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The Growth Phases of Pleuropneumonia and Agalactia on Liquid and Solid Media

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THE GROWTH PHASES OF PLEUROPNEUMONIA AND AGALACTIA ON LIQUID AND SOLID MEDIA.

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(PLATES XXXI.-XXXIV.)

WITHOUT some brief chronological survey of the efforts which have been made in the past to unravel the growth cycles of these filterable but readily cultivable organisms, it would be impossible to appreciate the standpoint from which the work here recorded took origin. Such survey seems all the more necessary if only to make clear how it came about that those few workers who have not been repelled by the inherent difficulties of the problem have reached such curiously discrepant conclusions from their observational data. And yet in spite of these discrepancies, largely ones of interpretation, a considerable measure of agreement has been reached, at least with regard to the initial phases of growth on liquid and solid media.

In this survey I shall be concerned mainly with the pleuropneumonia organism which was cultivated for the first time in collodion sacs in the peritoneum of the rabbit and ultimately in Martin's broth with added serum, by Nocard, Roux, Borrel, Salimbeni and Dujardin-Beaumetz in 1898. The organism of agalactia is a more recent discovery dating from Bridré and Donatien's work of 1923 and it is indeed fortunate from the point of view of systematic morphology that the pleuropneumonia organism no longer occupies an isolated position. Nocard and his collaborators referred to their organism simply as "*un microbe d'une extrême ténuité.*" Its exact form they found it difficult to determine even after staining and it is strange that a similar remark could have been made many years later by Bechhold (1926). Morphological studies began in 1909-10 with Bordet's account of his observations made with the Giemsa stain which he decided, on grounds with which the present writer agrees, was the "*colorant de choix.*" Bordet describes and figures fine undulating elements, spheres and rings. The spheres or globules appeared a little later than the filaments and his opinion was that the former were derived from the latter. What impressed him was a possible analogy with the resting globular forms known to occur in old cultures of cholera and he suggested that the

organism belonged most probably to the vibrionic group though motility, an important characteristic of this group, was not established in pleuropneumonia. Bordet's drawings illustrate chiefly the spirillar or filamentous forms with minute swellings in their substance, together with small globular and ring forms. There is no evidence that he saw any of the larger yeast-like elements now known to be characteristic of pleuropneumonia growths. Immediately following Bordet's came the communication of Borrel, Dujardin-Beaumetz, Jeantet and Jouan (1910). These authors examined the growth by dark-ground methods, by Giemsa staining, and by some form of *surcoloration* preceded by a mordant. The latter became their method of choice. Giemsa they regarded as too feeble a stain and one by use of which important details were liable to be missed. Their observations were made on centrifuged broth cultures, small amounts of the deposits being spread like blood films. The study of colonies on solid media they regarded as "*à peu près impossible*." With dark-ground methods they observed isolated granules, diplococcal forms and groups of "cocci" in a scarcely visible sheath, while at later stages, filamentous and ramifying filaments and star-shaped forms or asters made their appearance. They attached considerable importance to this "mucous" sheath which they claimed to demonstrate by *surcoloration* and within this sheath they believed the microbe divided in various directions in space forming chains and asteroid forms. About the third or fourth day asteroid forms were particularly numerous but as the culture aged, extraordinary polymorphic involution forms were noted and figured. These plaques they considered to arise from coalescence of mucoid substances secreted by the ageing microbes. The authors' numerous microphotographs are not very satisfactory but they certainly show in addition to the forms described by Bordet, large globular masses and indications of the so-called involution forms which other methods have since more clearly defined. Their final judgment was that the organism is essentially of coccal nature whose peculiar method of division is determined by its enclosure in a viscous sheath and they hint at some morphological resemblance with *B. radicicola* of the Leguminosæ. The features on which they laid stress are reflected in the name given by them to the organism, *Asterococcus mycoides*, really only the first of a series of names suggested for this parasite.

A brief and unsatisfactorily illustrated paper by Martzinowski followed in 1911 in which he compared the forms obtained in lung smears of affected calves with those observed in Giemsa-stained preparations of cultures from filtrates. In the lung smears he noted elongated cocci and swollen rods often united by filaments resembling vibrios and spirilla. These same forms were present in the cultures in addition to large globular formations and involution forms. In spite of the polymorphism exhibited, Martzinowski preferred to class

the organism as a cocco-bacillus and he gave it the name of *Cocco-bacillus mycoides peripneumoniæ* which, he said, would suit it perfectly ("*lui conviendrait parfaitement*"). Freiberger's contribution in the following year (1912) was in the nature of a criticism of the findings of Borrel and his colleagues, particularly their dark-ground findings. From his own observations by dark-ground methods of various uninoculated media with and without the addition of serum and of various animal sera, he decided that in all probability the peculiar findings of Borrel were in no way specific. He suggested further that certain of Borrel's forms which were not repeated in uninoculated media were due to particular dispersions of the protein constituents of the medium resulting from growth of the invisible virus. The paper deserves citation not for its intrinsic value but for the fact that it unwittingly draws attention to the undoubted danger of exaggerated reliance on dark-ground findings and the dispensing with data obtained by other methods. The suggestion that not only the dark-ground findings but also those described by Bordet, Borrel *et al.* in stained preparations were probably not genuine formed elements of growth but medium artefacts and the like was made again by Frosch (1922) and even repeated by Dahmen (1929), whose chief complaint, however, was that the Bordet findings were hardly likely to be genuine as they could not be brought into genetic relationship with each other—a difficulty which has faced all who have hitherto worked on this subject, whatever their observational data have been. Frosch (1923) was the first to attempt the examination of edges of colonies on solid media by the aid of ultra-violet light photography. Dark-ground methods he criticised on the ground that they yielded only diffraction-figures and not geometric pictures of elements of doubtful nature. The photographs which illustrate his paper are not satisfactory. They show, but dimly, round oval or polygonal bodies together with plaques of homogeneous structure which Frosch recognised as the essential elements of the growth. Mycelial-like structures were also noted and he finally reached the conclusion that the organism was related to the yeasts. He therefore proposed for it the name *Micromyces peripneumoniæ bovis contagiosæ*.

A study on similar lines was made by Barnard (1925) in connection with his work with Gye on the cultivation of "viruses" from avian and mammalian growths. Unfortunately he makes no reference to previous studies, relying entirely on dark-ground examination of fluid cultures and on photographs of edges of growths on cover-slips taken by ultra-violet light. These photographs resemble generally those made by Frosch. They show, dimly, masses of spheroids and particles which, in his view, constituted the only elements visible in fluid cultures by dark-ground illumination. The ultimate viable particle capable of reproducing the growth had a diameter of $0{\cdot}2\,\mu$. This particle was, as a rule, intimately associated with a larger spheroidal body which had

developed from the particle and which was capable again of forming further particles from its substance. These particles would wander off from the main body although a fine filament connecting them to the parent could be detected by ultra-violet light photography. The particles which had wandered off sometimes appeared as clusters or chains occasionally intermingled with spheroids.

Essentially, therefore, Barnard's conception of the growth cycle was one of simple budding. He makes no mention of the presence of vibrionic or filamentous forms in dark-ground specimens. The study by Barnard's colleague Smiles (1926), using also dark-ground methods only, revealed a cycle essentially different from that of Barnard. An initial corpuscle (spherical or aspherical or granular) elongates to a cylinder which breaks up into two or three small spherical or granular forms. Chains and groups of spheres result and such groups are connected by long filaments "of low visibility." It is clear that no satisfying conception of the growth phases can be obtained by such methods. It is notable, however, in the light of the now well-known findings in stained preparations, that Smiles detected the presence of these interconnecting filaments. Bechhold and Sierakowski (1926) attempted to render the pleuropneumonia elements visible by gilding them and subsequently examining them in the ultramicroscope. The authors must have been singularly unfortunate in their efforts to stain the elements as they make the strange statement: "*Im gewöhnlichen Mikroskop bei Hellfeld lässt sich kaum etwas erkennen auch nicht nach Färbung.*" The work of Orskov (1927), though incomplete, is important as it was an attempt, successful so far as it could go, to get an idea of the early happenings at the edge of young cultures on solid media. It was undertaken primarily to secure evidence in support or otherwise of Barnard's conception of the growth cycle and one of the strains he used was obtained from Mr Barnard. He used a powerful source of light and examined the growing edge through a slip placed over a cube of growth taken from a Petri plate. He noted and figured the production of a ramifying "mycelium" springing from a single small "battonet" or oval body, the extremities of the branches bearing swellings. This was the appearance in a small compact colony of 20 hours' growth. As the colony aged, larger elements and filaments containing swollen elements in their interior appeared. These swollen elements he considered to be degenerate forms in process of autolysis and he believed that such forms had lost the power to germinate when transferred to fresh media. Autolysis, he considered, might take place quite early and at different times in different strains. Orskov's method of attack did not permit him to follow satisfactorily the later stages in the growth of the colony, but his demonstration of the production of a filamentous "mycelium" from the primary oval battonet accorded ill, as he reports, with Barnard's conception, while it went far to explain the vibrionic and spirillar and ring forms described in 1910 by Bordet.

Quite recently extensive studies have been made by Nowak (1929, 1930) and Wroblewski (1931), both of whom relied entirely on staining methods (*surcoloration* following the use of a mordant) applied mainly to growths in serum broth. The former employed growth material obtained by centrifugalisation of broth cultures. By examination of the broth cultures in this way at different stages they attempted to build up the cycle of the organism. Both authors' papers are accompanied by numerous microphotographs of the various elements encountered. Nowak's examination of the "elementary corpuscles" from which the primary growth proceeded led him to suppose that they did not possess the rigid consistence and structure of ordinary cocci, but rather that they represented droplets of a thick protoplasmic fluid incompletely consolidated and stabilised. The description is an apt one. This elementary body germinates, producing a true ramifying mycelium containing in its interior round or oval deeply stained corpuscles whose nature and function he does not profess to explain. The asteroids of the early french workers he considered to be simply elementary corpuscles in process of germination. By the side of the ramifying filaments are vibrionic and spirillar forms and rings with attached filaments. Sometimes a tress of filaments was observed like a floating actinomyces colony. Other features noted were spherical masses which appeared to have arisen from condensation of deeply staining portions of the mycelium. By the sixth day the mycelial filaments disappear, having broken down to fragments which become transformed to elementary corpuscles again. Nowak's scheme is shortly: elementary corpuscle—buds—filaments containing deeply stained portions—fusion of latter to large globular masses—finally degradation of mycelium to primary elementary corpuscles. He would suggest for the organism the name *Mycoplasma peripneumoniæ*.

Wroblewski's study was on very similar lines and based on day-to-day examination of broth cultures stained by carbol-fuchsin preceded by a mordant which gave rise to no precipitates. I can refer here only to the chief features of his work. Starting from a seeding from a 6-day culture containing mainly elementary bodies or "spores" of $0{\cdot}3\,\mu$ diameter, these at 24 hours are found to have pushed out one or two dendrites which become ramifying filaments showing deeply stained granules at their extremities. He gives minute descriptions of the various elements seen on successive days of culture, but the main fact which emerges is his agreement with Nowak and with Orskov as to the germination of the "elementary corpuscle" during the early phase of growth in liquid medium into ramifying filaments presenting at their extremities and here and there in their substance deeply stained chromatic masses. These ramifying filaments are amply illustrated. At 48 hours and later he draws attention to the occurrence in films of two other types of spheres ($0{\cdot}5\,\mu$ to $2{\cdot}5\,\mu$), the smaller of homogeneous and the larger of granular structure. The former he

does not hesitate to enrol as female elements (*oogonies*) and he has observed them surrounded by minute granules emitting fine filaments (referred to as *spermites*). The larger and more granular spheres he interprets as *spermatocysts* and he has seen them emitting from their interior fine filaments terminated by granules. The author, rather irresponsibly it must be feared, uses mycological terms for dubious and intriguing elements met with in fluid media whose connection with each other or with the main cycle cannot possibly be elucidated by the method of investigation employed. In the author's opinion the filaments of the mycelium after forming *exospores, endospores, oogonia* and *spermatocysts* undergo autolysis. The most characteristic elements of the growth are, in his view, the star-shaped conidia and he would propose for the organism the name *Asteromyces peripneumoniæ bovis.*

Hosakawa and Kawamura (1931)* have made a morphological study of the elements present in young serum-broth cultures. They emphasise the essential filamentous and branching nature of the first phase of growth resulting from pullulation of "spores" and their brief paper is accompanied by a very interesting series of drawings of these branching forms with their terminal and intra-filamentous condensations, presenting a remarkable resemblance to characters in a japanese text. Their figures are identical with those illustrated in plate XXXI., fig. 1 of *this communication*. The effect of alterations in *p*H of fluid medium and the temperature of incubation, on the morphology of the elements is discussed also in a brief but illustrated paper by Mizuguchi, Kano and Sahingu (1932).*

Summary of position.

To sum up, the morphology of the elements in pleuropneumonia growths has, so far, been studied in liquid cultures mainly and to a much less extent in solid media. Dark-ground and staining methods have been applied to liquid cultures while attempts have been made to examine both by ordinary light and by ultra-violet light the early growths on solid media. So far as the actual elements are concerned the account given by Bordet in 1910 of the polymorphic elements in liquid and solid culture media has been, in the main, amply confirmed by most subsequent workers. Other seemingly important and characteristic forms have been noted since by staining methods. Dark-ground methods and ultra-violet light photography have not been fruitful in illuminating the genetic relationship of these elements, and in fact no method used hitherto would be likely to throw light on any but the early phases of growth. These early phases as observed by Orskov, Nowak, Wroblewski and Hosakawa and Kawamura are now quite clearly understood and as we shall see they are amply corroborated by the results obtained by the method of attack pursued by the writer

* For a translation of these papers from the japanese I am indebted to Dr D. Kato.

and illustrated in this communication. It is the object of this paper to bring these early phases into relation with the later phases of growth as determined by impression preparations of growth on solid media. Previous workers have opined that the characteristic embedding of pleuropneumonia colonies beneath the surface of the agar precluded this method of attack, but it will be admitted that, if this difficulty can be overcome, the data so obtained cannot fail to clarify the growth cycle in all its phases and at the same time throw light on the nature of the polymorphic elements observed in liquid media.

Methods employed in the present work.

At the outset of the enquiry it was considered necessary to orient oneself with regard to the features of pleuropneumonia growths in liquid media (horse serum broth almost exclusively). Films were prepared directly from cultures at different ages from 15 to 18 hours onwards and also from the centrifuged deposits of such cultures. The seedings for such cultures were derived from uncentrifuged serum broth cultures and from Berkefeld V and L 2 filtrates of these.

The seedings in many cases were deposits from filtrates centrifugalised at high speed (14,000 r.p.m.). Material for examination was spread with platinum loop in a thin layer in the central portion of a slide or, more frequently, placed near one end and drawn along by aid of another slide. The preparation is fixed in absolute alcohol and stained in the inverted position in a vaseline-sealed Petri dish overnight with weak Giemsa (1 drop to 1 c.c. of a mixture in equal parts of tap and distilled water). Differentiation is performed with tap water only, followed by distilled water.

Impression preparations of growth on solid media.

The difficulty arising from the self-embedding habit of the organism was for the most part successfully overcome by the use of slides pressed down with some firmness over the growth area and then carefully raised without disturbing the texture of the agar. The serum agar plates after being poured are dried for 18 hours in the incubator. The seeding in appropriate dilution is dropped on a marked area and allowed to dry on or is carefully spread without disturbing the agar surface. It is not to be imagined, however, that successful preparations yielding valuable details of growth could always be guaranteed. To secure such meant the discarding of many blank or unsatisfactory slides. It was an advantage if the slide on removal had no adhering fluid film, or, if such film was present, that it should be unbroken.

Staining of the impressions by the long Giemsa method presented no difficulty and yielded perfect and extraordinarily delicate pictures of the elements and their interconnections in the colonial growth at various periods. As is well known, the typical pleuropneumonia colony

when fully developed possesses a granular centre and a clear periphery of strictly circular contour. In the early stages the growth has a granular appearance with quite irregular contour (see plate XXXIV, figs. 23, 25). By seeding thickly over a small circle of agar one can watch the development within the first 24 hours of these irregular clumps and provided they are not too numerous for the food supply, their gradual acquisition of a clear circular ring of active peripheral growth. If spread too thickly colonial growth may be checked at the granular stage (see plate XXXIV, figs. 21, 23). It will be understood, therefore, that impressions at early stages will show, if successful, the structure of the granular centres while those of later stages will show, according to the pressure exerted, either the structure of the peripheral active growth only or, in addition, that of the more deeply embedded central portion. Some squashing of elements undoubtedly occurs, especially in impressions taken at the later stages of growth when involution is in progress, but even in the case of the younger elements of the active peripheral growth ring, the pressure may be sufficient to display the essentially plastic and deformable character of the chromatic elements to which most workers have drawn attention. In the making of films from liquid media a difference can easily be detected between the shape of the corpuscular elements and their processes according as the material is distributed thinly in the centre of the slide and allowed to dry or is drawn from a minute drop placed at one extremity. In the latter case the elements and their processes are found to be aligned in the direction of the drawing slide.

The events in serum broth.

This demands only a brief description inasmuch as the interconnections of the elements cannot be elucidated except, as already stated, in the earliest phases. Preparations about the 15th or 18th hour, which should be made from undisturbed cultures show, when stained by Giemsa, a predominance of thread forms with deeply stained thickenings in their substance and at one extremity. Spherical and ring forms are much less numerous. Dark-ground examination at this stage also reveals the fine thread forms. That these are simply dislocated elements belonging to an elaborately branched "mycelium" springing originally from an initial corpuscle, is revealed by fortunate preparations such as those illustrated in plate XXXI, figs. 1 and 2.

They are also identical with those illustrated profusely by Nowak and Hosakawa and Kawamura. By the 48th hour though filaments are still present, the rounded or quadrangular forms or rings together with more bizarre types, predominate (see plate XXXI, figs. 3, 4, 5; plate XXXIII, figs. 18, 19; plate XXXIV, fig. 20). Tresses of filaments with large yeast-like elements entangled within them or lying in juxtaposition are frequently seen in stained deposits of centrifuged cultures and certainly give the impression of being of the nature of floating colonies.

The significance of the large yeast-like elements among the other polymorphic forms during the active growth phases from 48 hours onwards gave rise to considerable speculation which was relieved only by the data yielded by impressions. From the 5th day onwards the majority of the elements assume the rounded or condensed shape and the thickening of their walls would indicate a reduction in plasticity. Specimens about the 10th or 12th day of growth show almost exclusively small densely stained spore-like bodies or rings with thickened walls. The smallest of these spore-like bodies would seem to be of the order of 0·3 μ, but in addition to these "spores" or initial corpuscles, there are usually seen in old cultures smaller particulate granules (0·2 μ or less) which appear to be derived from "mycelial" debris. As Bordet maintained, therefore, the general form of the elements changes as growth progresses, from the filament or thread to the condensed chromatic round or quadrangular body. At all stages, however, representatives of these small condensed forms can be found and it is known that at all stages pleuropneumonia growths yield viable filtrates.

Seedings from Berkefeld V and L 2 filtrates have frequently been made and the former have on the whole been most successful in initiating growth. Stained preparations of deposits from centrifuged V filtrates have shown not only the small spore-like initial corpuscles but even short threads attaining a length of 4 μ. Deposits from L 2 filtrates have revealed as a rule only the small densely stained "spores."

It may be convenient here to refer to the difficult problem of estimating the size of these primary filterable elements which can initiate growth. Barnard, as already mentioned, and Elford (1929) refer to the smallest element capable of reproducing growth as the "particle." The "spheroidal" bodies which, with the particles, constitute, according to these workers, the sole elements present in 3-day serum broth cultures incubated at 37° C. may attain considerably larger dimensions, being really the result of growth of the particles. I am concerned here, however, only with the size of the "particle" or the chromatic body which I have generally referred to as the initial corpuscle present in candle filtrates. Barnard in 1925 gave the particle a diameter of 0·25 μ. Later, Elford, by filtration of 3-day serum broth cultures through graded collodion membranes, estimated the size of this particle as falling within the range 0·1 μ - 0·15 μ, while the spheroids formed from these according to Barnard's scheme and from which they could be separated by filtration, fell within the range 0·175 μ - 0·25 μ. Dr. Elford informs me (July 1933) in a private communication, that the corresponding figures which Barnard, on microscopical grounds, would now assign to these two bodies are respectively 0·2 μ and 0·2μ - 0·5μ. Barnard's largest particle, therefore, would seem to approximate to the smallest spheroid and from my own observations of the initial corpuscles which pass through candle filters

and are directly observed to initiate growth, I would assign to them a figure of the order of Barnard's smallest "spheroid," but not one of the order of Elford's "particle." In 3-day fluid cultures and at later periods there are invariably present minute stainable particles of this order of size, frequently occurring in heaps. In agalactia cultures they are even more prominent and they would appear to consist essentially of "mycelial" detritus. If growth, as Elford holds, can be initiated by "particles" of the minute size he assigns to them, the suggestion may be hazarded that some of the larger plastic spheroids have slipped through. In view of the plastic nature of these pleuropneumonia elements, a fact to which all workers have drawn attention, it is reasonable to doubt whether the measurements of these elements can be made with the same degree of precision we are entitled to expect in the case of the more rigid elementary bodies of which similar studies have been reported.

The growth phases as revealed by impression preparations.

These phases I propose to describe quite shortly in the light of actual specimens, many of which are illustrated by drawings or photographs in plates XXXI-XXXIV.

Phase 1. *The germination of the initial corpuscle.* (Impression at 40 hours from agar inoculated with deposit of centrifuged broth filtrate.) Numerous deeply stained chromatic corpuscles are seen with ramifying pinkish filaments of varying length proceeding from them and containing in their substance here and there small round or oval chromatic bodies. These filaments in some areas have coalesced, forming tangled tresses dotted with small chromatic nodes.

This phase is illustrated partly in plate XXXI, fig. 6, from agar and in plate XXXI, figs. 1, 2, from 15-hour serum broth cultures.

It is precisely that illustrated by Orskov, Nowak, Wroblewski and Hosakawa and Kawamura. In consonance with it are the detached vibrionic forms described by Bordet and the solid or annular forms of many observers. Tresses of filaments interspersed with chromatic nodes are also characteristic features found in stained deposits of broth cultures at 18 hours.

Phase 2. Impression at 24 hours. Seeding from active broth culture (plate XXXI., fig. 6). Terminal and endomycelial chromatic nodes in the interlacing mycelial filaments swell up to large oval structures at first homogeneous but later showing partition to round or oval or quite irregular bodies of varying size. At this early stage, pale blue amorphous small plaques of blue-staining matter appear in the neighbourhood of these formations. In the next stage we shall see that the fully formed elements appearing in the young colony, which represents really a condensed collection of mycelial filaments with their accompanying nodes, possess a blue-staining sheath or capsule in which the chromatic node is embedded.

Phase 3. The young colony in the granular (central) phase (plate XXXI, fig. 7). Here the young colony is seen to consist of many closely tangled mycelial threads interspersed with their chromatic nodes. The results of these unions constitute the granular foundation of the colony (plate XXXII, fig. 8). The chromatic nodes in this condensed mycelium enlarge to bodies of very varying size, each surrounded by a pale blue-staining sheath. The young colony, therefore, before the clear peripheral ring develops, consists of round or oval "nucleated" corpuscles together with residual granular "mycelial" matter. Even in the smallest corpuscle the chromatic dot can be distinguished (see plate XXXII, fig. 10).

Phase 4. *The fully developed young colony.* (48 hours.) Excellent impressions have been obtained of this stage. The lighter impressions bring to view the peripheral vegetative elements, leaving the granular centre undisturbed. The details of structure of these actively growing elements are extraordinarily delicate. Their concentric arrangement will also be noted (see plate XXXII, fig. 9). It was suggested in a preliminary account of the cycle by the writer (Ledingham 1933) that these elements, threads, rings, spheres, asters, etc. arose by some process of partition from the larger elements described in phase 3. Further study, however, does not support this mode of division. These elements are produced from the "nucleated" bodies of the granular centre in precisely the same way as the original buds from the initial corpuscle, viz. by a process of frequently repeated pseudopodial budding. These rapidly produced chromatic elements of varied shape are, in fact, connected together like beads in a closely strung necklace. Their concentric arrangement is determined, doubtless, by the physical forces which govern the spherical colonial form. When first laid down these elements of the peripheral layer exhibit great diversity of shape and size, as might be expected (plate XXXIII, figs. 14, 15, 16) if they are endowed, as we believe, with high plasticity. As the colony ages, however, the tendency is for the chromatic thickenings and adjoining filaments to condense and harden to a more rounded or oval or quadrangular form (plate XXXII, fig. 12). The condensation may proceed until the body is reduced to the dimensions which permit its passage through candle filters.

Involution and degenerative changes. Impressions of colonies from the 5th day onwards reveal further changes in the elements, some frankly degenerative, others indicating simply alterations in structure and staining properties. While many of the larger "nucleated" bodies, which in impressions form a sort of palisade to the granular residuum of the colony, retain their form and staining properties perfectly, even at late periods of growth, and exhibit very clearly the continuity of their chromatic nodes with the primitive "mycelium" (plate XXXII, fig. 13), others show up as pale blue-staining homogeneous masses, their chromatic portions having disappeared. The asteroid forms are often

numerous. These are simply bodies of quadrangular and many-cornered shape with intensely stained, almost pyknotic, protoplasm and presenting several pseudopodial processes wandering out over the agar surface (plate XXXIII, fig. 17). There are, however, in addition to the asters, many bizarre forms similarly provided with pseudopodial processes. In the same field with such sprouting elements are seen the smallest corpuscles with pseudopodia proceeding from them. Such germination does not as a rule proceed to further development on the exhausted agar. There is no reason to doubt the viability of these bizarre pyknotic forms in old cultures in view of their ability to throw out pseudopodial filaments on the agar. Whether they are capable on transference to fresh media to initiate colonial growth is not certain. Probably they can do so. In any case, when drops of an active 3-day serum broth culture containing these large forms are placed on a small circle of agar and allowed to dry, one gets a rapidly growing confluent growth with a thick circular band at the periphery where the major portion of the corpuscles have come to rest. Such seedings simply continue growing on the agar from the stage they had reached in the broth and only later may such growth become permeated by "mycelial" filaments arising from the smallest corpuscles. In such ring growths the participation of the large "nucleated" elements deposited on the agar has often been noted.

Strains used in this work. The strain ("PP") obtained from the National Institute for Medical Research, Hampstead, was used for the most part. It was the strain employed by Barnard and Elford and by Orskov and has for many years been cultivated on artificial media. Two strains of more recent origin were examined, one of japanese and the other of chinese origin. No essential differences were observed between the elements in these newer cultures and in the old strain "PP." It may just be noted that the elements of the japanese strain were consistently smaller, more compact, and more angular than those of "PP" so that from the appearance of the cells in impressions one could readily decide which strain was under study.

Agalactia.

For the culture of agalactia, which has been examined by similar methods, I am indebted to Professor Bridré of Tunis who, with Donatien, recovered in 1923 from infected sheep the polymorphic organism—"*filtrable mais non invisible*"—the general resemblance of whose elements to those of pleuropneumonia they fully recognised. Nowak and Wroblewski (1930) and Wroblewski (1931) have studied the morphology of the elements in serum-broth cultures of agalactia by methods similar to those employed by them in pleuropneumonia. In their joint communication of 1930 they refer to the filterable elementary corpuscle (or *goutelette*) as resembling that of pleuropneumonia, but they found a difficulty in establishing in early fluid

cultures the pullulation of this initial body though, at later stages of growth, filaments were present in abundance. Wroblewski gives a detailed account of his findings at different stages of growth in serum broth. At 24 hours few elements were seen consisting of small granules of 0·25 μ diameter and rings with filaments terminated by granules ranging from 0·3 μ to 0·5 μ. By the third or fourth day in addition to the small granules are seen rings of 1·5 μ diameter, which "emit" very fine filaments reaching a length of 25 μ. Other filaments bear small spheres or spherules which "emit" fine threads interpreted as *spermites.* The presence of homogeneous non-granular bodies is also recorded and these he interprets as female forms (*oogonies*) following in general the scheme adopted by him for the corresponding forms in pleuropneumonia. For Wroblewski the most characteristic element in the agalactia cultures was the "conidial" ring provided with peripheral granules or *exospores* and he consequently proposed for the organism the name *Anulomyces agalaxiæ.* The numerous microphotographs illustrating his paper include long filamentous forms, like those figured in Bridré and Donatien's original paper, annular rings with granules (interpreted as annular conidia with exospores) together with larger yeast-like bodies enrolled as *spermatocysts.*

My own observations.

Serum-broth cultures at 37° *C.* Unlike pleuropneumonia agalactia shows little evidence of active proliferation at 24 hours, only clumps of initial corpuscles being seen in centrifuged deposits. At 48 hours numerous small rings appear, some being provided with fine attached filaments. By the fourth or fifth day large rings and threads and clusters of yeast-like bodies are numerous together with tangled filamentous masses representing most probably floating colonies. At later dates (10th and 12th days) threads are few and the mass of the culture consists of deeply stained small initial corpuscles. Except for their smaller size and more delicate reaction to the Giemsa stain the cultural elements of agalactia resemble closely in morphology, polymorphic variety and in sequence of appearance those of pleuropneumonia. Good film preparations, however, of grown cultures and of centrifuged deposits of cultures are not obtained with such regularity as in the case of pleuropneumonia. The agalactia fluid cultures are more granular and growth is accompanied by a profuse production of crystalline matter (both acicular and biscuit-shaped) whose nature has not yet been ascertained. These crystals occur also in growths on solid media (see plate XXXIV, fig. 24).

Impressions of growths on serum-agar. The seedings used have been Berkefeld V filtrates and deposits of filtrates as well as uncentrifuged serum-broth cultures. Microscopically visible colonies of the granular type (without peripheral rings) do not appear before 24 hours and then only if the seeding has been rich in elements. The fully developed

colonies with clear peripheral rings do not appear till the third or fourth day or even later when the seeding has been well spread. They resemble closely those of pleuropneumonia but are of much smaller dimensions. Impressions of the earliest stages on serum-agar have been obtained only when thick seedings have been dropped on the agar or lightly spread over a small area. It has not been possible to follow so clearly as in pleuropneumonia the initial pullulation and subsequent development of the initial corpuscles which pass candle filters. The structure of the early granular colony (at 24-48 hours) has, however, been clearly defined as a filamentous feathery mass interspersed with deeply stained beads. The elements are so closely packed in the young colony that the participation of individual vegetating corpuscles in the total mass is obscured. There is no reason to doubt, however, that the course of events up to the stage of the granular colony is precisely similar to that of pleuropneumonia. When the colonies are fully developed about the fourth day (plate XXXIV, fig. 24), there has been no difficulty in demonstrating clearly the structure of the peripheral clear ring which consists, as in pleuropneumonia, of concentrically arranged "moniliform" filaments produced from the larger "nucleated" yeast-like bodies by a similar process of pseudopodial budding. The picture is quite similar to that of pleuropneumonia at a similar stage, the only essential difference being the smaller size of the elements.

Preparations from the fifth day show great reduction in the dimensions of the rings and spheres down to deeply stained minute forms of the filterable order. At late stages degenerate and involution forms similar to those in pleuropneumonia are always present together with collections of minute pink-staining particles derived from the effete "mycelial" matter in the centre of the colony.

It has not been possible in this communication to provide illustrations of the stages in the growth of agalactia ascertained by impression technique. Allowing for differences in detail the same general features emerge as in pleuropneumonia.

Discussion.

There can be little doubt that to the reader who has not actually studied these organisms microscopically the impression derived from illustrations of their polymorphic structure must at first seem a perplexing one and such impression may indeed be intensified if he happens to be familiar with and perhaps to attach undue importance to the peculiarly discrepant reports of their morphology in the literature. For this feeling of perplexity the enrolment of pleuropneumonia (and of agalactia) in the submicroscopic or virus order which has been customary since the discovery of the pleuropneumonia organism and its artificial culture, must be held largely responsible.

The fact that some elements of an organism may be of dimensions near the limit of microscopic resolution and capable of filtration through candle filters though retaining the power to reproduce growth, while others may be far above this limit, as Bordet showed in 1910, should have been in itself sufficient to justify the removal of pleuropneumonia from the virus group and its enrolment in the family of frankly microscopic organisms possessing filterable but viable elements. The expression "filterable phase" is to be avoided as we have no reason to suppose that in the case of pleuropneumonia and agalactia the filterable elements, though they function as true spores, are so completely differentiated, as true spores are, from other and larger elements in the parent culture. The filterable elements would seem rather to represent particularly condensed but still plastic and unconfined masses of protoplasm common to other and larger elements which fail to pass candle filters and it may be added that to this plasticity of protoplasmic structure many authors have attributed the ease with which these small condensed masses and even short threads are enabled to pass candle filters.

In spite of their very apparent polymorphism for which the plastic nature of the protoplasm and prolific growth are largely responsible, these organisms are essentially of simple structure. Commencing with the filterable viable element we note its spore-like capacity to pullulate to filamentous and ramifying elements. The protoplasmic substance of these filaments, whether at their extremities or in their course, retains the power to elaborate the more deeply stained chromatic and consolidated nodes from which further moniliform growth proceeds The term "moniliform" I use only for convenience to express the beaded character of the growing filament. Unlike those of a moniliform streptobacillus the "beads" exhibit the greatest variety in size and shape, particularly during the early stages in their development. In early colonial growths, as I have described and figured, these chromatic condensations may assume considerable dimensions, bizarre shapes and a quite characteristic differential reaction of their outer and inner parts to the Giemsa stain, and their further pullulation by a unipolar or multipolar pseudopodial budding process furnishes the great pass of polymorphic units, rings, spheres, filaments, etc. present in cultures at the period of maximal growth.

The origin of the blue-staining sheath surrounding the fully developed chromatic node and its entrant filament demands consideration. Though it is especially prominent in the larger fully developed forms which remain in close apposition to the granular centre of the colony, it can be demonstrated in suitably stained preparations in nodes of even the smallest dimensions and in which consolidation of the chromatin has taken place. In the earliest phase of the young colony, when concentration and consolidation of the thickenings in the primary branching filaments is taking place,

blue-staining masses make their appearance alongside the purple chromatic elements. There seems no reason to suppose that the blue-staining material is anything more than the achromatic residual portion of the primary protoplasmic substance when the chromatic element has become separated off and consolidated. It is a feature which can be demonstrated by the Giemsa stain in fully developed rods of any moniliform streptobacillus when the chromatic beads have retracted and become pyknotic, leaving a blue-stained matrix. In referring to the large sheathed forms as yeast-like and "nucleated" it is not implied, therefore, that we are dealing with elements of essentially fungal nature.

It would doubtless be desirable to give such systematic rank to these two organisms which would adequately reflect the essential features of their morphology and growth processes so far as it has been possible to unravel them, but I do not propose here to do more than offer certain suggestions on this aspect of the subject. As I have indicated, various genera, old and new, *Vibrio, Asterococcus, Coccobacillus, Micromyces, Mycoplasma* and *Asteromyces* have been tentatively and perhaps rather irresponsibly proposed by previous writers on the strength of their observations of pleuropneumonia morphology and the general impressions made upon them by such studies. I should, however, regard the determination of order and family as of more urgent importance than the determination of genus or the coining, if necessary, of a new genus. My own studies of pleuropneumonia and agalactia lead me to the conclusion that both organisms may quite appropriately be enrolled in the order of *Actinomycetales*, Buchanan (1918), and in the family of the *Actinomycetaceæ* belonging to that order.

The occurrence in both pleuropneumonia and agalactia of branching filamentous forms, particularly in the early stages of culture, seems of itself sufficient to justify enrolment in that family. The difficulty arises when we come to consider generic rank. Of the four genera, *Actinobacillus, Leptotrichia, Actinomyces* and *Erysipelothrix,* included by Buchanan in the family of the *Actinomycetaceæ, Actinomyces* alone would seem to share certain broad features in common with pleuropneumonia and agalactia. The various species of *Actinomyces,* both parasitic and saprophytic, exhibit a remarkable degree of pleomorphism: The elements may assume the form of branched filaments, rods, cocci and vibrios; in liquid cultures floating colonies of tangled radiating mycelium may be found; adhesion of colonies to underlying solid media is a common feature; as growth progresses, filamentous elements tend to break down to rod-shaped elements and coccoid bodies (so-called conidia); in the animal body and in some species in artificial culture also, there is a tendency to the production of swollen "clubs" at the extremities of filaments and such clubs in certain suitable culture media may develop a hyaline sheath; from any of these

polymorphic elements development may proceed and there is no evidence that capacity to regenerate growth is confined to any particular element and, in the case of the club, only the entrant filament has the capacity of initiating growth (Bayne-Jones 1925).

The importance of filterability as a differential character in pleuropneumonia and agalactia is probably of doubtful value in view of the frankly microscopic elements to which the filterable element can give rise. In any case it would seem at present to be of no greater or lesser importance than the similar feature in *Leptospira* and, in both, the explanation of the filterability may be found to lie in an unusual plasticity of protoplasmic structure.

A new genus must certainly be set up to include these two organisms as species, but whatever generic name is proposed, its distinguishing characters are not likely to diverge very fundamentally from those of *Actinomyces*. The determination of an appropriate name for such new genus must, however, await further consideration and further research.

Summary.

The morphology and growth phases of pleuropneumonia and agalactia have been studied in liquid and solid media and the conclusion has been reached that these organisms may be placed, provisionally, in the family *Actinomycetaceæ*. The question of appropriate genus is reserved for further consideration.

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Plate XXXI

Fig. 1.—Serum-broth. 18 hours. Giemsa. Filamentous and branching elements with terminal and internodal thickenings and rings, representing the first stages in the vegetation of the filterable corpuscle. × 1500.

Fig. 2.—Serum-broth. 15 hours. Centrifuged deposit. "Actinomycotic" rosette springing from initial deeply stained corpuscle in centre. Giemsa. × 1500.

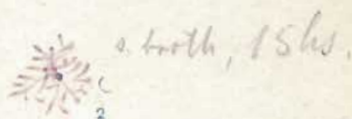
Figs. 3 and 4.—Serum-broth. 4 days. Composite picture to show filamentous forms with and without terminal nodes. Giemsa. × 1500.

Fig. 5.—Serum-broth. 15 days. To illustrate variety of elements, filaments, spheres, rings and yeast-like bodies with thickened walls. Rings produced by curls in filaments. Budding processes. Giemsa. × 1500.

Fig. 6.—Serum-agar. Impression at 24 hours to show vegetation of initial spores to filamentous branching filaments interspersed with chromatic nodes. Coalescence of filamentous and nodal matter and swelling of latter to form basis of young colony. Giemsa. × 1500.

Fig. 7.—Serum-agar. Impression at 2 days. Young colonies in the granular state with "mycelial" matter in centre and young "nucleated" masses formed from the nodes at the periphery. Giemsa. × 1500.

Pleuropneumonia



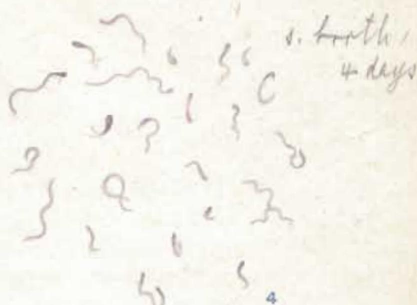


Plate XXXII

Fig. 8.—Serum-agar. Impression at 2 days. Early colony with granular "mycelial" centre and "nucleated" cells at periphery. Giemsa. × 1500.

Fig. 9.—Serum-agar. Impression at 2 days. Light impression (granular centre only faintly shown) illustrating the actively pullulating elements in the peripheral clear ring of the colony. (Compare plate XXXIII, figs. 14, 15, 16, at same stage.) Giemsa. × 1500.

Fig. 10.—Serum-agar. Impression at 3 days. To show condensation of nodular elements. Each node has a blue-staining sheath in which the chromatic element is embedded. Giemsa. × 1500.

Fig. 11.—Serum-agar. Impression at 5 days. Involution of "nucleated" elements forming palisade to colony, with residual blue-staining sheaths. Giemsa. × 1500.

Fig. 12.—Serum-agar. Impression at 7 days. To show structure of elements dislocated from main mass of colony. Great reduction in size of elements and condensation of chromatin. Giemsa. × 1500.

Fig. 13.—Serum-agar. Impression at 6 days. Composite drawing of "nucleated" bodies with chromatic elements surrounded by blue-stained sheath. Giemsa. × 1500.

PLEUROPNEUMONIA

8

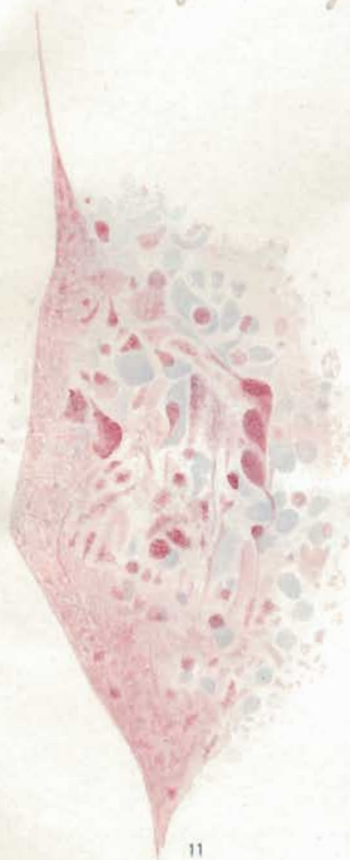
Agar, 2 days

9

Agar 3 days

10

Agar, 3 days



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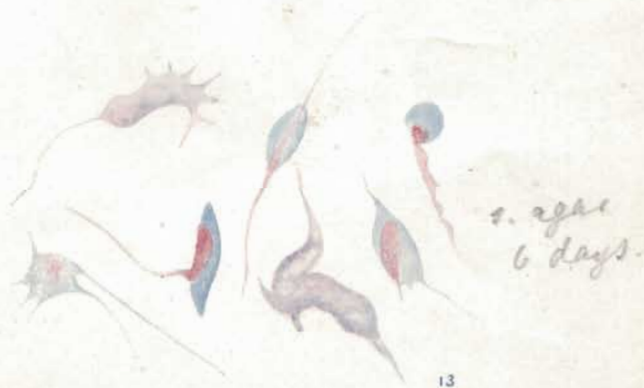
12

agar 4 days.



13

agar 6 days.

Plate XXXIII

Fig. 14.—Serum-agar. Impressions at 2 days. To show the elements in the active peripheral growth ring of the fully developed colony. Giemsa. × 2000.

Fig. 15.—Serum-agar. Impression at 2 days. Note concentric arrangement of moniliform filaments and residual yeast-like bodies. Giemsa. × 2000.

Fig. 16.—Serum-agar. Impression at 2 days. Active peripheral growth with pseudopodial budding of the large yeast-like bodies. Giemsa. × 2000.

Fig. 17.—Serum-agar. Impression at 3 days. Later stage showing deeply stained pyknotic asteroid forms with pseudopodial filaments. Giemsa. × 1000.

Fig. 18.—Serum-broth. 4 days. Showing condensed rings and spherical elements with few filaments. Giemsa. × 1500.

Fig. 19.—Serum-broth. 5 days. Condensed initial corpuscles mainly. Disappearance of filamentous forms. *Giemsa. × 1200.*

Pleuropneumonia

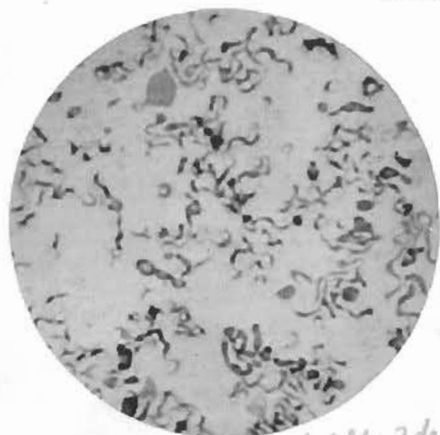


Fig. 14.

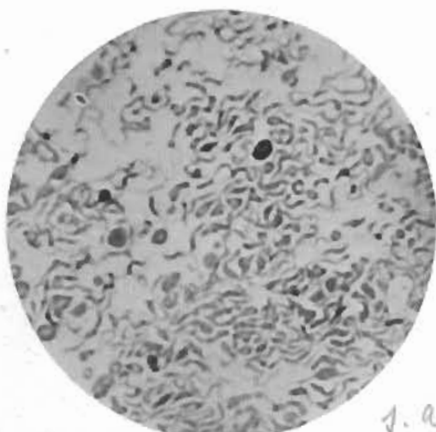


Fig. 15.

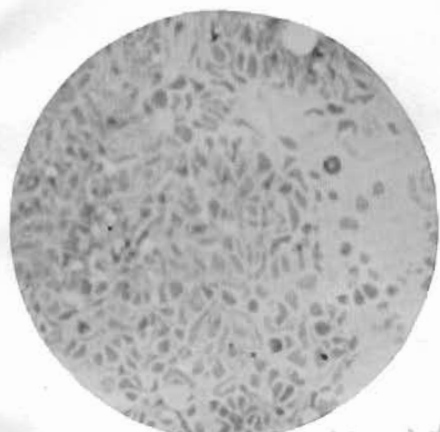


Fig. 16.

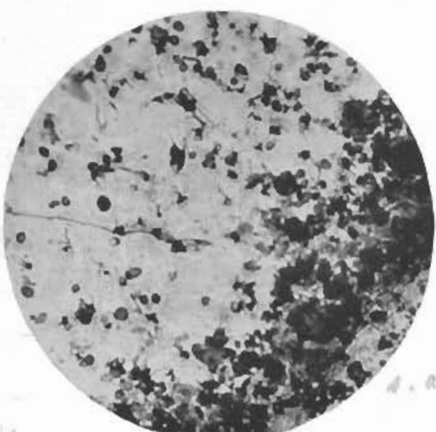


Fig. 17.

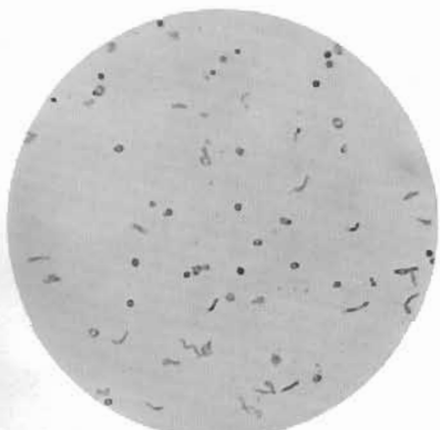


Fig. 18.

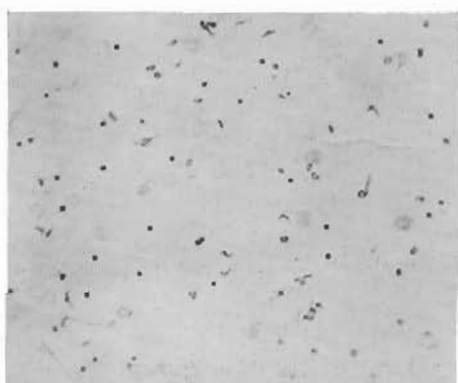
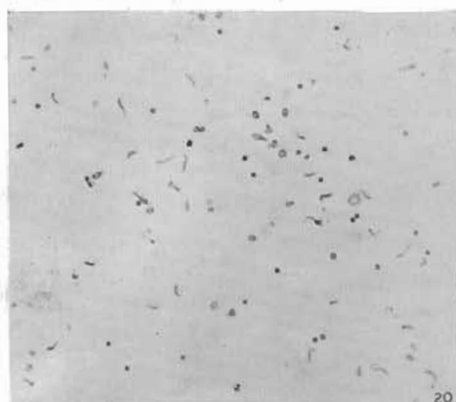
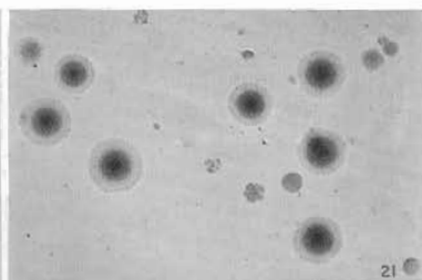
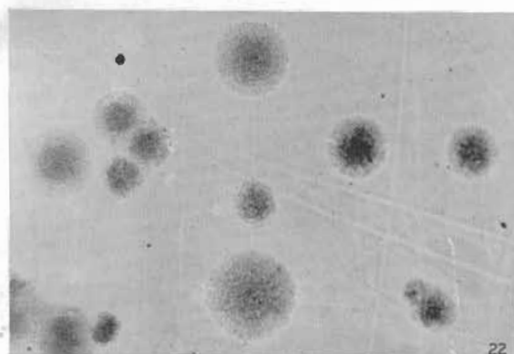


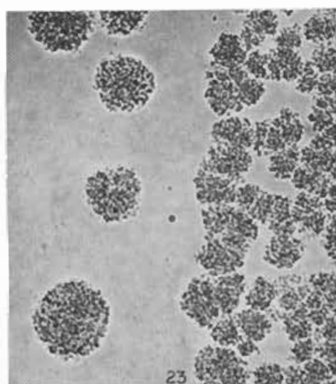
Fig. 19.

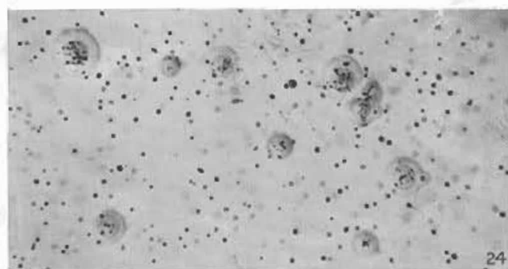
PLEUROPNEUMONIA











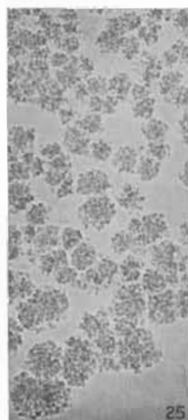


FIG. 20.—Serum-broth. 5 days. Another field from preparation illustrated in plate XXXIII, fig. 19. Giemsa. × 1200.

FIG. 21.—Fully developed colonies of pleuropneumonia (strain "Shanghai") and others which have not yet acquired the clear peripheral rings. × 70.

FIG. 22.—Colonies of pleuropneumonia. Strain "PP." Note peripheral rings containing two granular centres. × 70.

FIG. 23.—Pleuropneumonia. 3 days. Thick seeding. No peripheral rings. × 70.

FIG. 24.—Colonies of agalactia on serum-agar. Note crystal formation. × 70.

FIG. 25.—Granular pleuropneumonia colonies from thickly seeded portion of plate illustrated in fig. 22. × 70.