SOME NEW OBSERVATIONS ON THE DEVELOPMENTAL CYCLE OF THE ORGANISM OF BOVINE PLEUROPNEUMONIA AND RELATED MICROBES

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(With Plates 4–7, containing Figs. 1–18)

I. Introduction

Since 1910, when Bordet studied the morphology of the organism of bovine pleuropneumonia in culture, many attempts have been made to unravel the life cycle of this microbe by various methods. Though on certain points no agreement has been reached and a full description of the whole cycle is still lacking, most authors are now in agreement with regard to the general appearance and structural detail of colonial and liquid growth, and the decision whether or not a newly discovered organism deserves admittance to the group of 'pleuropneumonia-like organisms' has presented little difficulty. The literature up to 1933 was critically reviewed by Ledingham (1933) in the introduction to his paper on pleuropneumonia and agalactia; since then the isolation of quite a lengthy series of pleuropneumonia-like organisms both parasitic and saprophytic has been recorded, and the reader may be referred for a review of this extended field to a recent article by Sabin (1941). Sabin gives the following criteria for identification: '(1) growth in cell-free culture media with the development of polymorphic structures including rings, globules, filaments and minute filtrable elementary bodies, usually 125 to 250 mμ in size which are the minimal reproductive units; and (2) the development on suitable media of characteristic minute colonies which may be as small as 10 to 20μ and as a rule not larger than 600μ.' We would add that the minute colonies owe their characteristic appearance to the possession of a dark centre and a clear peripheral zone. The polymorphism for which, as Ledingham (1933) says, 'the plastic nature and prolific growth are largely responsible', has been noticed by most workers who have studied the morphology of pleuropneumonia. Some have been satisfied to describe the various appearances only, others have attempted to explain the widely varying forms by the not very convincing assumption of different modes of reproduction (Turner, 1935) or by the formation of spores as well as sexual phases (Wroblewski, 1931). There seems to be full agreement on one important point, viz. the production by all pleuropneumonia-like organisms of large numbers of small granules which have been called 'elementary corpuscles' (Nowak, 1929), 'elementary bodies'

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(Tang, Wei, McWhirter & Edgar, 1935; Sabin, 1941), 'spores' (Wroblewski, 1931) and 'conidioids' (Turner, 1935). It is agreed also that these filtrable granules are the 'minimal reproductive units' giving rise to the polymorphic forms which again produce in a way hitherto not understood the initial reproductive units. The different elements occurring in pleuropneumonia such as the elementary corpuscles, the filaments, the disks (often described as rings) and the bodies of bizarre shapes have been demonstrated, but the precise sequence and interconnexion of events has not yet been elucidated by the various techniques so far employed. Frosch (1923), Barnard (1925) and Dahmen (1929) have photographed the organism by ultra-violet microscopy, but have carried out no systematic study of its morphology. It is surprising from a purely microscopical and physical point of view that the use of ultra-violet photography has not yielded any additional information to that already elucidated by visual methods. Smiles (1926) and Turner (1935) relied wholly on the dark-ground method to demonstrate the phases of growth which they observed, but this method has been criticized by Freiberger (1912), Frosch (1923) and Ledingham (1933), largely on the ground that diffraction phenomena play a big part in the formation of dark-ground images. The only serious attempt to study the organism on solid media by transmitted light has been made by Ørskov (1927). This method is impracticable owing to the very low absorption of visible radiation by the elements of pleuropneumonia. So far the most satisfactory way of examining the growth of pleuropneumonia on solid media has been carried out by the impression technique, by means of which Ledingham (1933) succeeded in demonstrating a variety of forms and in showing the existence of elements consisting of a protoplasmic matrix containing consolidated chromatinic structures. He noted that a good deal of deformation occurred in impression preparations and that consequently many had to be discarded. Klieneberger (1934) used a modified impression technique with fixation through the agar in an effort to overcome the deformation of the elements, but it was later realized by her that the desired result was not fully achieved by the method used at that time. She has since succeeded in improving her method for the preparation of so delicate an organism as pleuropneumonia. With this improved method *the formation of the elementary corpuscles inside the boundaries of the small and large bodies* could for the first time be clearly demonstrated, and the corresponding stages on solid, semi-solid and liquid media correlated with each other. At the time it was felt that the new observations made with this technique should be supported by evidence that the structures so resolved were capable of demonstration in living material. Recourse was therefore had to the microscopical method of annular oblique incident illumination developed at the National Institute for Medical Research (J. S.), and this has been successfully applied to the study of living pleuropneumonia cultures on solid or semi-solid media. During the progress of this work it was found that the structures observed by the new staining technique were indeed capable of demonstration in the

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living organism, and this paper is devoted to an account of the new findings. Proof has also been obtained that the fixing does not alter the forms or sizes of the elements and artefacts are absent. The results obtained by these two different methods of examination support the view expressed by Ledingham (1933) that these organisms are essentially of simple structure.

II. Methods employed in the present work

A. *Preparation of stained specimens*

All pleuropneumonia-like organisms consist at certain stages of highly attenuated protoplasm. Since they are easily distorted they do not lend themselves to the ordinary technique of making preparations no matter by what means the transfer of culture from broth or solid media to the microscope slide or cover-slip is made. To overcome this difficulty the organisms are in the improved method grown under a cover-slip with a sufficient amount of appropriate medium to allow normal growth, whilst exposure of the micro-culture to physical forces such as pressure, effect of evaporation or tearing is avoided. After the desired development has taken place the micro-cultures are fixed in situ without being touched. To demonstrate the growth as it occurs on solid and liquid media the following procedure was adopted here.

(*a*) *Demonstration of growth on solid media*

The liquefied medium is poured into a Petri dish. When it has set the inoculum is gently spread on its surface. Squares of medium are cut out with a sterile knife and placed on sterile cover-slips with the inoculated side towards the slip. For ease of further manipulation, during which there must be no slipping between medium and cover-glass, the surface dimensions of the slips should be greater than the contact face of the block of medium. The cover-glass with adherent squares uppermost are placed in sterile dishes containing filter paper and are incubated in a moist chamber. After the required time of incubation fixation is carried out as follows. Without disturbing the medium and cover-glass three drops of Bouin's solution are placed on the agar, each being imbibed by the medium before the next is added. The cover-glasses and adherent squares which have imbibed the fixative are kept in a moist chamber for from 8 to 20 hr.; the agar square is then removed from the slip by means of a knife in one gentle movement. It usually peels off easily and the fixed growth sticks firmly to the cover-slip. The latter is now quickly transferred to water which is changed twice in the course of 10 min. It is then stained in Giemsa solution overnight (100 c.c. of equal parts of distilled water and tap water plus 3·5 c.c. of Giemsa). After staining, the cover-glasses are again washed, dehydrated, differentiated in mixtures of acetone and xylol, and finally mounted in neutral Canada balsam. It is essential that during the whole process the cover-glasses should never be allowed to become dry. The dehydrating mixtures used are: (1) water and acetone *aa*, (2) acetone, (3) acetone 19 parts plus xylol 1 part, (4) acetone 14 parts plus xylol 6 parts, (5) acetone 6 parts plus xylol 14 parts. Dehydration is followed by four changes of xylol before mounting. It is important to remove the acetone completely, otherwise the stain will fade quickly. It should be mentioned that fixation through agar was first introduced into bacteriology by Kuhn (1931). The method described here represents a distinct improvement on the modification of the agar-fixation technique as described by one of us (E. K.) in 1934.

(*b*) *Demonstration of growth in liquid media*

It may be pointed out that when the medium is semi-solid and very soft the organism will develop under the cover-glass as if the growth was taking place in a liquid medium. Thus, to demonstrate the development in the latter the following procedure is adopted.

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A plate is prepared with semi-solid medium containing about 0·7 % agar. The medium should be as soft as possible, but it must still be capable of setting on cooling. Several drops of freshly inoculated liquid medium are then gently spread on the cold agar; small squares are cut out with a sterile knife and placed on sterile slips. These are treated as described under (*a*).

B. *Preparation of living specimens*

The examination of micro-cultures on solid or semi-solid media cannot be carried out satisfactorily by the usual methods of direct illumination when using objectives of high numerical apertures, for the thickness of the medium on which the organism is grown must be many times greater than that of preparations suitable for examination by direct or dark-ground methods with the existing wide-cone illuminators necessary for work with these objectives. On the other hand, attempts to grow the organism on layers of medium sufficiently thin to allow the use of these methods are unsuccessful, as development of the growth is retarded and ceases at an early stage. These difficulties are ruled out by illuminating the object from above and using beams of light symmetrical with respect to the axis of the objective. Since the living object on the surface is demonstrated best in a dark field, oblique beams should be used. To illuminate the object evenly from all sides annular beams should be introduced. Therefore the method of annular oblique incident illumination[1] has been used for this part of the investigation.

During the earlier stages of the work preparations were made by placing inoculated squares of agar between a cover-slip and a slide. In order that no light directly reflected by the surface of the medium should enter the objective, this surface should be mounted perpendicular to the axis of the microscope. Since it was rarely possible to cut out blocks of media from Petri dishes so that the upper and lower surfaces were parallel, this method had to be abandoned and the following method of preparing the micro-cultures was adopted. Parallel-faced metal rings (external diameter 2·0 cm., internal diameter 1·6 cm.) are dipped in hot wax and sealed to a sterile microscope slide, thus forming a small dish the depth of which may be conveniently taken as 0·2 cm. The medium is poured into this dish from a pipette until the surface is slightly above the level of the ring. When it has set a small loopful of inoculum is placed on the exposed surface of the medium on which a round cover-slip is well pressed down by means of a hot metal ring until it comes into contact with the waxed ring which at the same time seals the cover to the micro-dish. When the first cultures thus produced were examined they were still seen on a slightly luminous background owing to light scattered by the medium, some of which entered the objective. To absorb this light a black dye, naphthalene blue black, was introduced into the medium in the proportion of 1:2000. The appearance of the image now corresponded to a true dark ground with the great advantage of accentuating differences in 'reflective power' and widening the range of intensities so that details of structure could be recognized by this method which could not be brought out by ordinary dark ground. The dye does not appear to have any effect on the development of pleuropneumonia-like organisms in the concentration used. With some of the more aerophil organisms of the group there seems to be a lag owing to the semi-anaerobic conditions which this method involves, but the microstructures of the colonies and organisms do not differ from those grown aerobically. There also seems to be no difference between the development on the surface of a plate culture and that produced between cover and agar medium.

It is of interest that when the described staining and fixing technique is used the density of the stain corresponds to 'reflective power' of the living bodies as seen under annular oblique incident illumination. As no evaporation of moisture can take place in the sealed-

[1] The illuminator used was an Ultropak (by E. Leitz) designed to work with an objective having a numerical aperture of 1·0 and an initial magnification of 75.

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up micro-dish, the growth does not move across the field while under examination and consequently photographic records which may require as much as 15–30 min. exposure can be obtained.

III. The growth phases of the organism of bovine pleuropneumonia

As a result of their highly attenuated nature already noticed by Nocard and his co-workers in 1898 the elements of pleuropneumonia yield to all kinds of physical forces. When grown on solid or semi-solid media the mutual pressure of neighbouring organisms determines their form, whilst variation in depth between cover-slip and medium allow the organisms to grow out along the lines of least resistance. Any unevenness on the surface of the medium will have a similar effect. The elements may be further shaped by external forces such as those due to evaporation causing slipping between medium and cover-glass. Then the elements become stretched in the direction of slip and may be drawn out into extremely thin filaments. This was noted by one of us (J. S.) when unsealed living micro-cultures were examined under annular oblique incident illumination. After examination the specimens had been put aside at room temperature. When they were re-examined a few hours later it was found that many of the elements had become stretched. In similar micro-cultures a slow passive movement across the field was seen to take place during the examination. In the same way, when the cultures are grown on slips with adherent squares for the purpose of preparing fixed and stained specimens shrinkage may occur from the sides of the block of medium, thus causing an outward pull. The elements of the cultures are then found to be stretched in a radial direction from the centre of the culture towards the periphery. This is strikingly illustrated in Fig. 6, which shows the colonies of a stained micro-culture of the sewage organism *A* (Laidlaw & Elford, 1936). As will be seen from the illustration, the stretched filaments (*a*) had continued to develop and formed many small bodies. Slight stretching had also occurred in the specimen shown in Fig. 1 (see arrows). It is important therefore to cultivate the micro-cultures in a moist chamber to reduce evaporation to a minimum, but unless they are sealed up the stretching effect cannot be entirely avoided. If it occurs, it usually does not take place in the centre of the preparation, but only in its peripheral parts. When impressions are made in the ordinary way the pressure of the cover-glass, even when allowed to drop gently over the colonies, is generally sufficient to cause the organism to become grossly deformed. When elements of structure are drawn out into filaments they are still viable and capable of further development. Since filaments may be produced by natural forces as well as in the course of preparing specimens for microscopical examination they should not be regarded as 'artefacts'.

When cultures are grown on media of different consistency the differences observed in size and shape of the elements are striking. All pleuropneumonia-like organisms tend, in course of development, to form large elements on the

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surface of solid media where they often spread out into big flat films (Figs. 1, 2, 8, 9, 11, 13, 14, 15, 16); in semi-solid media the elements are smaller and more rounded (Fig. 3). In liquid media the largest elements are small spheres (Figs. 4, 5), the youngest stages being so attenuated that they may be drawn out into filamentous forms by such thermodynamic forces as Brownian movement and convection currents. Our observations seem to indicate that this occurs in all liquid cultures of pleuropneumonia owing to the particularly thin and plastic nature of the organism. In the experiments carried out on semi-solid media in which external influences have been reduced no filamentous forms occurred, and the bewildering variety of sizes and shapes of elements was much less evident. When polymorphism is thus diminished it is easier to follow the developmental cycle in which two different processes of proliferation are apparent, viz. by segmentation followed by the formation of elementary corpuscles within the boundaries of ripe bodies. The cycle as indicated by our observations is outlined in what follows and presented diagrammatically in Fig. 17. The development on solid media is shown in the diagram arranged vertically under A, that on liquid media under B.

Proliferation by segmentation

It is generally agreed that the organism can develop from a granule A (1), B (1). This grows into a small resolvable spherical element. It may continue to grow on as a more or less round body, A (2), or, according to circumstances, may take on an irregular shape and even be drawn out into a filament, B (2). After enlargement the small, soft element may form various numbers of segments according to its environment and accidental shape. In A (3) and B (3) the formation of three segments is indicated. These parts or segments may continue to be connected with each other or may become separated. In either case they may form new segments as further growth takes place, A (4) and B (4). All pleuropneumonia-like organisms have a tendency to form small elements in liquid media, but, as described above, grow larger and flatter on the surface of solid media with the increasing consistency of the latter. Breaking up or segmentation starts right at the beginning of growth in pleuropneumonia and continues during the early stages. The individual segments may be minute or of considerable size and may take any shape. Thus, for example, a *Staphylococcus*-like growth can be produced on solid medium if the segments are small and break up into bodies of approximately the same sizes. A streptococcal appearance is often produced in broth when the filamentous forms divide up. While surface growth on solid medium may consist of very large forms, the elements that grow into the depth of the medium are usually small, round bodies. The early stages of pleuropneumonia consist of thin protoplasm and seem to lack a limiting membrane; this is indicated in the diagram by their representation in half-tone. In A (2) to A (4) and B (2) to B (4) of the diagram the most commonly produced early stages of proliferation are outlined.

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Proliferation by formation of elementary corpuscles

The other method of multiplication in pleuropneumonia is the formation of minute granules within particular elements of the culture. This development may occur in comparatively young growths. The time of its occurrence is dependent on the amount of nutritive substance available and the amount of inoculum used. As the development of the attenuated early stages continues some elements appear to become more concentrated; this is indicated in the diagram by representation in black tone. These elements take the stain more deeply and show greater brilliance under annular oblique incident illumination, A (5) and B (5). These bodies, which at first are fairly uniformly stained throughout, form ultimately what appears to be a membrane which takes the stain deeply, A (6) and B (6). At that stage the bodies usually contain darker patches and deeply stained structures, which are particularly brought out in the stained specimens. The pictures seen suggest that concentrated material, perhaps of nuclear character, has clumped irregularly within these bodies (Fig. 2). The next step is that the deeply staining structures divide up, thus forming elementary corpuscles within the membrane-enclosed elements, A (7) and B (7). The appearance of these bodies which are flat on solid media and like spheres in the liquid, is a regular feature in the development of pleuropneumonia-like organisms. The granules or elementary corpuscles stain deeply when newly formed, thus proving that they consist of concentrated material. They show up brilliantly under annular oblique incident illumination. In some species as the *L 3*, *L 5* and *L 6* organisms (Klieneberger, 1940) the granules are almost equidistantly arranged in the bodies on solid medium (Fig. 8). In pleuropneumonia and *Asterococcus canis* their distribution is not so regular (Figs. 1, 3, 13, 14, 15, 16). The distribution of these elementary corpuscles within the element is of interest. At first sight they appear to be scattered diffusely throughout the mass of the cytoplasm, but on closer inspection many of them will be found to line the inner surface of the limiting membrane. Since in pleuropneumonia and various *L* types the bodies are very thin in depth, only one layer of granules is seen, but with *Asterococcus canis* two layers, separated by a structureless one, are frequently observed when examined under annular oblique incident illumination. These observations suggest that the elementary corpuscles are primarily formed in the peripheral zones of the protoplasm close to the membrane. Later, in the case of cultures on solid media, the membranes disappear and the free elementary corpuscles start the cycle again if the nutritive conditions of the medium allow continued growth.

Returning to the development of pleuropneumonia in fluid media we find that at the stage where granules begin to develop in the spherical bodies they also appear to lie close to the membrane. This is shown in Figs. 4 and 5 for pleuropneumonia and in Fig. 7 for the *L 6* organism, where chains of small spherical elements are seen containing two or more granules. In the spheres

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the granules are often situated in different planes and therefore cannot all be seen at one time. At a later stage these chains become broken up and are replaced by individual spheres in which the granules appear as if they belong to the cell wall. Still later empty spheres are found having particles attached to their external surface by means of thin filaments. This suggests that the particles which are originally seen within the body are finally shed in some way or other through the membrane in the liquid culture.

Whatever the process is by which the elementary corpuscles are liberated to commence a new cycle it seems clear that granules develop into forms the shape and size of which depend on the age of the culture and the nature and properties of the medium, and later these forms produce the elementary corpuscles inside their boundaries.

IV. Examples of different growth phases illustrated by photographs of stained and living specimens of pleuropneumonia and related organisms

In the growth of pleuropneumonia-like organisms the initial stages are the most difficult to demonstrate; they are nearly always extremely small and often at the borderline of visibility; they take the stain only delicately, and in correspondence with this are of very low visibility in the living state; it has, however, been possible to demonstrate them by the methods described above. In the photographs of stained specimens (Figs. 4, 5) young and intermediate developmental stages of pleuropneumonia occurring in serum broth are found, and they may be compared with Figs. 1 and 2, Pl. XXXI of Ledingham's paper. Some of those shown in these two illustrations (Figs. 4, 5) are represented diagrammatically (Fig. 18) to show the successive stages of the cycle. Delicate young filamentous forms (*a* and *b*) become segmented. The segments swell up (*c*); they become more concentrated and then stain deeply; they seem to be connected by fine filaments at this stage (*d*). The next stage in which the segments or bodies have formed membranes and show darker and lighter patches, is shown in (*e*). Bodies containing different numbers of ripe granules are found at (*f*). The differences between the forms and sizes of bodies to be found in fluid media and at the edge of colonies on solid or semi-solid media is clearly demonstrated by the illustrations on Pl. 1. Figs. 1 and 2 show the appearance of the surface outcrop of colonies after 4 and 3 days' incubation respectively. In the younger growth the deeply stained structures (*a*) within the limiting membranes are irregular in size, the smaller bodies containing only one or two such elements (*b*). Their deep staining suggests that they are consolidated masses of substance, the nature of which may be nuclear. This stage (Fig. 2) precedes that of regular granular formation (Fig. 1 *a*, *b*) in all pleuropneumonia-like organisms. On semi-solid media (Fig. 3) the bodies, though larger than those found in liquid media, are never so large as those on solid media described above, nor are the granular elements as numerous (*a*). There seems to be a close relationship between the

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sizes of the bodies and the number of stained elements within them. The above general description of granule formation in pleuropneumonia can be applied to the *L 6* organism (Klieneberger, 1940) shown in Figs. 7 and 8. The difference in size between the bodies grown in liquid and on solid media and the distribution and number of stained granules within them are well demonstrated in these two photographs of stained preparations. When grown on solid media (Fig. 8 *a*) the distribution of granules is found to be more homogeneous than is the case with pleuropneumonia. Segmentation of the larger forms of pleuropneumonia-like growths which have grown out from the central mass of the colony is demonstrated in the case of sewage *B* organism (Laidlaw & Elford, 1935) in Fig. 9. As will be noted the large bodies have stained differently, and this suggests different stages of development, the younger ones being faintly stained (*a*) as compared with the older (*b*). The large bodies of the peripheral zone have not yet formed membranes, but the granular nature of the central mass of the colony is evident.

Fig. 10 illustrates the typical appearance of a vigorously growing young colony of pleuropneumonia at about 3 days' incubation when examined under annular oblique incident illumination. The growth is for the most part confined within the medium (*a*), and at this stage the development of a surface growth consisting of a single layer of organisms has not yet begun. The outline of the colony is irregular (*b*), but much less so than at earlier stages of growth. The organisms have either granular or small spheroidal shapes, the latter being relatively constant in size. A colony of *Asterococcus canis* I (Shoetensack) at 2 days is illustrated in Fig. 11. As with pleuropneumonia the main mass of the colony is embedded in the serum agar, but at the stage illustrated a surface layer has developed to form a broad peripheral zone, thus giving to the colony a uniform appearance in all directions. Very large forms are developing in the periphery in place of the granular and small spheroidal material to be seen over the central mass. Some of these seem to be homogeneous (*a*), whilst in others segmentation appears to be taking place (*b*). Secondary development of organisms occurs from the zone immediately within that containing the large forms, and these secondary elements (*c*) push themselves forwards between the large bodies. This type of picture is influenced by the accidental mode of growth in each case, and although there are a number of different characteristic appearances for the peripheral surface growth they all show that the mutual pressure of the bodies on one another largely determines their shape and orientation. This is evident in Fig. 13, where the large bodies of *Asterococcus canis* (*a*) spread out around the colony. Here the development of granules has taken place after 4 days' incubation, and it will be seen that they are not evenly distributed. Many of them are closely packed near the boundaries of the large bodies.

Two interesting phenomena are observed in the *Asterococcus canis*. It is common to find that some of the bodies contain a fluid in which the granules vibrate and eventually aggregate in some part of the body. When, however,

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the granules are not free to vibrate they are either distributed in a single layer, in which case the bodies are very large, or in two layers, when they tend to be smaller. The second phenomenon in *Asterococcus canis* is peculiar to the type of illumination used, viz. in many of the large bodies the granules appear to be coloured a brick red. Those shown in Fig. 13 were of this kind. There is no doubt that these 'chromatic' granules owe their colour to selective scattering of a portion of the visual spectrum and not to any absorption properties. In contrast to this phenomenon in *A. canis* which requires further investigation, the corresponding granules of pleuropneumonia appear white when illuminated by the whole of the spectrum emitted by an incandescent source.

The development of pleuropneumonia on solid media and the size which the bodies may assume are influenced not only by the consistency of the medium, but also by the amount of serum added to the agar; the less the serum content the larger are the bodies in the young stages of surface outgrowth and the colonies grow more slowly. In a 3-day colony (Fig. 12) on rich medium (1 : 5 serum agar) there is a narrow zone of surface outcrop in which the largest bodies (*a*) have reached their maximum size. They have not yet developed granules inside their membranes. Those granules (*b*) that can be noticed lie nearly always in the interspace between three adjoining bodies. Such granules are due to the secondary development mentioned above, and as they develop they give rise to the appearances of secondary growth seen at (*a*) in Fig. 15 and (*b*) in Fig. 16. In the 4-day culture (Fig. 14) granular elements have begun to develop inside large bodies (*a*), whilst a new fringe of smaller forms (*b*) often of granular nature, is forming at the periphery. The influence of the mutual pressure of the bodies is evident from the fact that they frequently tend to be elongated (*c*) in a radial direction. When incubation of a colony at this stage is continued secondary development from inner zones tends to mask the outlines of the large forms near the periphery of the colony (Fig. 15). In the edge of this 5-day old colony the granules in the larger bodies are more distinct. The bodies themselves are separated by granular and small spheroidal forms of the secondary growth (*a*). This separation of large bizarre bodies by smaller elements is often clearly seen when surface growths of two colonies become confluent (Fig. 16). Bodies (*a*) in such confluent areas of growth are usually larger and more polymorphic than in other parts of the colonies. This can also be seen from the illustration of the stained pleuropneumonia growth in Fig. 1, but whereas in the latter abundant development of granules has taken place, this stage has scarcely been reached in the growth illustrated in Fig. 16.

V. DISCUSSION

Two methods of demonstrating the life cycle of pleuropneumonia have been used here, viz. fixation through the agar followed by a staining and differentiating technique which does not allow the specimen to dry during the process, and observation of living material under annular oblique incident

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illumination. The first is akin to an impression method, but avoids distortions because the specimen is grown between an agar layer and the cover-slip under conditions which reduce evaporation to a minimum. The fixing and staining method is particularly suitable for the demonstration of pleuropneumonia-like organisms which can thus be studied on media of different consistency. The technique of annular oblique incident illumination is a dark-ground method. Its advantages over the ordinary dark ground are as follows: in the first place genuinely developed living material on solid and semi-solid media can be studied; secondly, there is no glare and a long scale of delicate differences in visibility helps to distinguish between different elements. Both methods used here demonstrate essentially the same details; both show that the life cycle of pleuropneumonia and related organisms is the same under different conditions of growth, though appearances differ widely. There are two modes of multiplication, viz. segmentation followed by the formation of elementary corpuscles within elements possessing limiting membranes.

The new observations described here emphasize the importance of taking into account external forces and influences which determine the forms of the organisms during their life cycle. Although various workers have concluded that the elementary corpuscle develops into a filamentous mycelium in the first stage of growth, such a phase has not been observed on semi-solid or solid media when external influences have been controlled. Filamentous structures in pleuropneumonia are not a necessary phase in the life cycle, but they occur as the results of external influences on a soft, amorphous, growing protoplasm. Segmentation is also widely influenced by external conditions. Like other plastic amorphous masses the bodies easily take the hour-glass shape and then separate at the thinnest point. On top of fairly dry agar the soft bodies develop undisturbed into huge flat films without breaking up into smaller forms. From the point of view of reproduction the large forms thus produced should not be regarded as single units. The number of granules in any one body would appear to be directly proportional to its size. Consequently the large bodies may well be regarded as a kind of 'symplasm' composed of as many units as there will be elementary corpuscles at a later stage of development. Large protoplasmic masses either surround themselves with a membrane as a whole, or they break up forming small bodies which singly produce membranes when they start on their reproductive process.

The second finding of importance is the actual process of granule formation itself. This seems to be preceded by a concentration of the protoplasm, followed by the formation of a membrane and the appearance of structures suggesting the presence of a nuclear apparatus. Distinctly stained chromatic structures have already been previously described and illustrated (Ledingham, 1933). The regularity of the formation of the elementary corpuscle inside bodies of a certain stage, in broth as well as on solid media, and their apparent uniformity in the stained and living specimen are very striking and certainly prove that the bodies, big or small, are not involution forms as suggested by

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Ørskov (1938), but represent an important stage in the life cycle of the organism. They have been found in all species so far examined, viz. in pleuropneumonia, *Asterococcus canis*, the *L 3*, *L 5*, *L 6* organisms in Laidlaw & Elford's sewage organisms *type A* and *B*.

How far the new observations described in this paper will help towards a clarification of the systematic position of pleuropneumonia and its congeners we would hesitate to state, but one feature at least of the growth cycle as now revealed must possess critical value in formulating decisions. The basic process of the developmental cycle must characterize the group as a whole. Apart from the plasticity of the elements which constitutes a most important character it has now been demonstrated that two modes of multiplication occur, segmentation and the formation within larger elements possessing limiting membranes of the ultimate reproductive units which are small enough to be filtrable. The larger elements may have been the result of segmentation or they may have developed directly from granules. We are in agreement with Sabin (1941) that inclusion among the Schizomycetes is hardly possible. His proposal of a new class Paramycetes and order Paramycetales containing two families, one Parasitaceae to include the parasitic members and the other Saprophytaceae to include the saprophytic species, may supply a temporary want, but we would suggest that, as further data become available, this vexed problem of classification and nomenclature be referred to the Nomenclature Committee of the International Society for Microbiology.

SUMMARY

A study of the growth phases of pleuropneumonia has been carried out by two new methods: (1) a new fixing and staining technique, (2) a new dark-ground method. The results obtained by the two methods are in good agreement, and the life cycle of the organism of bovine pleuropneumonia and related microbes as revealed by means of both methods has been described.

ADDENDUM

It may be of interest to note that a collection of the following organisms is kept at the Lister Institute, Elstree, Herts, and a smaller collection of the same strains at the National Institute for Medical Research, Hampstead.

List of strains

Strain	Source	Isolated by	Passage at 16. x. 1941
L 1 old	*Streptobacillus moniliformis*	Klieneberger	440
L 1 rat 30	,,	,,	284
L 1 M 43	,,	,,	172
L 3 Ash	Lung lesions of rat	Klieneberger	223
L 3 5254	,,	,,	222
L 3 lung 78	,,	,,	182
L 3 lung rat 2	,,	,,	25
L 3 M 20	Lung lesions of mouse	Edward[1]	39

[1] Edward (1940).

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List of strains (continued)

Strain	Source	Isolated by	Passage at 16. x. 1941
L 4 72 gland	Enlarged gland of rat	Klieneberger[1]	237
L 4 Woglom I[2]	'Pyogenic virus' lesions	,,	93
L 4 Woglom II	,,	,,	84
L 4 rat II Collier[3]	'Joint of arthritic rat' sent by Dr Collier from Batavia	,,	68
Asterococcus canis I	Dog	Shoetensack[4]	
Coarse	Mutants of *A. canis 1*	Klieneberger	139
Granular			130
L 5	Mouse brain (rolling disease)	Klieneberger	77
L 6	Mouse brain (splenectomized mouse)	,,	79
M 55	Swollen joint of mouse	Jahn[5]	—

Three strains of bovine pleuropneumonia from different sources.
One strain of pleuropneumonia from the goat isolated recently by Dr Longley (Nigeria).
One strain of agalactia of sheep.
Eleven strains from sewage, soil and water isolated by Laidlaw and Elford and by Seiffert.[6]

We wish to thank Mr F. V. Welch for his help in the various technical problems which arose during the work.

REFERENCES

Barnard, J. E. (1925). *Lancet*, **2**, 117.
Bordet, J. (1910). *Ann. Inst. Pasteur*, **24**, 161.
Collier, W. A. (1939). *J. Path. Bact.* **48**, 579.
Dahmen, H. (1929). *Handb. Path. Mikroorg.*, Kolle, Kraus, Uhlenhuth, 3rd edit. **9**, 1.
Edward, D. G. ff. (1940). *J. Path. Bact.* **50**, 409.
Freiberger, G. (1912). *Z. InfektKr. Haustiere*, **12**, 455.
Frosch, P. (1923). *Arch. wiss. u. prakt. Tierheilk.* **49**, 35, 273.
Klieneberger, E. (1934). *J. Path. Bact.* **39**, 409.
Klieneberger, E. (1938). *J. Hyg., Camb.*, **38**, 458.
Klieneberger, E. (1940). *J. Hyg., Camb.*, **40**, 204.
Kuhn, P. & Sternberg, K. (1931). *Zbl. Bakt.* Orig. **121**, 113.
Laidlaw, P. P. & Elford, W. J. (1936). *Proc. Roy. Soc.* B, **120**, 292.
Ledingham, J. C. G. (1933). *J. Path. Bact.* **37**, 393.
Nocard, Roux, Borrel, Salimbeni & Dujardin-Beaumetz (1898). *Ann. Inst. Pasteur*, **12**, 240.
Nowak, J. (1929). *Ann. Inst. Pasteur*, **43**, 1330.
Ørskov, J. (1927). *Ann. Inst. Pasteur*, **41**, 473.
Ørskov, J. (1938). *Zbl. Bakt.* Orig. **141**, 229.
Sabin, A. B. (1941). *Bact. Rev.* **5**, 1.
Seiffert, G. (1937). *Zbl. Bakt.* Orig. **139**, 337.
Shoetensack, H. M. (1934). *Kitasato Arch.* **11**, 277.
Shoetensack, H. M. (1936). *Kitasato Arch.* **13**, 269.
Smiles, J. (1926). *J. Roy. Micr. Soc.* **46**, 257.
Tang, F. F., Wei, H., McWhirter, D. L. & Edgar, J. (1935). *J. Path. Bact.* **40**, 391.
Turner, A. W. (1935). *J. Path. Bact.* **41**, 1.
Woglom, W. H. & Warren, J. (1939). *J. Hyg., Camb.*, **39**, 266.
Wroblewski, W. (1931). *Ann. Inst. Pasteur*, **47**, 94.

[1] Klieneberger (1938). [2] Woglom & Warren (1939).
[3] Collier (1939). [4] Shoetensack (1934, 1936).
[5] Klieneberger (1940). [6] Seiffert (1937).

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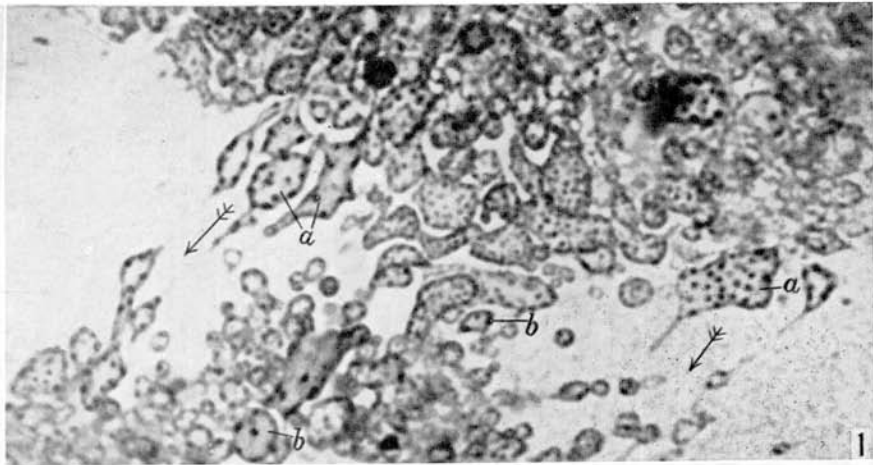

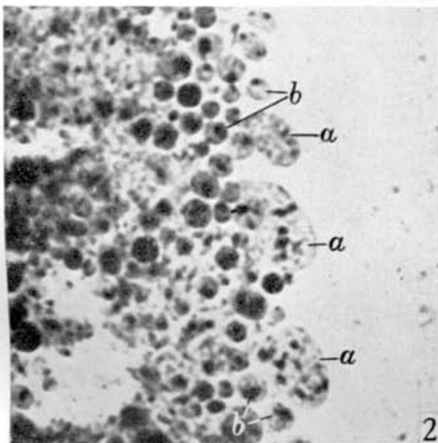

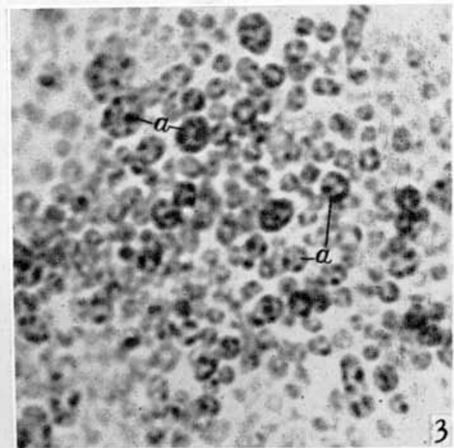

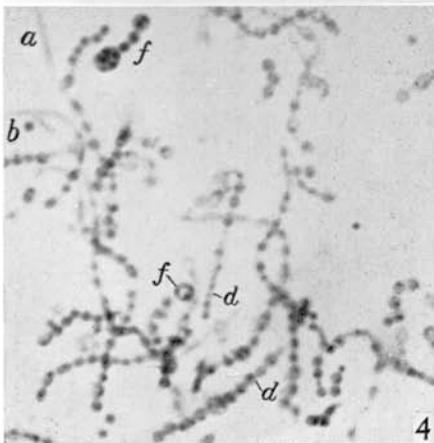

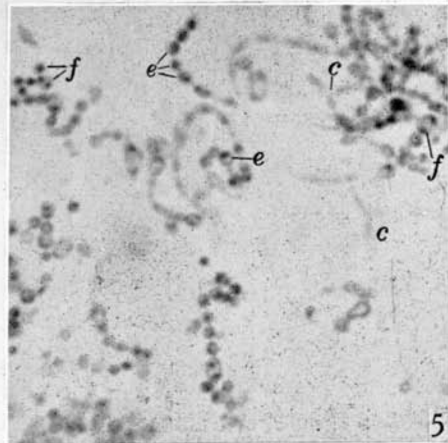

Figs. 1–5

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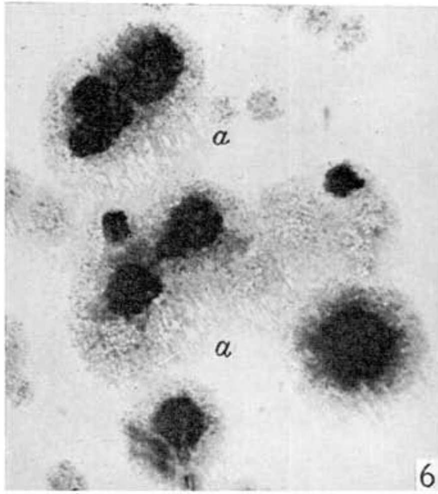

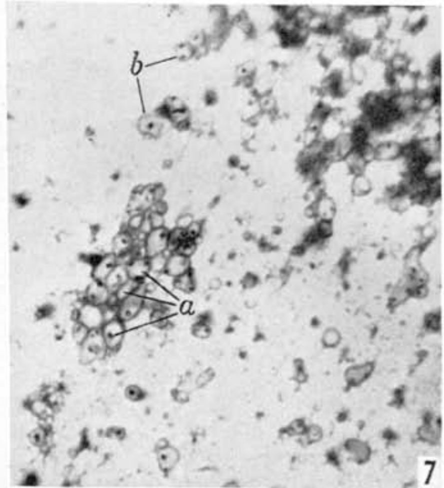

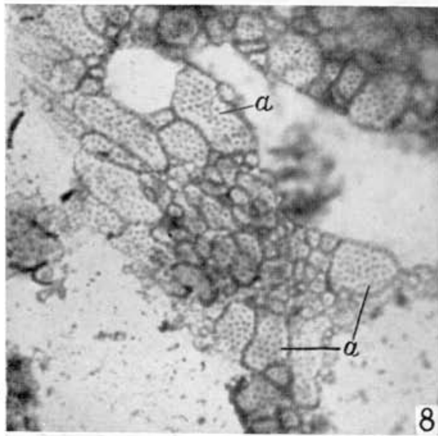

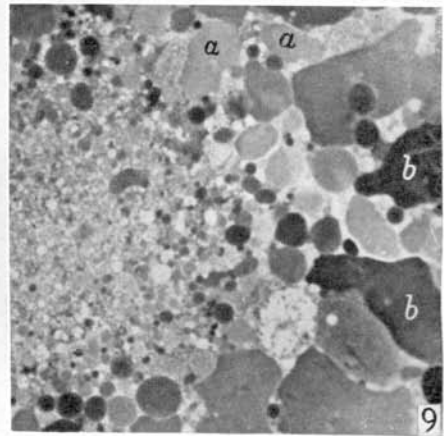

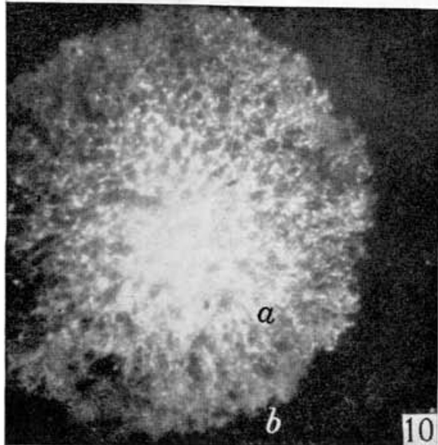

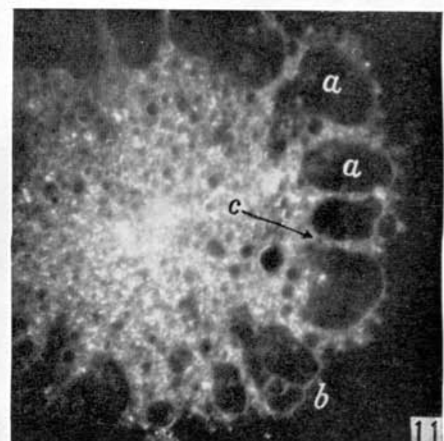

Figs. 6–11

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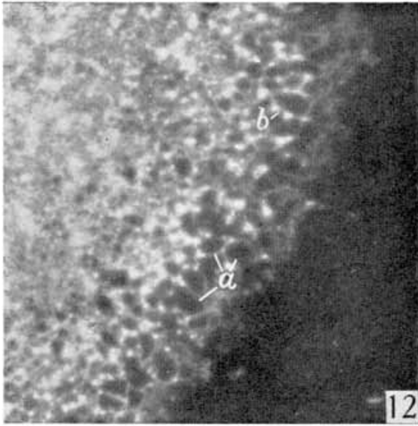

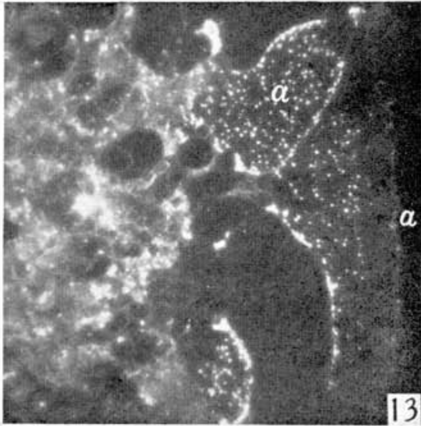

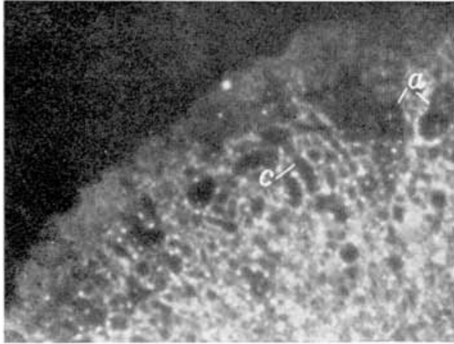

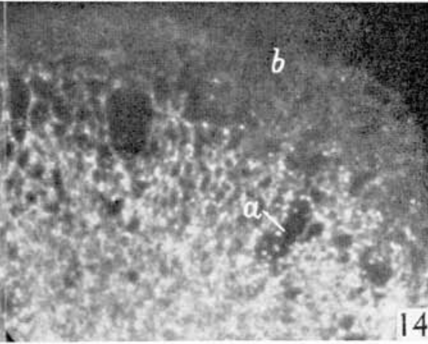

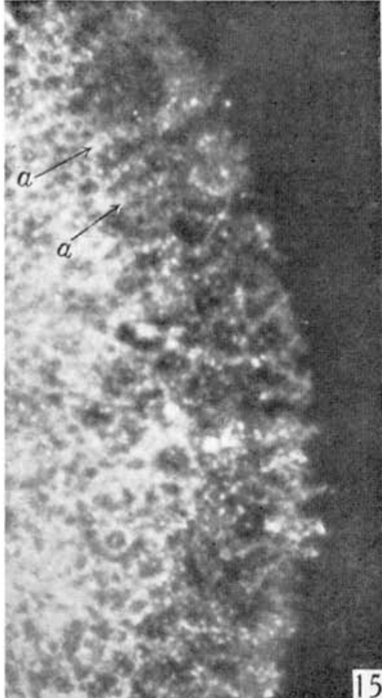

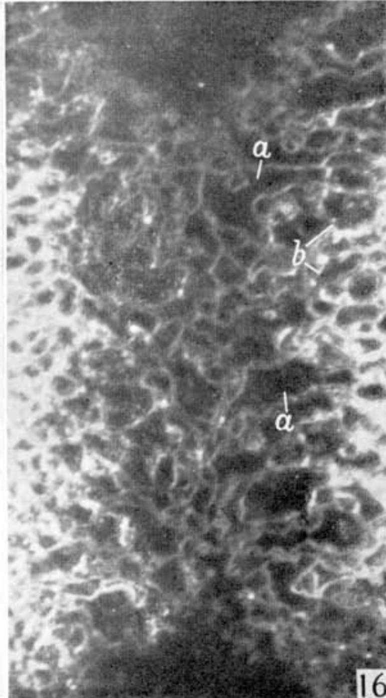

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A B

(1)

(2)

(3)

(4)

(5)

(6)

(7)

Fig. 17

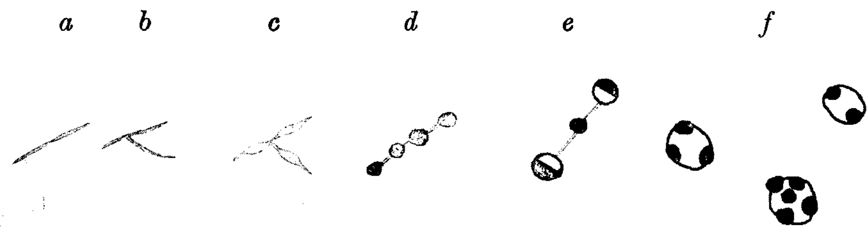

Fig. 18

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EXPLANATION OF PLATES 4–7

Fig. 1. Organism of bovine pleuropneumonia, 3-day growth on solid medium, fixed and stained specimen. Magn. ×1875, N.A. 1·40.

Fig. 2. Organism of bovine pleuropneumonia, 2-day growth on solid medium, fixed and stained specimen. Magn. ×1875, N.A. 1·40.

Fig. 3. Organism of bovine pleuropneumonia, 3-day growth on semi-solid medium, fixed and stained specimen. Magn. ×2250, N.A. 1·40.

Fig. 4. Organism of bovine pleuropneumonia, 3-day growth on liquid medium, fixed and stained specimen. Magn. ×1875, N.A. 1·40.

Fig. 5. Organism of bovine pleuropneumonia, 3-day growth on liquid medium, fixed and stained specimen. Magn. ×1875, N.A. 1·40.

Fig. 6. Sewage organism *A*, 2-day growth on solid medium, fixed and stained specimen. Magn. ×200.

Fig. 7. *L 6* organism, 3-day growth on liquid medium, fixed and stained specimen. Magn. ×1250, N.A. 1·40.

Fig. 8. *L 6* organism, 4-day growth on solid medium, fixed and stained specimen. Magn. ×1250, N.A. 1·40.

Fig. 9. Sewage organism *B*, 1-day growth on solid medium, fixed and stained specimen. Magn. ×1250, N.A. 1·40.

Fig. 10. Organism of bovine pleuropneumonia, living colony, 3-day growth on solid medium. Magn. ×675, N.A. 1·0.

Fig. 11. *Asterococcus canis*, coarse, living colony, 2-day growth on solid medium. Magn. ×675, N.A. 1·0.

Fig. 12. Organism of bovine pleuropneumonia, living colony, 3-day growth on solid medium. Magn. ×1350, N.A. 1·0.

Fig. 13. *Asterococcus canis*, coarse, edge of living colony, 3-day growth on solid medium. Magn. ×800, N.A. 1·0.

Fig. 14. Organism of bovine pleuropneumonia, composite photomicrograph, edge and part of central growth of living colony, 4-day growth on solid medium. Magn. ×1350, N.A. 1·0.

Fig. 15. Organism of bovine pleuropneumonia, edge of living colony, 5-day growth on solid medium. Magn. ×1350, N.A. 1·0.

Fig. 16. Organism of bovine pleuropneumonia, living confluent growth between two colonies, 4-day growth on solid medium. Magn. ×1350, N.A. 1·0.

Fig. 17. Diagram of the development of the organism of bovine pleuropneumonia on solid (A) and liquid (B) media.

Fig. 18. Diagram of successive growth stages as found in Figs. 4 and 5.

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