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Studies on morphology and multiplication (proliferation) of pleuropneumonia-like organisms (PPLOs, now classified as mycoplasmas) and on bacterial L-phase (L-form bacteria)

II. Electron microscopy

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Complementary to the light microscopic observations on the proliferation of the pleuropneumonia-like organisms (PPLOs, now mycoplasmas) and L-form bacteria (Kandler and Kandler, 1945) we performed electron microscopic studies on the same strains.

The first electron microscopic images of PPLOs showed rod-like bacteroid shapes in addition to the spherical forms (Weiss, 1944; Smith *et al.*, 1948). Most likely, these strains are not true PPLOs but instead L-forms mixed with bacteria. Various other authors agree that all organisms have a disc-like or vesicle-like form; they can be present as individual bodies or they can be organised in chains or larger clusters (Ruska *et al.*, 1947; Liebermeister, 1953a; 1953b; Dienes, 1953). The electron microscopic images by Freundt (1952) are exceptional in that they also show extensive thread formation.

Liebermeister (1953a) suggests that all non-spherical shapes are artefacts from preparation of the samples. Especially the organism causing pleuropneumonia is assumed to be particularly labile and can only be imaged using the buried glass slide technique ("Aufwuchsverfahren", Liebermeister, 1953a). He proposes that proliferation occurs by budding or by breakup into multiple bodies, because both small and large bodies are found next to each other (Liebermeister, 1953a). Gerber (1953) considers only the homogenous spherical bodies as genuine and proposes that the vesicles containing one or more granules are lysed forms. On the other hand, Liebermeister finds the latter to be present preferentially in older cultures, whilst the former are frequently seen in young cultures. However, he did not study the development of changes in morphology during the development in detail. It could be shown that PPLOs can not be distinguished from L-form bacteria by electron microscopy: both formed spherical bodies of higher or lower electron density and no mycelium-like threads (Dienes, 1953).

The aim of this study was to shed light on why the individual bodies have different morphology. Our light microscopic data already showed that the spherical form can be found in certain culture conditions and that during the growth phase different budding forms can be found. These are occasionally organised in chains or clusters, arguing against Gerber's opinion (1953) that these shapes are only artefacts due to the preparation of the samples for electron microscopy. Therefore our experiments focused on studying the individual developmental stages of PPLOs.

Material and Methods

The same strains as described previously were used (Kandler and Kandler, 1954). Preparation of electron microscopy samples from cultures grown on plates is inefficient, therefore we used cultures grown in liquid medium. We used Seiffert-Bouillon (personal communication), containing: meat peptone (tryptic digest) 0.75%; yeast extract 0.5%; NaCl 0.3%; K_2HPO_4 0.2%; glucose 0.2%; horse serum 10-20%. The broth was filter-sterilised, because autoclaving rendered the liquid turbid. The cultures were inoculated by transferring a small piece of agar from a culture grown on plates or from a liquid pre-culture into the new medium, then the cultures were incubated at 37°C. For the preparation of the samples, the cultures were pelletted at 6,000 g, washed twice with dH_2O and then resuspended in dH_2O. A drop was transferred onto the cellulose lacquer foil on a microscope slide. After drying, the preparations were vaporisation coated with TiO_2 and viewed under the electron microscope (for electron microscopy techniques see original German paper).

Results

First, several resuspension buffers were tested.

The cell pellets were resuspended in phosphate buffer pH 7.5 of different molarity, in veronal-acetate buffer or in NaCl solutions of various concentrations. However, none of these buffers resulted in better preparations than resuspending the sample in dH_2O. Highly concentrated solutions such as m/15 phosphate buffer led to a precipitation of the organisms: it was almost impossible to resuspend the pellet and the suspension appeared very cloudy. The electron microscopic image of such samples only showed smaller or larger clumps of organisms. Therefore, for all our experiments we used dH_2O to resuspend the cell pellets.

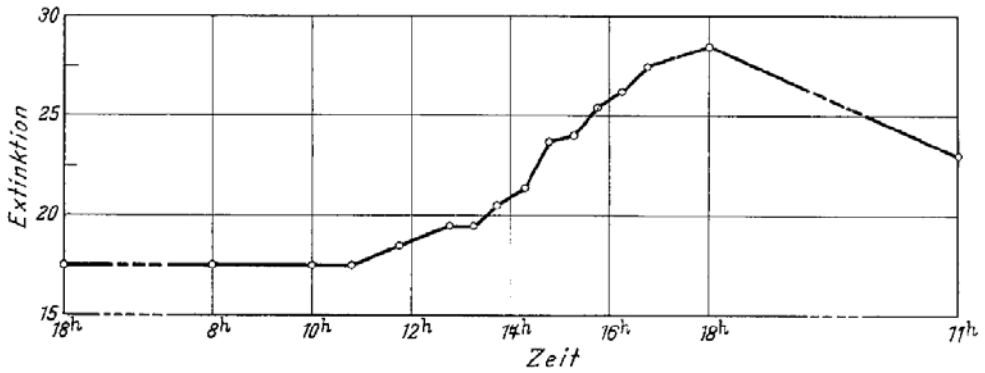

Fig. 1. Photometric measurement of turbidity in growing “Findlay” culture

Generally, visible growth could be observed approximately 20 h after inoculation. The turbidity increased rapidly during the next hours before stagnating. After about 48 h, the turbidity decreased slightly and the organisms formed visible clumps at the bottom of the flask. A spectrophotometrical growth curve of the strain “Findlay” is shown in Fig. 1, confirming the observations made by eye: the extinction increased from the 18th to the 24th hour and slightly decreased after about 48 h. The turbidity of the culture was very homogenous. Occasionally, the strains grew less well and growth only started after 36 h, nevertheless the curve was consistent with the one shown in Fig. 1.

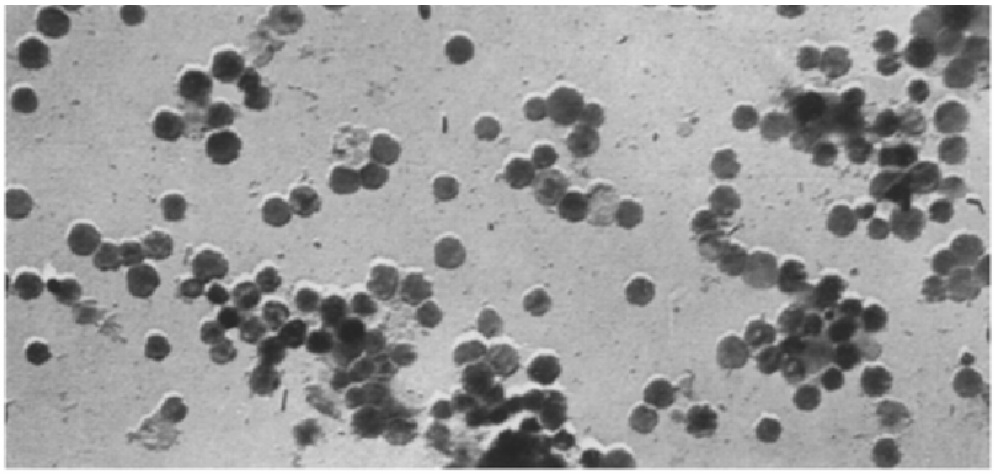

Fig. 2. “Urin”, 40 hrs, electron micrograph, magnification 4500x

We usually obtained clear images from samples taken during the growth phase (before decrease of optical density). Clusters of spherical bodies, chains reminiscent of streptococci and many individual spherical bodies (Fig. 2) could be observed. The majority of the spheres are completely homogenous and even high magnification does not show any internal structures (Fig. 3). Additionally, larger bodies of low contrast were visible, which appeared to be empty. Often, the high contrast particles are attached to the low contrast vesicles as if they originated from them. The average diameter of the high contrast bodies shown in Fig. 2 is 0.66 ± 0.073 µm (100 individual measurements). The size is surprisingly uniform with only 11% deviation, particularly considering that these organisms have been described as highly polymorphic in literature.

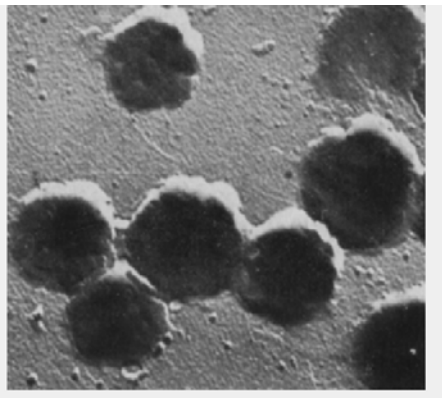

Fig. 3. “Urin”, 40 hrs, electron micrograph, magnification 13000x

Different observations can be made in images acquired from cultures in earlier or later developmental stages. Fig. 4 shows a cluster of dense and rather undefined centres that are held together by a matrix of low contrast. These centres have an average diameter of 0.4 – 0.6 µm and are therefore slightly smaller than the free bodies shown in the previous figure. This is reminiscent of plasmodia, consistent with the forms seen by phase contrast microscopy (Kandler and Kandler, 1954). It is conceivable that these clusters can disintegrate into individual bodies shown in Fig. 2.

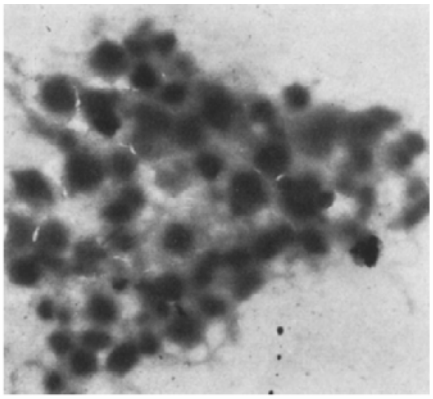

Fig. 4. “Findlay”, 35 hrs, electron micrograph, magnification 11000x

Preparations from older cultures yet again look different. Fig. 5 shows a very vague dissolving sheath next to which there are small particles of high contrast that have high density in their periphery and low density in their centres. These particles have a diameter of 0.4 – 0.5 µm, whilst the small granules within have a diameter of only 0.15 – 0.2 µm. Therefore, these particles could represent the smallest units capable of proliferation, as found in previous filtration experiments (Seiffert, 1937a; 1937b; Elford, 1938).

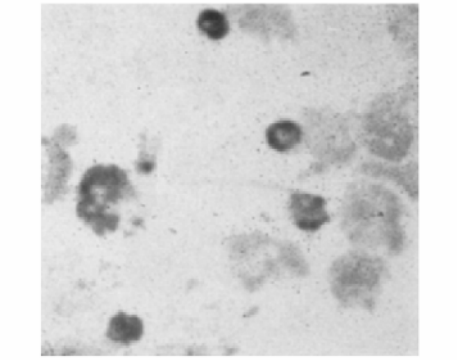

Fig. 5. “L-Seiffert”, 10 days,
electron micrograph, magnification 11000x

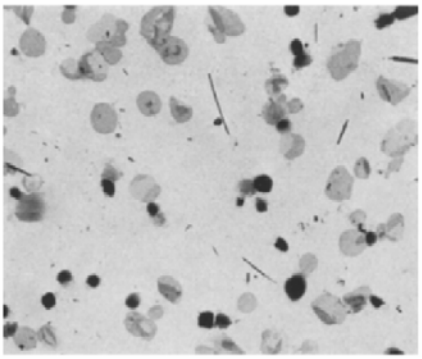

Fig. 6. “Urin”, 4 days, culture without serum,
electron micrograph, magnification 3700x

All images shown so far had been obtained from cultures grown in the presence of 20% horse serum. To test if the serum concentration has an effect on morphology, we grew three strains *(Urin, Findlay* and *Laidlaw A*) in media containing varying amounts of serum and prepared samples as described above after 4 days of incubation. In these experiments, turbidity started after about 2 days and was significantly lower in the absence of serum. Without serum many empty sheaths with few high-contrast particles were visible (Fig. 6). The diameter of the sheaths was 0.8 – 1.5 µm whilst the high-contrast particles measured 0.5 – 0.8 µm across. Addition of as little as 1 % serum already reduced the amount of empty sheaths present and led to a decrease of size of the dark particles. This phenomenon was even more pronounced in the presence of 5% serum (Fig. 7). Here, the high-contrast particles have a diameter of only 0.4 – 0.6 µm. When grown in the presence of higher serum concentrations (20% and 40%), again more empty sheaths were found and the small

particles increased slightly in size. Therefore, we used an intermediate serum concentration of 10% for the following experiments.

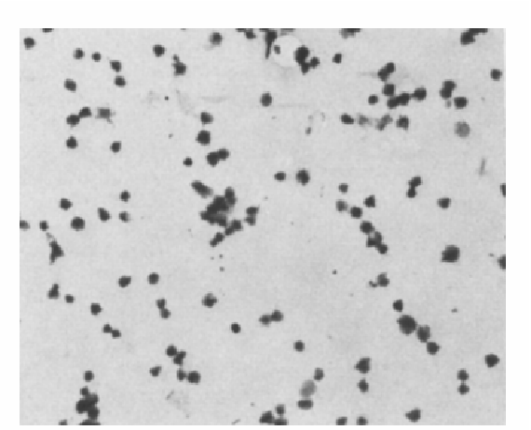

Fig. 7. “Urin”, 4 days, culture with 5% serum, electron micrograph, magnification 3700x

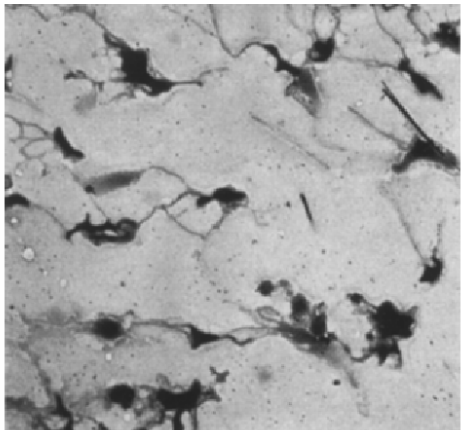

Fig. 8. Organisms causing pleuropneumonia, 2 days, suspended in distilled water, electron micrograph, magnification 4900x

Now we investigated the pleuropneumonia causing organism using the same methodology that had proven successful for the PPLO strains. However, Liebermeister (1953a) reports that this organism becomes highly deformed when drying on the microscope slide films. Indeed, we were unable to obtain good images from cultures at the beginning of the stationary growth phase, showing only distorted threads (Fig. 8). Yet, in the phase contrast microscope the organisms appear as uniform spheres after washing in dH_2O. When samples from the same cultures were prepared 24 h later however, similar spherical bodies as observed previously for the PPLOs were observed (Fig. 9). The average diameter of these bodies is 0.74 ± 0.11 µm (60 particles measured), therefore the size variability is higher (15%) than for the PPLOs shown in Fig. 2.

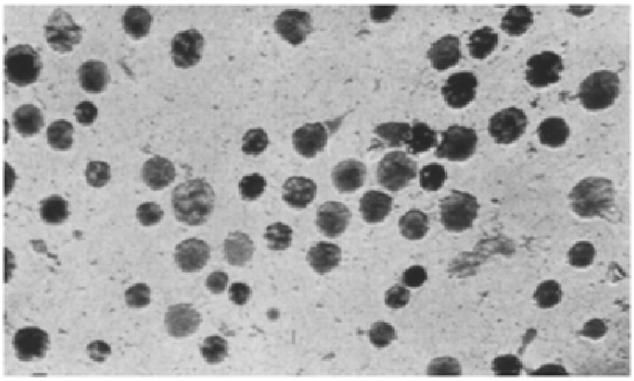

Fig. 9. Organisms causing pleuropneumonia, 3 days, suspended in distilled water, electron micrograph, magnification 4900x

Measurements of different fields of the same preparation consistently gave an average diameter of 0.73 ± 0.11 µm (54 particles measured). Also in an independent sample the average diameter was consistent, with 0.73 ± 0.112 µm (60 particles measured). Thus, both size and variability seems to be constant. Even though the numbers are larger than for the above mentioned PPLO strain *“Urin”*, more measurements would be required to determine if the difference is significant or not.

Here, we just want to point out that PPLOs have a certain rather uniform size during particular stages of their development.

One might argue that the PPLO forms shown in Fig. 4 and 5 are artefacts and only the uniform shapes seen in Fig. 2 and 3 represent authentic forms. Nevertheless, it is conceivable to interpret the differences as developmental stages, which would be consistent with the light microscopic observations (Kandler and Kandler, 1954). To address this question, we performed a time course experiment. An 8 day old culture, containing small high-contrast granules with a diameter of 0.2 – 0.35 µm surrounded by an irregular low-contrast matrix of often thread-like appearance (Fig. 10), was used to inoculate fresh medium. After 6 h, the morphology has changed completely, and large clusters become visible, within which subunits are already noticeable (Fig. 11). This formation is doubtlessly equal to the situation shown in Fig. 4, only here the contrast is lower and the image different due to the oblique sputtering. The small spheres giving strong shadows are not PPLOs but parts of the medium.

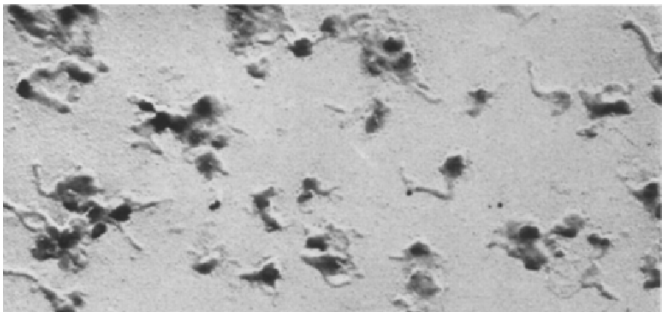

Fig. 10. “Findlay”, 8 days, electron micrograph, magnification 7000x

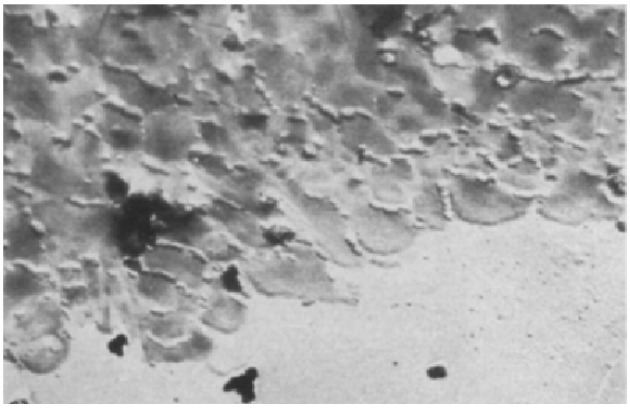

Fig. 11. “Findlay”, 6 hrs, electron micrograph, magnification 7000x

In other images these media particles are less frequent because the sediment from 10 ml medium usually is, if many organisms are present, resuspended in several ml dH_2O. In this case however, only few organisms had grown, therefore the pellet was resuspended in a lower volume and the contamination is less diluted.

After 17 h, the clumps had mostly disappeared and dissolved into relatively uniform, individual spheres (Fig. 12), comparable to what is shown in Fig. 2, 3 and 9. Only some smaller clumps containing discernible smaller bodies within are still present. The

disintegration of the clumps into individual organisms is probably responsible for the increase in optical density after 18 h. Gerber (1953) already concluded that this effect must be due to breakup into multiple progeny cells, however, this could not be confirmed by our data (Kandler and Kandler, 1954). Thereafter, the main growth phase appears to be over and only a few organisms can continue to proliferate, resulting in further increase in optical density of the culture.

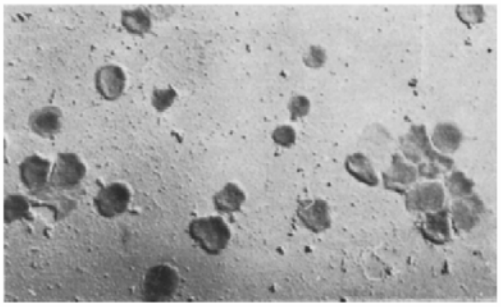

Fig. 12. “Findlay”, 17 hrs, electron micrograph, magnification 7000x

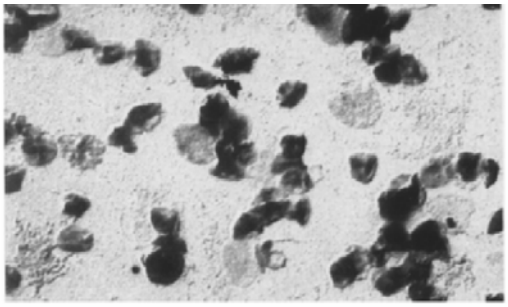

Fig. 13. “Findlay”, 36 hrs, electron micrograph, magnification 7000x

36 h after inoculation, the organisms are still individually discernible or aggregated in small clusters, but they are less uniformly shaped and appear heterogeneous and polygonal (Fig. 13). Because by phase contrast microscopy the organisms still look spherical, this effect might be due to artefacts during drying of the samples. However, considering that this sample was prepared in the same way as the previous samples, the change in morphology might also be due to the development of instability. Part of the matrix seems looser and thinner, while other parts appear more condensed. After 17 days, this process has proceeded to a stage where most of the plasma is visible as a vacuolated vesicle with one or more granules in its periphery (Fig. 14). These granules have a diameter of 0.2 – 0.3 µm and are thus equivalent to the bodies in the starter culture at the beginning of this experiment (compare Fig. 10), the differences in the images is due to changes in the vaporisation technique.

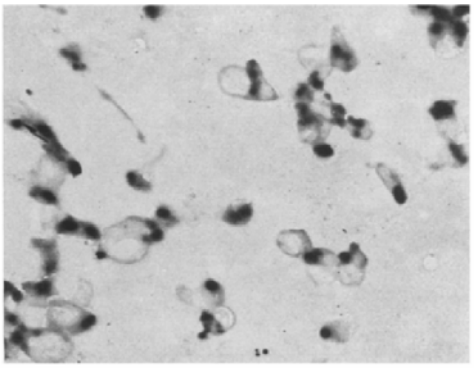

Fig. 14. “Findlay”, 17 days, electron micrograph, magnification 7000x

These granules are probably the small viable PPLO forms identified in the filtration experiments and can be interpreted to have a similar role as bacterial spores. They are filled with the nuclear substance and some plasma, whilst the remainder of the plasma degenerates and disappears. Contact of these bodies with new favourable substrate might

enable them to germinate, leading to the formation of new plasma material and loosening of the nuclear substance. In this process, some of the old plasma remains and is referred to as sheath above. However, this germination process could not be visualised directly, because the granules are below the resolution limit of the light microscope and electron microscopy does not allow visualisation of live cells. The initial stages of the organisms used for light microscopy are different from these spore-like forms; rather, they are equivalent to the uniform, large vegetative bodies shown in Fig. 2 and 3, the size of which has been shown to be dependent on the agar concentration in the medium (Kandler and Kandler, 1954). The light microscopic studies showed that these vegetative bodies proliferate by budding and that in this process plasmodium-like intermediates appear. These observations can be confirmed by the present electron microscopic study, where occasionally also budding stages could be found (Fig. 15).

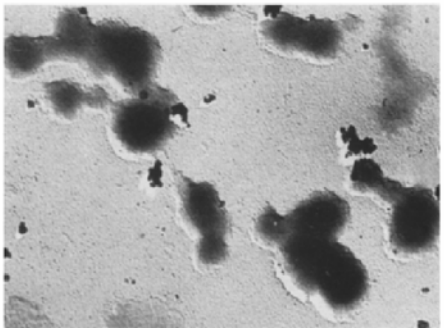

Fig. 15. “Mist” (dung), 40 hrs, electron micrograph, magnification 6100x

On solid medium the individual organisms tend to stick together in clusters until they are separated by mechanical forces during re-streaking on fresh plates. In liquid medium, the organisms appear to separate from each other more easily. In this process, an outer plasma layer might be formed, due to which the shape can be maintained when the sample is dried on the cellulose lacquer foil. The organisms causing pleuropneumonia differ from other PPLOs in that they are delayed in developing this layer, resulting in a stable shape only at a later time point. The above mentioned finding that young cultures of pleuropneumonia causing organisms form thread-like artefacts whilst older cultures are visible as uniform spherical bodies was reproducible. After a few days, also this strain showed the same loosening and the formation of dense granules as described for the PPLOs above. No membrane comparable to a bacterial membrane and no mycelial structures could be found, which is consistent with previous observations (Liebermeister).

Unfortunately, we were unable to grow the L-forms of *Bacterium proteus* or *Vibrio cholerae* in liquid cultures. Therefore, these organisms could not be studied by electron microscopy. Yet, previous studies could not determine any major differences between L-forms and PPLOs in electron microscopic images (Tulasne and Bringmann, 1952; Dienes, 1953).

However, we could image intermediate stages during the L-form development of *B. proteus*. This was achieved by adding penicillin (1000 IU cm^3) to a culture after incubating for several hours. The bacteria started swelling at one or more sites and high-contrast

inclusions, probably nucleus equivalents, became visible within. In appropriate conditions, these can bud into L-forms, as has been shown previously (Stempen and Hutchinson, 1951; Dienes and Smith, 1944). Fig. 16 and 17 show bacteria that have started to form vesicular outgrowths.

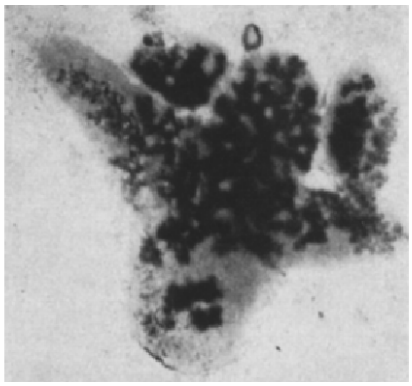

Fig. 16. *B. proteus*, young culture in nutrient fluid with 1000 IU/cm^3 penicillin, electron micrograph, magnification 7000x

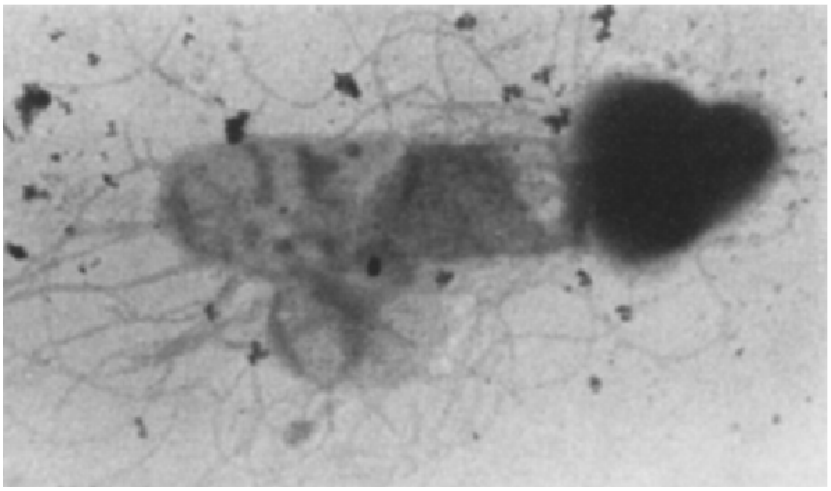

Fig. 17. *B. proteus*, young culture in nutrient fluid with 1000 IU/cm^3 penicillin, electron micrograph, magnification 10000x

Discussion

Consistent with previous results (Ruska *et al*., 1947; Liebermeister, 1953a; 1953b; Dienes, 1953) and with our light-microscopic studies (Kandler and Kandler, 1954) this work could confirm that individual organisms have a spherical shape. Flattening of the sphere observed by electron microscopy is probably due to drying of the sample. As a result from proliferation by budding, the individual bodies are usually connected to a cluster or a chain. Particles from young cultures of the pleuropneumonia causing organism are often thinning into threads and drying on the slide seems to lead to distortions. However, not all non-spherical forms should be considered artefacts, as Liebermeister (1953a) and Gerber (1953) had suggested. Irregularly shaped bodies as the ones shown in Fig. 15 could instead be interpreted as budding stages.

A systematic study of the correlation between age of a culture and the morphology of the individual organisms was performed. This was a valuable addition to the light microscopic studies, where budding from relatively homogenous particles of a diameter of

0.5 µm into clusters or chains could be observed, but where the origin of the small particles (0.3 µm) identified in earlier filtration studies had remained unsolved. This paper now shows that in stationary cultures the initially homogenous bodies become partially condensed. Simultaneously, the surrounding matrix becomes less dense and loses contrast. Then, one or more high-contrast granules (diameter 0.2 µm) become visible in the older bodies. The matrix gets vacuolated and is likely to dissolve, otherwise it can be observed as empty sheaths in the preparations. The small granules, which probably can be filtrated, might be a kind of resting (spore-like) form of the PPLOs.

The presence of these small granules is again reminiscent of the elementary bodies of the large viruses (Edward, 1954; Ruska and Poppe, 1947; Dienes, 1953). Particularly striking is the comparison of the images in this paper with images of incomplete viruses. Whilst for a long time, only uniform bodies were considered viruses. Over the past years, results suggested that, after inoculation with a virus, particles with a different morphology can be isolated (compare Henle, 1953). These particles have the same agglutination properties as the virus, but are not pathogenic. They were interpreted as viral proliferation forms and denominated incomplete viruses. Recently, these incomplete viruses causing avian plague could be isolated and characterised (Schäfer *et al.*, 1954). The electron microscopic images are very similar to our observations with the PPLOs, with the difference that the viral particles are 2.5 fold smaller. Schäfer suggested that the elementary bodies arise from compaction within the larger bodies, which is in agreement with our observation on the PPLOs. Based on our light and electron microscopic date, it is likely that PPLOs proliferate by similar means as the large viruses. Thus, the so far classic form of a virus would be equivalent to the spore-like stage, it then germinates and becomes surrounded by a matrix inside a host cell, explaining why isolation of viruses in such a labile state is difficult. Only under nutrient starvation conditions the substance required for proliferation – probably nuclear substance – condenses, and typical viruses are formed again. In such a scenario, the only difference between PPLOs and viruses would be that the PPLOs retain enough matrix material in the spore-like forms to be able to grow on relatively simple medium. The viruses on the other hand are parasitic forms that depend on the metabolic activity of a host cell. Due to the increased amount of matrix material, the spore-like forms of the PPLOs are larger than the usual virus dimensions.

These thoughts lead to the question of the systematic position of PPLOs and their relation to bacterial L-forms. To date, there are two views:

1. PPLOs are the most primitive group of organisms known, and both bacteria and viruses originate from this group. Therefore, L-form bacteria would be a recapitulation of an earlier developmental stage, according to the biogenetic law.
2. PPLOs are not a class on their own but represent stable L-form bacteria that have lost the ability to convert back into the normal bacterial life style. It is possible that viruses developed by step-wise loss of metabolic systems in adaption to a parasitic life style.

Currently it is not possible to decide which of both views is more accurate. However, these phylogenetic speculations might encourage further investigations about the characteristics of PPLOs and L-form bacteria.

Summary

Here, electron microscopic studies are shown, in conjunction with the light microscopic studies presented previously. It could be shown that 1-2 days after inoculation, relatively uniform disc-shaped bodies can be isolated from liquid culture. Large clusters can be found in young cultures, which later on separate into individual organisms. In older cultures, the matrix is less stable and drying on microscope slide foil results in the formation of irregular shapes. These shapes contain small granules of high-contrast, which might represent resting (spore-like) forms of PPLOs and are possibly equivalent to viral elementary particles.

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