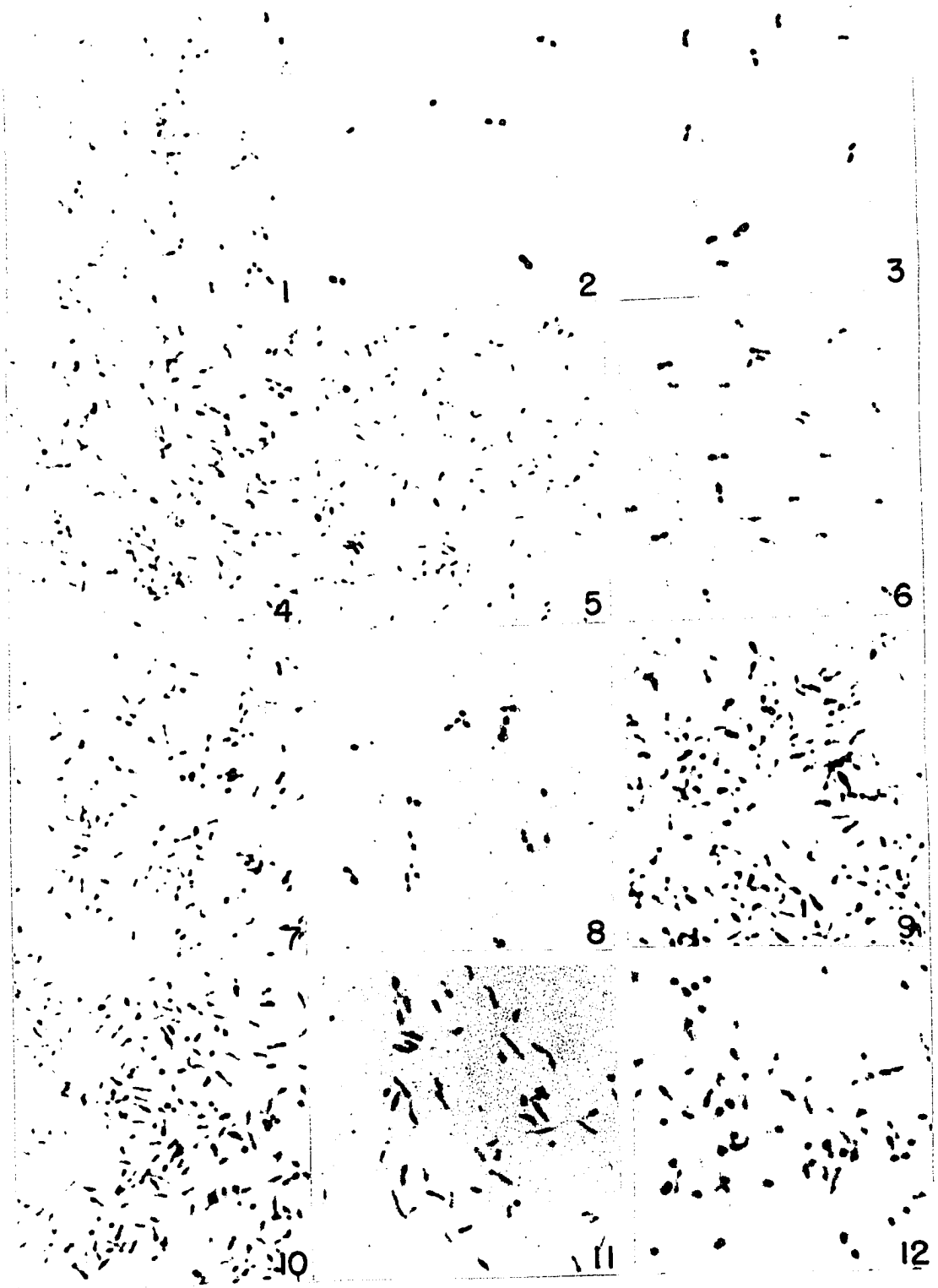

Figure IX.

Shaken Peptone Cysteine Broth Inoculated with Shaken Peptone Cysteine Broth Culture Strain Schu.

observed. Thereafter, until after 48 hours, short and long bipolar staining bacillary forms were found to comprise the majority of the various forms observed. It is noteworthy that as early as 12 hours after inoculation dumbbell filamented coccoid, and various bizarre forms were not infrequently observed. Seventy-two hours and thereafter coccoid and filamentous forms were dominant and bizarre forms were frequently observed.

Figure X. Shaken peptone cysteine broth inoculated with shaken peptone broth culture strain Schu. The morphology of the organisms present in the inoculum used was the same as that described for figures VII and VIII. Coccoid and filamented coccoid forms predominated. Four hours after inoculation it was noted that bipolar staining bacillary forms were in the majority. No further appreciable change in morphology was observed until after 48 hours. At that time filamented forms became increasingly greater in number. Filamented and coccoid forms were in the majority thereafter; also an increase in bizarre forms was readily observed.

Figure XI. Shaken peptone broth inoculated with shaken peptone broth culture strain Schu. As noted in figure X a change from coccoid to bacillary form had occurred during the first 4 hours of growth. No further

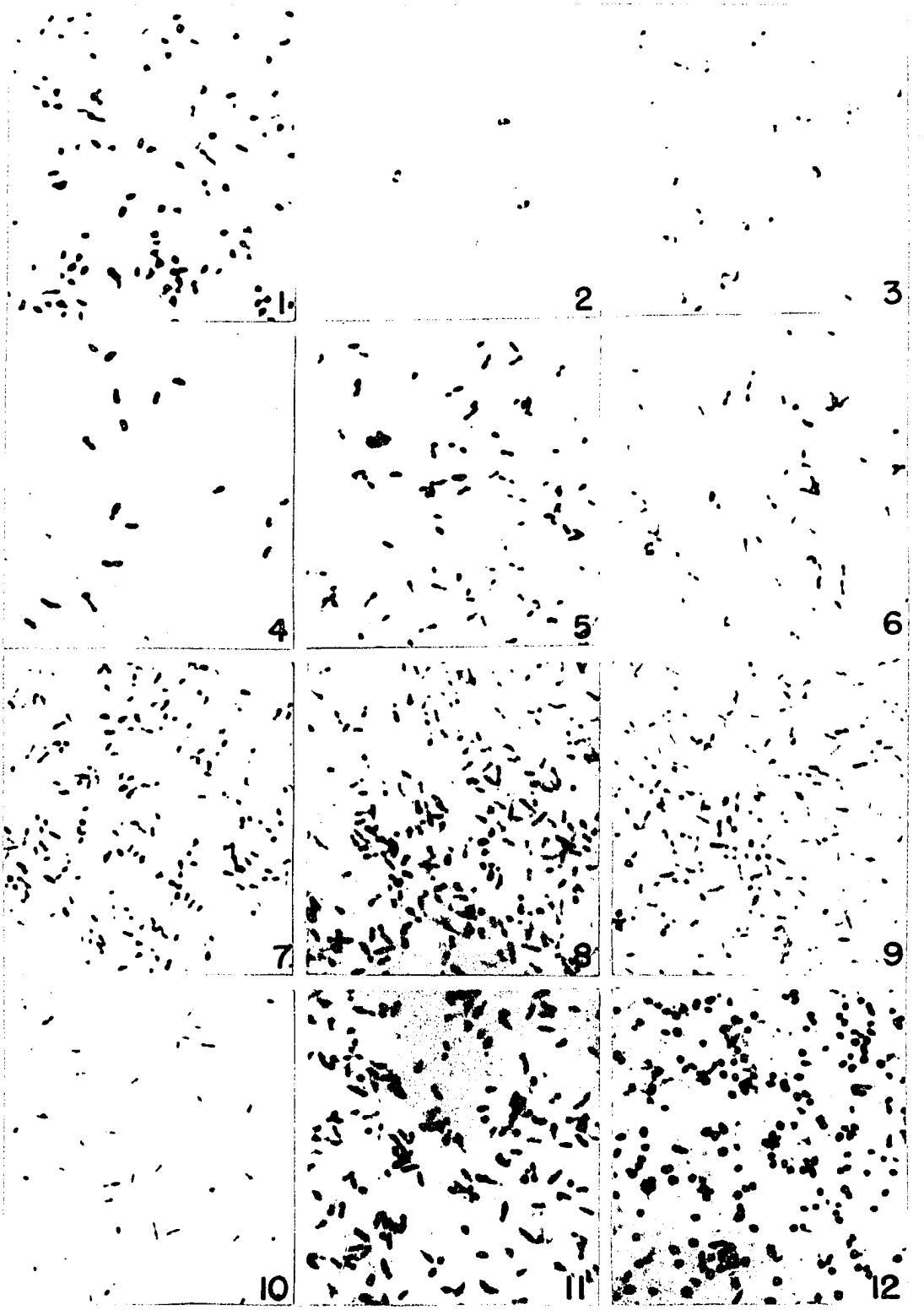

Figure X.

Shaken Peptone Cysteine Broth Inoculated with Shaken Peptone Broth Culture Strain Schu.

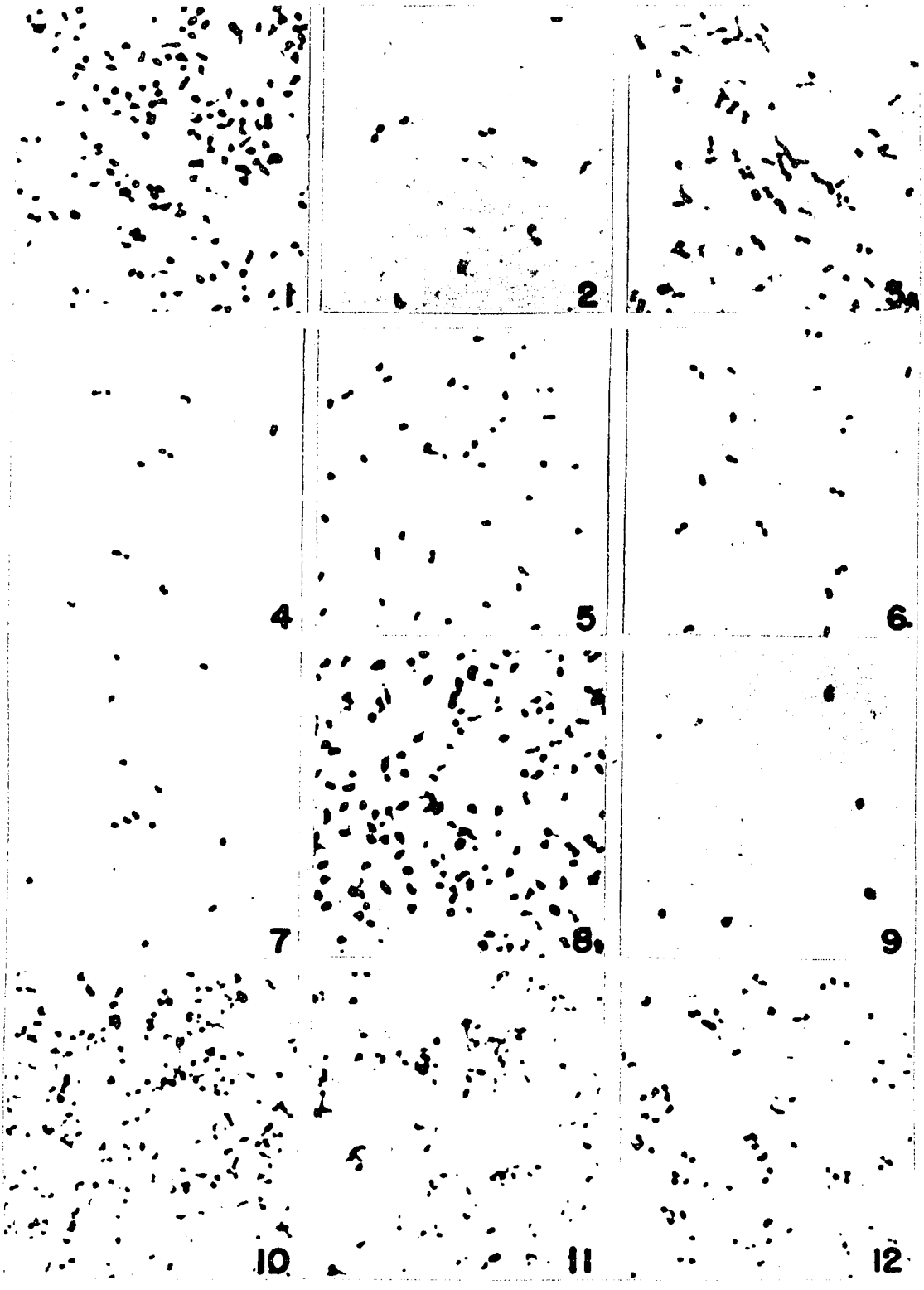

Figure XI.

Shaken Peptone Broth Inoculated with Shaken Peptone Broth Culture Strain Schu.

definite change in morphology was noted until 16 hours. At that time an increase in number of coccoid forms became evident. Large and small coccoid forms predominated until after 48 hours. With increasing age the filamented and bizarre forms became more prominent in number.

Figure XII. Shaken peptone cysteine broth inoculated with shaken peptone broth culture strain 38. It will be observed that the cells used as inoculum were of a variety of forms. Though the bipolar staining bacillary form predominated, coccoid and diploid forms were numerous. As growth proceeded after inoculation there was an increase in the number of large bipolar bacillary forms. However, no dramatic change in morphology was observed until after 20 hours. Note the variety of forms 24 hours after inoculation. (Photomicrograph 7.) With increasing age an increasing number of filamented forms appeared. However, after 96 hours large coccoid forms predominated, though filamented and bacillary forms were frequently observed.

Figure XIII. Shaken peptone broth inoculated with shaken peptone broth culture strain 38. As in figure XII, the cells used as inoculum consisted mainly of bipolar staining bacillary forms. However, coccoid and diploid forms were numerous. No definite change in morphology with the exception of the appearance of several large

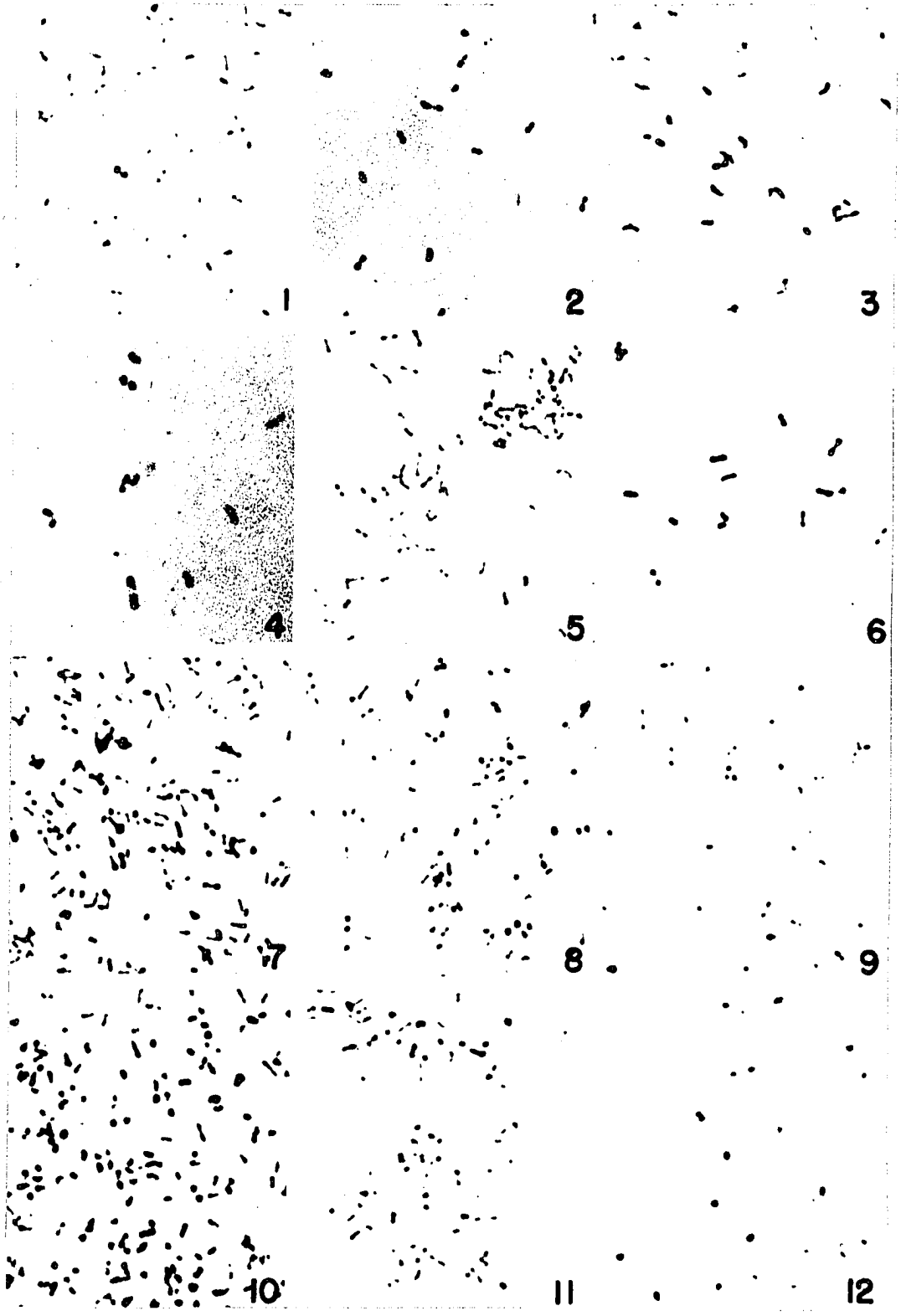

Figure XII.

Shaken Peptone Cysteine Broth Inoculated with Shaken Peptone Broth Culture Strain 38.

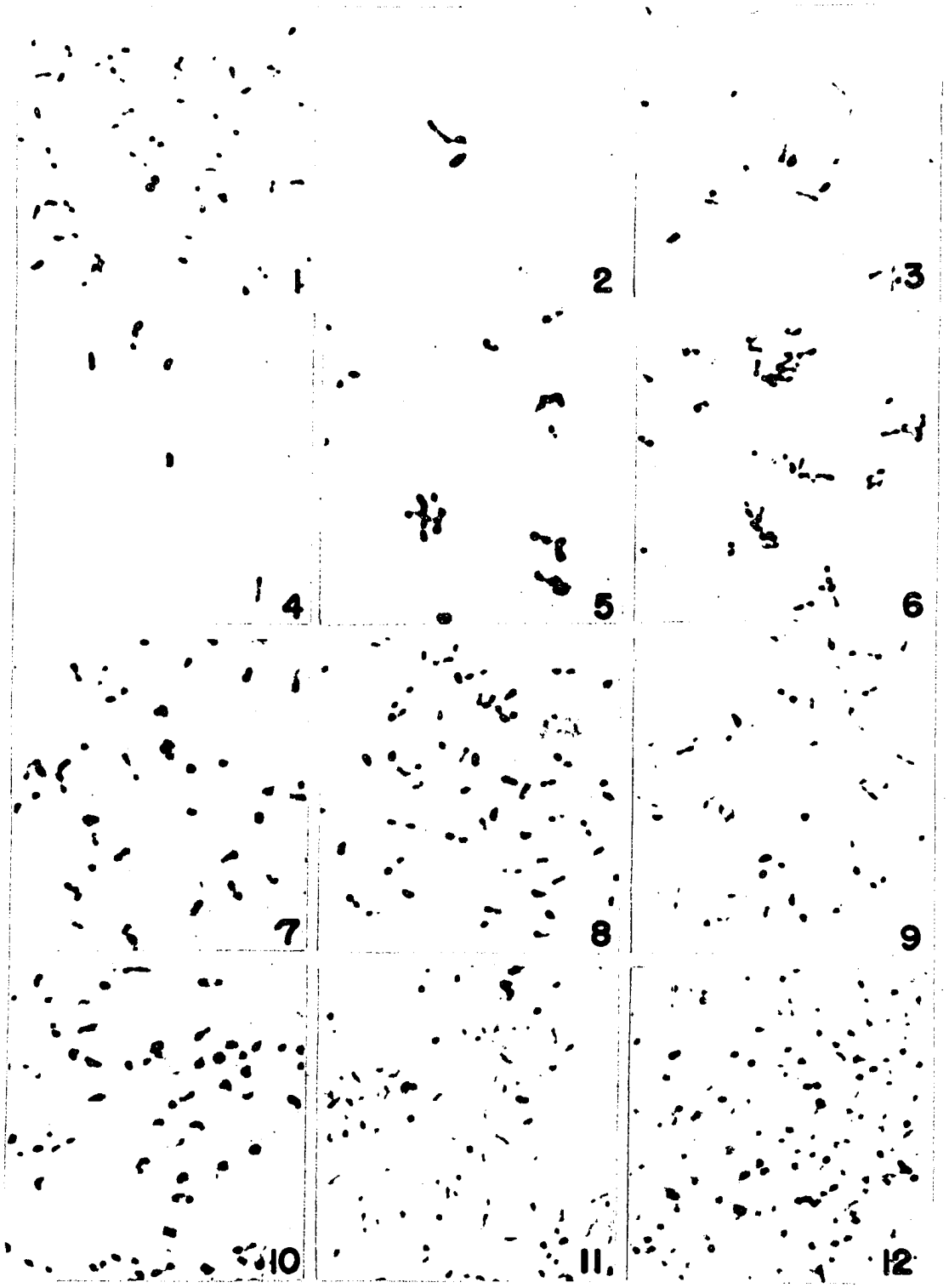

Figure XIII.

Shaken Peptone Broth Inoculated with Shaken Peptone Broth Culture Strain 38.

bipolar staining bacillary forms occurred until after 36 hours. With increasing age more filamented and large irregular forms were observed. At 96 hours filamented forms predominated. Thereafter, however, fewer filamented forms were observed and large coccoid and irregular forms became more numerous.

Figure XIV. Strain Schu cultivated on dextrose cysteine blood infusion agar. The cells used for inoculation consisted mainly of coccoid forms. Four hours after inoculation of the new medium large coccoid forms increased in number. As growth proceeded a definite alteration in morphology was observed. Eight hours after inoculation short bacillary forms predominated and coccoid forms were relatively few in number. However, after that time an increase in coccoid forms became evident. Twenty-four hours after inoculation large and small coccoid forms predominated and persisted throughout the period of examination. Beginning at forty-eight hours there appeared more and more filamented and irregular forms. With increasing age a greater number of large coccoid forms were seen.

Electron Microscopy. Most frequently observed during this study with both liquid and solid media was the small coccoid form approximately 0.45 to 0.5 u in diameter, although large coccoid, large and small bacillary, oval,

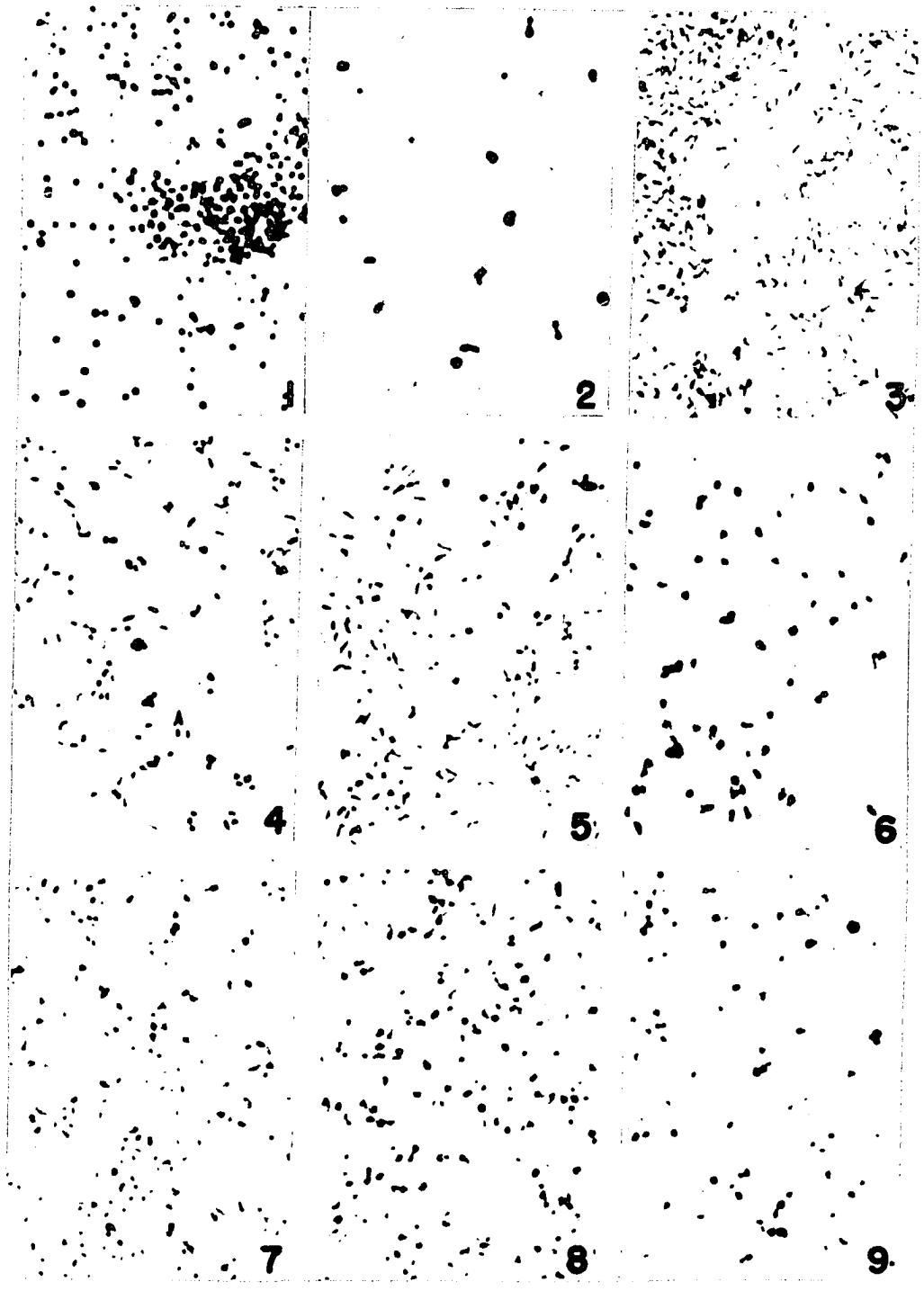

Figure XIV.

Strain Schu Cultivated on Dextrose Cysteine Blood Infusion Agar.

minute, and filamented forms were often seen. Less frequently observed were bean-shaped, dumbbell, bizarre, and so-called "involution" forms which usually represented but a small portion of the total population. Finely filamented forms were more frequently observed in cultures incubated for 96 hours or longer. Occasionally delicate filaments attached to the cell, or broken and free, were observed. Frequently these filaments contained more dense minute concentrations described and termed "minimal reproductive units" by Hesselbrock and Foshay (1). Areas of greater electronic density described by these authors as characteristic peripheral chromatin concentrations were often seen. The various morphological forms appeared singly, in diploform, or in short or long chain formation. No morphological differentiation could be made between the strains used in this investigation.

During the course of this study minute forms as small as 110 mu in diameter were observed. Examination of the electron micrographs revealed that although electron micrographs were in sharp focus as evidenced by the edge of the collodion film seen in various preparations, the cells generally presented a hazy, nebulous appearance. Bacterium tularense seemed to possess an extremely delicate outer limiting structure (cell wall) of very low electronic

density in contrast to the cell walls of various other organisms described by Wámoscher (22) and reported to be "extremely solid, elastic, extensible, and enormously resistant to pressure". The extremely delicate structure of the cell wall of Bacterium tularense is best illustrated in electron micrographs showing large coccoid and large bacillary forms the cytoplasm of which is unevenly distributed or has shrunken, allowing the cell wall to be more clearly visible. Usually it was difficult or impossible to differentiate the limiting edge of the delicate cell wall from the surrounding area. Ruptured cells and their extruded cytoplasm were frequently observed. Although semitransparent cytolyzed cells were often observed and carefully examined, jagged lines of fracture which would indicate the presence of a solid or rigid cell wall were not demonstrated.

Figures XV to XXIII depict the morphology of strains Schu and Memp as observed with the R.C.A. Type B Electron Microscope.

Electron micrographs were taken at a magnification of 6,800 diameters with the exception of number 25, the original magnification of which was 8,100 diameters. All electron micrographs were enlarged 2 to 4.25 times their original magnifications. The scale of magnification is

given by the one micron scale appended to each figure unless otherwise indicated.

Description of Electron Micrographs Made with the Aid of the R.C.A. Type B Electron Microscope and Presented in Figures XV to XXIII.

1. Saline suspension prepared from a 24 hour dextrose cysteine blood infusion agar culture of strain Schu and reincubated at 37 C for 20 hours. Cluster of coccoid forms showing peripheral arrangement of areas of greater electronic density.

2. Same. Coccoid form with irregular protoplast of greater density which has shrunken from the cell wall.

3. Same. Smaller coccoid form showing extremely delicate cell wall.

4. Saline suspension prepared from a 96 hour dextrose cysteine blood infusion agar culture of strain Schu and reincubated at 37 C for 5 hours. Filamented coccoid form with small spherical body at tip of filament.

5. Peptone broth culture of strain Schu incubated at 37 C for 24 hours. Two coccoid forms and adjacent bacillary form are shown.

6. Same. Large and small coccoid and oval forms. Smallest coccoid form at lower left is approximately 210 mu in diameter.

7. Same. Note so-called "involution" form at left.

8. Same. Filamented bacillary form.

9. Distilled water suspension prepared from a

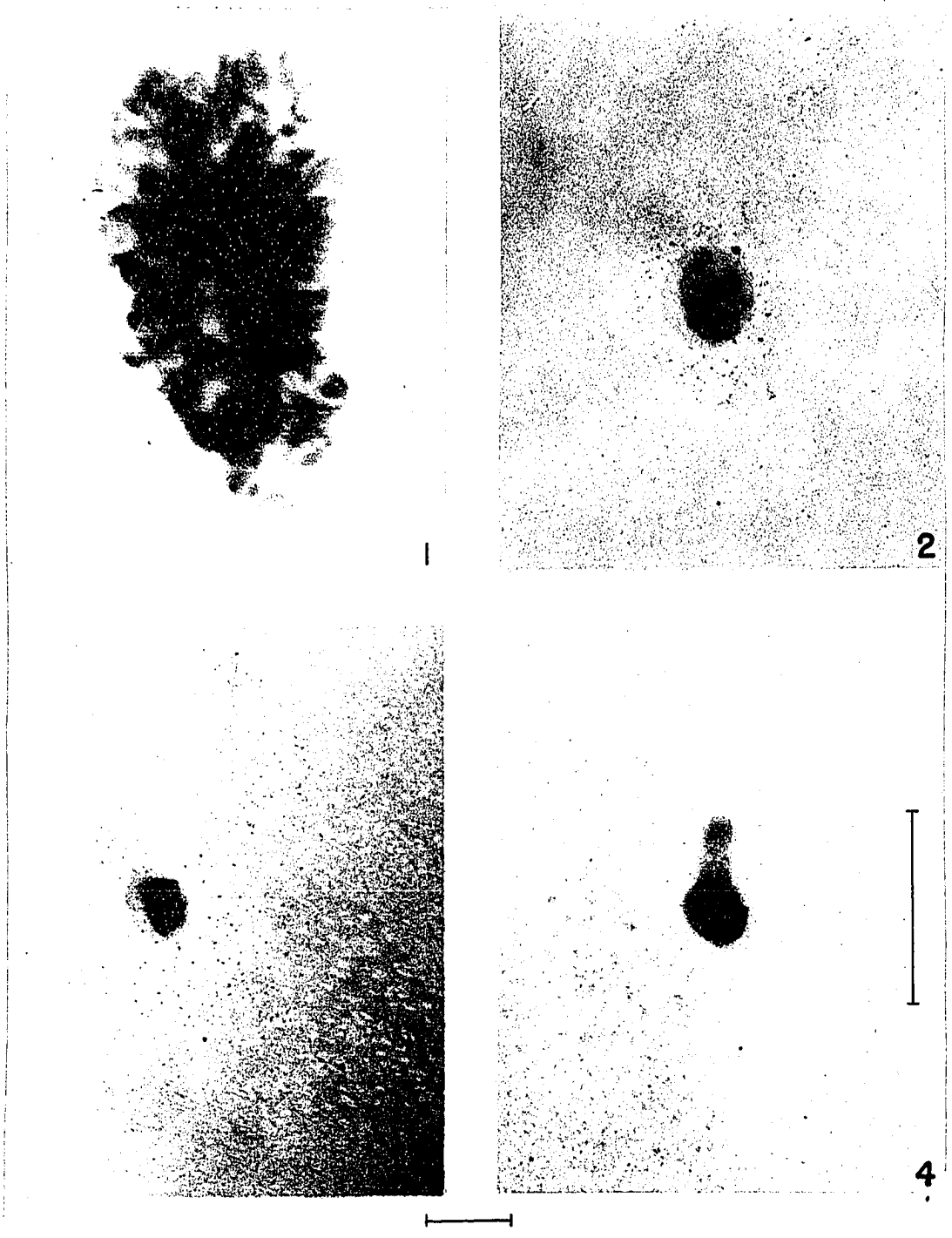

Figure XV.

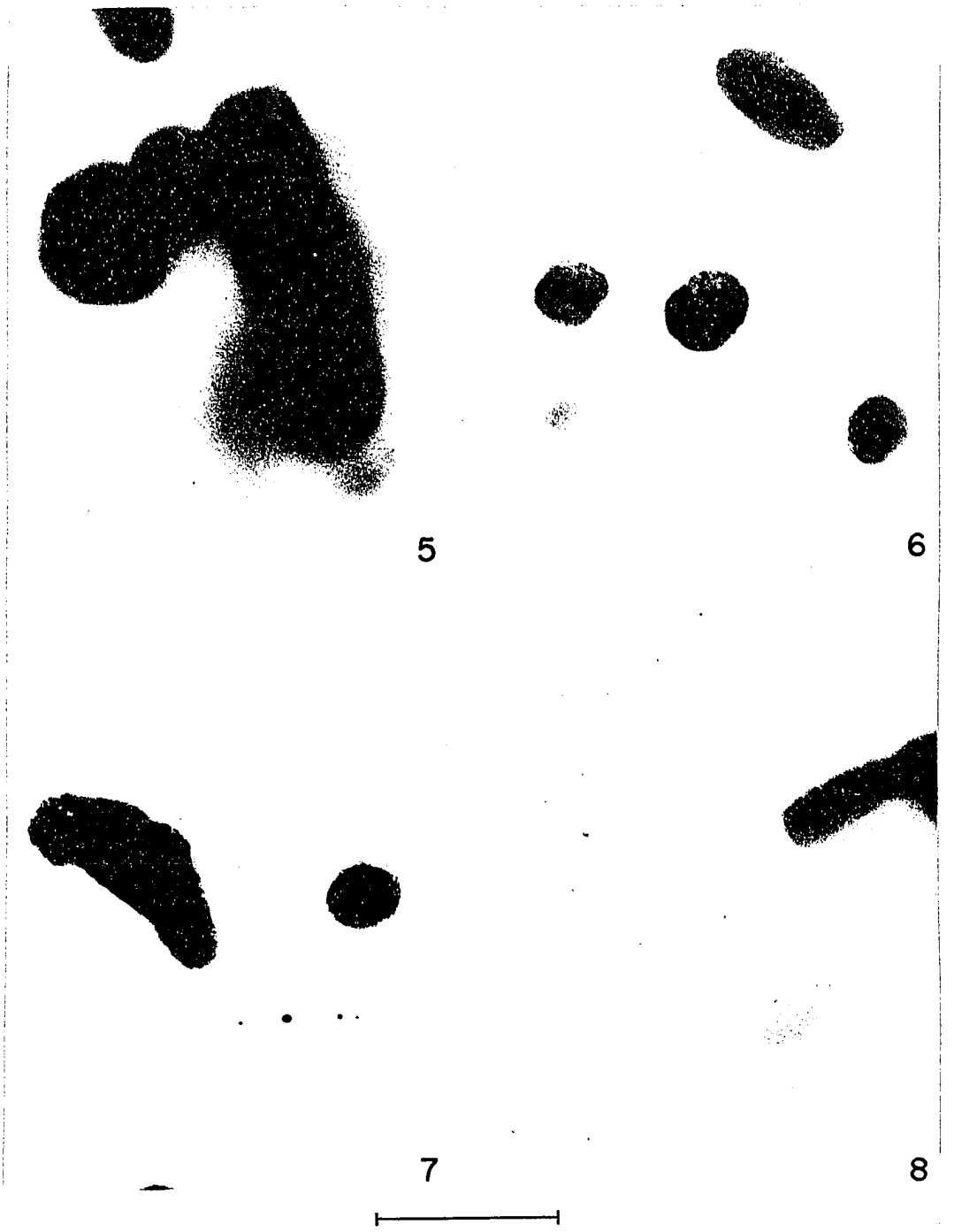

Figure XVI.

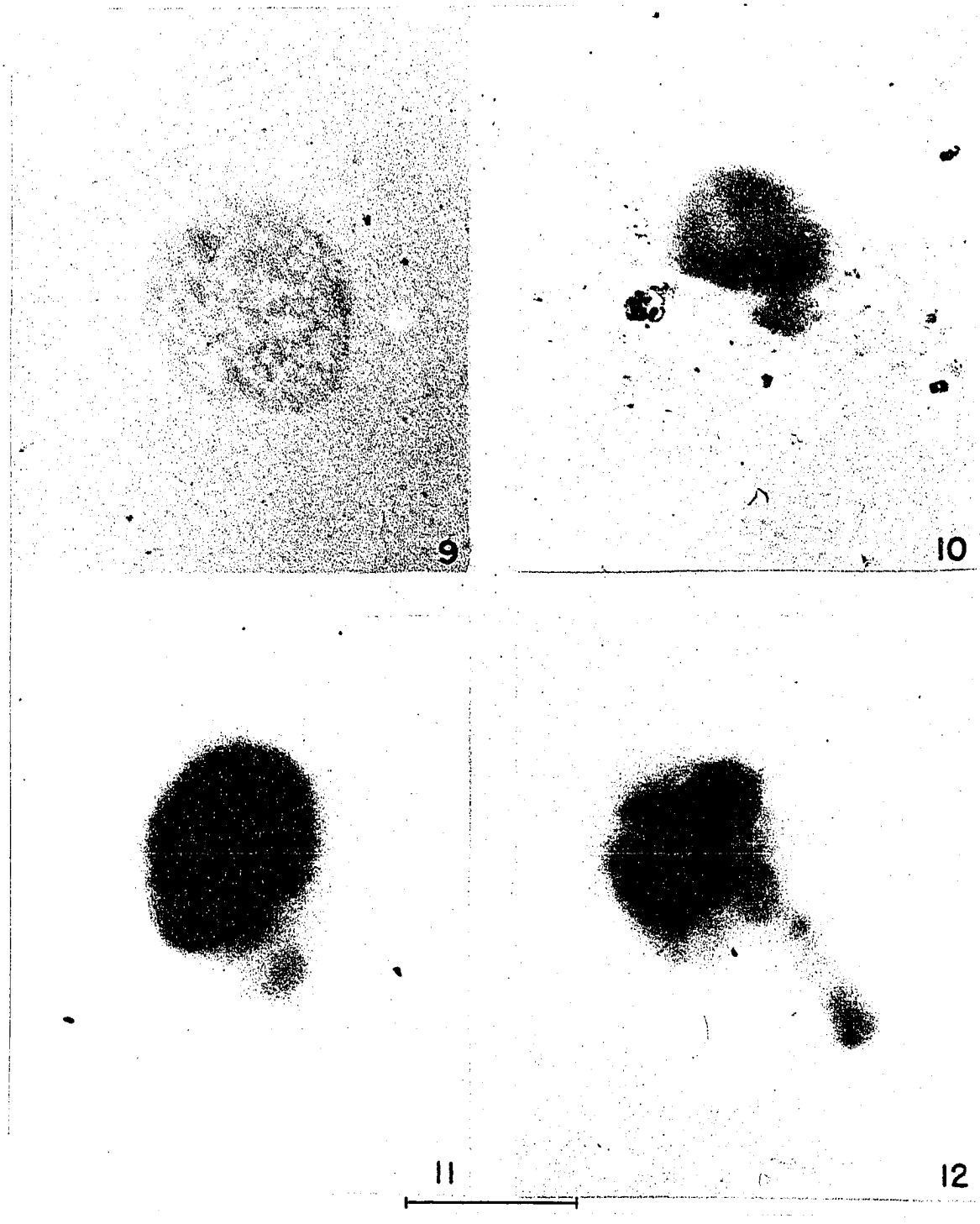

Figure XVII.

24 hour dextrose cysteine blood infusion agar culture of strain Schu and reincubated at 37 C for 5 hours. Disintegrating coccoid form. Note "punched-out" appearance.

10. Distilled water suspension prepared from a colony of strain Schu on DCBA which had been incubated for 72 hours and allowed to remain at room temperature overnight. Coccoid form suggesting early budding.

11. Gelatin hydrolyzate culture of strain Schu incubated at 37 C for 8 days. Another coccoid form suggesting budding.

12. Same. Granular coccoid form with filamentous projection.

13. Same. Unusual shapes probably produced by traumatic distortion of cells.

14. Same. Chain of coccoid forms. Note distribution of cellular debris produced by cellular rupture.

15. Same. Cluster of coccoid and oval cells emphasizing the lack of density of Bacterium tularense.

16. Same. Contrast sharpness of collodion film edge with nebulous character of coccoid cell shown in electron micrograph. Note that a part of the folded film edge can be seen through the semitransparent cell.

17. Same. Cluster of coccoid, crescentic, and oval forms.

Figure XVIII.

Figure XIX.

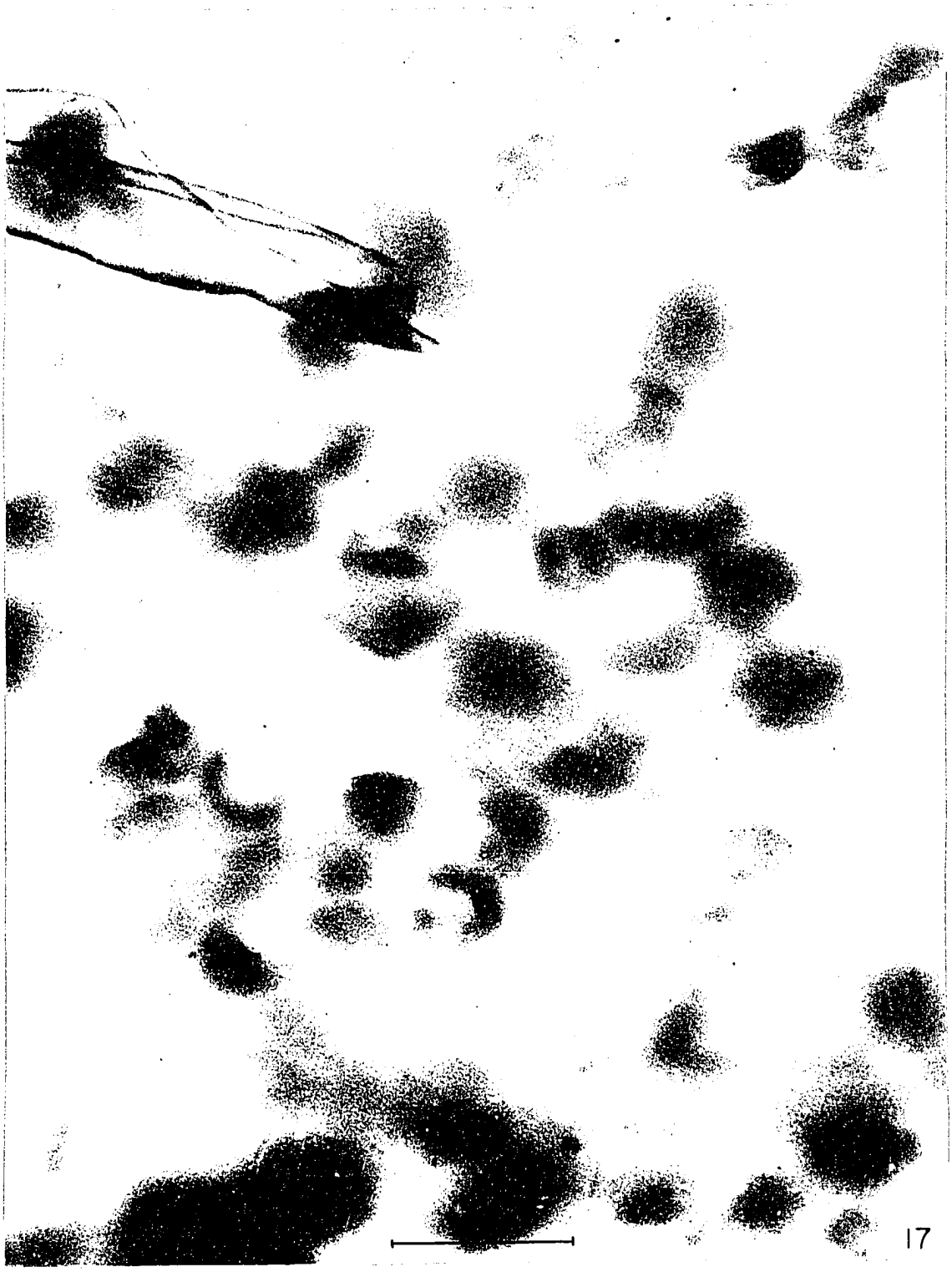

Figure XX.

18. Soybean hydrolyzate culture of strain Memp incubated at 37 C for 96 hours. Long filamentous structure.

19. Same. Two minute forms. The smaller is approximately 110 mu in diameter.

20. Same. Oval form with adjacent partially disintegrated cell.

21. Same. Large bacillary dumbbell shaped structure with more dense spherical concentrations at terminal ends.

22. Same. Coccoid forms showing peripheral areas of increased density.

23. Same. Group of bacillary forms. Note the bipolar arrangement of areas of greater density within a number of cells.

24. Distilled water suspension of Staphylococcus aureus. Note density and definite outline of these cells in contrast to cells of Bacterium tularense.

25. Washed Rickettsia rickettsiae. Note definite morphology and cell wall.

26. Distilled water suspension of Eberthella typhosa "R". Contrast the cell wall and density of this organism with the cell wall and density of Bacterium tularense. Protoplasts of these forms have shrunken from the cell walls.

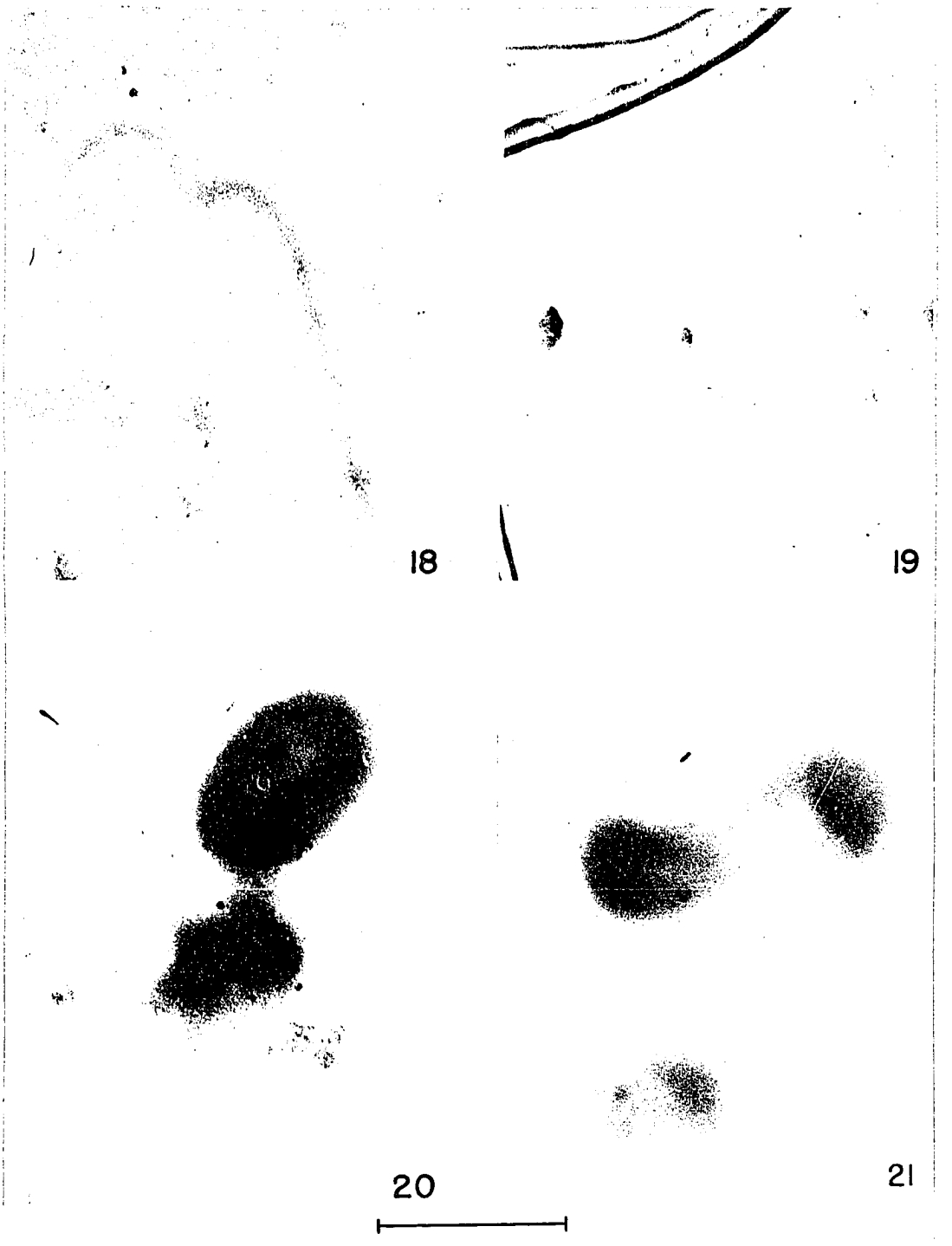

Figure XXI.

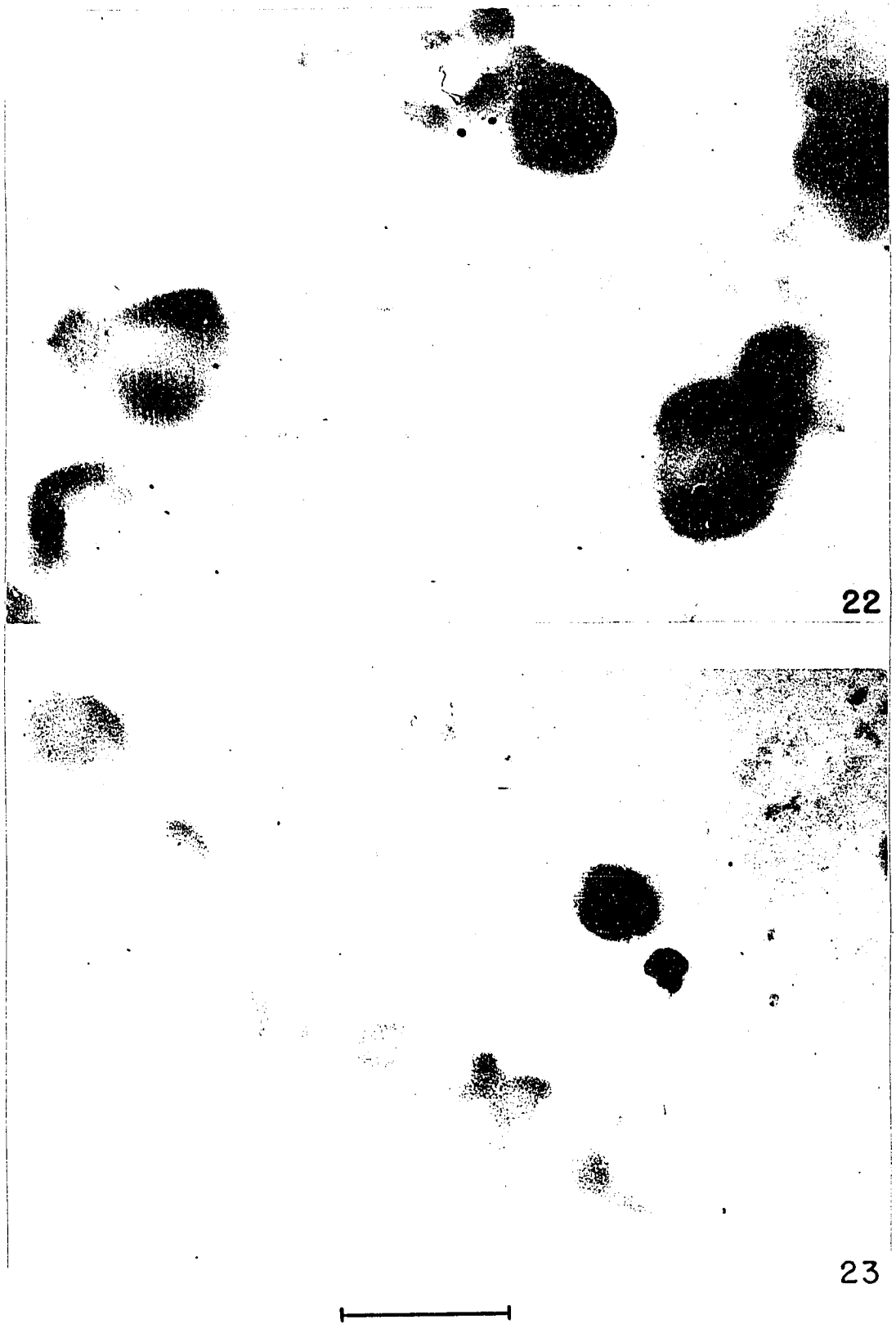

Figure XXII.

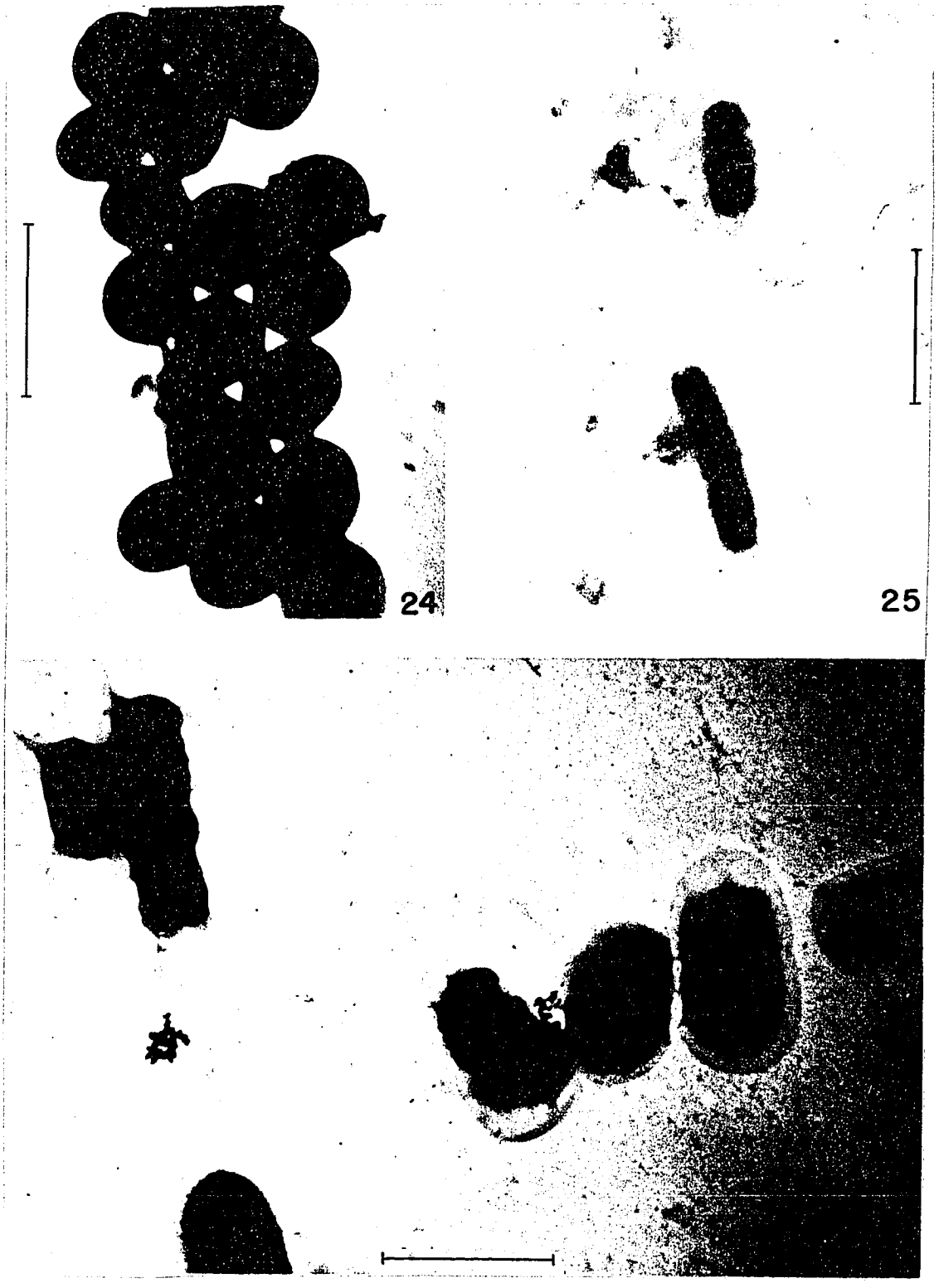

Figure XXIII.

Electron micrographs made with the Type EMU Electron Microscope equipped with an objective aperture presented more contrast than those made with the Type B instrument without an objective aperture. This greater contrast is attributed directly to the use of the objective aperture. It is well known that contrast in an object illuminated by a beam of electrons is not due to differential absorption but rather to an unequal amount of scattering of electrons at different points in the preparation and that the image responsible for an electron micrograph is formed by focusing the electrons that penetrate the specimen and its substrate. Williams and Wyckoff (11) have explained that contrast in the final image is a result of the failure of lenses to focus accurately the electrons which are scattered through relatively large angles while passing through specimen and substrate. Should the widely scattered electrons be permitted to strike the photographic plate, a generally distributed fogging results. However, if a small objective aperture is employed widely deflected electrons are excluded and only those which are able to be focused with precision by the objective and projector "lenses" are permitted to pass. In this manner greater contrast is achieved.

The shadow casting technique of Williams and Wyckoff

(11) was employed in the preparation of a number of specimens and several desirable effects were observed. It was found that maximum contrast was achieved by use of this technique. Also the thicknesses of various forms could be estimated from the lengths of the shadows which they had cast. The three dimensional effect aided greatly in the detection of minute forms of the organism. Filaments which produce a minimum of contrast against the background of the specimen were made clearly evident by the shadow casting technique.

Several specimens were prepared according to the modified replica technique recently described by Hillier and Baker (10). This method of specimen preparation permits study of the exact physical relationship of cells in a growing colony and makes possible a correlation between changes in morphology and the processes of cell division. However, observation of several specimens of Bacterium tularense, strain 38, prepared according to this method indicated that the technique was of limited value in the examination of this organism. It was exceedingly difficult to obtain a monocellular layer and subsequent examination revealed that organisms in carefully prepared specimens were grossly distorted. Failure to obtain good preparations seems to be related

directly to the buttery consistency of the colony, the lack of rigidity, and small size of the organism.

Replicas prepared according to the slide method proved somewhat more successful. However, the study of the exact physical relationship of cells in the growing colony was not possible by this method.

Despite the fact that special techniques were employed to enhance contrast of the organism no significant difference in morphology was observed which would differentiate strain 38 from strains used in the study with the Type B instrument. As in the previous study, large and small coccoid and bacillary, oval, minute, and filamented forms were seen most frequently. Bean-shaped, dumbbell, bizarre, and so-called "involution" forms were also noted occasionally. Several forms observed in specimens prepared from 72 hour colonies strongly suggested multiplication by means of budding and others by the process of binary fission.

A greater number of filamented forms were observed in specimens prepared from 120 hour colonies than from 72 hour colonies. As previously noted, filaments frequently contained one or more minute dense concentrations.

Utilization of the shadow casting technique, where-

by relatively thick organisms collect a thicker deposit in the direction of the oncoming metal atoms and at the same time shield other areas to produce what might be called "permanent" shadows of themselves, confirmed the initial observation by Hesselbrock and Foshay (1) that many bacillary forms of the organism are taeniform or ribbon-shaped. The dense terminal ends of bipolar bacillary forms seemed to possess greater thickness than the almost transparent ribbon-like central portions of the organism as evidenced by the shadows cast. A few coccoid forms appeared flattened but the majority, including minute forms, cast shadows the dimensions of which indicated their spherical shape.

The increased contrast produced by the objective aperture permitted even more precise examination of the cell wall than was possible in the previous study. Despite the contrast realized it was difficult to distinguish the limiting edge of the cell wall from the surrounding area. Thus previous observations concerning the lack of density and the delicate construction of this structure of Bacterium tularense were confirmed. Capsules were not demonstrated.

In general, previous observations of the morphology of Bacterium tularense made with the aid of the Type B

instrument were confirmed by this study in which special techniques were employed to enhance contrast. It is noteworthy that all forms which have been shown to make up the complex morphology of Bacterium tularense were seen in almost every preparation examined with either the Type B or the Type EMU instrument, and that no morphological differentiation could be made between the strains examined.

Figures XXIV to XXIX depict the morphology of strain 38 as observed with the R.C.A. Type EMU Electron Microscope equipped with an objective aperture.

Electron micrographs were taken at a magnification of 6,100 diameters and enlarged to 18,300 diameters. The scale of magnification is given by the one micron scale appended to each figure.

Description of Electron Micrographs Made with the Aid of the R.C.A. Type EMU Electron Microscope Equipped with an Objective Aperture and Presented in Figures XXIV to XXIX.

1. Distilled water suspension prepared from 120 hour colony of strain 38 cultivated on DCBA. Cluster of irregular coccoid cells. Note "ghost-like" cells within cluster which exhibit a lack of electronic density. A bacillary form possessing irregular areas of greater electronic density is shown at the lower left.

2. Same. Note large coccoid form at lower left and adjacent bacillary "ghost" cell. Protoplast of the coccoid form has shrunken thus revealing the cell wall. The lack of density of the bacillary "ghost" cell emphasizes the delicate character of the cell wall of Bacterium tularense. An oval form is seen above, and below is found a bacillary form containing irregular areas of greater density. The protoplast of this cell has shrunken from its almost invisible cell wall.

3. Same. Left: Coccoid form displaying medial cross sectional line of low density. Below a crescent-like form can be seen. Right: Bacillary form possessing bipolar areas of greater density. Note delicate cell wall of this cell.

4. Same. Left: Coccoid form with small semicircular peripheral area of less density. It is possible that the smaller coccoid form adjacent to this area represents a budding process of the larger form. Right: Oval cells

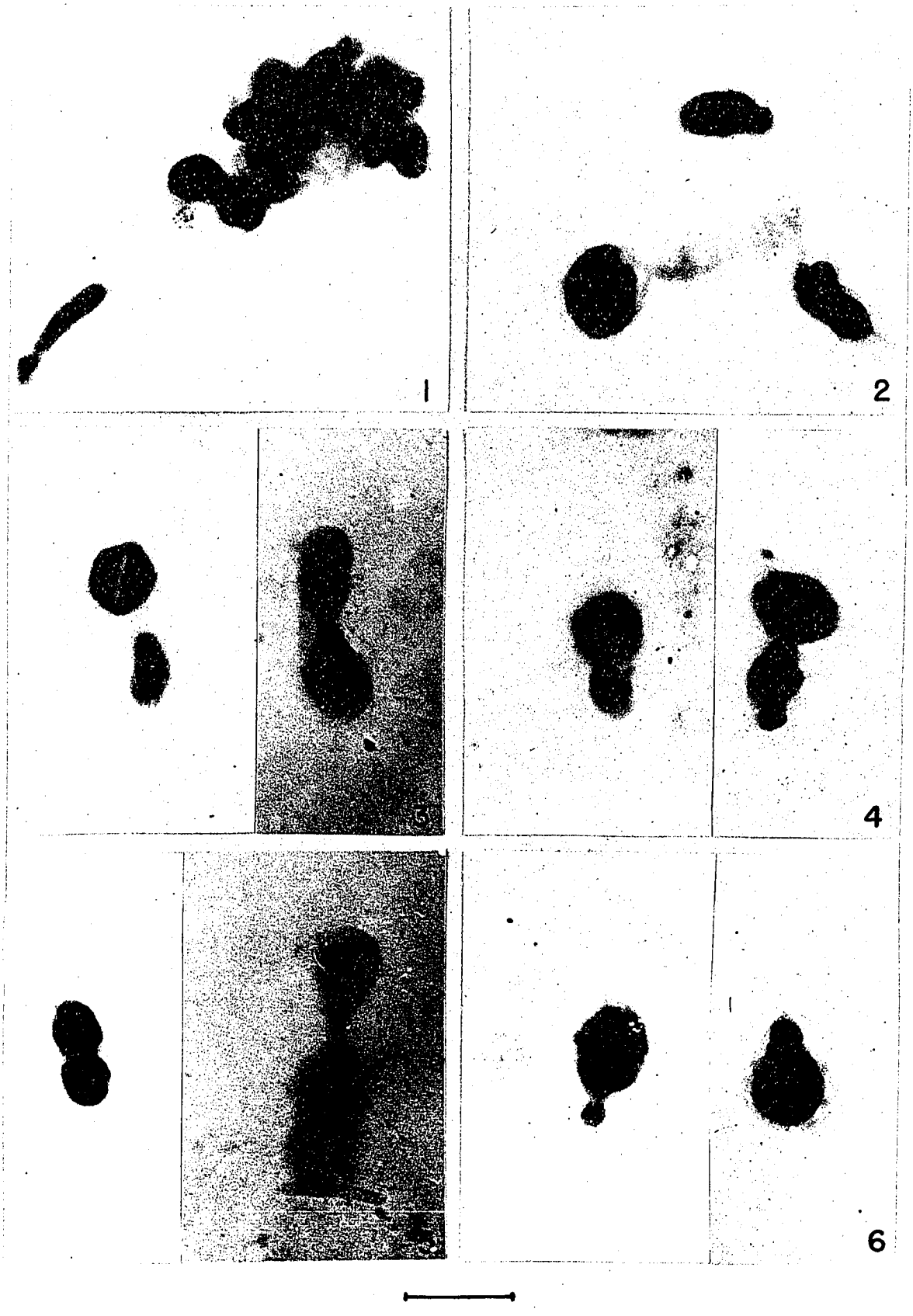

Figure XXIV.

showing irregular areas of varying density.

5. Same. Left: Short bacillary form exhibiting dense bipolar concentrations of cytoplasm. Right: Note the filamentous-like attachment between the two topmost cells.

6. Same. Budding coccoid forms. Note delicate continuous cell wall of form at right.

7, 8, 9, 10. Same. Filamented coccoid forms. Note dense minute concentration within filament extending from dense peripheral area of coccoid form shown in electron micrograph number 8.

11. Same. Long filamentous linkage between cells. Note dense minute concentrations within linkage. Form at lower right shows small semicircular peripheral area of less density than remainder of cell.

12. Gold shadowed replica of 120 hour colony of strain 38 cultivated on DCBA. A true relationship of the cells of the colony is not shown, and the cells appear highly distorted. The method of replica preparation employed was unsatisfactory.

13. Gold shadowed replica prepared from a 120 hour colony of strain 38, the cells of which were suspended in distilled water and allowed to dry on a clean glass slide. Note coccoid depressions imprinted in the formvar film by

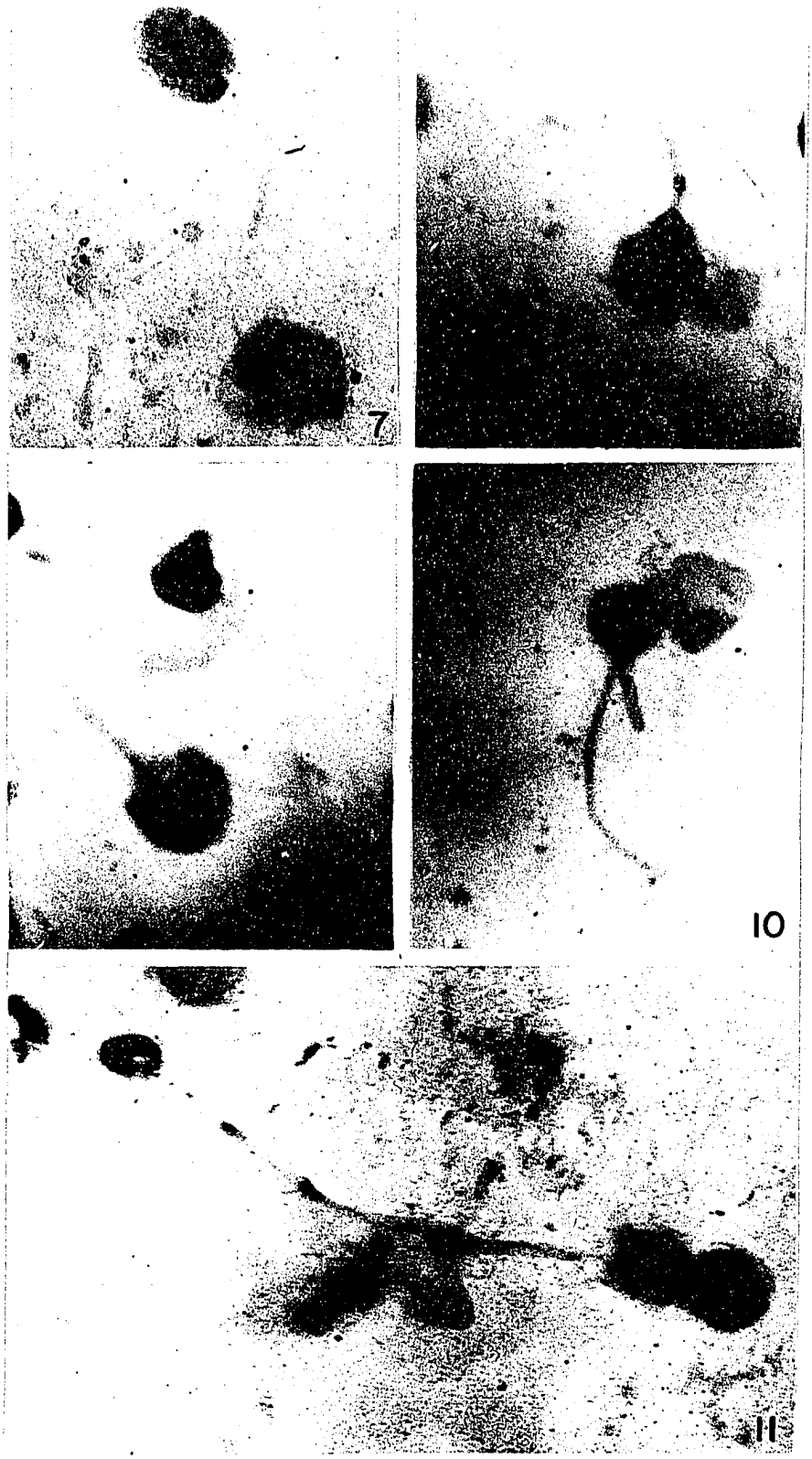

Figure XXV.

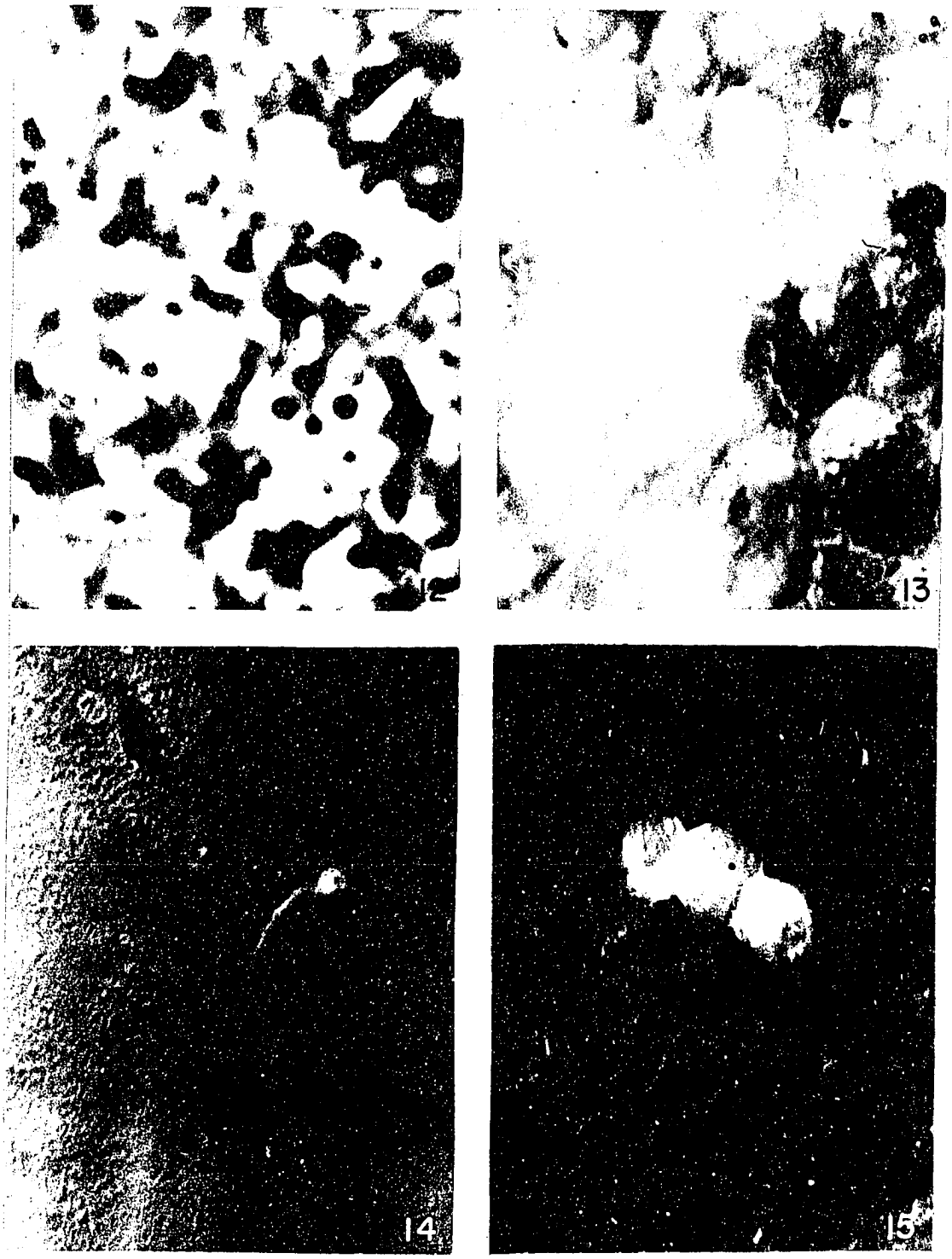

Figure XXVI.

cells which adhered to the slide.

14. Gold shadowed preparation of a distilled water suspension of cells obtained from a 120 hour colony of strain 38 cultivated on DCBA. The minute filamented coccoid form is approximately 220 mu in diameter.

15. Same. Cluster of 3 coccoid forms. Note filament arising from one of the cells.

16. Gold shadowed replica prepared from a 72 hour colony of strain 38, the cells of which were suspended in distilled water and allowed to dry on a clean glass slide. Note the coccoid depressions imprinted in the formvar film by cells which adhered to the slide. A single coccoid cell and its corresponding shadow is shown at the upper right.

17. Gold shadowed preparation of a distilled water suspension of cells obtained from a 72 hour colony of strain 38 cultivated on DCBA. Long and short bacillary forms and an oval form are shown. The sharp dense edges of the shrunken protoplasts can be readily differentiated from the cell walls of these forms.

18. Same. Note that coccoid and bacillary forms appear to show a wrinkled cell surface.

19. Same. Coccoid forms and their corresponding shadows.

20. Same. Underexposed electron micrograph of bacillary

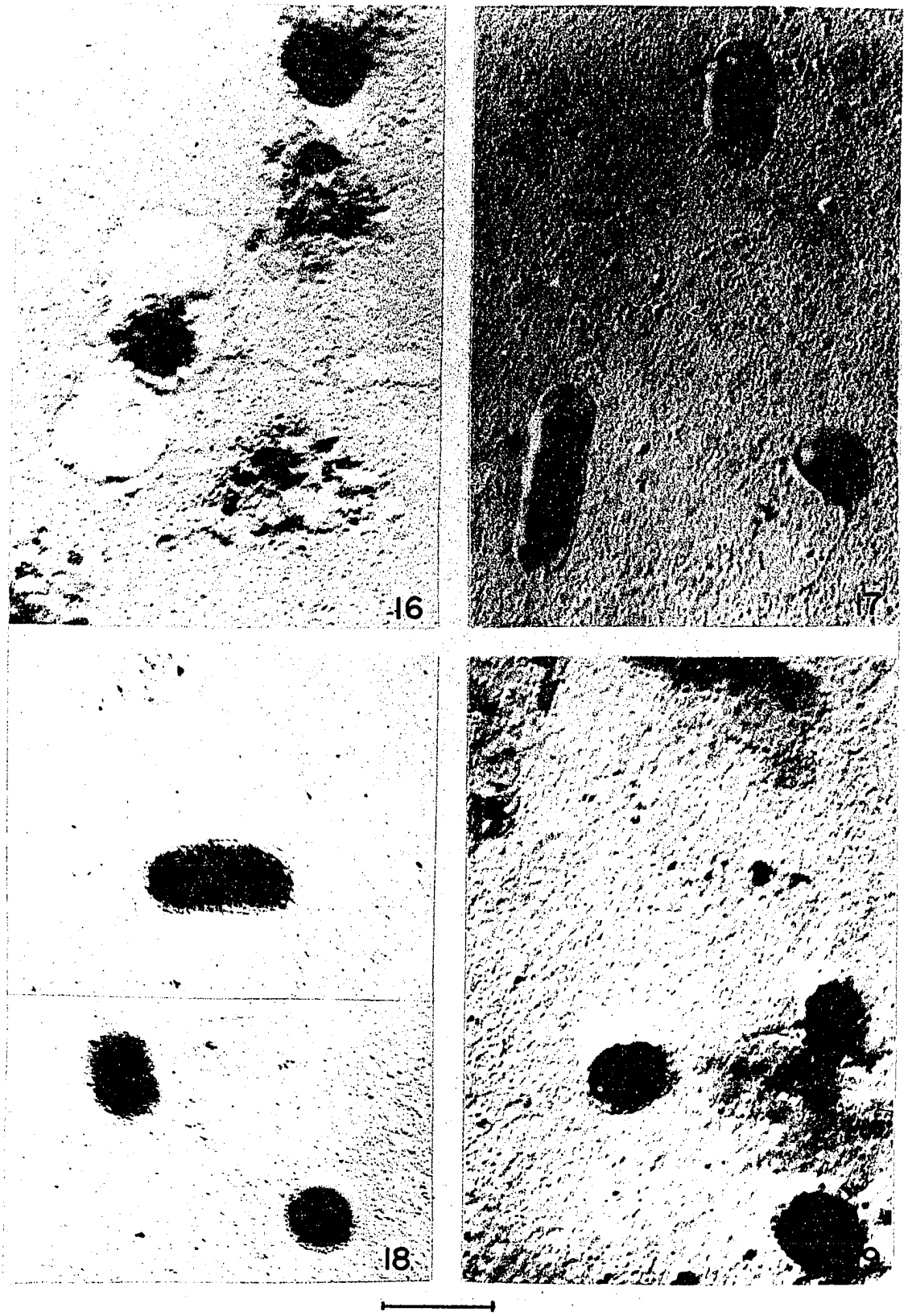

Figure XXVII.

form showing granular internal structure.

21. Same. Large disintegrating coccoid cell.

22. Same. Minute coccoid form. This cell is approximately 220 mu in diameter.

23. Same. Cellular form strongly suggesting multiplication by binary fission. Also note the minute coccoid form of approximately 220 mu in diameter.

24. Same. Short bacillary forms. The protoplasts of these forms have shrunken from the terminal ends of the cells.

25. Same. Top: Bipolar bacillary form. Note delicate cell wall and filamentous linkage between bipolar concentrations of dense cytoplasm. Bottom: Another cellular form strongly suggesting multiplication by binary fission.

26. Same. Extreme left: Coccoid form and adjacent irregular filamented coccoid form. Extreme right: Minute coccoid form approximately 270 mu in diameter.

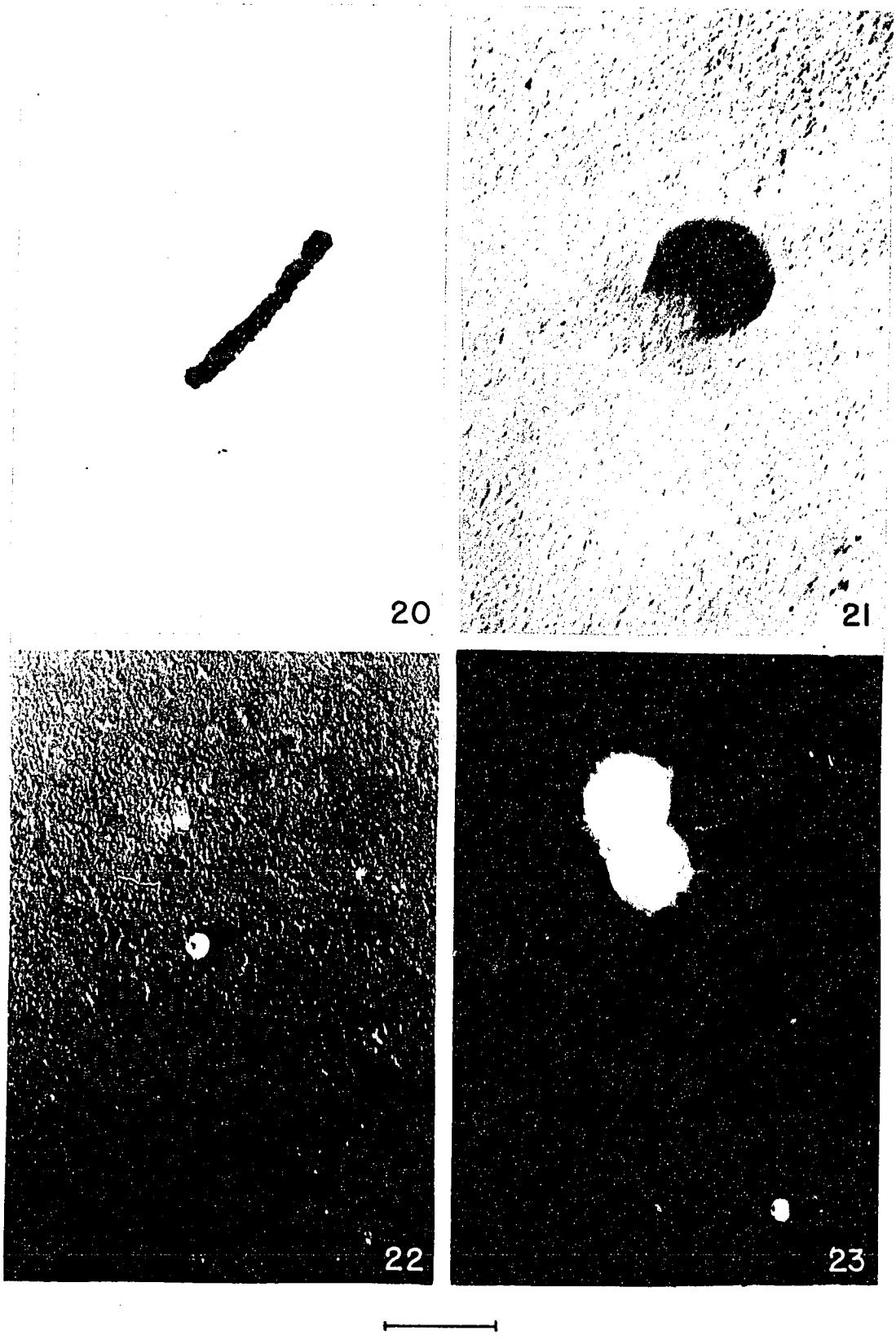

Figure XXVIII.

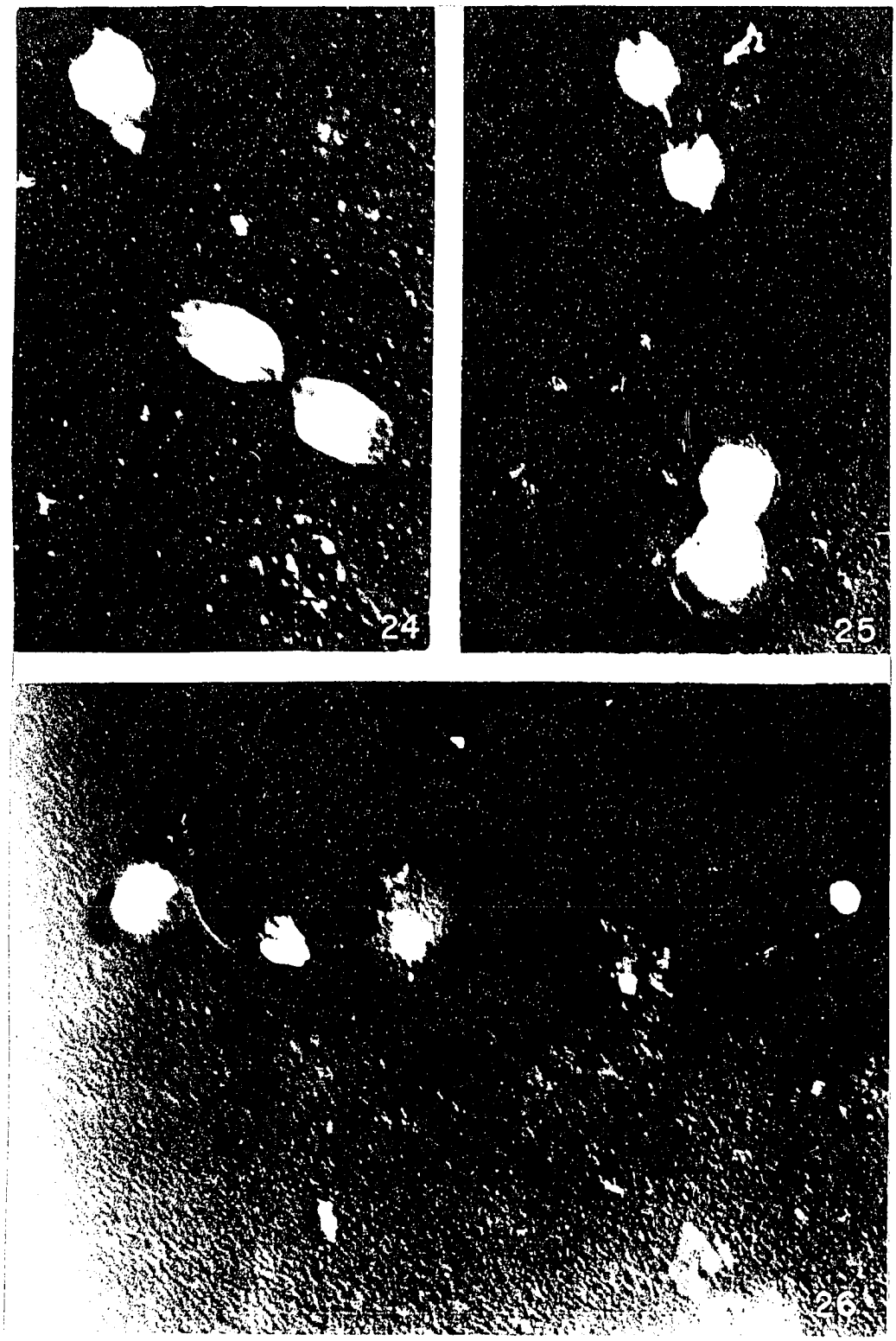

Figure XXIX.

DISCUSSION

It is well established that many physical and chemical properties of the environment, such as the surface tension and reaction of the medium, the abundance of oxygen supply, concentration of nutrients and electrolytes, and the temperature of incubation affect not only the shape and dimensions of bacterial cells but also the comparative rates at which protoplasm synthesis, cell division, and cellular disintegration takes place. Henrici (23), Sherman and Albus (24), and Winslow and Walker (25) have all pointed out that a number of properties can be correlated with various growth phases of the bacterial cell. For example, in the early growth period there is usually observed increased length and slenderness of the cell which indicates a greater magnitude of some axially disposed force opposing the surface tension of the medium, increased staining intensity with the basic dyes, change in the electrokinetic mobility indicating an isoelectric point of the protoplasm more on the acid side, increased metabolic activity, and increased susceptibility to injurious agents and procedures.

In discussing the effect of environment on the physicochemical characteristics of bacteria Dubos (26) stated that, although most bacteria can adapt themselves to an extremely wide range of osmotic pressure, the hemorrhagic septicemia group is affected by large concentrations of electrolytes

which cause the appearance of forms abnormal by their size and pleomorphism. It is appropriate to point out again that during the present study special effort was made to demonstrate the cultural suitability of the media employed. None of the media contained large or excessive concentrations of electrolytes. However, before the systematic study of the morphology of Bacterium tularense by Hesselbrock and Foshay (1) much of the information concerning the morphology of the organism had been based upon isolated observations without much regard to the suitability of media, the age of the cells, or the morphological modifications which occur during the growth cycle of the organism.

The present study of the morphology of Bacterium tularense by light and electron microscopy confirms the morphological observations of Hesselbrock and Foshay (1) and suggests that in the course of growth of the organism a progressive modification of morphological form takes place. It would seem that the complex forms observed are the normal expression of the fundamental growth cycle of the organism and not the result of the direct and reversible effect of an unfavorable physicochemical environment. It is quite illogical that such consistency of complex morphological form as was observed by various techniques could be attributed to artifact formation.

The very minuteness and pleomorphism of some forms of

Bacterium tularense which render observation by light microscopy so difficult makes electron microscopy particularly desirable. In contrast to light microscopy this method of examination provides a veracious image rather than just an indication of the presence of minute structures. The electron microscope has practically eliminated the previously all-important problem of resolving power as a major factor in bacterial cytology, and has made possible micrographs of minute particles and fine detail not visible by means of the light microscope.

However, the properties of electrons necessitate, to a certain extent, restrictions upon the types of observations possible with the instrument. For instance, the material to be examined is dried under atmospheric conditions, subjected to the vacuum of the electron microscope, and during examination bombarded with electrons which move at a high velocity. Another disadvantage is the low power of penetration, which is a function of the wave length of the electron stream and the density of the object. The penetrating power is inversely proportional to the wave length and, for a set wave length, decreases as the density of the specimen increases. Therefore, the ability of electrons of a fixed wave length to penetrate an object of

a certain density is directly related to the thickness of that object. No single wave length is suitable for all purposes. Structures of low density, such as the cell wall, clearly visible at the usual 60 kv are often invisible at higher voltage. Only at 150 kv, possible only with an experimental electron microscope, were Knaysi and Mudd (27) able to observe the nuclei of Staphylococcus flavocyaneous. Knaysi (28) has pointed out that another limitation of the electron microscope is the inability of this instrument to give a clue to the chemical nature of the structures it demonstrates, since it detects only differences of opacity to high velocity electrons. The previously mentioned conditions necessary for electron microscopy have raised questions concerning the significance and interpretation of the images portrayed in electron micrographs, especially with respect to possible alteration in size, internal structure, and to the introduction of artifacts. In a comparison of the morphology of Bacillus megatherium with light and electron microscopy, Dubin and Sharp (29) found no significant difference between measurements of the size of unstained bacteria dried in air and photographed with an optical microscope and measurements of the comparable portion (inner dense substance) of the identical bacteria later seen in electron micrographs. However, measurements of the size of

the organism obtained by Benian's Congo red method were less than those of the total bacterial substance observed in electron micrographs indicating that Benian's method fails to outline the whole bacterial cell.

It is noteworthy that in the present study the general morphology of Bacterium tularense, as depicted by light microscopy, was confirmed by electron microscopy.

The great resolving power of the electron microscope and the application of special techniques designed to enhance contrast of the specimen made possible precise observation of the complex morphology of Bacterium tularense. The observation that this organism possesses an extremely delicate outer limiting structure (cell wall) of low electronic density, in contrast to the cell walls of various other organisms, suggests that the physical properties of this structure may be responsible for the high mortality and extensive fragmentation of the organism incurred by sonic vibration, as shown by Downs, Coriell, Eigelsbach, et al. (30), and the high mortality resulting from desiccation, including lyophilization, as noted by Penfield and Snyder (31).

Knowledge of the morphology of the organism permits tentative explanations of the above occurrences and makes possible suggestions directed toward fertile lines of in-

vestigation. It has been possible to confirm observations by Hesselbrock and Foshay (1) concerning the existence of minute forms, and apparent multiplication by budding. These investigators (32) obtained evidence that small buds and minute forms, which they termed minimal reproductive units, were of different protoplasmic constitution than the nuclear chromatin since malachite green stained all nuclear chromatin green, but all buds and minute forms were stained brick red. These observations indicated chemical differences and hence possible differences in antigenic constitution. Foshay (33) found that supravitally stained preparations of restored lyophilized cultures contained many larger forms in the process of disintegration. They stained only lightly while minute forms remained intact and stained as well as before lyophilization. It is quite possible therefore that minute forms are more resistant to physical or chemical injury. In the present study a change from bacillary to coccoid morphology was observed during the growth of the organism. It does not seem illogical that differences in antigenicity and resistance to physical and chemical injury may exist between these morphological forms.

In 1945 Larson (34) reported that the aqueous phase obtained by ether extraction of a crude Bacterium tularense yolk sac vaccine demonstrated a marked immunizing ability

in an investigation in which the white rat was used as the test animal. In this article it was stated that the sediment resulting from centrifugation of the aqueous phase for 1 hour at 4,000 rpm contained many organisms but that the supernatant fluid contained negligible numbers of bacteria. Both the former and the latter were found to be approximately as active as the whole aqueous phase when employed to produce active immunity in rats. No mention was made of the method employed whereby the presence of "negligible" numbers of bacteria in the supernatant fluid was determined. Apparently filtration was not performed. On the basis of the complement fixing action of the supernatant fluid of the aqueous phase and the results of the previously mentioned immunization experiments, Larson concluded that he had demonstrated a specific soluble antigen in the aqueous phase of an ether extracted yolk sac vaccine. In the writer's experience and in that of Downs (35) the viable count on DCBA of the supernatant fluid of a 24 hour peptone cysteine broth culture of Bacterium tularense centrifuged at 5 C for 1½ hours at 4,000 rpm revealed the presence of between 8 and 9 million organisms per ml. It is possible at least that the supernatant fluid described by Larson as having contained negligible numbers of bacteria actually contained a sizeable number of bacteria per ml many of which were probably minute

forms. It is quite understandable that these forms could not be readily observed in stained smears. It is possible that a sufficient number of organisms were present in Larson's preparation obtained from the supernatant fluid of the aqueous phase to provoke the animal protection which he has attributed to the presence of a specific soluble antigen. In the interpretation of studies of this nature the complex morphology of Bacterium tularense and its implications should be recognized.

A comparison of photomicrographs and morphologic descriptions of certain forms of pleuropneumonia organisms as published by Ledingham (36), Klieneberger (37), Turner (38), Sabin (39), and Dienes (40) with those of certain forms of Bacterium tularense emphasizes the striking similarity in the morphology of these two organisms. Hesselbrock and Foshay (1) have reported that certain forms of a recently isolated strain of Streptobacillus moniliformis examined by them could not be differentiated morphologically from certain forms of Bacterium tularense. These investigators believe that Bacterium tularense is more closely related to the pleuropneumonia group than to any other group clearly defined at present but that certain of its unique features and morphologic units set it apart from this group as it is now constituted.

Dienes (40) has stated in regard to the morphology of the pleuropneumonia group that round forms, both small and large, are indistinguishable in appearance, staining reaction, and physical properties from analogous forms which appear in small Gram-negative bacteria in Pasteurella, in Bacterium tularense, and in Hemophilic bacteria. It was suggested that the pleuropneumonia group fits into the system of bacteria near the genera Pasteurella and Hemophilus.

The morphology of Bacterium tularense as depicted by light and electron microscopy is in agreement with the systematic study by Hesselbrock and Foshay (1) and emphasizes the complex morphology of this organism. Morphological observations made in the present investigation lend support to the recommendation by these investigators that on the basis of the unique features and morphology of this organism, the more noncommital name, Bacterium tularense, is the one of choice and should be used until more knowledge is accumulated concerning this organism and its near relatives.

SUMMARY

Determination of the minimal inocula required to initiate growth of Bacterium tularense, strain Schu, together with growth curve studies revealed that peptone broth and peptone cysteine broth are suitable for cultivation and subsequent morphological examination of the organism. Peptone cysteine broth is particularly well adapted for the cultivation of Bacterium tularense, strain Schu, and will initiate growth from an inoculum of approximately 1 organism per ml of medium.

Smears prepared at various intervals during the growth curves of strains Schu and 38 and stained by Giemsa's method revealed that young cultures are composed principally of bacillary forms. As growth continues coccoid forms increase in number and, subsequently, filamentous forms also become more numerous. With increasing age large coccoid, bizarre, and so-called "involution" forms are more frequently observed. Peptone cysteine broth cultures maintain a predominance of bacillary forms for a proportionally greater time than do peptone broth cultures. Although bacillary forms seem to predominate at some period during the early growth of the organism in all media, large and small coccoid, oval, dumbbell, bizarre, and even the so-called "involution" forms are also present in practically every preparation.

The morphology of Bacterium tularense as determined

by the electron microscope with and without the aid of special techniques to enhance contrast is in agreement with the study of the morphology of the organism by light microscopy. In general, all the forms which comprise the complex morphology of Bacterium tularense as demonstrated by light microscopy were observed with the electron microscope. Several forms of the organism strongly suggest multiplication by means of budding and others by the process of binary fission. The suggestion of the existence of minute morphological units of less than 300 mu in diameter by Foshay and Hesselbrock (8) was confirmed. The typical cell possesses little opacity to the electron beam and presents a semitransparent nebulous appearance. Critical examination for the presence of a cell wall revealed an extremely delicate structure of very low electronic density which possibly accounts for the low survival rate when subjected to sonic vibration or the lyophilization process.

Results of the present investigation lend support to the recommendations by Hesselbrock and Foshay (1) that for the present at least the name, Bacterium tularense, is the one of choice and that all other names for this organism should be invalidated.

BIBLIOGRAPHY

1. Hesselbrock, W., and Foshay, Lee. 1945 The morphology of Bacterium tularense. J. Bact., 49, 209-231.

2. McCoy, G. W., and Chapin, C. W. 1912 Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, Bacterium tularense. J. Infectious Diseases, 10, 61-72.

3. Ohara, H., Kobayashi, T., and Kudo, J. 1935 A study on the pleomorphism of Bact. tularense ("Yato-Byo" bacteria, Ohara) and their special flagellum. Tohoku J. Exptl. Med., 25, 520-530.

4. Galli-Valerio, B. 1938 Observations sur l'agent Specifique de la tularemie (Coccobacterium tularense (McCoy et Chapin) Galli-Valerio). Schweiz. med. Wochensch., 68, 1206-1209.

5. Ota, I. 1936 Morphologic studies of Yato-Byo bacteria and Bacterium tularense, especially flagella staining. Kokumin Eisei, 13, 207-309.

6. Kobayashi, T., Kawakami, F., and Ota, I. 1936 Staining of Bacterium tularense and flagella by Victoria blue (4R). Kokumin Eisei, 13, 43-52.

7. Ohara, H. 1940 Bacterium tularense and "Yato-Byo" bacteria. Proc. Third Internat. Cong. Microbiol., 678-679.

8. Foshay, L., and Hesselbrock, W. 1945 Some observations

on the filtrability of Bacterium tularense. J. Bact., 49, 233-236.

9. Eigelsbach, H. T., Chambers, L. A., and Coriell, L. L. 1946 Electron microscopy of Bacterium tularense. J. Bact., 52, 179-185.

10. Hillier, J., and Baker, R. F. 1946 The mounting of bacteria for electron microscope examination. J. Bact., 52, 411-416.

11. Williams, R. C., and Wyckoff, R. W. G. 1946 Applications of metallic shadow-casting to microscopy. J. Appl. Phys., 17, 23-33.

12. Snyder, T. L., Penfield, R. A., Engley, F. B., Jr., and Creasy, J. C. 1946 Cultivation of Bacterium tularense in peptone media. Proc. Soc. Exptl. Biol. and Med., 63, 26-30.

13. Tamura, J. T., and Gibby, I. W. 1943 Cultivation of Bacterium tularense in simplified liquid media. J. Bact., 45, 361-371.

14. Nicholes, P. S. 1946 On the antigenic structure of Bacterium tularense. Ph. D. Thesis, University of Cincinnati.

15. Downs, C. M., Coriell, L. L., Chapman, S. S., and Klauber, A. 1947 The cultivation of Bacterium tularense in embryonated eggs. J. Bact., 53, 89-100.

16. Kirchner, F. 1930 Ein einfaches Verfahren zur Strukturuntersuchung beliebiger Substanzen mittels Elektronenwellen. Naturwissenschaften, 18, 707.

17. Marton, L. 1940 The electron microscope. A new tool for bacteriological research. J. Bact., 41, 397-413.

18. Zworykin, V. K., Morton, G. A., Ramberg, E. G., Hillier, J., and Vance, A. W. 1945 Electron optics and the electron microscope. John Wiley and Sons, Inc., New York.

19. Schaefer, F. J., and Harker, D. J. 1942 Surface replicas for use in the electron microscope. J. Appl. Phys., 13, 427-433.

20. Fisher, R. A., and Yates, F. 1943 Statistical tables for biological, agricultural, and medical research. Oliver and Boyd, Ltd., London.

21. Francis, E. 1942 Fermentation of sugars by Bacterium tularense. J. Bact., 43, 343-346.

22. Wamoscher, L. 1930 Versuche uber die Struktur der Bakterienzelle. Z. Hyg. Infektionskrankh., 111, 422-460.

23. Henrici, A. T. 1928 Morphologic variation and the rate of growth of bacteria. C. Thomas, Springfield, Illinois.

24. Sherman, J. M., and Albus, W. R. 1924 The function of lag in bacterial cultures. J. Bact., 9, 303-305.

25. Winslow, C. E. A., and Walker, H. H. 1939 The earlier phases of the bacterial culture cycle. Bact. Revs., 3, 147-186.

26. Dubos, R. J. 1946 The bacterial cell. Harvard University Press, Cambridge, Massachusetts.

27. Knaysi, G., and Mudd, S. 1943 The internal structure of certain bacteria as revealed by the electron microscope - a contribution to the study of the bacterial nucleus. J. Bact., 45, 349-359.

28. Knaysi, G. 1944 Elements of bacterial cytology. Comstock Publishing Company, (Cornell University) Ithaca, New York.

29. Dubin, I. N., and Sharp, D. G. 1944 Comparison of the morphology of Bacillus megatherium with light and electron microscopy. J. Bact., 48, 313-329.

30. Downs, C. M., Coriell, L. L., Eigelsbach, H. T., Plitt, K. F., Pinchot, G. B., and Owens, B. J. 1947 Studies on tularemia. II. Immunization of white rats. J. Immunol. (In press)

31. Penfield, R., and Snyder, T. 1947 Lyophilization of Bacterium tularense. (To be published)

32. Hesselbrock, W. 1943 Newer aspects of the biology

and morphology of Bacterium tularense. Ph. D. Thesis, University of Cincinnati.

33. Foshay, L. 1945 Personal communication.

34. Larson, C. L. 1945 Immunization of white rats against infections with Pasteurella tularensis. Pub. Health Rep., 60, 725-734.

35. Downs, C. M. 1946 Personal communication.

36. Ledingham, J. C. G. 1933 The growth phases of pleuropneumonia and agalactia on liquid and solid media. J. Path. Bact., 37, 393-410.

37. Klieneberger, E. 1934 The colonial development of the organisms of pleuropneumonia and agalactia on serum-agar and variations of the morphology under different conditions of growth. J. Path. Bact., 39, 409-420.

38. Turner, A. W. 1935 A study of the morphology and life cycles of the organism of pleuropneumonia contagiosa bovum (Borrelomyces peripneumonia Novum genus) by observation in the living state under dark-ground illumination. J. Path. Bact., 41, 1-32.

39. Sabin, A. B. 1941 The filtrable microorganisms of the pleuropneumonia group. Bact. Revs., 5, 1-68.

40. Dienes, L. 1945 Morphology and nature of the pleuro-

pneumonia group of organisms. J. Bact., 50, 441-458.