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NEGATIVE STAINING OF A NON-HAEMADSORBING STRAIN OF AFRICAN SWINE FEVER VIRUS

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ABSTRACT

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Since the application of negative staining, preceded by fixation, prevents the disruption and distortion of the capsid of the African swine fever virus, improved contrast and evaluation of the appearance and size of virus particles in the electron microscope is possible and, in addition, the icosahedral shape of the virus is demonstrable. The mature virus particle contains at least 2 capsid layers and an outer envelope.

Résumé

LA COLORATION NÉGATIVE D'UNE SOUCHE NON HÉMADSORBANTE DU VIRUS DE LA PESTE PORCINE AFRICAINE

L'application de la coloration négative précédée de la fixation a évité la désintégration et la déformation de la capside du virus de la peste porcine africaine. Grâce à cette technique, on a obtenu un meilleur contraste et une meilleure appréciation des images et de la taille du virus au microscope électronique; de plus, la structure icosaédrique du virus a été démontrée. Le virion complet est formé d'au moins deux couches capsidiques avec une enveloppe périphérique.

INTRODUCTION

The replication of African swine fever virus (ASFV) in cell cultures was studied at the ultrastructural level by Breese & De Boer (1966) and by Moura Nunes, Vigario & Terrinha (1975). They showed that the virus replicates in the cytoplasm and is released with an outer envelope by budding from the cell membrane. According to Vigario, Terrinha & Moura Nunes (1974), the envelope may be of importance in haemadsorption since non-enveloped virions failed to produce this effect. Using cellspread preparations in combination with negative staining, Almeida, Waterson & Plowright (1967) compared the morphology of ASFV with Tipula iridescent virus (TIV). The information available from their 2 micrographs on ASFV is limited, however, and needs confirmation. In this laboratory, electron microscopy of negatively stained samples of haemadsorbing and non-haemadsorbing strains of ASFV was used on various occasions between 1973–1975 for the purpose of diagnosis and identification (Pini & Hurter, 1975; Pini, unpublished observations, 1976). In this study an attempt was made to improve the morphological appearance of the virus particles in the electron microscope but, since the ASFV particles appeared to be pleomorphic and unstable, the deduction of the number of morphological units was not possible. Certain features of the virus particles which have not been described before are discussed.

MATERIALS AND METHODS

The electron microscopic observations reported in this study refer to the non-haemadsorbing strain 24823 of ASFV. A line of monkey kidney (LLC-MK_2) cell monolayers was infected with 1×10^6 ID_{50} of a virus stock at the first passage in buffy coat cell cultures. Twenty-four hours later, when generalized cytopathic effects were observed, the cells were harvested and sedimented for 5 min by centrifugation at 300 *g*.

Sectioning

The material to be sectioned was fixed for 1 h in 3% glutaraldehyde in 0,1 M sodium cacodylate buffer, washed twice for 15 min in sodium cacodylate buffer and post-fixed for 1 h with 1% OsO_4 in Millonig's buffer (Millonig, 1961). After being dehydrated in increasing ethanol concentrations and cleared in propylene oxide, the specimens were embedded in Epon (Luft, 1961). Thin sections were picked up on uncoated copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963).

Negative staining of cell spread preparations

A. The cell pellet obtained by centrifugation was drained of all medium and mixed with distilled water to produce lysis of the cells. Negative staining was then carried out by either of the following 2 procedures:

(a) The method of fixation described by Nermut, Frank & Schäfer (1972) and Vernon, Lawrence, Cohen, Durso & Rubin (1976) was applied in the manner described below:

A formvar-carbon-coated grid was floated on a drop of the lysed cells for 1 min, transferred to a drop of 1% OsO_4 or 3% glutaraldehyde for another minute and floated for 10 seconds on a drop of 3% phosphotungstic acid (PTA), pH 6,2. The excess fluid was drained with filter paper and the specimen examined in a Siemens Elmiskop 1A or 102. Drying of the grids before staining was not attempted.

(b) A drop of the resulting suspension was mixed with PTA and a drop of this mixture was placed on a grid, drained and dried.

B. Preparations were also made to restrict cellular lysis by fixing cell pellets with OsO_4 or glutaraldehyde, decanting the fixative, adding a drop of distilled water and mixing the suspension with PTA. This mixture was then applied to a grid. Non-infected cell cultures, treated as described above, were used as controls.

Instrumental magnification was calibrated with a cross-ruled grating replica (Fullam, 54 800 lines/inch). Measurements of particles were done on a Nikon profile projector, model 6C.

RESULTS

When negatively stained cell spread preparations were used for diagnostic purposes [Method A (b)], only relatively small numbers of virus particles in

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various stages of disruption and showing structural surface subunits could be seen (Fig. 1). As shown in Fig. 2, virus particles which are apparently empty were demonstrable to a lesser extent. No other forms of particles were observed.

Contrary to previous results obtained with other strains of ASFV, (Els, unpublished observations) isolate 24823 [Method A (b)] produced large numbers of apparently compact forms of virus particles (Fig. 3). However, the majority of these seemed to be distorted, with a loss of the hexagonal outline seen in thin sections. Many disrupted particles similar to those in Fig. 1 were also found. In an attempt to preserve the hexagonal outline and the arrangement of the subunits so that their number could be deduced, the Method A (a) of fixation described under Materials and Methods was applied.

Some significant differences were observed when the method of PTA staining without fixative [Method A (b)] was compared with staining after fixation [Method A (a)]. With the former method (Fig. 1, 2 & 3), most of the virus particles were collapsed, disrupted or distorted, while with the latter, the virus particles were well preserved, had a clear hexagonal outline, were more uniform in size and were generally more densely stained (Fig. 4).

This fixation of cell-spreading preparations prior to staining [Method A (a)] also confirmed the icosahedral shape of ASFV. In Fig. 5, micrographs of particles which appear as transparent superimposed icosahedrons are compared with models in similar orientations. The outline of an icosahedron is drawn in on the micrographs to illustrate the correspondence of the orientations. That the ASFV particle has the form of an icosahedron is thus not inferred from a hexagonal outline alone.

With fixation included in the staining procedure, the disruption of virus particles and the display of regularly repeating units, as illustrated in Fig. 1 & 10, were eliminated. Some suggestion of a capsid structure on the virus particles could be seen occasionally, but individual capsomeres were not clearly delineated (Fig. 8 & 9) and their number could not be determined.

Without fixation the majority of particles observed were broken and unsuitable for the deduction of a capsomere number because of their pleomorphic shape and the superimposition of sub-units. Many particles were found showing morphological units (Fig. 10), but the particles themselves rarely revealed a distinct hexagonal shape.

In addition to the compact forms of virus particles (Fig. 4 & 5), several virus particles were found enclosed in an envelope (Fig. 6, 8 & 9). To confirm that this envelope was not accidently acquired during the staining process, preparations where cellular lysis was restricted were made (Method B under Materials and Methods). By this technique virus particles were found within preserved microvilli (Fig. 7). The corresponding situation in positively stained sections is illustrated in Fig. 12.

Measurement of 50 fixed particles gave an average diameter of 212 nm from vertex to vertex and 196 nm from side to side (Fig. 4 & 5). The average of these 2 measurements, 204 nm, agrees well with the reported value of 200 nm. On the other hand, 50 particles stained with PTA alone varied in size from 175 nm to 300 nm, with an "average" of 215 nm. Since they represent collapsed particles, an increase in size can be expected. When the diameters of 10 non-enveloped particles in thin sections were measured, a value of about 190 nm was found.

In the cell spread preparations described above, tubular structures of varying length and thickness with a subunit arrangement similar to that of the ASFV particles were observed. One example is shown negatively stained in Fig. 11, while that in Fig. 13 represents the equivalent structure found in thin sections. They may represent aberrant forms of ASFV particles.

Discussion

Using negatively stained cell spread preparations, Almeida *et al.*, (1967) demonstrated the presence of 2 forms of ASFV viz. compact structures, hexagonal in outline and often displaying 2 surrounding membranes, and less stable particles lacking the bounding membrane but showing subunits. They compared ASFV to TIV and suggested that the particles of both viruses are of the compound cubic type, have icosahedral forms built up of regular subunits and are stabilized by the addition of outer membranes. Furthermore, they suggested that the outer membrane is a lipoprotein one, probably acquired on passage through the cell membrane.

In our hands, however, virus particles of the compact form (Fig. 4 & 5) show a loose envelope (Fig. 6, 8 & 9) enclosing 2 membranes which appear to be an integral part of the icosahedral shell, and it seems more appropriate to interpret them as capsid shells or layers. In Fig. 4 (inset) & 6 b, an empty particle with the 2 capsid layers can be seen and the subunits are regularly arranged on the outer shell rather than on the inner layer. Furthermore, Fig. 7 shows a compact particle within a preserved microvillus. Therefore it is concluded that the envelopes shown in Fig. 6, 7, 8 & 9 seem to be a further acquisition taking place at the cell membrane during budding, the modality of which is illustrated by means of thin sections in Fig. 12.

The presence of a 3rd internal thin layer, regularly found in empty particles and shown by some virions in Fig. 4 & 4 (inset), still remains to be explained, however. Some particles indicated by arrows in Fig. 13 show a similar thin layer beyond the dense outer shell. The explanation of these layers on the capsid and any correlation to the unit membrane model proposed by Stoltz (1973) for icosahedral cytoplasmic deoxyriboviruses requires further investigation.

From this study it has not been possible to ascertain the total number of capsomeres comprising the capsid of ASFV particles. The particles in Fig. 10 show regular repeating subunits with indications of a triangular arrangement but, owing to the pleomorphic shape and probable superimposition, 2 neighbouring 5-co-ordinated morphological units could not be identified. The fact that fixation did not improve the resolution of subunits suggests the possibility that the capsid may be covered by some amorphous layer which would effectively prevent the penetration of negative stain between subunits. Treatment with a nasal decongestant on *Sericesthis* Iridescent Virus, as was done by Wrigley (1969), may lead to a more detailed substructure from which the number of capsomeres could be deduced.

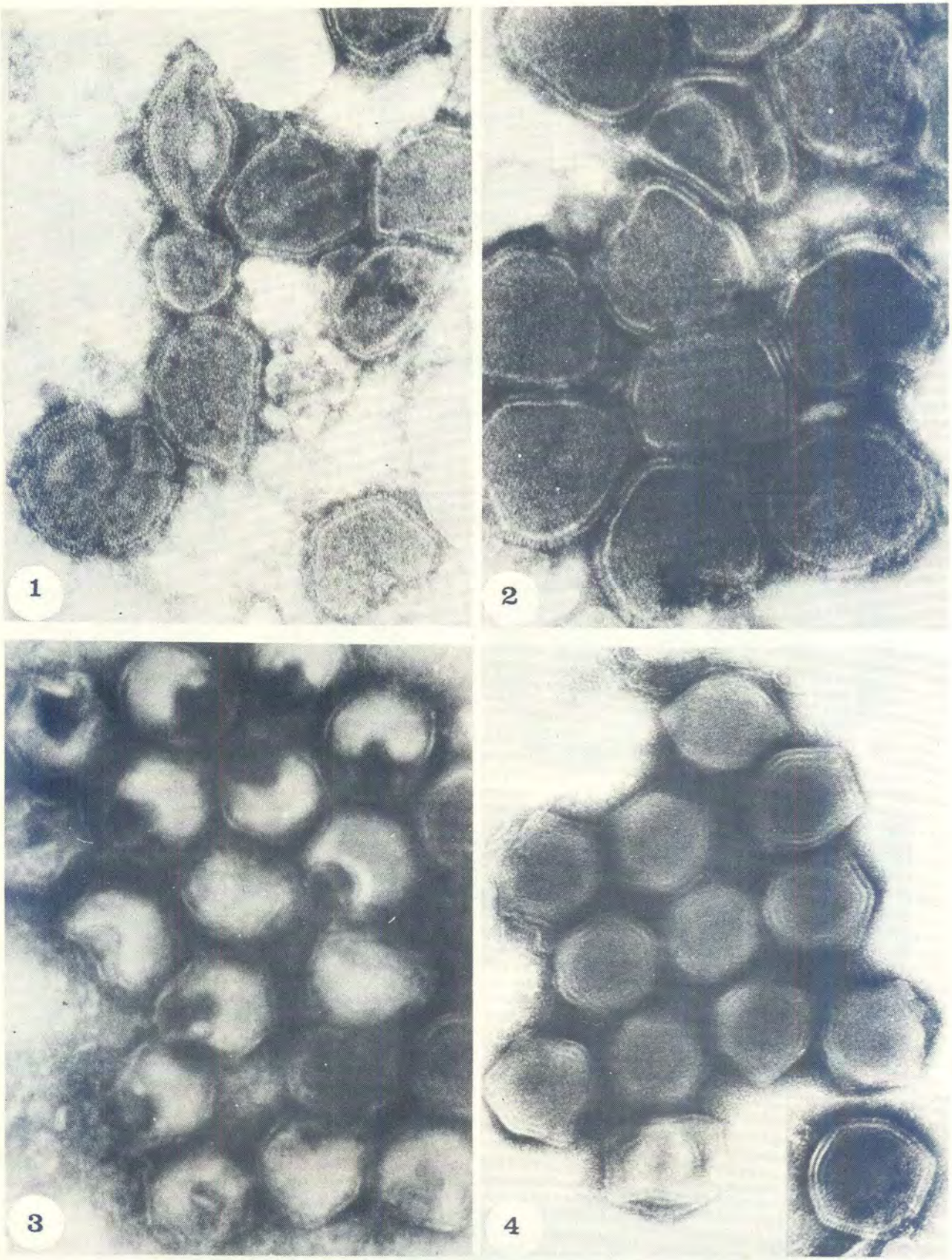
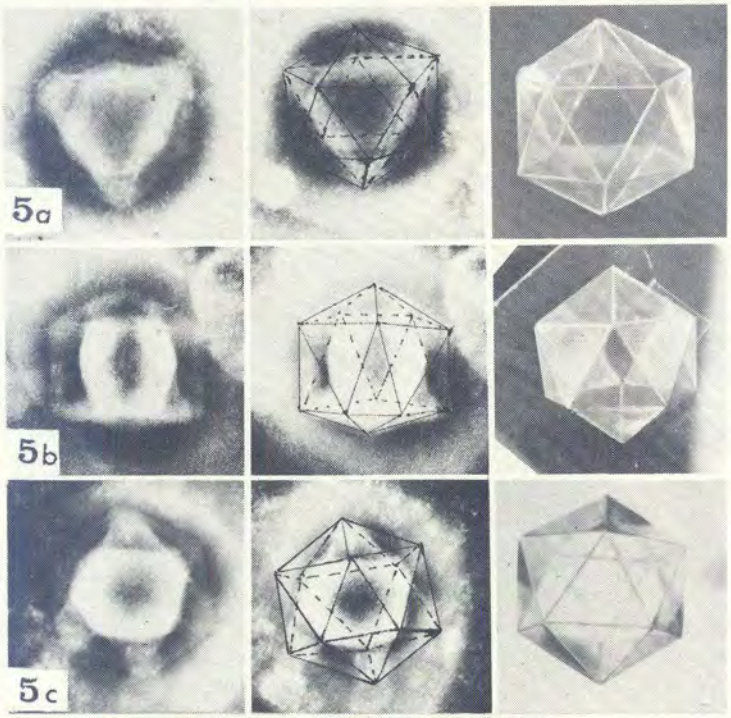


FIG. 1–2 Negative staining without fixation of ASFV particles. Various disrupted and distorted forms of virus found in samples tested for diagnostic purposes. The particles in Fig. 2 appear to be "empty" particles. × 100 000

FIG. 3 Negative staining without fixation of compact forms of ASFV strain 24823. Some negative stain penetrating the outer shell of ASFV particles leads to the appearance of a 'membrane' surrounding a central core. This outer shell should not be identified as an envelope. × 100 000

FIG. 4 Negative staining preceded by fixation with OsO_4. Particles appear to be in a better preserved condition and show a clear hexagonal outline. Some virus particles show the presence of 2 or 3 layers. × 100 000

Inset: "Empty" virus particle with evidence of subunits on the edge of the multilayered capsid. Fixed preparation. × 100 000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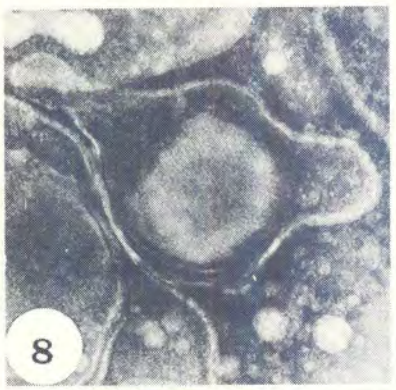
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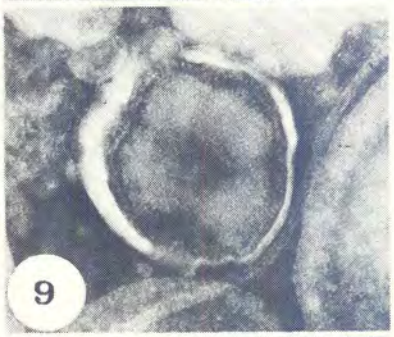
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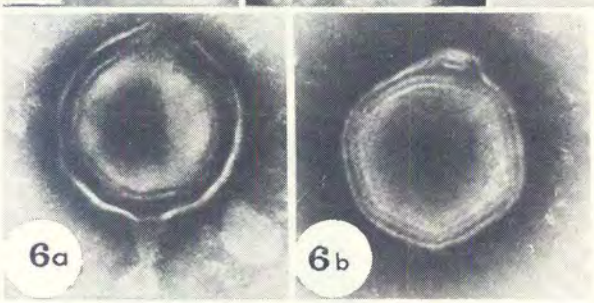
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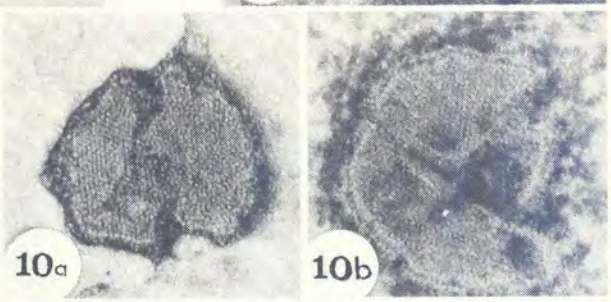
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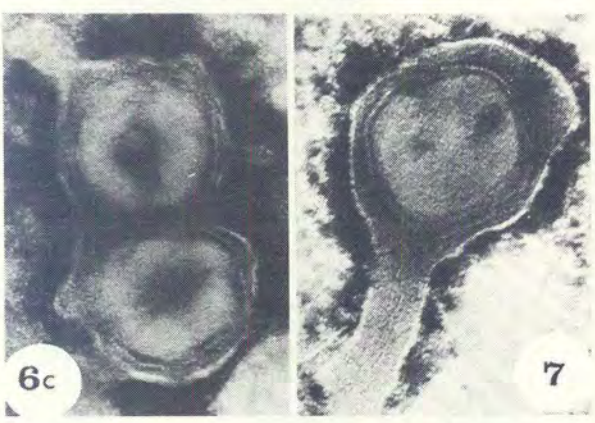
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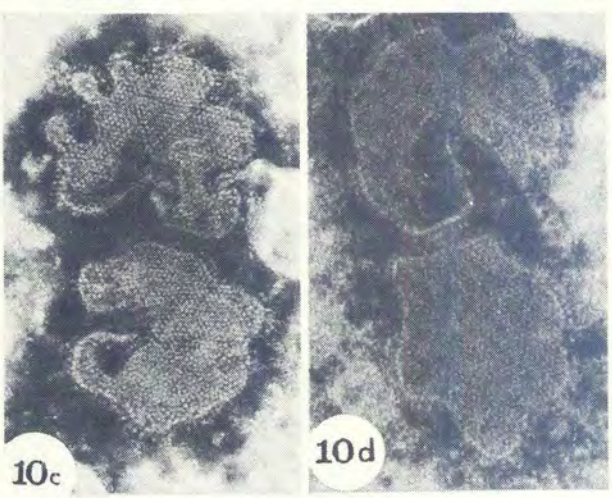
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10d


11

FIG. 5a, b & c Selected examples of single virus particles. The icosahedral structure of the virus particle is compared with that of a model in a corresponding orientation. Such particles were always observed in areas of relatively dense stain deposition. Fixed preparation. × 100 000

FIG. 6a, b & c Enveloped virus particles. In (b) the multilayered nature of the capsid inside the envelope is visible. In (c) two particles are contained in one envelope. Fixed preparation. × 100 000

FIG. 7 Enveloped virus particle inside a microvillus. Fixed preparation. × 100 000

FIG. 8 & 9 "Compact" form inside envelopes showing some arrangement of subunits not clearly delineated. Fixed preparation. × 100 000

FIG. 10a, b, c & d Selected virus particle with evidence of regular repeating units and some indication of triangular facets. Unfixed preparation. × 100 000

FIG. 11 Tubular structure of cylindrical form of ASFV with evidence of the same subunit arrangement as on complete virus particle. Indication of icosahedral profile at one end. × 100 000

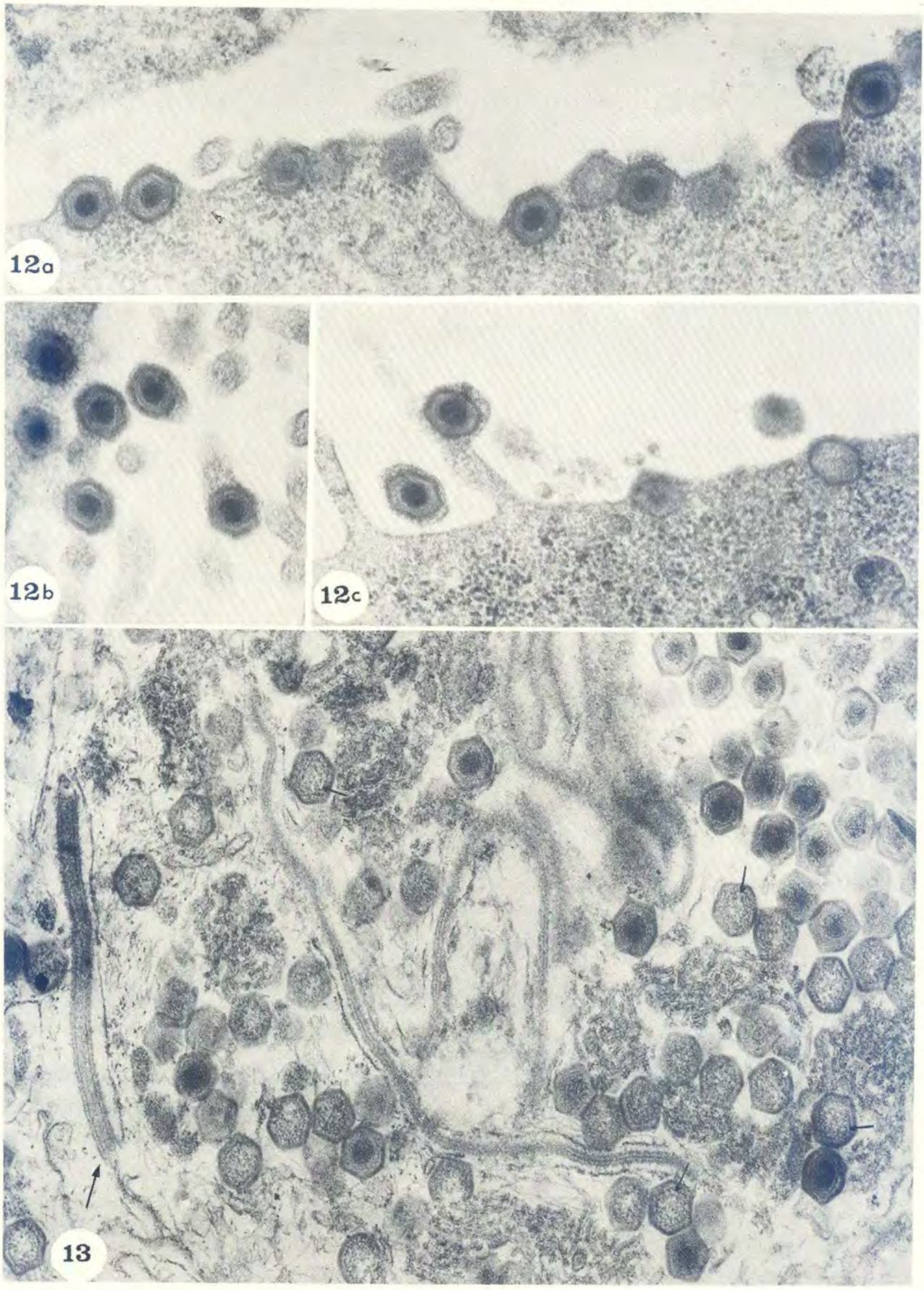

FIG. 12–13 Micrographs of thin sections of LLC-MK_2 cells infected with ASFV 24823 strain. × 50 000

FIG. 12a Virus particle in the process of obtaining an envelope by budding off at the cell membrane

FIG. 12b & c Virus particle obtaining an envelope by budding off in a microvillus of its host cell

FIG. 13 Replicative area of the cytoplasm of the infected cell. Note tubular structure (arrow) (compare Fig. 11). Various hexagonal- and pentagonal-shaped particles indicating an icosahedral shape and membranes of viral origin can be seen

In conclusion, it seems that staining with PTA alone and drying afterwards may have a deleterious effect on virus particles and cause their collapse, and prior fixation probably minimizes the disruption of viral capsids during application of negative stain and subsequent drying. Fixation of lysed cell suspensions seems to preserve the natural characteristics of the viral population contained in the cells. A comparison of the appearance of fixed virus particles with that of the virus stained with PTA without fixation indicates that there has been an improvement in the microscopic evaluation of the appearance and size of ASFV particles. Only one type of particle (including empty particles) seems to be released from lysed cells, but its appearance depends on the method of specimen preparation.

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