The Journal of Pathology and Bacteriology

Vol. XLI., No. 1

616 . 999 . 23 : 576 . 809 . 4

A STUDY OF THE MORPHOLOGY AND LIFE CYCLES OF THE ORGANISM OF *PLEURO-PNEUMONIA CONTAGIOSA BOUM* (*BORRELOMYCES PERIPNEUMONIÆ* NOV. GEN.) BY OBSERVATION IN THE LIVING STATE UNDER DARK-GROUND ILLUMINATION.

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(Plates I.-VI.)

I. Introduction.

The introduction by Loeffler and Frosch (1892) of the conception of "filterable viruses" (as typified by the causal agent of foot and mouth disease) into animal pathology, following upon Pasteur's failure (1883) to demonstrate by existing techniques visible or cultivable micro-organisms in virulent pleuritic exudate from cases of *pleuropneumonia contagiosa boum*, together with the demonstration by Dujardin-Beaumetz (1900) of the filterability of the causal agent through Pasteur-Chamberland "F" candles, has led to a general if uncritical tendency, which appears to have hardened into a habit, of describing it as a "filterable virus": thus Dahmen and Ziegler (1929) refer to pleuropneumonia as a disease "die . . . durch ein filtrierbares Virus . . . verursacht wird." Elford (1929, p. 140) states that "The bovine pleuro-pneumonia organism is generally recognised as a member of the filterable virus class . . ."; and Walker (1930) that ". . . it is caused by a filterable virus. . . ." Nevertheless, ever since the morphological

researches of Bordet (1910) and Borrel and his collaborators (1910), it has been evident that the organism is far from ultravisible,* and that certain phases are indeed larger than many bacteria and obviously not filterable; and, of course, as Nocard and his collaborators showed in 1898, it grows fairly readily in nutritive media not containing living tissue.

Reflection thus shows that it is no filterable virus *sensu stricto.* Burnet and Andrewes (1933-34) prefer to regard it and the closely related organism of agalactia of goats as virus-like but cultivable micro-organisms. Later in this article we shall suggest that even this moderate view is perhaps tinged with tradition and that attempts to ascribe viral affinities to the pleuropneumonia organism might henceforth well cease.

In spite of a recent review of the literature on pleuropneumonia by Ledingham (1933*b*) in this *Journal*, we consider that the different method of approach justifies a re-discussion of the earlier work, especially in so far as it bears upon the present investigation. Nocard and collaborators (1898) reported that, at a magnification of about 2000, examination of cultures grown in collodion sacs inside rabbits revealed, in contrast with control uninoculated sacs, enormous numbers of extremely small, refractile, rapidly oscillating points, the structure of which could not be determined by staining. Tartakowsky and Dschunkowski (1901) described in suspensions of colonies on serum agar more or less large elements that stained badly, and on very meagre grounds suggested that the organism be classified in Fischer's *Allococcaceæ* group of the bacteria ("Protisten"). Lipschütz (1908) reported the presence of extremely small spherical forms, single or in pairs or short chains of three or four elements, or as agglomerations; he wisely suggested that the terms "ultramicroscopical," "submicroscopical" and "invisible" with reference to this organism be discarded as inaccurate, but proposed its inclusion in the *Strongylosomata* or *Strongyloplasmata*, genera introduced by him for the reception of the filterable viruses. As late as 1930 he included it in his *Chlamydozoa-strongyloplasmata* group under the organotropic viruses.

In 1910 Bordet, after observations on Giemsa-stained preparations from cultures on blood-smeared glycerol potato-extract agar and in blood peptone broth, suggested that it be grouped among the vibrios, spirilla or spirochætes. In the same year Borrel, Dujardin-Beaumetz, Jeantet and Jouan published an extremely interesting account of the morphology of the organism separated from serum Martin's broth cultures by centrifugation and stained with Giemsa or preferably with carbol fuchsin after treatment with Loeffler's mordant †; they described and photographed the characteristic forms, being particularly struck by the "asterococcal" forms, *i.e.* bodies appearing by their staining methods as cocci with radiating arms. The interesting thing is that they studied the organism by dark-ground illumination, and the drawing attached to their article testifies that they saw most of the forms revealed by staining; observation by transmitted light, however, disclosed only indefinable points. No attempt was made to formulate a life cycle for the organism, but from the "asterococcal" forms

* Dujardin-Beaumetz (1900) referred to it simply as an organism "à la limite de la visibilité."

† The suitability of this method of staining has been reaffirmed by Borrel (1932).

and the alleged presence of a thick mucous capsule the name *Asterococcus mycoides* was proposed.

The findings of Borrel *et al.* have been strongly challenged, particularly by Freiberger (1912), who claimed to have found similar bodies in control uninoculated media. Titze, Giese and Wedemann (1922-23)and Seelemann (1922-23), on the contrary, admitted that differences were present, but, considering that the forms seen might be due to some sort of " reaction products " of the " virus," hesitated to identify them. Gramss (1922-23) could not distinguish by dark-ground examination between pleuropneumonia cultures and solutions of egg white. Frosch (1923 *a* and *b*) similarly had no success with this method ; and indeed took pains to suggest why it should be foredoomed to failure, claiming, for example, that although it makes small objects visible it gives no geometrical pictures of them but merely diffraction effects, from which neither the true shape nor size can be recognised, that it is restricted to objectives of relatively low aperture, and that even its possible use in determining motility is vitiated by the disturbing presence of serum particles (" Mikronen "). These objections will be answered later. Apparently Ledingham (1933*b*) also placed little reliance upon dark-ground observation. Bechhold and Sierakowski (1926) could find practically nothing in cultures by direct illumination or even by staining, but noted spherical filter-passing bodies by examination under the ultramicroscope. Cultures concentrated by ultrafiltration yielded small flocculi, allegedly due to auto-agglutination, which were then visible as amorphous material even by transmitted illumination. They concluded that it must be the optical refractive properties that prevented its visibility under the microscope and proceeded to apply the cumbersome and entirely unnecessary gold-intensification method. Beller and Tahssin-Bey (1927), although noting most of the previously described forms in Giemsa-stained preparations, could find by dark-ground examination only punctiform objects. Wróblewski (1931*a*) did not hesitate to state that " die Peripneumonieerreger ist im Dunkelfeld . . . unsichtbar " ; later (1931*b*) he reported his inability to distinguish by dark-ground examination between cultures and uninoculated controls, and also assumed the explanation of its alleged invisibility by dark-ground examination to be the similarity of its refractive index with that of the medium in which it was examined,* although he was puzzled on reflecting that if this were the case, one should not be able to observe opalescence or " swirls " in the cultures.

On the other hand certain workers have had more success with dark-ground observation. Barnard (1925) was able to observe by this method (using monochromatic green light of the mercury arc) the forms that he thereupon photographed by transmitted ultraviolet light. Smiles (1926) made some accurate but incomplete observations on certain phases, and Futamura (1929) confirmed by dark-ground examination his observations upon stained preparations. Elford (1929, p. 140) examined his various ultrafiltrates similarly and described " particulate " and " spherical " bodies.

Life cycles have been suggested based upon (*a*) superstained preparations of fluid cultures by Nowak (1929) and Wróblewski (1931*b*), (*b*) Giemsa-stained impression preparations of surface colonies on serum agar by Ledingham (1933 *a* and *b*), (*c*) examination of the early stages and edges of living surface colonies by transmitted blue light by Ørskov (1927), (*d*) photography by ultraviolet light of the edges of living surface colonies by Barnard, and of fluid cultures in addition by Frosch (1923*b*), and (*e*) dark-ground observation of living fluid cultures by Smiles.

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* Frosch found the refractive index of his medium to be 1·34.

Most workers have recognised or postulated the existence of very small filterable bodies in cultures. Briefly, the cycles proposed are as follows. According to Nowak, the filterable body (" elementary corpuscle ") becomes the asteroid body, and by further development a much-branched mycelium that fragments into pieces which differ from the endomycelial granules and become in turn elementary corpuscles. The significance of the endomycelial granules and of the " ring-shaped bodies " was unexplained. A certain amount of conjugation of filaments occurs giving rise to large bodies and masses of spheres. He suggested the name *Mycoplasma peripneumoniæ*. Wróblewski claimed that the elementary corpuscle first germinates to produce two long, branched filaments at the ends of which an asteroid body forms : each then develops into a branching mycelium in which other asteroid bodies (" conidial rings ") provide sites for further branching. The final asteroid bodies (" conidial asters ") are provided with terminal granules (" exospores ") while " endospores " are produced within the mycelium. In addition, a sexual conjugation between male cells (" spermites ") and female cells (" oogonia ") occurs. The name *Asteromyces peripneumoniæ bovis* was suggested. Ledingham (1933*b*) stated that the initial corpuscle pullulates to form a branching mycelium at the end of which or along which appear large consolidated deeply-stained nodes from which further similar growth occurs. He claimed that from these large swellings most of the forms described by earlier workers are budded off by a pseudopodial process. Ørskov observed the direct development of the initial corpuscle into a branching mycelium. Barnard claimed that the filterable particle enlarges to form a sphere from which other spheres are budded off, the process being repeated indefinitely. Frosch (1923*b*) considered that it reproduced by a sort of budding similar to that of yeasts, attempted to explain the mycelial forms, etc., in harmony with this hypothesis and suggested the name *Micromyces peripneumoniæ bovis contagiosæ*. Smiles stated that the " granular form " develops into a spherical form that elongates to form a cylindrical body and breaks into small spherical or granular forms, the cycle being repeated indefinitely. There is thus much disagreement among observers concerning the life cycle.

In the present work the attitude has been adopted that more success was likely to follow direct observation of the unfixed organisms in fluid cultures than the study of colonies on the surface of serum agar : the long incubation necessary for full development (4 days), with the resulting desiccation, the fact that the organism is often not fully aerobic but micro-aerophilic (in " shake " cultures it frequently grows best in a zone a few mm. below the surface) and is therefore probably growing under dysgenic conditions, the adherence to and growth into the medium, and the possibility that staining might produce artefacts or distort the organisms all combined to convince us that in spite of justifiable criticisms of fluid cultures, they are more suitable than solid for the purpose. There are strong objections to impression preparations that require for their making considerable force just short of splitting the medium ; for there must be a possibility of distorting or bursting certain forms. Furthermore, it is questionable whether the surface organisms removed by this technique represent the normal forms and methods of reproduction inside the colony ; certainly they are

the most exposed to desiccation and other dysgenic influences and in any case only a selection of them adheres to the slide. We are aware, on the other hand, of the objections that can be raised to a reliance upon dark-ground examination of cultures in liquid media ; *e.g.* the apparent size is increased, the image of very small objects tends to appear disc-like and the vitiation of the thin layers of culture medium limits the amount of growth under the coverslip.

We contend nevertheless that with experience and suitable apparatus an ability to interpret the forms seen is acquired which establishes the method as a valuable and satisfactory one. The illustrations accompanying this paper should support the contention.

With the exception of Smiles, no one has attempted to work out by dark-ground observation the morphology and life cycle of this organism in the living, unfixed, growing state, and no one has succeeded hitherto in photographing it satisfactorily either by dark-ground illumination or even by bright-field illumination with ultra-violet light.

II. Technique.

In this work the organism has been grown almost exclusively in a new medium, referred to as V.F.-O.S. broth, which is based upon the V.F. broth used by us in our studies on infectious necrotic hepatitis of sheep * and which will be described in detail elsewhere. It is essentially a peptic digest (pig stomach) of ox liver and muscle, adjusted to *p*H 7·4, enriched with 10 per cent. of ox serum and sterilised by filtration through a Seitz E.K. disc instead of by heat. In this medium the organism of pleuropneumonia produces, from a small inoculum (a loopful), rich cultures within 24 hours, which are frequently quite turbid and in which characteristic swirls (" ondes soyeuses ") are produced by gentle shaking. It appears to be the best medium for the organism of pleuropneumonia that has yet been evolved. From it may be prepared an agar, V.F.-O.S. agar, preferably by allowing sterilised agar gel slopes to imbibe V.F.-O.S. broth.

As much depends upon the making of satisfactory preparations this will be discussed in some detail. In all work of this nature, slides and coverslips must be scrupulously clean and free from defects, a requirement we have often had great difficulty in fulfilling.† A very small droplet of the culture or suspension to be examined is placed in the centre of a slide which is then inverted and gently lowered on to a $1\frac{1}{2}$ in. $\times \frac{3}{4}$ in. coverslip placed on a pad of clean filter paper. For visual work the preparation is pressed firmly down on the filter papers in order to remove excess fluid, and the edges of the coverslip luted with paraffin wax to prevent currents from carrying away the objects studied. For photographic recording the above technique leaves too thick a layer of fluid between coverglass and slide to prevent the microbes, many of which are extremely sensitive to Brownian movement, from oscillating during exposure. The preparation must be so thin that they are either lightly pinched between the coverslip and slide or are attracted

* Turner, A. W. 1930. Black disease (infectious necrotic hepatitis) of sheep in Australia ; *J. Council Sci. and Indus. Res. Australia,* iii. 117 (Bull. no. 46).

† Bechhold and Sierakowski similarly complained that slides must be tested for suitability (" auf optische Leere "), and that only about one-third of a given lot will be usable.

to the glass and consequently immobilised, when exposures up to many minutes may readily be given. To produce such thin preparations, smaller droplets of fluid are taken so that on applying the coverslip the film barely reaches the edges. The preparation is placed on a thick pad of filter papers, coverslip upwards, covered with a clean filter paper, and a glass rod or tube is rolled backwards and forwards rather firmly above it; in this way all removable excess fluid is squeezed out and absorbed in the paper, at which stage inspection should show a vivid series of Newtonian rings between slide and coverslip, which is then luted as before. It is a very interesting fact that the organisms usually appear to advance along the edges of the droplet during the lowering of the coverglass, thus tending to concentrate not at its centre but at its edges: if too large a droplet is taken there is a danger that most of the organisms will be swept away and washed into the absorbent paper; if too little, the unavoidably enclosed air bubbles may set up currents by thermal expansion or contraction. Furthermore, if the pressure applied is excessive mycelial forms in an advanced stage of endomycelial fragmentation may be considerably broken up. However, the photographs accompanying this paper demonstrate that it is possible to make satisfactory preparations.

Observations have been carried out mostly with Zeiss equipment, comprising the cardioid dark-field condenser, 60× apochromatic oil-immersion objective of N.A. 1·05 and compensating oculars from ×3 to ×30, thus giving a range of magnifications from 180 to 1800 diameters, generally with the binocular but occasionally the monocular tube, which is of course used when photographing. Occasionally and with success the Beck focussing dark-field condenser has been used. The source of light has been either a Phillips 75 watt 2·5 amp. A.C. point-light globe or better the Zeiss 100 watt 2-2·5 A.C. point-light. Correct centring and adjustment are of course essential.

In order to photograph preparations a very simple technique has been adopted (Turner, 1933). A Leitz Leica (miniature) camera, with the Elmer F 3·5 lens of focal length 5 cm. at full aperture and focussed at infinity, is supported horizontally over the focussed microscope (which is consequently also focussed at infinity) on a wooden tripod with adjustable legs; the lens is placed as near as possible to the ocular without pressing upon it and is centred over it as accurately as possible, after which the exposure is given. As is known, the Leica camera produces negative 35×25 mm. on cinematograph film. Although Agfa superpan and occasionally Kodak superpanchromatic films have been used for most of the photographs, and are essential when the exposure must be short, experience has shown that for favourable subjects the Agfa fine-grain orthochromatic film, which is much slower, can be used with advantage to the definition of the subsequent enlargements. The image on the film is about one-fifth the magnification conventionally ascribed to a given combination of objective and ocular; thus the image produced by a 60× objective and K30 ocular, conventionally rated at 1800 diameters (*i.e.* if projected on a screen 25 cm. from the equivalent focus of the ocular), being now brought to focus from parallel emergent rays by a lens of focal length 5 cm., is recorded at a magnification of approximately 360. However the negatives are of such fine grain and are so sharp that ample enlargements are readily made from them up to 7 or 10 diameters, giving maximum final magnifications of 2520 to 3600 diameters respectively. The combination of the extremely sensitive Agfa superpan film and the great increase in brightness of the image due to the relatively low magnification actually upon the film allows satisfactory negatives to be produced in as short a time as one second. In general, depending upon the brightness of the image, an exposure of one to five seconds has been sufficient for Agfa superpan film, one half-minute for orthochromatic fine-grain film.

Many scores of strains have been used in this work, all isolated by us in Queensland, and therefore most of them have been recent subcultures, very often first subcultures from pleuritic exudate or affected lungs; the oldest strains used have been isolated only about two years. These facts may be significant in comparing our findings with those of workers in Europe who have perforce used old laboratory strains.

The method of study has usually been to use either old cultures in V.F.-O.S. broth (about three weeks' continuous incubation) or L2 filtrates of them, in each case separated from the medium by centrifugation at 4000 *r.p.m.* for 1½ to 3 hours. The centrifugates have then been resuspended in V.F.-O.S. broth, incubated, and samples withdrawn at intervals for examination; or samples of the suspension have been put up as thin preparations under luted coverslips, examined by dark-ground illumination and selected forms observed at intervals, the microscope and preparation being incubated in the constant temperature room.

Advantages claimed for this technique are (1) that it makes possible the examination and photographing of living micro-organisms and avoids the use of fixatives and stains, (2) that it allows the germination, growth, changes of shape and a large part of the life cycles to be followed microscopically and (3) that it takes full advantage, in a very simple way, of the corrections for spherical aberration in the objective, which in a focussing camera could only be obtained by adjusting the tube-length of the microscope.

Probably on account of the restricted removal of harmful metabolic products by diffusion the necessarily thin preparations unfortunately do not usually grow as well or as completely as the same suspensions incubated in test tubes, and it is therefore not possible to follow the complete growth cycle of an individual organism.

Measurements have been made partly by means of the Zeiss compensating eye-piece screw micrometer and partly from photographs. It is recognised that there is probably considerable error in the estimations of the small measurements and that, as pointed out by Beck (1932), the apparent size under dark-ground illumination is probably larger than the real. However similar criticism applies, in some degree at least, to stained preparations examined by transmitted light; the superstaining (mordanting) techniques and the Romanowsky stains increase the size of micro-organisms, which, in the case of pleuropneumonia, are still beneath the limits of accurate measurement. No doubt measurement from ultra-violet dark-ground photographs is desirable.

III. The Life Cycles of the Organism of Pleuropneumonia.

Continued observations over three years have revealed that this micro-organism has at least five methods of reproduction. It is proposed to refer to them as genethodes (Gr. *genesis* = reproduction; *hodos* = way) and to the phenomenon of multiple methods of reproduction as polygenethodism.

The final viable product of the growth of the pleuropneumonia organism, produced from the earliest stages of growth and found to the exclusion of other phases in old cultures, is the small filter-passing spheroidal body that functions as a resting stage or spore. For this we have adopted the term "conidioid," to distinguish it on the one hand from "conidium," which in the closely related

actinomycetes usually refers to viable pieces of filament or mycelium of irregular length and in the hyphomycetes has a special connotation, and on the other hand from the term "gonidium," which is applied to the viable particles formed by spontaneous fragmentation of whole cells of certain bacteria such as *Azotobacter* (Löhnis and Smith, 1916) and *Rhizobium radicicola* (Thornton and Gangulee, 1925-26). Since gonidia are frequently small enough to be filterable, it is possible that the term might be extended to the spheroids of pleuropneumonia; however, for the present, it is proposed to avoid pressing the analogy by adopting a distinctive term "conidioid." They are bright, small, spheroidal, ovoid or polyhedral bodies usually from 0·2 μ to 0·4 μ in diameter, some even reaching 0·8 μ, and they frequently have an indentation that varies in size and shape, being in some cases small, in others wide and shallow and with a tendency to irregular edges (diagram I), when the conidioids have a distinct saucer-shape. Their indentations are easily perceptible as they roll over. The invaginated polygonal varieties, although larger than the true filterable viruses, have a superficial resemblance to the viruses photographed by Barnard (see the excellent ultra-violet dark-ground photographs in Burnet and Andrewes, 1933-34).

They frequently adhere to the slide, thereby becoming immobilised, and are then more easily photographed (pl. I, fig. 23). It should be noted that they have a fairly definite outline and give no indication of being "gouttelettes d'un protoplasme épais" as they are described by Ørskov in stained preparations. One is tempted to believe that the indefinite outline described by workers who have studied preparations stained particularly by the rather brutal superstaining method (Loeffler's mordant and hot carbol fuchsin) may be artefacts due to the staining of a viscous, mucinous capsule; however, on account of their small size and the existence of diffraction rings it is difficult to ascertain whether this exists. These bodies are apparently the "cocci" of Borrel, the "corpuscles élémentaires" of Ørskov, Nowak, and Wróblewski, the "Elementärkörperchen" of Frosch, the initial corpuscles of Ledingham, and the particulate and granular forms of Elford and Smiles. They traverse bacteria-proof Chamberland "F" and L3 and Seitz "EK" filters.

Short statement on the methods of reproduction. It appears that the organism of pleuropneumonia has at least five "genethodes" or methods of reproduction :—(I) by endomycelial fragmentation, giving rise, after dispersion, to conidioids that germinate to produce more filaments and mycelia; (II) by the production of discules that give rise to mycelia, usually by multipolar germination, or that eventually in old cultures consume themselves in the production of exoconidioids, which become conidioids; (III) by a peculiar method of budding from spherules; (IV) by constriction or pinching off of

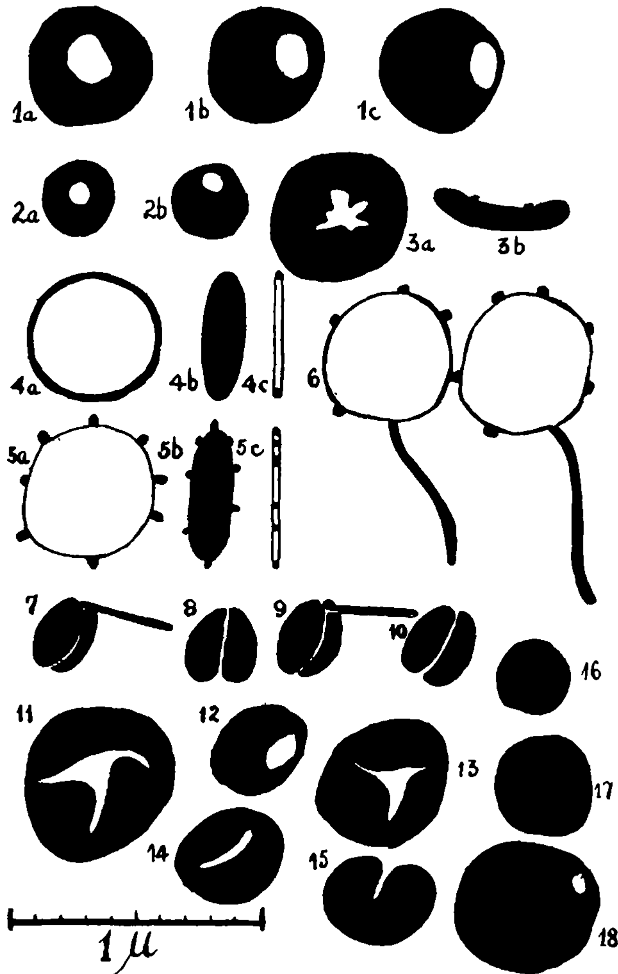


DIAGRAM I.

FIGS. 1*a*, 1*b* and 1*c*.—An indented conidioid showing successive positions of the indentation as it rolls over from left to right.

FIGS. 2*a* and 2*b*.—Similar but smaller indented conidioid, rolling from beneath to above.

FIGS. 3*a* and 3*b*.—A saucer-shaped form, possibly intermediate between conidioid and discule, shown from above and in profile. The indentation is apparently shallow and with irregular edges.

FIGS. 4*a*, 4*b* and 4*c*.—Successive positions of a discule as it rolls over from left to right.

FIGS. 5*a*, 5*b* and 5*c*.—Similar successive views of a germinating discule (young asterodiscule).

FIG. 6.—Two asterodiscules joined by a common germinal discule. They probably arose as contiguous discules in the same mother filament.

FIGS. 7 and 9.—Two conidioids apparently split medially, with a short rodlet attached (" facies impudica ").

FIGS. 8 and 10.—Two as above without attached rodlets.

FIGS. 11 and 13.—Two conidioids with irregularly triangular indentations.

FIGS. 14 and 15.—Two conidioids with longitudinal indentations, fig. 15 in profile.

FIG. 12.—Conidioid with oval indentation, approaching profile.

FIGS. 16 and 17.—Two conidioids without indentations.

FIG. 18.—Large conidioid with commencing indentation.

All blocks in this article sent to Dr. Lightfoot, Sydney, Australia on 21/4/35.

oval spore-like bodies from large filaments that may arise from peculiar clusters of club-like bodies; and (V) by constriction or pinching off of spherical, oval, cylindrical, disc-like or irregular bodies from flexible cylindrical forms that arise directly from conidioids. It must not be imagined that these various genethodes, described in detail below, occur in any fixed order or in definite waves. Genethodes I, III and V are those first seen when a suspension of conidioids begins to germinate; then genethode II appears, and examples of each are commonly seen in the one culture. Furthermore, there is a certain amount of overlapping of genethodes and transition from one to the other (*e.g.* from V to II or III). An attempt has been made to indicate this in the schema on p. 21 (diagram IV). In the past these peculiarities have made the interpretation of the various forms and their codification into a life cycle very difficult, and have doubtless been the main cause of the rival hypotheses submitted. It is considered that the present investigation has been greatly facilitated by the fortunate discovery of a rich culture medium and by the choice of dark-ground observation of micro-cultures as the chief technique. In the following paragraphs we give a more detailed description of these observations.

Genethode I. When a suspension of conidioids is incubated at 38·5° C. in V.F.-O.S. broth germination usually occurs in the following manner after an interval which may be as short as five minutes if they are young but which is usually a few hours. If recently formed they may appear as hollow spheroids with one or more superficial highly refractile granules (pl. I, fig. 1), in which case they first become apparently more solid and wholly refractile (pl. I, fig. 2). In either case they ultimately extrude one or two delicate, flexible, poorly refractile germinal tubes which increase in length and may begin to branch dichotomously even within two hours (diagram II). They stain quite well by Romanowsky stains or superstaining and are not artefacts due to the use of dark-ground illumination. A remarkable feature given at this early stage is the beginning of the characteristic endomycelial or "streptococcoidal" fragmentation, which is already advanced in some forms about two and a half hours after germination and is one of the reasons for the presence of filterable, viable forms of pleuropneumonia in cultures from the earliest stages. The majority of the germinal tubes increase in length, width and brightness, and soon reach the normal apparent width of between 0·1 and 0·2 μ; the original conidioid is absorbed and disappears. Both dichotomous and sympodial branching rapidly proceed and the organism continues to increase in brightness, so that usually a much branched mycelium is formed of relatively enormous size (pl. I, figs. 3, 4 and 5; pl. II; pl. IV, figs 1, 2 and 6; diagram III),

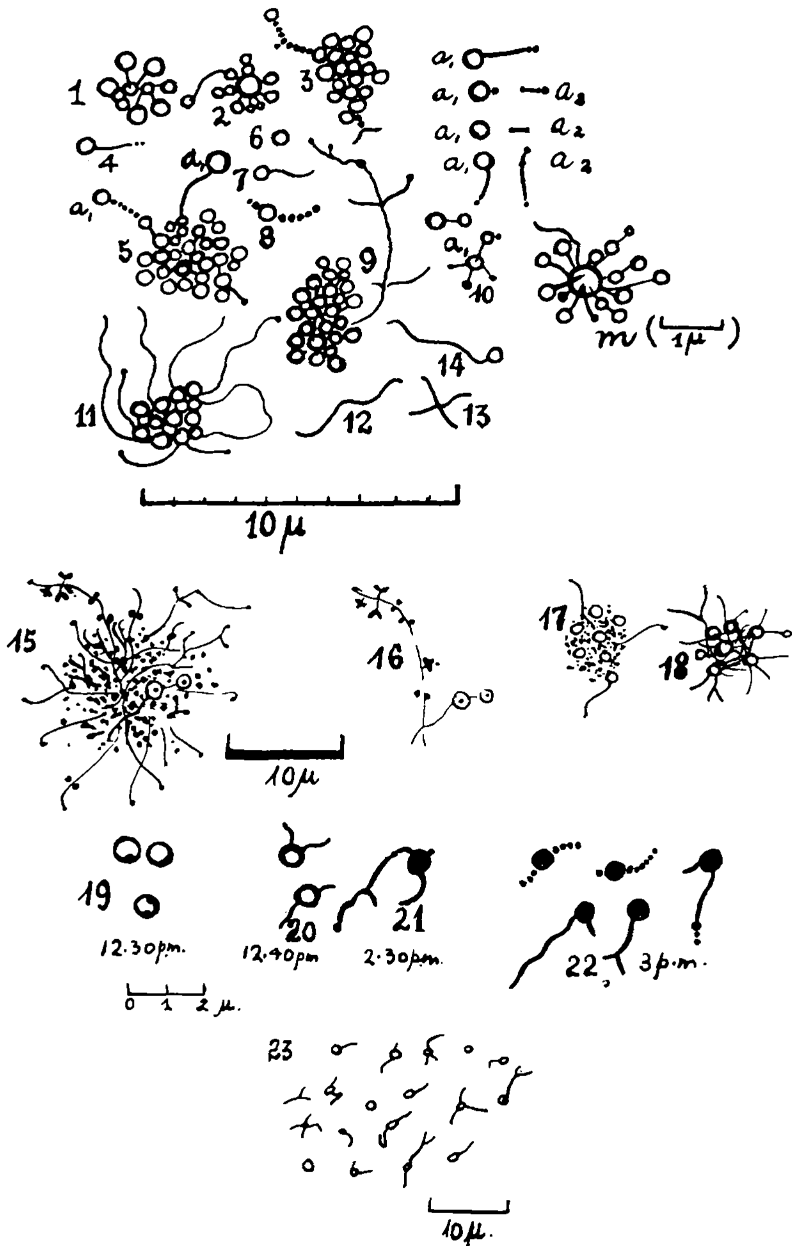


DIAGRAM II.

FIGS. 1-14.—Reproduction by genethode III (budding). Note the eventual growing out of filaments. a_1 = discules seen from above. a_2 = discules seen in profile.

FIGS. 15-18.—Forms simulating but definitely not sexual conjugation or fertilisation. Fig. 16 shows in detail two of the constituent filaments of the tangled mass in fig. 15. One of them has two spherular bodies on it.

FIGS. 19-22.—Germination by genethode I, illustrating rapidity of germination and precocious fragmentation.

FIG. 23.—Germination of proteiform discules which are frequently formed in early stages of growth according to genethode V. Note that germination is mostly uni- or bipolar, occasionally tri- and quadripolar, and that the central portion is gradually absorbed.

the longest we have measured being approximately 190 μ long, exclusive of the long filament (240 μ) described on p. 18. Since these measurements were recorded from preparations made by placing a coverslip without special care over a loopful of culture,

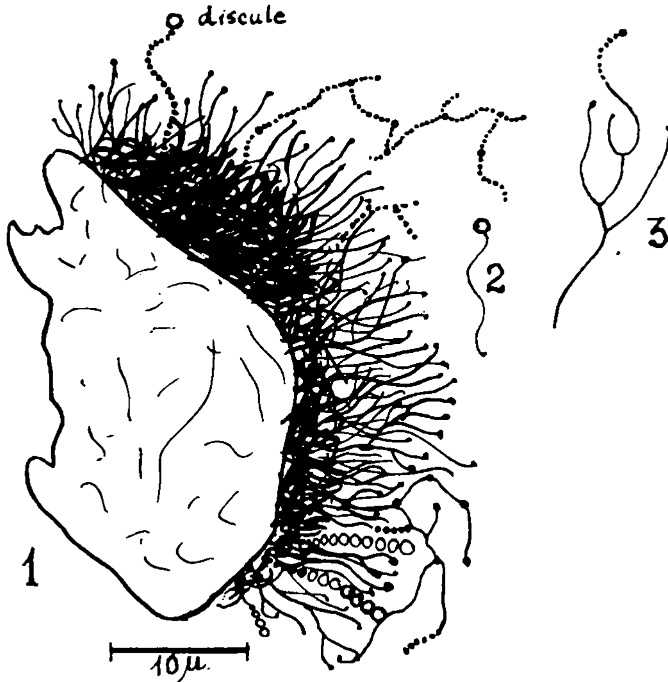


DIAGRAM III.—FIGS. 1-3.

FIG. 1.—Micro-colony of *Borrelomyces peripneumoniæ* which grew up on a small gritty particle, acting as nidus, adherent to coverslip. From an 18-hour culture in V.F.-O.S., put up, by technique ensuring sterility, as a luted preparation between slide and coverslip, and then left for a further 36 hours at room temperature. It grew as a tangled mass of filaments, from about 10 to 20 μ in length, some being largely transformed at the free ends into chains of spherular, wholly refractile bodies up to 0·5 μ in diameter ; one long branched filament about 45-50 μ in length had undergone total endomycelial fragmentation, with enlargement of certain of the elements ; one terminal element of a totally fragmented filament was definitely a discule.

FIG. 2.—Spherular body to which is attached a fine flexible dull thread with terminal granule.

FIG. 3.—Constituent filament of micro-colony shown in greater detail ; it is branched and the ends are provided with a small bright granule ; one branch has undergone considerable endomycelial fragmentation distally.

it is probable that in the undisturbed state the organisms of pleuropneumonia, when grown in V.F.-O.S. broth, can reach even greater lengths. Sometimes the tendency to branch is not so marked, an interval of 40 μ (pl. III, fig. 1) or even 85 μ being occasionally observed between branches ; on the other hand in certain delicate, poorly refractile filaments the branching may tend to occur at

nodes (pl. IV, figs. 7 and 10). We have observed that sometimes not all filaments appear to be of the same width ; but since in these cases the unusually thin ones are always poorly refractile and the unusually thick always highly refractile, the apparent difference in width may be an illusion due to the peculiarities of dark-ground illumination (pl. IV, figs. 7, 8, 9, 10 and 12).

After growth for two days or more, the ultimate fate of most of the mycelium is total fragmentation into coccoid elements some of which may remain for a time apparently enclosed within a sheath of some kind, for their Brownian oscillations appear to be limited laterally. By this process, referred to as endomycelial fragmentation (pl. I, figs. 9 and 12 ; pl. II ; pl. IV, fig. 1), the mycelium becomes converted into fragile streptococcus-like chains of apparently spherical elements about 0·15 μ in diameter (proconidioids), which in turn, on becoming free or while still in the filaments (pl. I, fig. 12), may become conidioids and are then able to germinate and repeat the cycle. Whether all these endomycelial elements are viable cannot be stated yet. Endomycelial fragmentation is regarded as the common method of reproduction or " genethode " of this polygenethodic micro-organism.

Genethode II. At various stages of growth, however, some filaments produce along their length (pl. III, fig. 1 ; diagram III, figs. 4 and 5) or at their ends (diagram III, figs. 1, 2 and 6) distinctly larger forms that rapidly take on the appearance of small plane discs with a brightly refractile rim and a non-refractile or poorly refractile centre. The size of the discules is usually from 0·4 to 0·6 μ when regular and entire, but discules measuring up to 1·6 μ have occasionally been seen. When the preparation is purposely made sufficiently thick, their disc-like nature is readily recognised as they roll over ; dark and non-refractile except for the rims, they suddenly reflect bright ovals of light as they reach a certain angle with the horizontal. On reaching the vertical plane they are seen in optical section apparently as thin, dark rods with a brightly refractile granule at each end (diagram I, figs. 4 *a, b* and *c* ; diagram II). They have been referred to by Frosch (1923*b*) as " doppelkonturierte Scheibchen " : by workers who have confined their attention to stained preparations their essentially disc-like nature has naturally not been appreciated and they have been described simply as " rings." The smaller ones can traverse an L2 filter. After their release from the filaments and discharge into the medium, they develop a much thicker and brighter rim.

They are capable of germination whether still in the filament or free. Chains of discules may form along filaments (diagram III, figs. 1 and 6) ; if several discules continue to adhere, their subsequent germination produces short fragile chains of up to four " asterodiscules," each adhering to its neighbour by a germinal tube

(diagram I, fig. 6; diagram III, figs. 10, 16 and 17). From a number of sites on the periphery (one to eight but usually five or six), short straight rodlets may grow out (pl. III, figs. 5 to 19 and 23; diagram III, figs. 7, 8, 9, 10 etc.), or the refractile material of the rim may first collect into bright granules (pl. III, fig. 32; diagram I, figs. 5 and 6; diagram III, fig. 18). Generally these

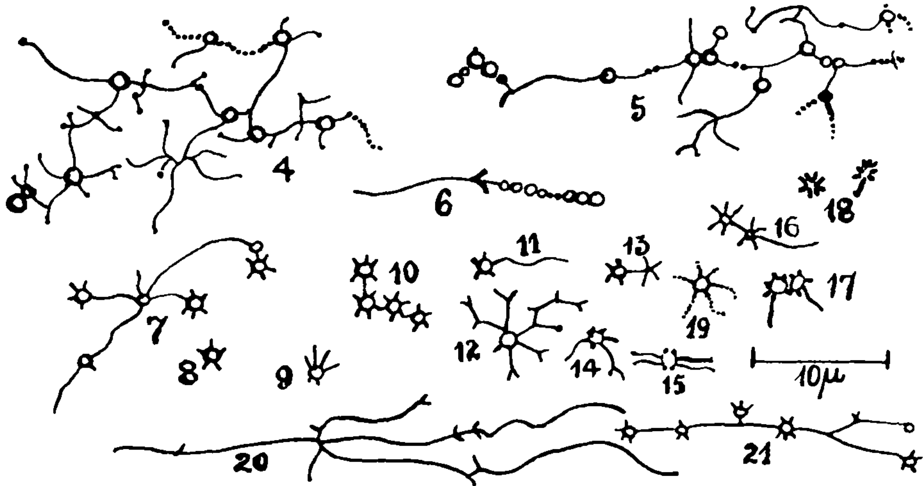


DIAGRAM III.—FIGS. 4-21.

FIGS. 4-21.—Phases of genethode II.
FIGS. 4 and 5.—Genesis of discules in a much-branched mycelium.
FIG. 6.—Row of terminal discules.
FIGS. 7 and 21.—*Discules germinating while still in the filament.*
FIGS. 8-19.—Germinating discules (asterodiscules).
FIG. 10.—Row of four asterodiscules, probably originating in a chain as in fig. 6.
FIG. 11.—One germinal tube is longer than the others.
FIG. 12.—Is developing well into a branching mycelium.
FIG. 13.—Two attached germinating discules the right-hand one of which is far advanced and has absorbed its central part.
FIGS. 14 and 15.—Two germinating discules.
FIGS. 16 and 17.—Two attached germinating discules.
FIG. 18.—Form of germination in which the periphery of the discule becomes non-refractile.
FIG. 19.—Final stage of auto-consumption, *i.e.* germinating with gradual absorption of the central portion and conversion of germinating tubules into rows of exoconidioids.
FIG. 20.—A good example of a large well-branched mycelium.

germinating discules or "asterodiscules," which resemble tiny cog-wheels and are the "asterococci" of Borrel, proceed to grow by increase in length of the radiating peripheral rodlets at the expense of the original discule, which shrinks correspondingly and becomes no longer identifiable (pl. I, fig. 16; pl. III, fig. 39). By a process of growth and branching a mycelium is formed (pl. I, figs. 16, 19 and 20). Reproduction *via* the discule is thus another

later alternative method of reproduction, another "genethode." The mycelia first formed from the conidioids and those formed by germination of discules are indistinguishable morphologically and there is no reason to regard them as physiologically different. Frequently, so-called micro-colonies are seen; these are tangled masses of delicate, trembling filaments usually radiating from a common centre: it is considered that they often arise from discules by multipolar germination, as shown in an early stage in pl. I, fig. 16, although they may also arise from masses of spherules (diagram II, figs. 9, 11, 17 and 18). Here reference may be made to certain forms that by superficial observation might be confused with endomycelial asterodiscules; they are produced by rapid dichotomous branching and are illustrated in pl. I, figs. 3, 4 and 5; pl. II; pl. III, fig. 37; pl. IV, figs. 1 and 11.

Another rare form that resembles the endomycelial asterodiscule in the ultimate result is shown in pl. I, fig. 7; without a preceding stage of discule formation, bright granules are extruded at certain places attached by invisible or scarcely visible threads. We are unable to state whether they become rodlets and behave subsequently as the germinal tubes of asterodiscules or whether they are cast off at this stage. Figs. 8, 15 and 17 of plate I show another uncommon form similar to and probably related to the last; the granules, which are particularly refractile, grow out not in one plane but in three dimensions on the end of short branches from various sites along the filaments. So many variations in detail have been seen in this organism, depending apparently upon the batch of medium and the strain, that there may be no essential difference between the last two forms and the typical germinating asterodiscules.

Discules occasionally germinate not by going through the "cog-wheel" stage, but by extruding from one to four flat, segment-shaped lugs; pl. III, figs. 28, 31 and 33); studied in the living state, they may sometimes be seen forming and being resorbed within a few minutes, a good example of the microbe's protean character. When development is to proceed the extruded segments elongate and become cylindrical tubes at the expense of the original discule, which is absorbed in the process. In a short time the discule has changed to a tri- or quadriradiate form with no detectable clue to its origin and presumably may then proceed to form a mycelium. A similar form of germination is particularly common with discules budded off from the cylindrical forms in genethode V; at two, three or four equidistant points the circumference approaches the centre and in a few minutes a dumb-bell-shaped, Y-shaped or cross-shaped form results. The discules found in pleuritic exudate of pleuropneumonia cases also frequently behave in this manner.

The mycelia of this genethode also eventually undergo endomycelial fragmentation into proconidioids, discules or both. When a young mycelium with many radiating filaments undergoes complete fragmentation it soon resembles a spherical mass of mostly coccoidal elements (pl. I, figs. 11, 13, 18 and 24) ; it is only by examination in the living state that their origin is revealed.

Summing up, the discule appears to have three functions. In young cultures (*a*) free discules may give rise to mycelia or (*b*) if formed along filaments (pl. I, fig. 6 ; pl. III, fig. 1) they may act as sites from which branching occurs (pl. I, figs. 19 and 20 ; diagram III, figs. 4, 5 and 7), in which case their function is probably not far removed from the first. As cultures age, discules disappear as follows (*c*) ; germination occurs from several points around the circumference, thus giving rise to asterodiscules, and the short radiating filaments are produced usually at the expense of the brightly refractile edge of the discule, which generally becomes invisible, although not always. As the discule is consumed or absorbed during this process (pl. III, fig. 35 ; diagram III, fig. 19), the extruded tubules undergo fragmentation commencing at the free ends, until at last they are totally converted into short radiating chains of bright coccoidal bodies that may be referred to as exoconidioids (pl. III, fig. 43) ; the discules that eventually disappear by this process thus act as conidioidophores. On becoming free, exoconidioids increase in size and develop into conidioids indistinguishable from those arising by endomycelial fragmentation. It is in this way that discules eventually disappear from old cultures.

Genethode III. This was first described by Barnard (1925) and embraces reproduction by budding. Many of the bright, thick-walled spherules, about 0·4 μ in diameter, that arise from conidioids in the early stages of germination of most strains reproduce by this method. From one or more points on the circumference bright granules arise that are extruded but remain attached by long delicate threads. The terminal granules enlarge to form daughter spherules and from them granddaughter spherules may be similarly formed. The result is a central spherule surrounded by satellite spherules still attached. They subsequently become free and continue the process. Finally the separate spherules may contract and consolidate and subsequently behave as conidioids, in that they germinate by extruding one or two ordinary germinal tubes and proceed to grow in the usual way (genethode I) ; or they may become transformed into the proteiform type of discule usually associated with genethode V (diagram III).

In V.F.-O.S. broth cultures, but most commonly in the water of hysteresis of cultures on the surface of V.F.-O.S. agar, the spherules are usually very dull, thin-walled and up to 0·5 μ in diameter. Advanced stages resemble masses of spherules which

PLATE I

FIGS. 1-20, 22 and 23.—Strain 279. Culture sown with heavy suspension of young conidioids from a 60-hour culture. ×3600.

FIG. 1.—Two recently formed conidioids photographed immediately before sowing.

FIG. 2.—Conidioids five minutes after sowing, showing early extrusion of germinal tubules and general increase in brightness (genethode I).

FIG. 3.—After 24 hours' incubation.

FIGS. 3, 4 and 5.—Fragments of the mycelium.

FIGS. 6 and 14.—Formation of bright endomycelial discules.

FIGS. 7, 9 and 12.—Endomycelial ("streptococcoidal") fragmentation.

FIG. 10.—Germination of discule giving a quinqueradiate form.

FIGS. 11, 13 and 18.—Origin of "masses of cocci" (Borrel) by endomycelial fragmentation of multiradiate "microcolonies."

FIGS. 8, 15, 17 and 22.—Rapidly growing mycelial fragments.

FIG. 16.—Quadriradiate germination of a discule giving rise to a branching mycelium ("micro-colony").

FIG. 19.—Short fragment of mycelium with three endomycelial discules undergoing germination *in situ*.

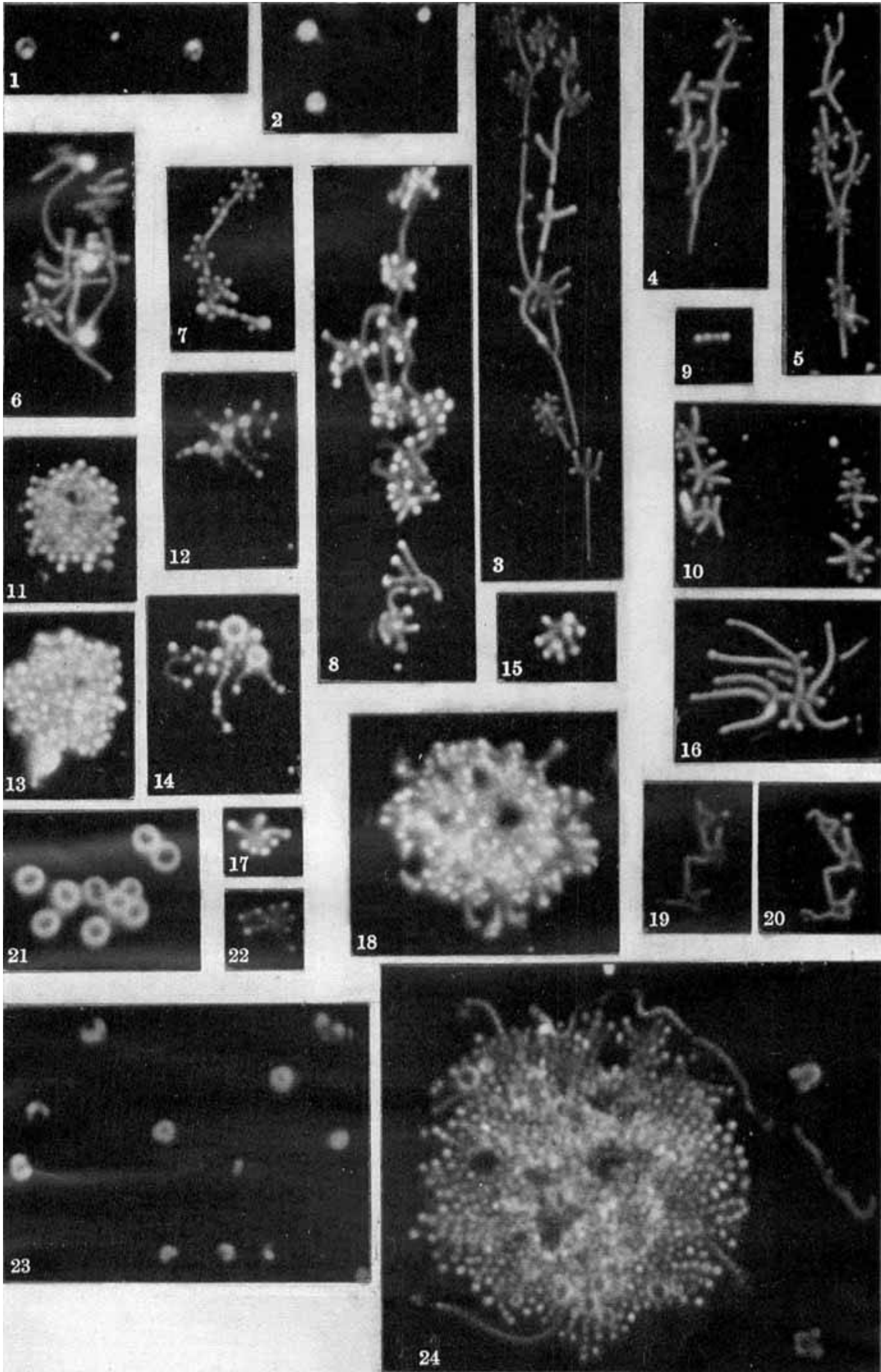
FIG. 20.—The same after 50 minutes' incubation.

FIG. 23.—Above culture after 11 days in incubator showing indented or invaginated conidioids of various sizes.

FIG. 21.—*Staphylococcus citreus* at same magnification as other figures, for comparison. Note dividing cells.

FIG. 24.—Strain 111*a*. 3-day culture in buffered V.F.-O.S. A radiating micro-colony in advanced endomycelial fragmentation. Note production of discules in or at ends of some of radiating filaments and note that two free discules are germinating by extrusion of two and four germinal tubes respectively; they reverted to discules on further incubation. ×2520.

ORGANISM OF PLEUROPNEUMONIA



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Strain 82. 24-hour culture showing much-branched mycelium with marked endomycelial fragmentation. Very few of the multiradiate forms betray their discal origin; most have either absorbed the central discule or have apparently germinated multiradially direct from conidioids. ×2520.

Plate III

Figs. 2 to 39, ×3600 : others as stated.

Fig. 1.—Strain 89. 18-hour culture; subsequently 4 hours under coverslip at room temperature. Long filament with endomycelial discules developing. ×2520.

Figs. 2 to 36.—Illustrating diversity in size and stages in reproduction of discules. From 3-day culture of strain 82, subsequently kept at room temperature for nearly one month.

Figs. 5, 6, 7, 8, 9, 10, 11 and 12.—Unipolar germination.

Figs. 14, 15, 16, 17, 18 and 19.—Bipolar germination.

Fig. 28.—Tripolar germination by extrusion of flat " lugs " or segments.

Fig. 32.—Multipolar germination with early branching.

Fig. 35.—Germination of discule, with its absorption and the fragmentation of germinal tubules into exoconidioids.

Figs. 36, 37 and 38.—Strain 82. 24-hour culture. Branching growth simulating that produced from discules.

Fig. 39.—Multipolar germination with complete consumption of discule.

Fig. 40.—Strain 83. 40-hour culture. Germination of discules. Note three dull spherular bodies, one large, one very small. ×1800.

Fig. 41.—Strain 112. Centrifugate of 24-hour culture. Note large "plastic" spherular body. ×1800.

Fig. 42.—Strain 111. Water of hysteresis of 43-hour culture on V.F.-O.S. agar slope. Large group of spherules (genethode II). ×1260.

Fig. 43.—Strain 288. 48-hour culture, then 54 hours under coverslip at room temperature, showing complete conversion of discules into exoconidioids, some of which (free) are developing into conidioids (top left-hand corner). This is the completion of genethode II. ×1800.

Fig. 44.—Strain 111*a*. 3-day culture. Radiating club-like bodies with pinching off of oval bodies. ×1800.

ORGANISM OF PLEUROPNEUMONIA

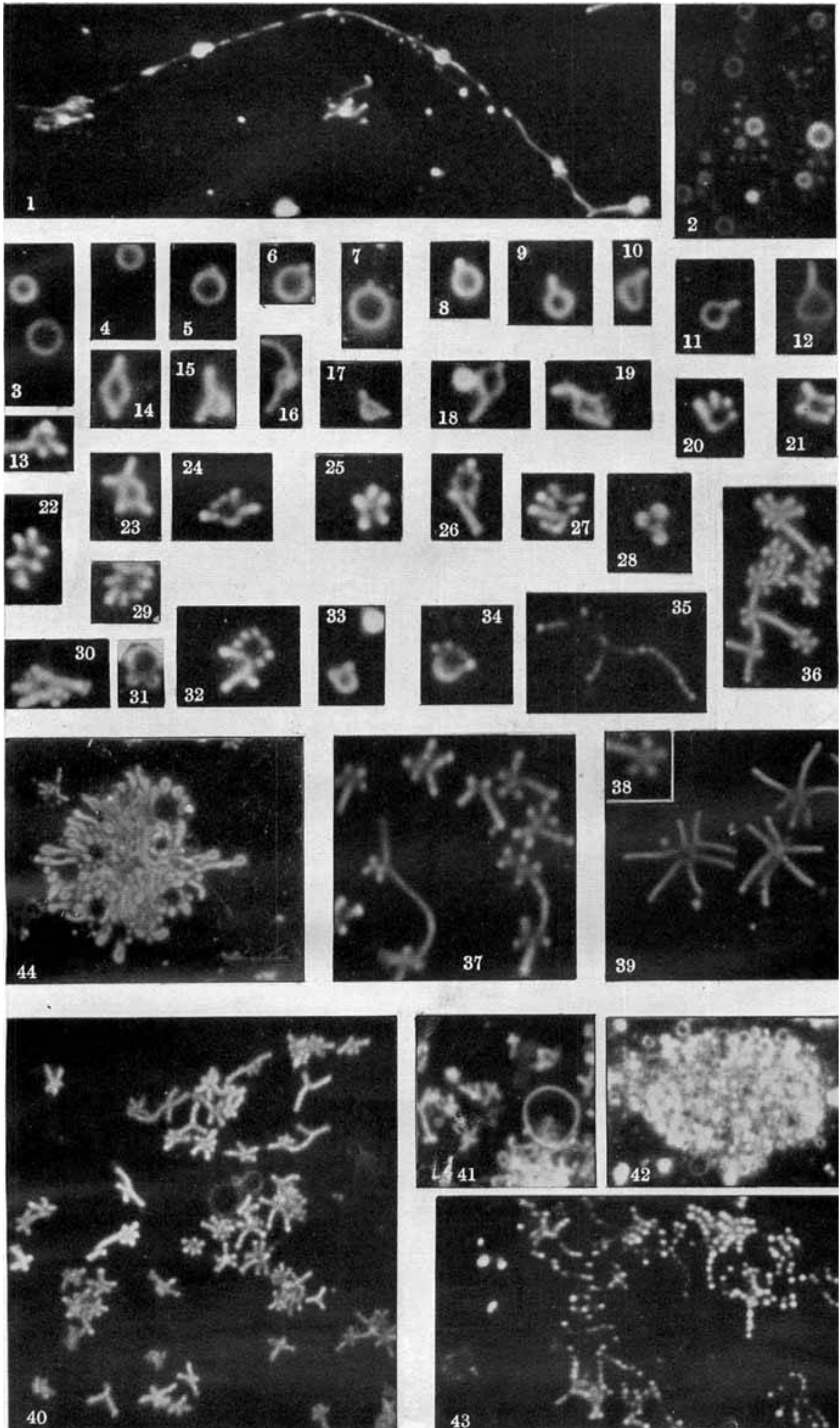

Plate IV

Illustrating various types of filaments

Fig. 1.—Strain 82. 24-hour culture. A branching mycelium showing an excellent example of endomycelial fragmentation resembling chains of streptococci. ×2520.

Fig. 2.—Strain 89. 20-hour culture. ×1800.

Fig. 3.—Strain 83. 42-hour culture. Endomycelial fragmentation. ×1800.

Fig. 4.—Strain 74. 30-hour culture. A form simulating sexual reproduction. A central spherule has protruded a number of radiating thin filaments. ×1260. (Thickness exaggerated by Brownian oscillations.)

Fig. 5.—Strain 83. 42-hour culture, showing two contiguous germinating discules ; no conjugation occurred. ×1800.

Fig. 6.—Strain 83. 42-hour culture. Long filament with elaborate tuft of branches at each end. ×1800.

Fig. 7.—Strain 89. 20-hour culture. Delicate, dull, nodally branching filament. ×1260.

Fig. 8.—18-hour culture, then six days under coverslip at room temperature. Thick bright form and many dull. ×1260.

Fig. 9.—Strain 74. 30-hour culture showing several bright forms and one dull. ×1260.

Fig. 10.—Strain 83. 42-hour culture. Delicate, dull, nodally branching filament. ×1260.

Fig. 11.—Strain 279. 24-hour culture. Branching fragment of mycelium. ×3600.

Fig. 12.—Strain 83. 42-hour culture. A thick, bright, branching filament. ×1800.

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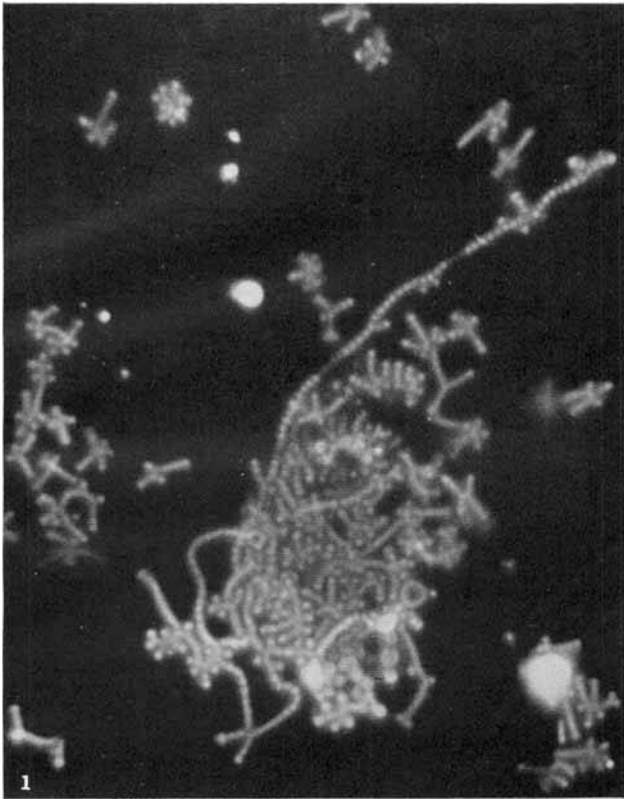

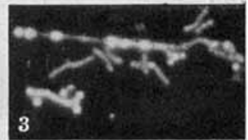

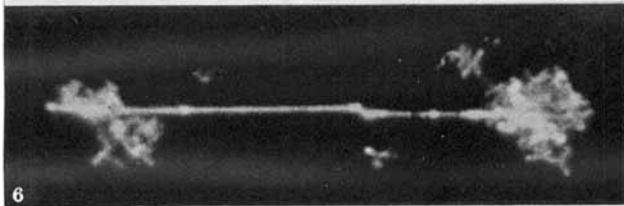

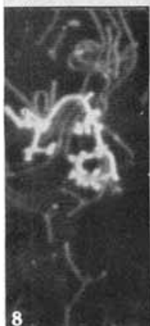

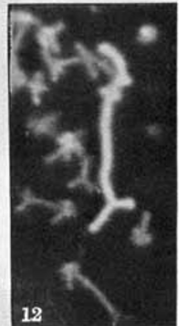

are difficult to photograph satisfactorily by our technique (pl. III, fig. 42). The proof that these remarkable forms are really a phase of the pleuropneumonia organism is given by the growing out from some of them of typical branched filaments, susceptible to endomycelial fragmentation and occasionally provided with terminal discules (diagram II, figs. 3, 5, 9 and 11). Spherules from which a long, delicate, dull filament has grown out are frequently seen (diagram III).

The cultures studied by Barnard apparently exhibited only this budding phase, at least at the stage when he examined them, and he was thus led to believe that it was the only method of reproduction. It is of relatively minor importance under our cultural conditions.

Genethode IV. Reference must now be made to an extraordinary phase which has been observed typically on only one occasion, but of which possible variants have been observed since. What its origin was we cannot say, but from its size it may represent a symplasm. An old culture of strain C. 74, some weeks old, was sown into V.F.-O.S. broth. As sometimes happens with old cultures, growth was delayed, but eventually after four days' incubation a rich bottom growth was observed. This commonly occurs with delayed growths, doubtless owing to the sedimentation of the conidioids, and on account of convection currents the bottom growth tends to rise as a semi-opaque wisp on removal from the incubator. A portion of the bottom growth was removed with a capillary pipette and examined under a coverslip. It consisted mostly of small oscillating bright points and fragments of mycelium including short branching forms, but early in the examination a peculiar form was observed upon which observation was thereupon concentrated, namely a mass of granular material fringed by a row of club-shaped bodies, the outline of the object, approximately 58 μ in diameter, having a remarkably regular hexagonal appearance. The clubs were approximately 10 μ long and 5 μ wide, although some were considerably smaller. It is obvious that this form did not exist as a hexagonal disc in the culture, but that it must have been considerably flattened and squashed during preparation; the most probable shape would be an approximately spherical cluster of club-shaped bodies radiating from a common centre.

Several of the "clubs" contained short chains (two to four) of oval spore-like bodies, apparently rising from the internal surface of their round ends. During the few minutes occupied in observing them, changes in the shape of the clubs were observed, such as a coalescence of neighbouring clubs. Apparatus was immediately assembled for photographing the object, but even during that short period the outline had changed to that of a more irregular hexagon (pl. V, fig. 1). To economise space, most of the illustrations of this

phase are given at a magnification of only 720 diameters (pl. V, figs. 1 to 6; fig. 7 is a dark-ground photograph of *Staphylococcus citreus* at the same magnification for comparison). Fig. 1 illustrates the appearance when first photographed at 11.10 A.M., fig. 2 its appearance at 11.45 A.M. *i.e.* 35 minutes later, at room temperature (24° C.). Comparison will reveal certain alterations, *e.g.* a large and a small club have coalesced to form a larger one (marked by white arrow). It was then removed, on the microscope, to the constant temperature room (38·5° C.).

At 12.25 P.M. (75 minutes under the coverslip) its appearance is shown in fig. 4. It will be observed that although considerable alteration in the shape of the clubs had occurred during this interval, there was no very great change in the short chains of oval bodies. However, when next examined at 2.35 P.M. (3 hours 25 minutes under the coverslip), after incubation, a remarkable change had occurred. Considerable swelling, coalescence and distortion had taken place in the fringe of clubs and the short rows of oval bodies were no longer detectable; on the other hand eight long unbranched filaments, the longest 160 μ long, proceeded from the region of the amorphous centre (fig. 5), floating gently as long streamers in a convection current. With two exceptions the filaments, which were about 0·8 μ wide, were almost completely divided into bright-walled oval spore-like bodies about 0·85 μ wide by 1·05 μ long; in one, division had reached its distal half only, but was proceeding very rapidly during observation and progressing towards the amorphous centre; a piece of fig. 5 showing the proximal end of the other, at its origin from the amorphous centre, is shown enlarged to 1800 diameters in fig. 11, and the same region photographed 10 minutes before in fig. 10. A comparison indicates the rapidity of the division. When next examined at 4.45 P.M. (5 hours 35 minutes under the coverslip) the appearance shown in fig. 6 was seen: alteration of the club-shaped fringe was far advanced so that they resembled pseudopodia; the number of filaments had increased to eleven, the largest having the extraordinary length of 240 μ; again the spore-like division had progressed but, in the filament illustrated in figs. 10 and 11, increase in length had apparently occurred at a greater rate than division, for the spore-like bodies again commenced further from the amorphous centre, approximately where they had commenced at 2.15 P.M. (compare figs. 12 and 10). Unfortunately an air-bubble in the preparation now began to approach the object (fig. 6). At this stage the preparation was replaced in the constant temperature room until 8 P.M. (8 hours 50 minutes under the coverslip), but the air-bubble had now completely engulfed and ruined the object. Only a single chain of oval spore-like bodies remained, and that was broken up during efforts to dislodge the bubble.

PLATE V

Strain 74. Illustrating genethode IV. From a delayed " bottom growth " after four days' incubation.

FIG. 1.—11.10 A.M. ×720.

FIG. 2.—11.45 A.M. ×720.

FIG. 3.—11.47 A.M. (deeper focus). ×720.

FIG. 4.—12.25 P.M. ×720.

FIG. 5.—2.35 P.M. White oblong enlarged in fig. 11. ×720.

FIG. 6.—4.45 P.M. ,, ,, ,, ,, ,, 12. ×720.

FIG. 7.—*Staphylococcus citreus*. ×720.

FIGS. 8 and 9.—Spore-like bodies 8 P.M. ×720.

FIG. 10.—Part of fig. 5. ×1800.

FIG. 11.— ,, ,, ,, 5. ×1800.

FIG. 12.— ,, ,, ,, 6. ×1800.

FIG. 13.—Part of fig. 9, ×1800 to allow comparison of germinating spore-like body with those in the chains above.

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The preparation near the bubble was now examined and a large number of spherical and oval spore-like bodies resembling those in the chains were found ; many of them were germinating by means of one or two short tubules, some of which were branching (fig. 13 at 1800 diameters). In addition, there were large numbers of conidioids, some germinating by means of bipolar tubules and many "protean" discules of the type produced according to genethode V ; some of these had assumed Y shapes, some cross shapes, some dumb-bell shapes. Next morning (9.20 A.M.) a complete examination of the preparation was made, but no forms not identifiable with known forms of pleuropneumonia were seen and there was no suggestion of bacterial contamination. The culture from which the club-fringed object was removed was examined exhaustively for bacterial or other contamination, but behaved consistently as a pure culture. From the above considerations it appears undeniable that the object was a phase of the pleuropneumonia organism, *i.e.* another genethode, although a very rare one : in spite of constant search in hundreds of preparations its like has not been seen since.

Nevertheless forms slightly resembling it have been seen. Strain 111 sown in buffered V.F.-O.S. broth (1 per cent. phosphate buffer salts for *p*H 7·4 incorporated) showed in a 3-day culture a peculiar body (pl. III, fig. 44, at 1800 diameters), 15 by 11 μ, consisting of a radiating agglomeration of small club-like bodies the largest of which were 3·3 by 0·7 μ with some filaments up to 7 μ long and 0·7 μ wide. The latter were constricting to form chains of oval bodies superficially resembling those described above, but resembling more strongly the oval segments pinched off according to genethode V (see below). Unfortunately an accident destroyed this preparation before its development could be followed.

Genethode V (diagram III, figs. 22-47). Many strains, including strain 4159 received from the Lister Institute, London, reproduce by another peculiar genethode that resembles in some respects genethode IV. When a suspension of conidioids (*e.g.* the centrifugate of a 25-day-old culture in V.F.-O.S. or a filtrate therefrom) is incubated in V.F.-O.S. broth many of them are transformed into bright, apparently thick-walled spherules about 0·4 μ in diameter. They elongate, resembling first coccobacilli and then cylindrical flexible bright rods about 4 μ long and 0·4 μ wide. Their resemblance to bacilli is at first disconcerting, but they are readily distinguished by their flexibility and protean peculiarities. A process of constriction at regular or irregular intervals results in the formation of short chains of spherical, oval, cylindrical, club-shaped or irregular elements that may completely separate or remain attached either by thin filaments of various lengths or by some

imperceptible attachment. This phase greatly resembles and is probably identical with that described by Smiles (1926), whose figures strongly resemble forms we have studied. It is unquestionably a method of reproduction; it must therefore be regarded as a fifth genethode. Upon becoming detached, these bodies may continue the process or they may then begin a method of reproduction according to genethode III, *i.e.* by budding; the initial spherules, about 0·4 μ in diameter, are bright and thick-walled. Delicate filaments are extruded on the ends of which bright granules appear which may be cast off at this stage or may develop into small clusters of spherules.

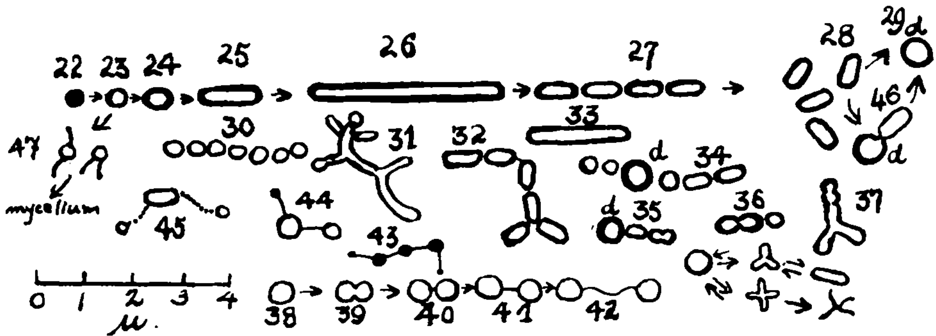


DIAGRAM III.—FIGS. 22-47.

FIGS. 22-47.—Phases of genethode V, showing stages in transformation of a conidioid (22) into spherule (23), coccobacillary form (24) and bacillary form (25, 26, 33) with binary fission (27, 28, 32), and subsequent development of discules by budding off from bacillary forms (46 and 35) or in filaments (34). Note branching (31, 35 and 37), also division of spherule to dumb-bell shape with subsequent withdrawal (38-42). At 30 a bacillary form has constricted into a chain of spherules, at 46 another has extruded two filaments that have subsequently fragmented and have spherules at the free ends. Fig. 44 shows reproduction of a spherule by budding (genethode III). At 43 is a stiff bent filament with refractile spheroidal bodies at intervals. Fig. 47 shows germination of spherules by extrusion of germinal tubes as described by Wróblewski (1931*a*).

Discules may arise during this genethode, by a simple process of budding or pinching-off, within or from the ends of the rod-shaped forms; on becoming detached they germinate or rather become transformed into tri- or quadriradiate centres for mycelial growth by a process of retraction of the circumference at three or four equidistant points as described on page 15. Another common transformation, by medial constriction, results in dumb-bell-shaped forms, which may separate to produce two spherules or even discules, or may draw away from each other and produce a short filament between them; this may subsequently undergo streptococcoidal fragmentation. This is the typically proteiform genethode: many of the changes of shape are reversible and even short

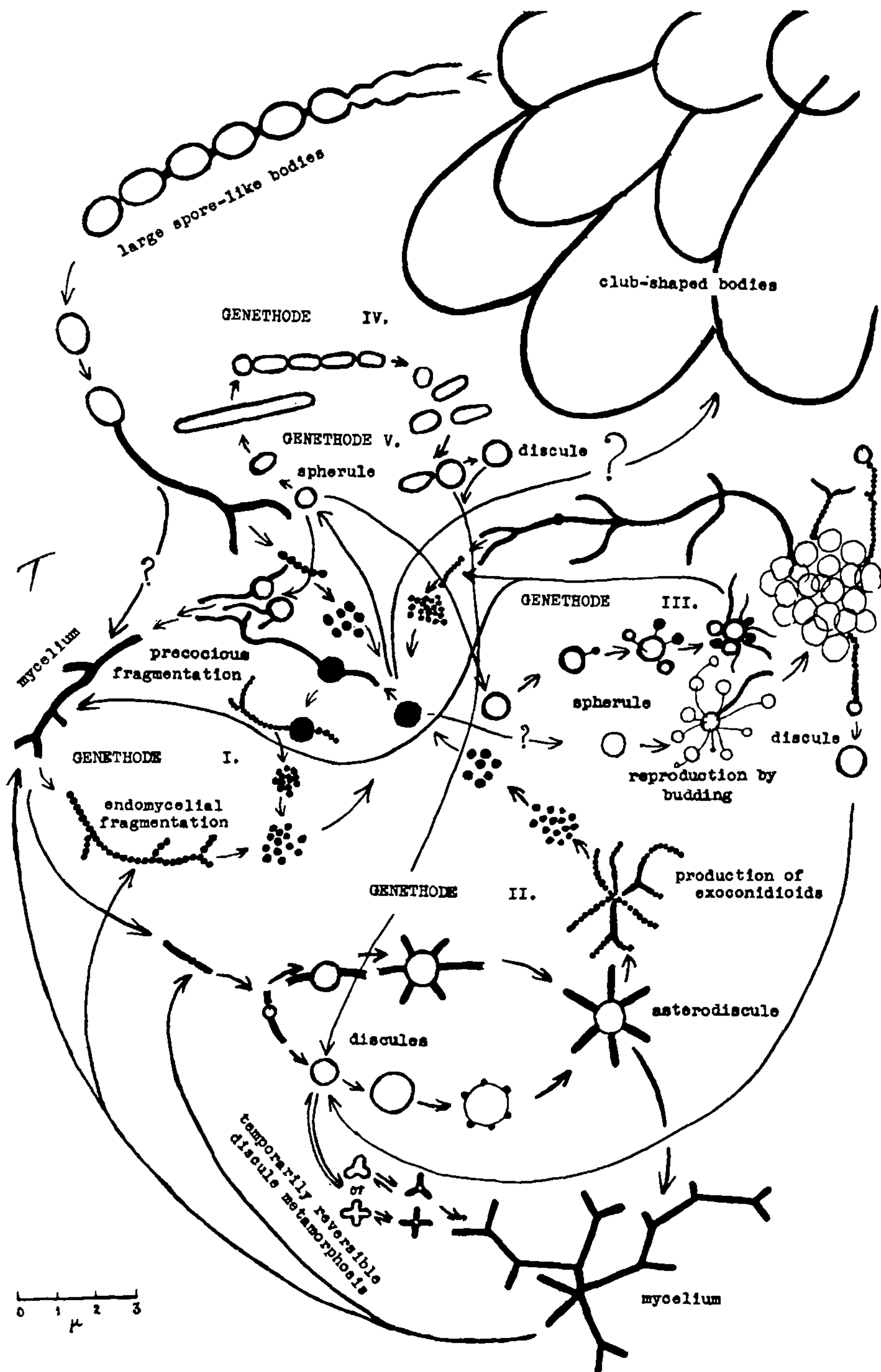


DIAGRAM IV.

Schema to represent the various genethodes of *Borrelomyces peripneumoniæ*. Although the genethodes are usually well defined, they are intimately related and certain variations are possible. The arrows indicate the direction of development. Certain of the phases in genethode V may be reversible.

rods or filaments that have undergone endomycelial fragmentation or constriction may revert to the undifferentiated form, at least temporarily.

Protean peculiarities.

A remarkable and characteristic feature, first mentioned by Ørskov, is the microbe's peculiar faculty of growing quickly or changing its shape quickly. Instances already indicated above are the rapid extrusion of the germinal tube from the conidioid that may occur within five minutes; the rapid endomycelial fragmentation in genethodes I and II, resulting in the complete streptococcoidal transformation of long mycelial filaments about 20 to 30 μ long within 5 or 10 minutes; the sometimes reversible transformation of discules into tri- or quadriradiate bodies or dumb-bell shapes within 20 seconds and the reversible fragmentation of filaments or rods in genethode V; and the pinching off of spore-like bodies from the long filaments (genethode IV) at the rate of one per half minute. Within three minutes a bright thick-walled cylindrical form of genethode V, 4 μ in length, has been observed to constrict into various shapes including a chain of four oval bodies, a bizarre swollen shape, back into an almost undivided rod and finally into a triradiate form.

Alleged " sexual conjugation " (Wróblewski).

We have been quite unable to confirm Wróblewski's claims (1931*b*) for a phase of sexual reproduction but have frequently studied in the living state forms which are probably similar to those on which he based his conclusions. In all cases they have been recognised by careful " optical dissection " to be of two sorts; (*a*) small micro-colonies of very delicate filaments with short, rather thick branches and occasional spherules along them and (*b*) small groups of thick-walled bright spherules, arising as a variant of genethode III from the spherules of genethode V, and from which fine, dull, unbranched filaments, terminated by a bright point, have grown out. It is easy to imagine how either of these two types of growth, when fixed and stained by superstaining techniques, might be interpreted as spherical cells (" oogonia ") surrounded by fine thread-like bodies (" spermites "). That is, however, a false interpretation. Furthermore, hours of observation under the microscope and at the optimum temperature have failed to reveal any indication of conjugation between contiguous phases of various sorts.

Appearances in old cultures.

Old cultures may show a mixture of certain of the above forms, but finally only the conidioids remain.

Before the final stage the following may be found.

(*a*) Very small, dull, actively oscillating points (Brownian movement), apparently roughly spheroidal or polyhedral, some being elongated and resembling tiny coccobacilli or small rodlets. The apparent size is very difficult to estimate, but is something of the order of 0·1 to 0·2 μ, many being much smaller. These particles are similar to the very rare ones found in control uninoculated broth and are probably the serum particles referred to by Frosch (1923*b*) as "Mikronen."

(*b*) Conidioids, both invaginated and not.

(*c*) Sometimes the conidioids may be apparently double, one being larger than the other and closely adhering to it, or it may be almost bisected by a split into two unequal portions; a tiny dull rodlet is frequently attached to one point of the division, the resulting form showing what might be called for reference the "facies impudica": it may represent an abortive attempt at germination, under unfavourable conditions, according to genethode I. The thin, dull rodlets about 0·5 to 1 μ long by about 0·1 μ wide that are occasionally seen, frequently with a small terminal granule, may be derived from them.

(*d*) Discules and asterodiscules gradually disappearing by fragmentation.

(*e*) Dull, regular, thin-walled spheres, about 0·5 to 0·6 μ in diameter, which may be similar to those of genethode III, or swollen involution forms.

(*f*) Various fragments of forms seen in young cultures may be present, *e.g.* small pieces of filament, branched or unbranched, dull or bright, with or without endomycelial granules.

(*g*) Long, rod-shaped bodies, of irregular diameter and with blunt or pointed ends are seen frequently in old cultures; they may be up to 2 or 3 μ long and about 0·2 μ in diameter and have the appearance of crystals of some sort.

Morphology of the living microbe in the tissues of the host.

The only attempt to study the living microbe in pathological material has been by Frosch (1923*b*), who took photographs of fresh pleuritic exudate from a case of pleuropneumonia by direct illumination with ultra-violet light; by this means he claimed to demonstrate the presence of large numbers of double-contoured, ring-shaped bodies and discs; the absence of the mycelial and asteroid forms described by Borrel etc. in cultures was regarded as throwing still more doubt on their specificity.

At this Station, where intensive work on pleuropneumonia has been going on for three years and where an infected herd has been established in proximity to the laboratory, very good opportunities for studying pathological material with the least possible delay after the death of the host have existed. It may be stated briefly that pleuritic exudate obtained *post mortem* and examined immediately after removal and before coagulation by the dark-ground technique described above, showed readily and constantly the presence of forms similar to those found in cultures. These have been mostly the mycelial phase with a certain number of discules either entire or germinating (asterodiscules). In addition the transformation of certain discules into tri- or quadriradiate forms (see schema, p. 21, genethode II) has been observed. Plate VI illustrates a series of photographs of "natural virus," as the pleuritic exudate is colloquially called: figs. 3 and 4 are two isolated discules undergoing germination with consequent resorption of the disc-like centre; the remaining photographs prepared from the centrifugate of the same material show leucocytic and other debris from the pathological fluid together with typical pleuropneumonia filaments, germinating discules etc. These cannot be confused with the pseudo-filaments sometimes seen by dark-ground examination of body fluids containing leucocytes or erythrocytes; they are unmistakably pleuropneumonia filaments similar to corresponding stages in cultures.

Exudate from the greatly distended lymph spaces of the interlobular septa of the lung contains similar forms, as does lung juice from lesions; in the latter case, however, an artefact that is readily distinguished from the parasite is caused by detached bronchial cilia.

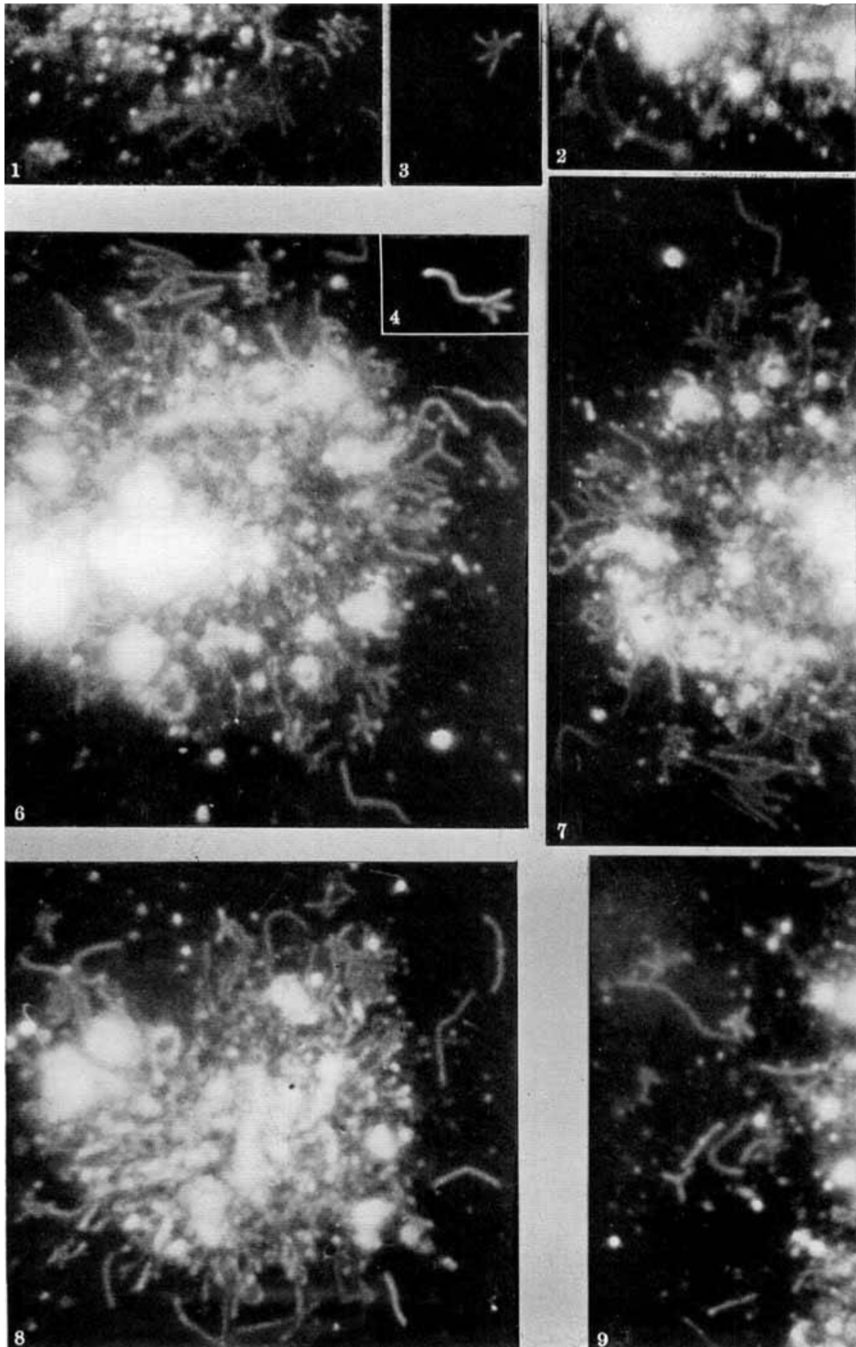
If pleuritic exudate be examined not in the fresh state but after storage for some hours, difficulty is experienced in finding recognisable pleuropneumonia forms; the eventual fragmentation of the filaments produces tiny oscillating points that can no longer be identified with certainty.

These examinations have supported our belief that the cultures examined and photographed during the above study represent the organisms found in the lesions of the natural disease.

IV. Phylogenetic relationships and nomenclature.

From the above observations it seems evident that the organism must be removed from the true filterable virus group, into which it has crept mainly through earlier imperfections in technique, and where it has since been tolerated until its true relationships could be demonstrated. It is certain that if its discovery had been delayed until the present day no one would suggest that it was a filterable virus; it would undoubtedly be regarded as a slender, ramifying, serophilic micro-organism, with peculiar optical properties

ORGANISM OF PLEUROPNEUMONIA



Borrelomyces peripneumoniæ in pleuritic exudate from an acute case of pleuropneumonia; figs. 3 and 4 from the exudate, others from centrifuged deposit of exudate (include much cellular debris); fig. 2 from a portion of the centrifugate in which originally only conidioids could be seen; after incubating for 18 hours branching filaments have grown out. All ×2400.

and an exaggerated faculty for producing filterable forms. Of the classifications and terminologies proposed for it we can quickly dismiss Tartakowsky's and Dschunkowski's *Allococcaceæ,* Bordet's *Vibrio, Spirillum* or *Spirochæta,* and Martzinowski's *Coccobacillus.* Nocard and Roux's *Asterococcus* rests upon an incorrect interpretation that the disc-like bodies are coccal in nature, and may thus be discarded. Lipschütz's inclusion of it in the *Strongylosomata* or *Strongyloplasmata,* which include the true viruses, cannot be accepted. There are three ways in which the organism of pleuropneumonia might be classified: (i) a new genus of the existing family *Actinomycetaceæ* (as suggested by Ledingham), (ii) a new family of the existing order *Actinomycetales,* or (iii) a new order of the class *Schizomycetes,* might be created.

The course to be taken will depend upon estimates of the importance of its peculiar characteristics and is thus a matter of opinion. What distinguishes it most clearly from other members of the class *Schizomycetes* is (1) its extreme pleomorphism, (2) the characteristic polygenethodism, or provision of alternative modes of reproduction, and (3) its peculiar protean or amœboid tendency to change its shape relatively quickly, which is seen nowhere else among the *Schizomycetes.* In spite of the suggestion of Ledingham to fit it and the organism of agalactia into the existing family *Actinomycetaceæ* (no doubt for the sake of simplicity), it is felt that their insertion even into the order *Actinomycetales* would strain the present definitions to breaking-point and that the cause of simplicity would be better served by leaving intact those definitions and creating a new order within the class *Schizomycetes.*

It is very difficult to form a sufficiently descriptive name that has not been already used or proposed for some other organism. There is little to recommend Wróblewski's *Asteromyces* as a special genus only for the pleuropneumonia organism, with *Annulomyces* as a separate genus for the agalactia organism. Ørskov's *Mycoplasma* connotes an alleged fluidity of the filterable conidioids which we have been unable to confirm. Frosch's *Micromyces* gives no clue to the main peculiarities of the organism. Since a descriptive nomenclature is beset with difficulties, we suggest a recourse to the otherwise undesirable "monumental nomenclature" and would perpetuate the name of Borrel, who with his colleagues first demonstrated its characteristic morphology, by erecting an Order *Borrelomycetales,* with Family *Borrelomycetaceæ* and Genus *Borrelomyces.* The two known species of the genus *Borrelomyces* would be *B. peripneumoniæ,* the causal organism of *pleuropneumonia contagiosa boum* and *B. agalactiæ,* the causal organism of *agalactia contagiosa caprarum.*

This would involve the extension of Bergey's classification as under.

Key to the orders of the class Schizomycetes (Buchanan).

1. Simple and undifferentiated forms (the true bacteria).
   ORDER I. *Eubacteriales.*
2. Specialised or differentiated forms.
   a. Plant-like.
   b. Mould-like.
      i. Reproducing by simple fragmentation.
         ORDER II. *Actinomycetales.*
      ii. Polygenethodic ; typically form filterable conidioids.
         ORDER III. *Borrelomycetales.*
   c. Sheathed.
      ORDER IV. *Chlamydobacteriales.*
   cc. Not sheathed.
   d. Sulphur bacteria.
      ORDER V. *Thiobacteriales.*
   dd. Slime-mould-like.
      ORDER VI. *Myxomycetales.*
   aa. Protozoon-like.
      ORDER VII. *Spirochætales.*

Description of order III. Borrelomycetales (*nov. ord.*). Parasitic forms. Regularly develop small filter-passing spheroidal or polygonal conidioids both from mycelium and from disc-shaped conidioidophores. Pleomorphic. Polygenethodic. Serophilic. Micro-aerophilic to aerobic. Non-motile. Gram-negative. Family *Borrelomycetaceæ* (nov. fam.) Genus *Borrelomyces* (nov. gen.). The characters of the family and genus are those of the order. The type species is *B. peripneumoniæ.*

Its inclusion within the *schizomycetes* is compatible both with its size and its filterability. Although its filterable phases or conidioids are small, even they are not of an entirely different order of size from certain other members of the class *Schizomycetes.* Thus according to Gotschlich (1929) the " Micrococcus der progressiven Absceszbildung bei Kaninchen " is only 0·15 μ in diameter, and according to St John-Brooks (1930) micrococcal forms exist with a diameter of only 0·2 μ. *Dialister pneumosintes* is 0·15 μ wide by 0·3 μ long. Even among the *Actinomycetes*, the recorded width of *A. asteroides* is 0·2 μ, of *A. farcinicus* 0·25 μ and of *A. metchnikovi* 0·3 μ, and a member of another genus of that family, *Erysipelothrix rhusiopathiæ*, is 0·2 to 0·3 μ in width and 0·5 to 1·5 μ in length. Compared with these measurements, the diameter 0·2 to 0·5 μ assigned by Barnard to the " particles " and " spheroids " respectively (quoted by Ledingham 1933*b*), and our own estimations

are not extraordinary; and of course the lengths of filaments measured by us (up to 190 or even 240 μ) become relatively gigantic. The true filterable viruses, on the other hand, range from 8 $\mu\mu$ for foot and mouth disease to 0·175 μ for Burnet's canary virus (Burnet and Andrewes, 1933-34).

Filterability is also compatible with membership in the class. If we accept the claims of a large number of workers for the existence of filterable forms of *Mycobacterium tuberculosis, Eberthella dysenteriæ, Eb. typhi, Escherichia coli* and *Staphylococcus aureus*, and of Sartory, Sartory and Meyer (1934) for a form of *Actinomyces bovis* capable of passing through collodion sacs implanted in the peritoneal cavities of guinea-pigs, the inclusion of the organisms of pleuropneumonia and agalactia as an order of the *Schizomycetes* becomes still less difficult and the view may be adopted that they are members of that class whose faculty for constantly and precociously producing enormous numbers of " filterable forms " has become developed to an extreme degree.

V. Discussion.

The above study of this interesting micro-organism has revealed a remarkable life cycle characterised by the provision of at least five methods of reproduction, for which the term genethodes is proposed. The list may not be exhausted even yet, for the " chromatic nodes " of Ledingham may represent another genethode. Whether they all occur in the animal body is at present unknown; in pleuritic exudate and lungs we have seen evidence only of genethodes I, II and possibly V.

The origin of most of the forms described herein can be traced in a satisfactory manner: the discules and the so-called asterococci or astral bodies (*i.e.* germinating discules) are seen to be derived, usually directly, from the mycelium, although occasionally they are pinched off from small cylindrical or irregular bodies. We have seen in V.F.-O.S. broth cultures no bodies reminiscent of Ledingham's chromatic nodes, but there appear to be distant analogies between them and genethode V in their faculty of budding off or pinching off discules.

Barnard's claim that the microbe reproduces from spherules by a peculiar sort of budding, *i.e.* by the extrusion of particles that remain attached to delicate threads and enlarge to become in turn spherules, has been confirmed but is believed to be relatively unimportant; the suggestion might be hazarded that this genethode represents, in the language of embryology, an " ancestral memory " or recapitulation of a method of reproduction that may have once been common in an earlier stage of the evolution of lower organisms and that has become developed and fixed in the yeasts.

Indeed *Borrelomyces*, with its discules that shed exoconidioids (*i.e.* conidioidophores), has resemblances to the moulds * also; and when one considers further the club-shaped bodies and the formation of oval " spores," resembling the oidial spores of certain fungi, by constriction or pinching off from filaments, one feels oneself in the presence of a form of life which is now engaged in stabilising itself and has more potentialities for subsequent fixation of its characteristics than any other with which one is familiar.

Unfortunately, through confusion with rinderpest, the early history of pleuropneumonia is too obscure to allow a determination of its first appearance, but it does not appear to have assumed epidemic proportions before 1693, when it was first recognised in Central Europe. It is therefore possible that the parasitic habit of the causal organism may not be more than a few hundred years old and it may figuratively be regarded as still going through its birth pangs. For this reason alone it and the related organism of agalactia, whose recognition is also of relatively recent origin, are full of interest to the bacteriologist and biologist.

Whether, in view of the evidence and speculation presented above, efforts to place them in the family *Actinomycetaceæ* or even in the order *Actinomycetales* are justifiable is at least open to question ; our own view is that a new order is necessary.

The conception of polygenethodism outlined above interprets and classifies the numerous morphological phases that have been described in the past and reconciles the various proposed life cycles : it suggests that the observations of Nocard, Borrel, Bordet, Frosch, Barnard, Smiles, Ørskov, Nowak and Wróblewski in fluid cultures were on the whole correct but incomplete and that Ledingham's observations, although confined mostly to surface growths on serum agar, are probably explainable as an extension of it : it brings some order and meaning into an otherwise apparently chaotic array of phases with extremes of shape and size.

The biological significance of this polygenethodism is far from clear. We can speculate whether it is an expression of the micro-organism's adaptability to alterations in its environment, whether it indicates the possession of unusual potentialities for future specialisation along perhaps one of the paths of development, whether certain of the genethodes represent " recapitulations " or whether it merely exhibits in a more obvious degree morphological and reproductive phenomena that are shared, less obviously, by at least some other members of the *Schizomycetes* : for *Borrelomyces*, although outstanding in this respect, is not unique ; it is rivalled by *Azotobacter* (Löhnis and Smith, 1916 ; Jones, 1920). Briefly

* Wróblewski (1931*a*) considered that "das Peripneumonievirus gehört einer speziellen, filtrierbaren Pilzsorte an."

they claimed that the typical oval cells, 2 to 3 μ by 3 to 5 μ, which are enclosed in thick gelatinous capsules, may give rise to the following forms, which can be isolated in pure culture and are therefore really variants: micrococcus forms 0·3 to 1·0 μ in diameter; oval wedge-shaped and rod-like cells, 0·3 to 0·75 μ long; rod-shaped cells, 0·3 to 0·5 μ by 0·75 to 1·0 μ, sometimes motile, which do not form endospores; similar smaller rods which produce endospores; large spore-forming rods; and branching fungoid forms. All these potentialities must be present in the "typical" cultures although there is no suggestion that the pleomorphic variants follow an orderly cycle. More important, three methods of reproduction, which would be covered by the suggested term "genethodes," appear to have been definitely established by them; (*a*) ordinary binary fission, (*b*) budding, and (*c*) fragmentation into filterable "gonidia." Reproduction by budding has also been observed by Stoughton (1929-30) in *B. malvacearum*, gonidial fragmentation in *Rhizobium radicicola* by Thornton and Gangulee (1925-26), and both methods in various members of the colon-typhoid group by Hort (1917). Indeed Kritschewski and Ponomarewa (1934), using an impression preparation technique and Giemsa staining almost identical with Ledingham's (1933*b*), claimed that *Eb. paratyphi B* is a highly pleomorphic organism; some of their photographs (*e.g.* fig. 8) strikingly recall his (Ledingham's) of pleuropneumonia. These workers reach the conclusion that "the doctrine of monomorphism contradicts reality and should be substituted by the statement that bacterial species are pleomorphic."

Evans (1932) has apparently been able to show "that a streptococcus, a filterable form, and an aerobic, Gram-positive, spore-bearing rod are phases in the life cycle of an organism isolated from cases of epidemic encephalitis and from the so-called herpetic and encephalitic viruses."

In the above organisms these pleomorphic forms and unusual methods of reproduction are apparently rare or at least not usually obvious; in *Borrelomyces*, polygenethodism appears to be constant and normal, and pleomorphism is very strongly developed.

VI. General summary.

1. The life cycles and morphology of the causal organism of *pleuropneumonia contagiosa boum* have been studied in the living state by dark-ground observation of macro- and micro-cultures in a new fluid medium, V.F.-O.S. broth.

2. The microbe is no filterable virus *sensu stricto*, but typically and constantly forms a relatively enormous branching mycelium, filaments of which may reach a length of at least 190 μ; certain unbranched filaments have reached 140 μ.

3. It owes its filterability to the constant and early production of filter-passing forms (" conidioids ").

4. It possesses at least five different methods of reproduction, for which the term " genethodes " is proposed: these are (1) by endomycelial fragmentation into coccoidal particles (proconidioids) that become conidioids; (2) by the formation in the mycelium of discules that develop into " asterodiscules " and shed " exo-conidioids," which become conidioids; (3) by a process of budding from spherules; (4) by the formation of oval, spore-like bodies from long filaments that arise from masses of club-like bodies; and (5) by the pinching off of variously shaped forms from cylindrical rod-shaped bodies that arise directly from conidioids. There is no evidence for any form of sexual conjugation.

5. Its polygenethodism, extreme pleomorphism and protean faculty of rapidly changing its shape prevent its satisfactory inclusion in any existing order of the *Schizomycetes*.

6. A new order *Borrelomycetales* to include it and the closely related organism of agalactia is proposed. The suggested terminology is *Borrelomyces* (nov. gen.) *peripneumoniæ*.

Addendum.

The recent work of Klieneberger (1934) forms a valuable contribution to the study of the architecture of colonies on serum agar. It is possible that on serum agar the organism does not reproduce by all its genethodes nor fully reveal its morphological potentialities. Unlike Ledingham (1933*b*), in whose laboratory she worked, she makes no mention of discules or asterodiscules, which we have frequently observed by dark-ground examination of living colonies on serum agar. She interprets Ledingham's " chromatic nodes " as superficial " globular bodies " with attached deep filaments that have been torn out of the medium and distorted in various degrees. The type of growth described resembles in some ways the microcolony in our diagram III in which were produced many branching filaments, the ends of which were transformed into rows of spherules and occasionally into terminal discules.

My thanks are due to my colleagues Mr A. T. Dann, M.Sc., who suggested the use of the Leica camera and took several of the microphotographs, Mr A. D. Campbell, B.V.Sc., and Mr A. T. Dick, B.Sc., who carried out a large amount of the cultural work and drew my attention repeatedly to cultures worthy of study; to Professor H. Hartung for valuable micro-photographical advice, and to Dr H. R. Carne and Professor J. C. G. Ledingham for the provision of strains of the pleuropneumonia organism from the Lister Institute.

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