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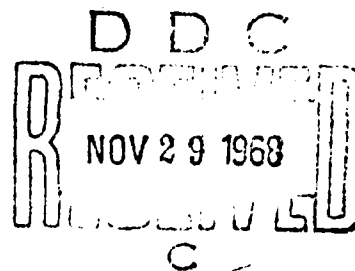
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THE ULTRASTRUCTURE OF MATURE ELEMENTARY BODIES
CAUSING ECTHYMA CONTAGIOSUM AND
STOMATITIS PAPULOSA

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With the use of improved methods for electronmicroscopic preparations, new insight has been gained into the ultrastructure of the vaccinia virus, especially as far as the structural composition of the DNA containing inner body or core is concerned (14, 23). While corresponding structural elements were also observed in the closely related forms like the smallpox virus, investigations so far on poxlike viruses causing contagious pustular dermatitis in sheep (Orf virus) (8, 15), bovine papular stomatitis (BPS-virus) (25, 16, 26) and even milker's nodules (paravaccinia virus) (17, 27, 13, 4) have only revealed filaments, a surrounding membrane, a peripheral protein layer and an inner body. So far no data is available on the ultrastructure of the core or the existence of double element in these viruses [cf. (9)]. Since these substructures are the ones which are of taxonomic importance for the pox viruses, it seems of interest to further investigate the morphology of the virions [for a definition of the "virion" concept see (2)] by examining two typical representatives, the Orf and the BPS-virus, to compare these to the latest findings on the vaccinia virus and thus to contribute to the elucidation of their taxonomic position.

MATERIALS AND METHODS

The Virus: We used the BPS strain described by Reczko (25) and the Orf strain isolated by von Liess (10). The

viruses were grown in tissue culture containing bovine testicular cells. The cells infected with the BPS virus were harvested five days, and those containing the Orf virus four days, after infection.

Embedding: A portion of the cells was fixed in the usual manner with buffered osmium tetroxide, formaldehyde, or with a 15:1 mixture of 80% ethanol and acetic acid, and embedded in Epon 812 and the sections stained with uranyl acetate.

Purification: The virus was freed from the remaining cells by ultra high frequency sound. The elementary bodies were then purified by differential centrifugation and with halogenated hydrocarbons and were then suspended in a 1:50 solution of McIlvaine buffer at a pH of 7.2. After being applied to a platinum-iridium carrier having a collodium film and a thin carbon layer, the elementary particles were further purified by the action of trypsin. Samples were also taken from these isolated particles and embedded after high speed centrifugation.

Negative Staining: The elementary bodies dried on the carrier were treated either directly or after previous treatment with fixing solutions, organic solvents or enzymes, with a 2-5% phosphorus wolframate solution for approximately one minute; the pH value of the solution was adjusted with sodium hydroxide and the preparation dried again.

Shadow casting: Other virus preparations were shadowed laterally from one or two opposite directions after previously having been treated with wolfram oxide as mentioned above.

Size Determination: Polystyrol-latex (particle diameter = 365 m μ) was mixed with the particles which were positively stained with osmium tetroxide or with acidic phosphorus wolframate and were then photographed at magnifications of 2,600 X and measured comparatively with a measuring microscope. In addition, the particles pictured in the sections were also measured under standardized magnification conditions and after negative staining.

Treatment with proteases: For the degradation of the virus protein by enzymes, the techniques used in the work with the vaccinia virus were used (21, 22). Crystallized pepsin was used in a 0.02% solution after ethanol-acetic acid fixation at a pH of 2.4 for a period of 20 hours at 37°C, and crystallized trypsin in a 0.02% solution of m/15 phosphate

buffer was used after ethanol-ether fixation or in the presence of 0.1 M thioglycolic acid after osmium tetroxide treatment, at a pH of 6-7 for a period of 4 to 24 hours at 41° or 37°C.

RESULTS:

Since we did not find any significant differences in the two viruses, all results in this investigation refer to both strains unless it is specifically stated otherwise. Even in the choice of photographs we have decided not to illustrate all observations with pictures of both the Orf and the BPS virion and have thus simply considered both of them to the same extent in our investigation. The normal morphology of the mature viruses is of primary importance. There are also some comments on the structure of anomalous forms as well as on the observed developmental stages; a short communication on this subject appeared previously (24).

Surrounding Membrane: In intracellular virions are often surrounded by a separate jacket located a small distance away from the virion proper; we do not consider this jacket to be a component of the virion itself. Isolated virions are surrounded by a four-layered membrane which is very easily recognized in sections, especially after osmium tetroxide fixation. In most cases, this jacket is not very clearly defined after the formaldehyde treatment, and after ethanol-acetic acid fixation it only consists of one layer. In the material fixed in osmium tetroxide we thus have the following four layers: the outermost layer with a dark outline, next a lighter zone, then an irregular, loosely arranged, dark layer and the innermost layer which is represented by a still heavier staining line (Fig. 15 and 22). As a whole, the membrane appears to be thicker than that of the vaccinia virus. This observation was confirmed by 80 measurements on the BPS and the vaccinia virions which were fixed in osmium tetroxide and embedded in Epon and photographed at a magnification of 100,000 X. In the BPS virus this membrane is about 20% thicker than in the vaccinia virus. The reason for this is the middle layer, which is only half as thick in the vaccinia virus and the irregular dark layer is missing altogether so that only a total of three lines appears. As in the case of the vaccinia virus, this membrane represents the last stage after exhaustive degradation by trypsin (Fig. 23).

The negative staining with acidic phosphorus wolfram-ate yield morphological type 1 pictured in Fig. 1-4, 9, and

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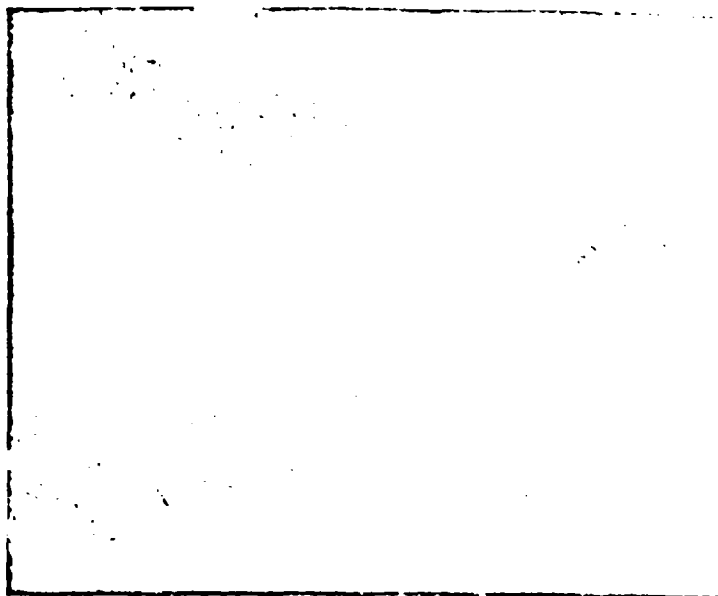
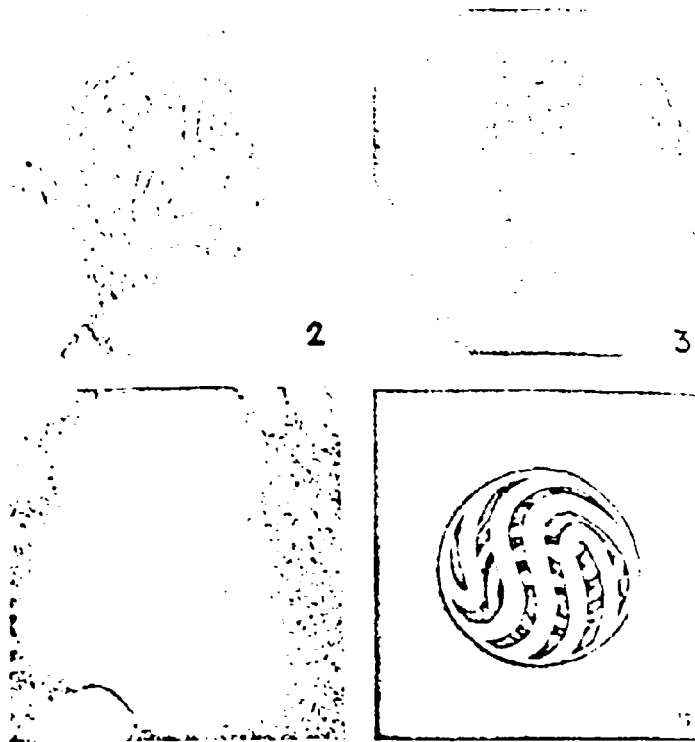


Fig. 1. Orf virions with crossing filaments. Negative staining with acidic phosphorus wolframate (morphological type 1). 150,000 X

10, which corresponds to morphological type 1 of the vaccinia virus (16, 14) and which is characterized by filaments present in the membrane. Occasionally without previous treatment, but somewhat more frequently after purification with trypsin and saponin, the outline of the elementary body, which also includes parts of the filaments protruding from the sides, can be discerned; it is possible that this outline represents the outer limits of the membrane (Fig. 10). On the inside of the filament a deeply staining axial region is present which has also been described by other authors (e.g. Fig. 1, 4, and 10). These filaments have a thickness of 120 Å and are thus significantly thicker than those of the vaccinia virus which have a thickness of 80-90 Å and can also be prepared much more readily. In addition to morphological type 2 and 3 (see below), a well formed type 1 is also found after staining with neutral and alkaline phosphorus wolframate; this type is much more distinct than in the vaccinia virus. After fixation with osmium tetroxide, uranyl acetate, formaldehyde and glutaraldehyde, treatment with trypsin and 3% acetic acid, the filaments are still very distinctly

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Figs. 2-5. Orf virions; negative staining with acidic phosphorus wolframate (morphological type 1).

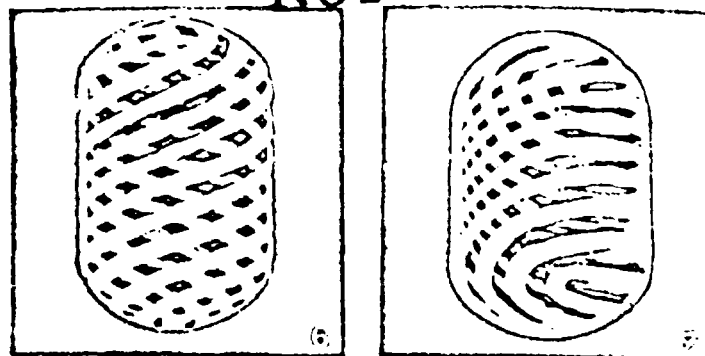
Fig. 2. Loop formation at the pole of a vertically oriented virion

Fig. 3. Criss-crossing pattern of a horizontally oriented virion

Fig. 4. Criss-crossing pattern in the left half of a virion in inclined position

Fig. 5. Models corresponding to the respective positions of the virions in Figs. 2-4. Representation of the filaments and the projection effect. (Four closed strands were drawn on a piece of glass tubing.) 150,000 X

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Figs. 6-7. Models corresponding to the respective positions of the virions in Figs. 2-4. Representation of the filaments and the projection effect. (Four closed strands were drawn on a piece of glass tubing.) 150,000 X

represented, but only very rarely do they still remain after treatment with absolute methanol or petroleum ether, and they do not show up at all after treatment with lauryl sulfate, benzene, ether or ethanol (Fig. 12). After fixation with gluteraldehyde the filaments are very stable during ethanol treatment, but they are very unstable after osmium tetroxide fixation. The filaments can no longer be observed in osmium tetroxide fixed material if they are subsequently treated with a reducing medium like a trypsin solution at a pH of 6-7, or with a pepsin solution at a pH of 2.4, in other words, under favorable conditions for proteolytic enzymatic activity.

While the filaments usually have a criss-cross arrangement in the images of negatively stained virions (Figures 1, 3, 4, 9, and 10), shadow casting plainly shows that when only one side is shown, the criss-cross arrangement is never observed (Figure 8), but occasionally elementary bodies are observed which show this criss-cross pattern of the filaments only on one side (Figure 4). However, observations of the empty jackets of damaged particles in unfixed trypsin-purified and subsequently shadow cast objects show distinctly that the filaments are part of the membrane as shown by the shorter shadows and the lower degree of staining of the empty jackets in Figure 11.

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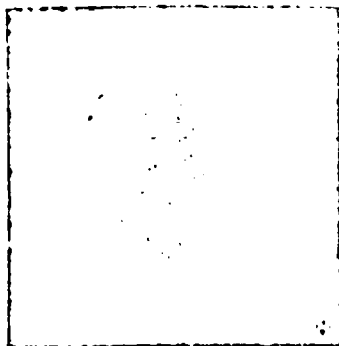


Fig. 8. Orf virion with filaments on the upper side; lateral shadow casting with wolfram oxide



Fig. 9. Orf virion with filaments. Loop formation at the poles. Negative staining with acidic phosphorus wolframate



Fig. 10. BPS virion. Filaments running almost parallel to the cross-sectional plane. Negative staining with acidic phosphorus wolframate. Pretreatment with 0.2% saponin. 150,000 X

All the filaments in the middle of the particle run around the virion in a parallel direction. In the Ori virus they form an angle of approximately 45 degrees with the cross sectional plane (Figures 1, 3, 4, and 9), while in the BPS virus they usually have a smaller inclination angle of about 10 degrees (Figure 10); however, in a few rare instances

virions were found which could not be distinguished from the Orf virus because the filaments had a relatively greater angle of inclination. At the poles, the filaments change direction in the form of a loop (Figures 2 and 9) and return to the other pole without interruption; however, free ends can be observed in a few of the separate areas. A postulated meridian running from pole to pole would transect 12 to 15 strands, some of which would be transected twice. This particular arrangement of the filaments holds for all virions. In view of the great variability however, any further statements are only of hypothetical nature and will thus be treated later on in the discussion.

The Double Element: Investigations so far have not shown whether the double element which is easily demonstrated with the various preparation methods and is present in all viruses of the vaccinia (20) or chicken-pox type (6), is also a definite component of the BPS and the Orf viruses. The double element, therefore, must be extremely small, if it is present at all; this has also been confirmed in our investigations. In longitudinal and cross section two small, thin, biconvex to planoconvex lenses can be recognized between the surrounding membrane and the peripheral protein layer in the central area between the two poles (Figures 14 to 16). Even in those images representing morphological types 2 and 3 (see below) obtained by negative staining with neutral or alkaline phosphorus wolframate, a corresponding superimposure of the double element can be discerned. After degradation of the protein layer and the inner body or core by means of trypsin, the double element can still be observed in the membrane (Figure 24). Cytoplasmic sections also show structures corresponding to the double element in the separate and otherwise empty membrane; this feature has also been observed in the vaccinia virus (13).

The Peripheral Protein Layer: As in the vaccinia virus, negative staining with neutral phosphorus wolframate yields morphological type 2. This type shows the peripheral protein layer with numerous radially arranged, elongated subunits (Figure 17) between the swollen membrane and the homogeneous, granular inner core. These subunits are the only structure which so far could be represented only with the negative staining method. The peripheral protein layer can also be recognized in the corresponding areas in morphological type 3 after staining with alkaline phosphorus wolframate (Figure 18) and on sections after all fixation methods. In particles fixed with ethanol-ether this layer is only degraded by pepsin at a pH of 2.4, resulting in membranes with an elongated inner core (Figure 25).

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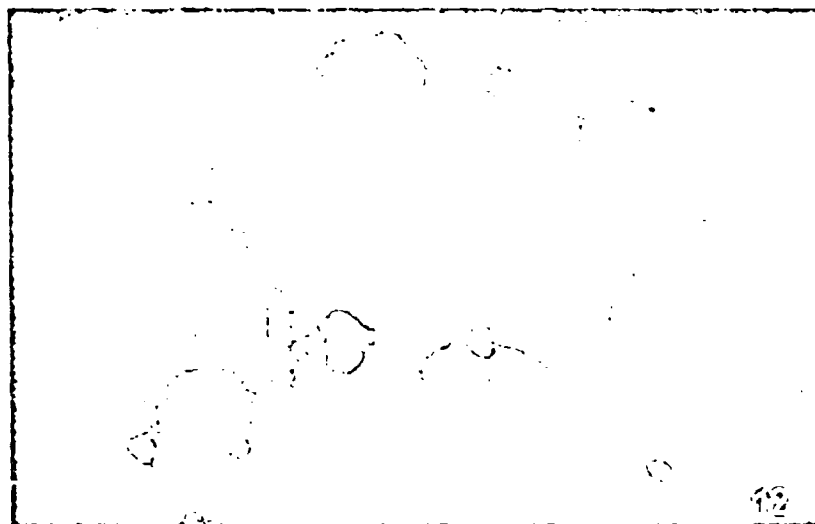
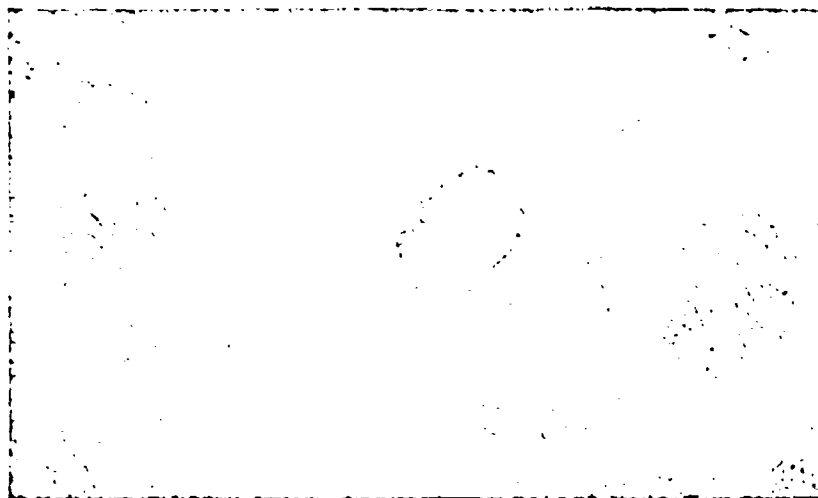


Fig. 11. Intact Orf virion between two empty membranes with filaments. Trypsin purification and shadow casting with wolfram oxide. Note the difference in contrast and the length of the shadow

Fig. 12. Orf virions with smooth surface. Destruction of the filaments with ethanol; shadow casting with wolfram oxide. 70,000 X.

The Ultra-Structure of the Inner Core: Negative staining with alkaline phosphorus wolframite at a pH of 8.0 to 10.5 yields morphological type 3, whose core ultrastructure

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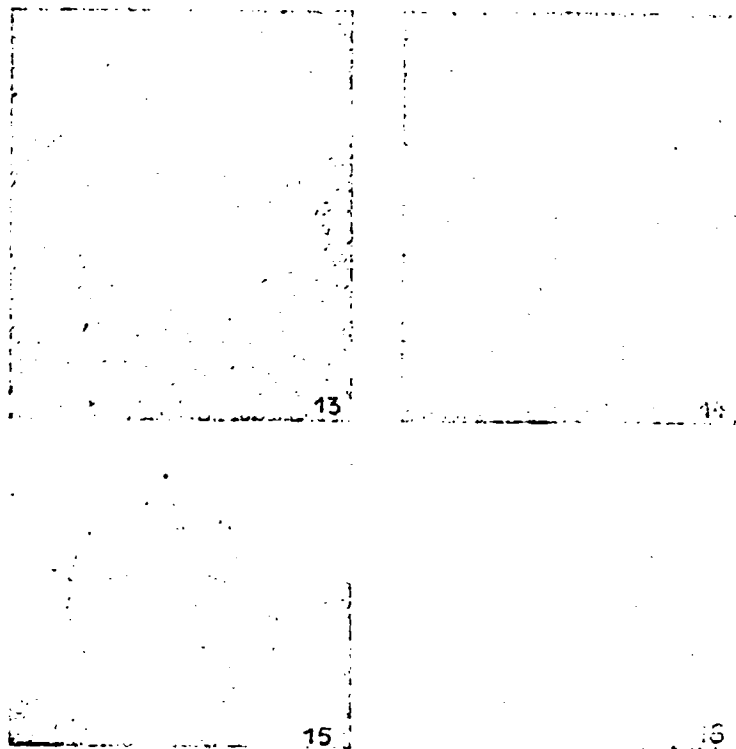


Fig. 13. Membrane of a BPS virion with double element in longitudinal section; apparently the result of intracellular enzymatic degradation. OsO_4 fixation

Fig. 14. Longitudinal section of an Orf virion. Distinct double element. Formaldehyde fixation

Fig. 15. Cross section of a BPS particle. Four layered membrane and double element. OsO_4 fixation

Fig. 16. Cross section of an Orf elementary body with double element. OsO_4 fixation. 180,000 X

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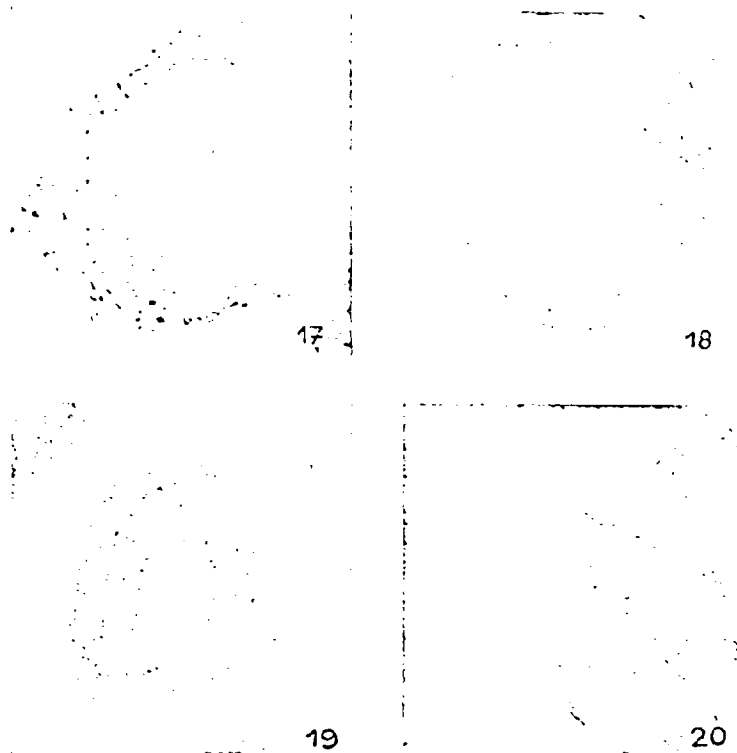


Fig. 17. EPS virion. Subunits of the peripheral layer and homogeneous, granular inner bodies; negative staining with neutral phosphorus wolframate. (morphological type 2).

Fig. 18. BPS particle. Triplet strands in the inner body; negative staining with alkaline phosphorus wolframate (morphological type 3)

Fig. 19. Horizontal section of an Orf virion with triplet strands; Formaldehyde fixation

Fig. 20. Cross sections of Orf virions with matrix and triplet; Formaldehyde fixation

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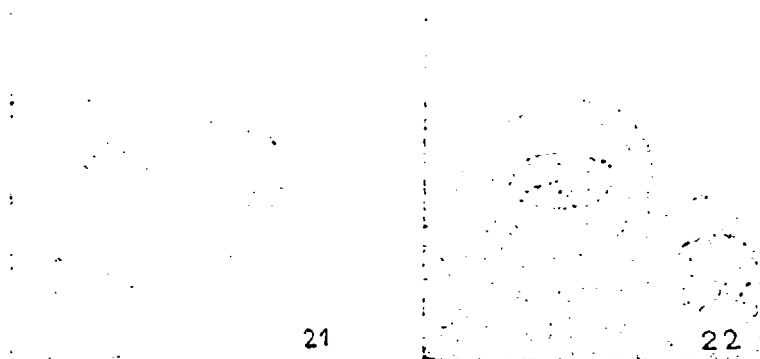


Fig. 21. Cross sections of Orf virions with matrix and triplet; Formaldehyde fixation

Fig. 22. Cross section of a BPS virion. Three-ring structures of the triplet with a light axis in a partially torn matrix; OsO₄ fixation. 150,000 X

corresponds best to the sectional images; this again is analogous to the results obtained with the vaccinia virus. In horizontal section or on negatively stained particles at appropriate superimposures, a triplet of tube-like strands having a length of approximately 1800 Å and a thickness of 300 Å and a differentially stained axis (Figures 18 and 19) can be recognized in a surprisingly electron dense matrix. The cross section or the corresponding superimposure after negative staining shows that in the matrix of the central area of the sections three-ring like structures are embedded which appear dark after ethanol-ether fixation and light after having been fixed with osmium tetroxide (Figure 20 to 22).

The Size and Shape of the Virus: The values obtained the size determinations agree well with those given in the literature. However, we have employed certain strategic criteria in order to determine whether a size difference between the two viral strains investigated by us actually exists. We have found a significant if only slight difference in length between the two viruses in the negatively stained preparations, but significant differences in length also occurred with repeated preparations of the same virus.

In numerous sections the virions do not appear round but elliptical. In 40 BPS particles measured at a magnification of 100,000 X, a longer axis of approximately 190 mμ was

obtained with the triplet rings, and a smaller one of approximately 160 m μ with the double element. Corresponding measurements on the Orf virus gave similar results. Since both of these values are significant and different, the cross-section is obviously not circular, and the outer shape of the virus can therefore, be compared with that of a flattened cocoon.

Virions With Anomalous Structure: Instead of the morphology described above, some one percent of the particles have an anomalous arrangement of the structural elements, as is also the case with the vaccinia virus in which case it occurs only on extremely rare occasions (14). In the middle area between the poles the peripheral protein layer protrudes into the inner core. The double element located at the foot of the invagination is not in the shape of a lense, but rather appears in the form of a cone with an outwardly directed base. This infolding may only be indicated very weakly, but it can also constrict the inner core almost entirely; if this occurs the inner core can only be recognized as a narrow peripherally located sickle in the cross sections.

Whenever such an anomalous inner structure occurs, the outer form is also changed in that the whole virion becomes somewhat shorter and wider. As the images show, this finding is not dependent on the method of preparation (Figures 26 to 31). At the present time an explanation for this anomalous arrangement is just as little possible as the answer to the question whether these forms have any particular communications concerning such an observation on similar anomalies in the virions of other pox viruses.)

Virus Development: The results of earlier authors on the developmental processes in the BPS and Orf viruses (25, 8) already indicate a certain parallelism to the pox viruses, but they still leave open the question concerning the structure of the immature elementary bodies. It is, therefore, of importance to state our observations in this area.

In the cytoplasm of the sectioned cells all known developmental stages of the virus belonging to the vaccinia group can be observed in their typical form. In addition to viral plasmazones, the immature elementary bodies, with the four-layered membrane described above the homogeneous plasma, are also found, but they contain only a few of the heavy staining nucleoids, as must be expected in single sections. The immature forms are somewhat larger than the virions.

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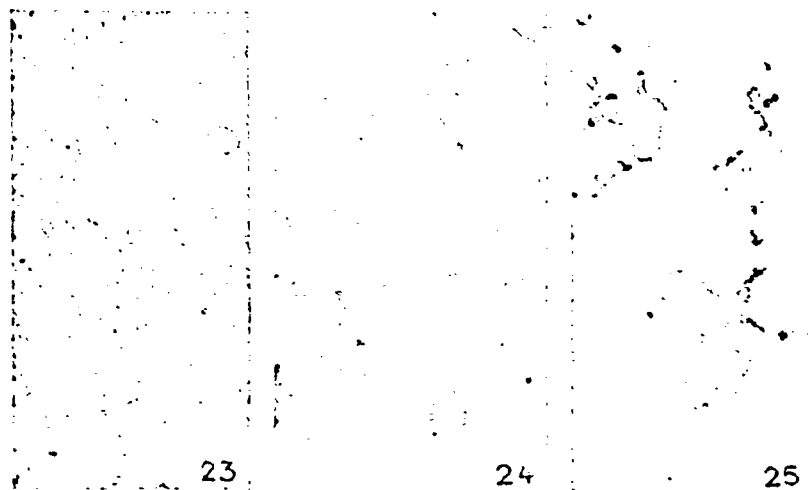


Fig. 23-25. Degradation of fixed Orf virions with proteolytic enzymes. Positive staining with OsO_4 and uranyl acetate.

Fig. 23. Empty membranes. Exhaustive trypsin degradation

Fig. 24. Membranes with double element. Partial trypsin degradation

Fig. 25. Degradation of the peripheral protein layer by pepsin. 30,000 X

In addition the cells show zones containing the round particles described and pictured by Reczko (25), which appear in the form of a light ring, which is interrupted by thread-like bridges and is located below the membrane and the plasma; in our opinion we are here dealing with especially fixation labile, primary immature states. In the developmental areas the immature as well as the mature forms are generally surrounded by a jacket consisting of several layers. After shadow casting and negative staining only mature elementary bodies, and occasionally empty membranes, can be definitely identified in the preparations.

DISCUSSION

Contribution to Negative Staining: In the representation of an object after negative staining we are dealing primarily with a transmission image which, in view of the great depth of field, does not permit us to assign the structures observed to the various planes in the object. The assumption that phosphorus wolframate only stains the upper or the lower side can hardly be verified, and the arguments for this hypothesis (7, 19) are not sound. On the basis of such images alone, it is therefore not possible to decide whether the criss-crossings of the virus filaments shown after negative staining are only found on one side, or whether they originate as a result of a superimposition of both sides (15). Only in images obtained by shadow casting can one determine that the filaments do not cross.

This example again shows that on the basis of images of negatively stained particles alone, statements concerning the number and arrangement of superficial structures, as for example in the case of the capsomeres of spherical viruses, can only be made with great reservation.

As we have already shown on the vaccinia virus, we are not dealing with functionally different virions in the case of morphological types 1, 2, and 3 obtained with the negative staining, but the small object presents a different image depending on the adjustment of the pH value of the phosphorus wolframate solutions used (14). After thus revealing the filaments by shadow casting, in other words, after observation in the electron microscope, morphological type 3 with all the fine structure of the inner core can still be produced by negative staining in the alkaline phosphorus wolframate. Since the image is dependent of the manner of negative staining, one is forced to substantiate all results obtained with this method with the use of other preparation techniques.

Size Determination: The results of size measurements in other viruses also revealed the dependence on the preparation methods used. The different results after phosphorus wolframate staining, especially under approximately identical conditions, are evidence for the questionable validity of the exactness of the procedures," Hall (5), in his work with biological material, observed significant size differences which arose as a result of the different preparations techniques. Thus it is not astonishing that in the reproducibility of the measurement results, difficulties arise in the even more complicated negative staining. It is, therefore,

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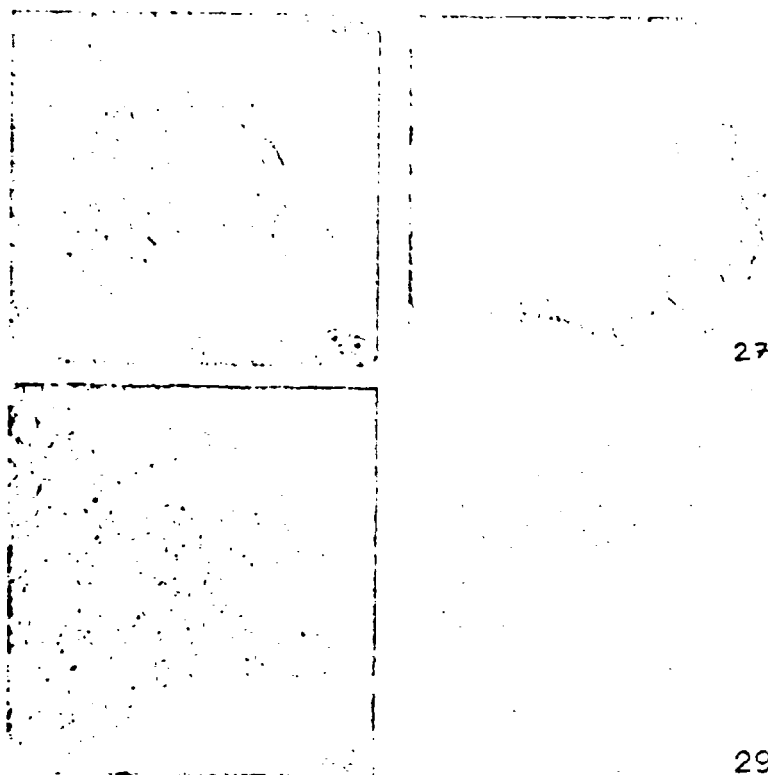


Fig. 26-29. Virions with anomalous structures

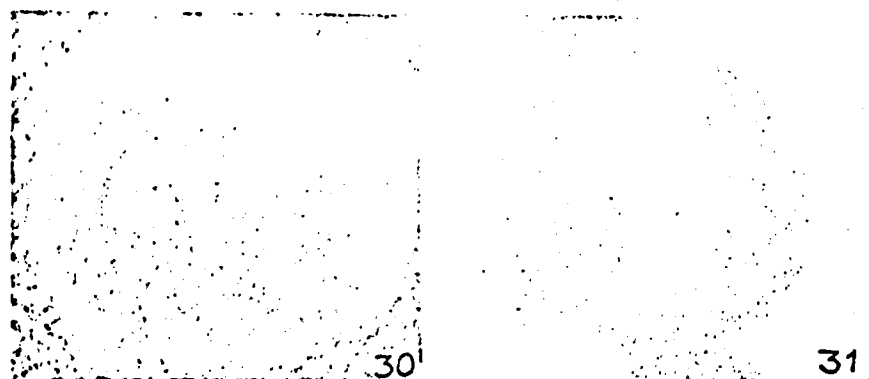
Fig. 26. Orf virion with filaments; negative staining with acidic phosphorus wolframate (morphological type 1)

Fig. 27. BPS virion; visible invagination of the peripheral protein layer. Negative staining with neutral phosphorus wolframate (morphological type 2)

Fig. 28. BPS particle. Pronounced invagination of the peripheral protein layer. Negative staining with alkaline phosphorus wolframate (morphological type 3).

Fig. 29. BPS virion in section. OsO_4 fixation

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Figs. 30 and 31. Representation of the Orf virions in the projection of sectional plane vertical to that shown in Figs. 28 and 29. Negative staining (morphological type 3) and section after formaldehyde fixation. 150,000 X

very probably that the significant differences found, are a direct result of the preparation methods used. In the comparison of the Orf and the BPS particles we were not able to determine any differences in size with the use of identical preparation techniques. Since the size determinations of osmium tetroxide stained particles is known to be relatively dependable, as shown by the many measurements performed on the vaccinia virus, we used this technique in our determinations and are reporting the length and the width of the Orf virus and the BPS virus as 300 m μ and 170 m μ respectively and consider this to be the average size for both viruses; a length-width axis ratio of 1.7 is thus calculated. The difference between these values and the true average lengths should be less than 20 percent. The deviations in the size of the virions are quite considerable; particles with dimensions up to twice the average value are sometimes encountered. All of the average sizes determined recently by Nagington et al. (16) on the Orf and the BPS virus, and those of Friedman-Kien et al. (4) and Moscovici et al. (13) in the virus causing milker's nodules, fall within the 20 percent range.

Virus Structure: The virions are bounded on the outside by the four-layer membrane containing the filaments, which are probably located in the irregular, loosely arranged middle layer since this one, as well as the filaments themselves, is larger than in the vaccinia virus. The ripple effect observed occasionally within the membrane in some sections cannot, in our opinion, be attributed solely to the

filaments. This is easily understood when one recalls the lability of the structure when treated with organic solvents even after prior fixation. Thus the behavior is not easily explained; one possible explanation, however, is the hypothesis that one might be dealing here with a lipo-protein.

The filaments surround the virion without crossing with small angles of inclination and return to the poles after forming a loop into the other direction. The variability in this arrangement is very great and for this reason the small model given in Figures 5 to 7 shows the latest findings only in diagrammatic fashion. It contains four parallel strands each of which is closed within itself; this is an assumption which, for the time being, is only to be considered an arbitrary since a decision on whether it is a case of one or several filaments, or whether or not these have free ends, is not possible at the present time. Nevertheless, there is good agreement between the light-optical and the electron-optical transmission image which still holds in the somewhat unusual slanting position shown in Figures 4 and 7.

Mainly because the filaments are revealed very distinctly, these viral filaments are well suited for further investigations concerning their chemical nature and functional significance.

The peripheral protein layer is located within the membrane, and between this protein layer and the membrane lies the double element which, in comparison with the vaccinia virus, is smaller in an absolute as well as relative sense. The protein layer surrounds the DNA containing inner core containing the three tube-like strands embedded in the core matrix. With an approximate length of 1800 Å and an approximate thickness of 300 Å these strands are smaller in the absolute sense than those of the vaccinia virus where they have a length of 2500 Å and a thickness of 400 to 500 Å (23). Thus in none of the structures described are we dealing with strain independent, absolute measurements, but they are all strictly specific.

Aside from the slight angles of inclination of the filaments in the BPS virus, no differences in the morphology, development and chemical behavior were observed in the virus strains investigated by us. Just in case it should turn that we are not even dealing here with two strains of the same virus, it should be mentioned that the taxonomic relationship would, indeed, be very close and be of a similar type as the

relationship between the smallpox, vaccinia, electro-melia and cow-pox viruses in the vaccinia group.

If the Orf and BPS viruses are compared with the vaccinia virus, some differences do appear. They are not block shaped but have the shape of a flattened cocoon, and with a size of 300 X 170 m μ they are significantly smaller than the vaccinia virus which has dimensions of 348 x 261 m μ (11). We consider this to be a true size difference even when the difficulties mentioned above are considered, and it is also directly evident when a mixture of both virus particles is applied to the same carrier. In addition, these measurements yield a value of 1.7 for the axis ratio of length to width, whereas the same value in the vaccinia particles amounts to only 1.3. The Orf and BPS viruses also have a thicker surrounding membrane with prominent filaments with a different arrangement pattern; they also have a more delicate double element as well as still thinner strands in the inner core.

No striking morphological differences are found in the inducibility of the three morphological types, the appearance of an anomalous form of the mature elementary particles in direct relation to the developmental stages and in the behavior during the action of proteolytic enzymes. The different behavior of the Orf virus described in Abdussalam and Cosslett (1) is readily explained by the unfavorable reaction conditions chosen by those authors during which the vaccinia virus would not be degraded either.

This comparison, however, simultaneously shows the far-reaching structural homology so that there can no longer be any doubt that the BPS and the Orf viruses belong to the group of the pox viruses.

Within this group these two viruses, together with the causative organism for milker's nodules -- which according to all the investigations up to this time, is very similar in nature -- form a subgroup which can be termed "para-vaccinia virus" which could be placed along side the vaccinia group and the chicken pox viruses.

SUMMARY

Electron-microscopical studies on the causative organism of contagious pustular dermatitis in sheep and bovine papular stomatitis, employing negative staining, shadow casting, thin sectioning and enzymatic analysis, revealed the typical

ultra-structure of the pox viruses. This ultrastructure had been studied previously in the vaccinia virus, but there are distinct differences in these structures between the vaccinia virus and the viruses described here. The surrounding membrane and the filaments are thicker, and the double element, the peripheral protein layer and the inner core with the triplet of tube-like strands are smaller than those of the vaccinia virus, and thus correspond to the reduced dimensions of the virions. No differences were found with respect to the anomalous forms of the virions, the behavior toward proteolytic enzymes and the developmental stages. Shadow casting showed that the filaments in the membrane do not cross but that the corresponding images result after negative staining by superimposure of the upper and lower sides. It is proposed that the Orf and the BPS viruses, as well as the virus causing milker's nodules, be classified as a sub-group called para-vaccinia viruses.

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