J. Hyg., Camb. (1967), **65**, 467
With 4 plates
Printed in Great Britain

A morphological comparison of Bittner and influenza viruses*

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(*Received* 20 *April* 1967)

It has been known for some time that negatively stained influenza virus and Bittner mouse mammary tumour virus resemble each other (Almeida & Ham, 1965). However, negative staining of intact virus delineates only the surface features of the particle and it seems possible that this external similarity does not extend to the inner components of these viruses, as they differ considerably both in physical properties and in their thin sectioned appearance (Moore, 1962; Morgan, Rose & Moore, 1956). Thin sections of mouse mammary tumours reveal two distinct forms which have been designated as virus (the types A and B of Bernhard) (Bernhard, 1958), while cells infected with influenza virus show only one form recognizable as virus, seen always at the surface of the cell and quite distinct from the type B Bittner particles. The present paper describes the results obtained when negative staining was used to study the internal component of both these viruses in particles that could be penetrated by the phosphotungstic acid (PTA). In addition, the negatively stained appearance of the mouse mammary tumour virus was compared with the picture that is obtained when the virus is studied by the thin sectioning technique.

MATERIALS AND METHODS

Bittner virus

Female C3H mice with spontaneously occurring mammary tumours were used as a source of Bittner virus. The animals were killed at ages varying from 9 to 18 months. The tumours were removed, and portions fixed in 10% formol saline for sectioning for light microscopy and in buffered osmium tetroxide for electron microscopy. Blocks for thin sectioning were embedded in Araldite according to conventional techniques (Luft, 1961). The remainder of the tumour was either used immediately for making extracts or frozen at $-20°$ C. for periods of up to 6 months. Extracts were made by homogenizing the tumours in a glass homogenizer of the tenBroek type using distilled water as a suspending medium. The suspension was spun at 10,000 rev./min. for 10 min. in the SS34 rotor of the Sorvall RC-2 centrifuge, and the supernatant was then spun for 30 min. at

* This investigation was supported by a grant from the Medical Research Council.

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15,000 rev./min. in the same rotor. This pellet was suspended in a small amount of distilled water (approx. 0·2 ml.) and negatively stained by mixing a small amount of this suspension with an equal quantity of 3 % phosphotungstic acid adjusted to pH 6. Carbon–formvar coated grids of 400-mesh were used and the specimens examined in a Philips EM200 electron microscope.

Influenza virus

The virus was grown by inoculating the PR 8 strain of influenza A virus into the allantoic cavity of 11-day embryonated eggs. After 3 days incubation the eggs were chilled and the allantoic fluid harvested. Some of this was used immediately to obtain a virus pellet while other portions were placed in vials and frozen at − 20° C. for periods of up to 6 months. After a clarifying spin of 3000 rev./min. for 5 min., the supernatant was centrifuged for 30 min. at 15,000 rev./min. in the SS 34 rotor of the Sorvall RC-2 centrifuge. The pellet was resuspended in distilled water and negatively stained as described for Bittner virus.

RESULTS

Light microscopy

Examination of sections stained with haematoxylin and eosin in the light microscope confirmed that the tumours displayed the histopathology associated with them (Moore, 1962). Duct formation was frequently seen, and the cells lining these ducts often displayed the eosinophilic juxtanuclear inclusions characteristic of the Bittner tumour.

Thin sectioning

Examination of thin sections of the mouse mammary tumours in the electron microscope again confirmed the features associated with such tumours (Moore, 1962). Type A particles, approximately 700 Å. in diameter, were frequently found in areas near membranes where type B particles, approximately 1000 Å. in diameter, were budding (Pl. 1, fig. 1). In several instances the morphology of the free type A particles could be seen within budding particles (Pl. 1, figs. 1, 2). Both immature (Pl. 1, fig. 2) and mature (Pl. 1, fig. 3) type B particles were present.

Negative staining

Bittner virus

Although the age of the mice at the time the tumour was taken differed by as much as 9 months and the state of the tumours varied from well differentiated to anaplastic, negative staining revealed virus particles in all the tumour extracts in a series of 25 (Pl. 2, fig. 4). The particles seen were identical with those described previously by others (Lyons & Moore, 1962; Parsons, 1963*a*). They were pleomorphic in form, generally in the size range 800–1000 Å., were bordered by a fringe 70 Å. long, and, although essentially pleomorphic, one particular form was frequently observed. This was a tailed particle with a head of approximately 1000 Å. and a tail of up to 3000 Å. in length (Pl. 3, fig. 6). Initially, no internal component was observed in the extracts of freshly excised tumours. However, tumours stored for periods of up to 6 months at − 20° C., when examined by negative

staining, were found to contain degraded particles whose number increased with time of storage. The envelope of such particles was penetrated by the phosphotungstic acid and this revealed the presence of an internal circular component (Pl. 3, fig. 8; Pl. 4, figs. 10, 11). Of the many degraded particles seen the outline of the internal body was always circular, and since the centre usually appeared darker than the rim it might be presumed to be a collapsed sphere. This central spherical body measured between 750 and 800 Å. in diameter and could be identified with the type A particles seen previously in negatively stained cell-spread preparations (Parsons, 1963*b*). This internal component revealed some surface structure in the form of poorly defined subunits, although these did not appear to be regularly arranged (Pl. 3, fig. 9). In places at the edge of the central body an appearance compatible with a two-layered structure could be seen (Pl. 3, fig. 9). Neither in general shape nor in subunit arrangement did the Bittner internal component resemble the capsid of any virus known to have cubic symmetry. The internal body was frequently seen in a state of disintegration (Pl. 4, fig. 10), but when this occurred no further structure was revealed. Disintegrating type A particles had a greater diameter than the intact forms (Pl. 4, fig. 10). Occasionally free-lying type A particles were seen in these preparations (Pl. 3, fig. 9).

Influenza virus

Pellets of the PR 8 strain of influenza when negatively stained revealed typical virus particles which, like Bittner virus, would be described as pleomorphic and having a distinctive fringe (Horne *et al.* 1960). The fringe on influenza virus was longer than that of the Bittner particles, measuring 100 Å. (Pl. 2, fig. 5). Filamentous forms were frequently observed but these could not be confused with the tailed particles described for the mouse mammary tumour virus, for although there is frequently a swelling at the ends of the influenza filaments they are not of the same dimension as the heads of the tailed Bittner virus particles (Pl. 3, fig. 7). Influenza particles in which the internal component was visible were not immediately identified on viewing these preparations.

Specimens of influenza virus that had been stored for varying times at $-20°$ C. were examined in the microscope, but unlike Bittner did not seem to lead to any increase in the number of penetrated particles. During this time it was discovered, almost accidentally, that if any of these influenza specimens, either fresh or frozen, were scanned with diligence a very small number of particles revealing internal structure could be found (Pl. 3, fig. 7). It was found that if a preparation of approximately 5×10^4 haemagglutinating units/ml. was used to prepare the grid then half an hour's scanning time at the electron microscope would reveal an average of two particles in which the internal component could be seen. This meant that we could establish a roughly quantitative basis for the assessment of breakdown in influenza virus preparations, and allowed us to confirm by a more objective means that storage at $-20°$ C. had not altered the influenza virus population.

It might be remarked that after establishing this finding a re-examination of freshly extracted Bittner virus particles showed that there too a small number of spontaneously degraded particles were present.

As has been previously described (Waterson, Hurrell & Jensen, 1962; Lovas & Takatsy, 1965; Apostolov & Flewett, 1965; Klimenko *et al.* 1966), the internal component of influenza virus is a coil formed from a strand approximately 60 Å. in diameter. According to our measurements the diameter of the whole coil varied between 400 and 600 Å. with the majority being close to 500 Å. It was only infrequently that a coil was sufficiently resolved for the number of turns in it to be counted, but in the most favourable instances the number of turns was found to be close to 10. In both influenza and Bittner virus preparations particles were observed that contained more than one internal component (Pl. 4, figs. 11, 12).

DISCUSSION

Until now it has been presumed that virus particles having similar external appearances might be expected to contain internal components belonging to the same symmetry group. For example, the many different types of para-influenza viruses, Newcastle disease virus, mumps, and the measles–rinderpest–distemper group, although differing in many of their properties, have within their similar envelopes indistinguishable helical structures. This has been established readily, as all of these viruses disrupt spontaneously and allow examination of the internal component in any negatively stained electron microscope specimen. However, Bittner and influenza viruses have not yielded so easily to study, because they are not spontaneously disrupted, and treatment with organic solvents, although disintegrating the virus, does not allow the internal component to be readily seen (Hoyle, Horne & Waterson, 1961; Lyons & Moore, 1965). The technique used in this study (storage at $-20°$ C.) was most successful in revealing the internal component of Bittner virus, but unsuccessful with influenza. However, a small number of spontaneously disrupted particles of influenza virus were eventually found, making it possible to carry out a comparative study of the two internal components.

It was rather surprising to find that influenza and Bittner virus, although externally similar, contained totally different internal components. It is taken for granted that as the influenza internal component is in the form of a strand of ribonucleoprotein (RNP) it will have underlying helical symmetry, although this has not as yet been satisfactorily resolved in the electron microscope. For this reason influenza virus is generally grouped with those viruses having compound helical structure (Waterson & Almeida, 1966). On the other hand, Bittner virus, also an RNA virus (Lyons & Moore, 1965), would now appear to have a spherical internal component. Until now a virus having an apparently spherical nucleocapsid has almost always been shown in the electron microscope to be built of regular, repeating sub-units arranged to give cubic symmetry (Almeida, 1963). We have not been able to show any regularity in the arrangement of the rather poorly defined subunits forming the internal component of Bittner virus, neither is there any suggestion of the hexagonal outline associated with cubic viruses. This may be because Bittner virus has a type of morphology not previously encountered in an animal virus; on the other hand, it may be that the Bittner particle does belong to

one of the two main virus symmetry groups, cubic or helical (Caspar & Klug, 1962), but we have not been able to see the symmetry-bearing component. For example there may be a ribonucleoprotein helix within this spherical component or there could be cubic symmetry basic to the surface structure that we have seen on the spherical component. It is interesting that when the superficially similar Rauscher mouse leukaemia virus was examined by negative staining, one group of workers (de-Thé & O'Connor, 1966) interpreted their micrographs as revealing helical structures while another group using snake venom to degrade the particles demonstrated a cubic component (Padgett & Levine, 1966).

Although the Rauscher virus does not have the distinctively fringed outer envelope exhibited by Bittner virus the overall morphology of the two particles is similar (Padgett & Levine, 1966). This gives rise to the rather interesting speculation that although the events of recent years, and most particularly those concerning such viruses as polyoma and SV 40, have tended to break down the barrier between oncogenic and lytic viruses (Howatson, 1962), there may be a group of RNA tumour viruses having their own distinctive morphology.

In addition to contrasting the internal structures of influenza and Bittner viruses we were also interested in correlating the negatively stained appearance of Bittner virus with that revealed by thin sectioning. Measurements on negatively stained preparations showed that the internal component, or type A particle, had an average diameter of 750 Å. and the enveloped particle varied within the size range 1000–1500 Å., values that are in good agreement with those obtained by thin sectioning. It is generally accepted that at least some of the type B particles are formed when type A particles move up to, and pass through, the cell membrane. Plate 1, fig. 1, shows a cell border where this is happening, and it can be seen that the internal component of the type B particle is identical with free-lying type A particles in the cytoplasm. The evidence that we present from negative staining shows that particles of a type previously described as type A by negative staining do in fact form the internal component of the more mature particle.

The number of responses to a virus that can be produced by a cell is probably quite limited and hence it is not surprising that a cell membrane with a similar morphological alteration should in one case enclose the type A particles of the mouse mammary tumour and in another case the RNP coil of influenza virus. Similarly, it is not surprising that the widespread viral changes on the surface of the influenza-infected cell would seem to be present also in the Bittner cell surface. For example, when budding particles of the type present in Pl. 1, fig. 2, are seen in negatively stained preparations (Pl. 3, fig. 6), it can be shown that the distinctive projection-covered membrane enclosing the head of the particle also extends the full length of the tail.

The correlation between negatively stained and thinly sectioned immature type B particles of Bittner now seems to be well founded, but the role of the mature type B particle still remains obscure. Negatively stained preparations revealed nothing that would correspond to the mature type B particle of thin sectioning. The only variation seen in the morphology of negatively stained type B particles in which the internal component was visible was a dispersion of the internal body with a

resultant increase in diameter (Pl. 4, fig. 10). Lyons & Moore (1965) suggest that the mature type B particle has a decreased infectivity, and it is possible that the mature type B particle is a degenerate form of the immature type B particle and that when the internal body has lost its integrity it is much more susceptible to the shrinking action of fixatives or dehydrating agents. However, this is merely speculation.

These findings suggest that it may be dangerous to associate viruses too closely on the basis of their external appearance alone. Influenza and the mouse mammary tumour provide an example of two such viruses, as they were found to contain completely dissimilar internal structures. The nucleoprotein component of influenza virus is in the form of a coiled coil approximately 500 Å. in diameter, while the Bittner virus contains a spherical component built up of poorly defined subunits that lack the characteristic pattern of cubic viruses, although this may still be present.

SUMMARY

By the negative staining technique both Bittner and influenza viruses are pleomorphic and have similar fringed surfaces. The present study revealed that Bittner virus had one characteristic form consisting of a head approximately 1000 Å. in diameter and a tail up to 3000 Å. long. On storage at −20° C. Bittner virus broke down to reveal a round internal component of 750 Å. diameter. Influenza virus did not break down on storage at −20° C. but a small number of spontaneously disrupted particles revealed that the internal component was in the form of a coil. The circular internal component of Bittner virus is presumed to be spherical and corresponds to the previously described type A particles as seen by negative staining. The complete enveloped particle corresponds to the type B particle of thin sectioning. It is suggested that the thinly sectioned mature type B particle may be a degenerate form of the so-called immature type B particle. In addition, it is suggested that certain murine RNA tumour viruses may have a morphology distinctive to them.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Part of the border of an acinar cell in a mouse mammary tumour. The cytoplasm contains several doughnut-shaped type A particles, two of which are near to the plasma membrane; in the case of one of them (arrowed) a change can be seen in the cell membrane where the type A particle has made contact with it. Superficial to the cell is another type A particle contained within a cell process and almost completely enrobed in an additional membrane. This enveloped particle would now be referred to as an immature type B particle. × 110,000.

Fig. 2. Another cell edge with several immature type B particles budding from it. At the top and right a number of type A particles can be seen in contact with the cell surface. The extracellular immature type B particles almost all retain a trailer of cell material. Note the constriction at the neck of these particles, a feature present also on the negatively stained particles. (Pl. 3, fig. 6.) × 110,000.

Fig. 3. At the bottom left of this micrograph immature type B particles can be seen budding. Farther out in the lumen of the acinus mature type B particles are present. These are typified by the shrunken nucleoids and less well organized appearance. × 110,000.

PLATE 2

Fig. 4. A group of negatively stained Bittner virus particles from a freshly prepared tumour extract. The particles are pleomorphic and display a distinctive fringed surface. Although there is a suggestion of internal structure in one or two of the particles no definite structure can be seen. × 200,000.

Fig. 5. A group of negatively stained influenza virus particles. As with the Bittner virus particles in Fig. 4 they are fringed, pleomorphic bodies. Like the vast majority of influenza particles none of these particles reveals any internal structure. × 200,000.

PLATE 3

Fig. 6. Although Bittner virus particles are pleomorphic the pattern illustrated here was frequently observed. The particles consist of a well-defined head of approximately 1000 Å. in diameter and a tail which may measure up to 3000 Å. in length. The viral envelope extends to the tail and indicates that the immature type B particles such as are seen in Fig. 2 have virally altered membranes not only around the head of the particles but also extending along the trailer of cell material. Once again these particles are from a freshly prepared extract and no internal component can be seen. × 300,000.

Fig. 7. This micrograph illustrates two features of an influenza virus preparation. The upper part of the figure contains the end of an influenza filament. Like the tail of the Bittner particles in Fig. 6 it is covered by a fringed membrane; unlike Bittner, however, there is no distinctive head at the end. Below this filament is one of the rare influenza virus particles which has spontaneously disrupted and allowed the phosphotungstic acid to delineate the internal structure. This is a hollow strand 60 Å. in diameter wound in a coil approximately 500 Å. in diameter. It is presumed that the strand forming the coil will itself be in the form of a coil or helix. × 300,000.

Fig. 8. The typical appearance of a Bittner particle from a tumour stored for several months at −20° C. About half the particles had changed in permeability and allowed penetration of the PTA. The internal component invariably appeared round as seen here, and is therefore presumed to be spherical in form. The fact that the centre almost always appeared darker makes it seem likely that the sphere is a collapsed one. × 300,000.

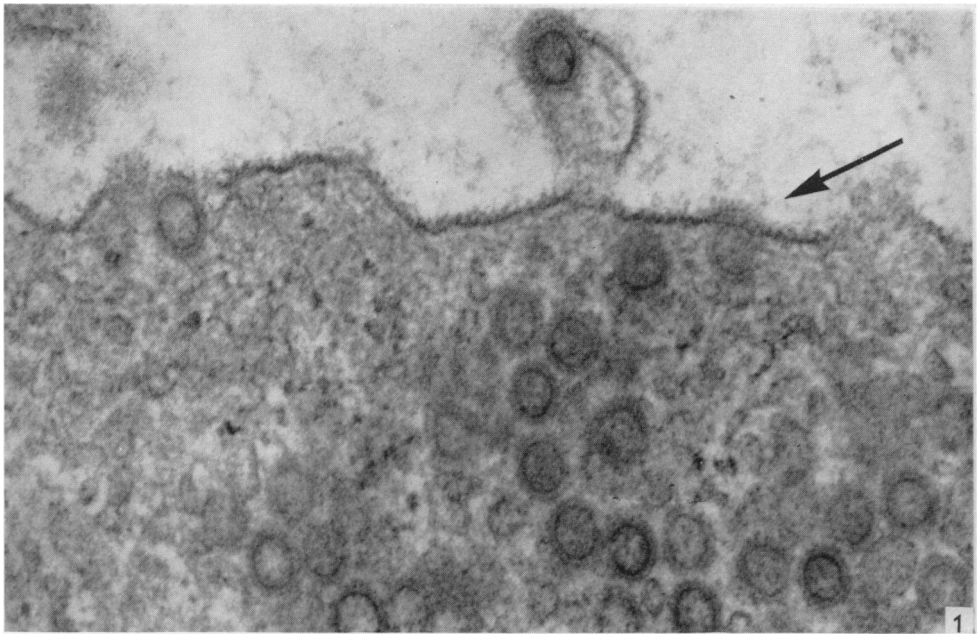
Fig. 9. Occasionally free type A particles were found in the stored preparations. Since these have no outer membrane it is easier to see that these bodies have at places (arrow) a two-layered structure around the periphery and are composed of rather poorly defined subunits. A structure like this, if it had cubic symmetry, would have a hexagonal outline rather than the form seen. × 300,000.

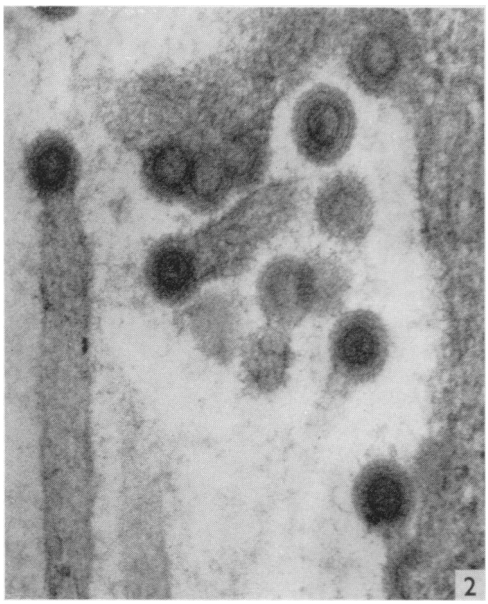
PLATE 4

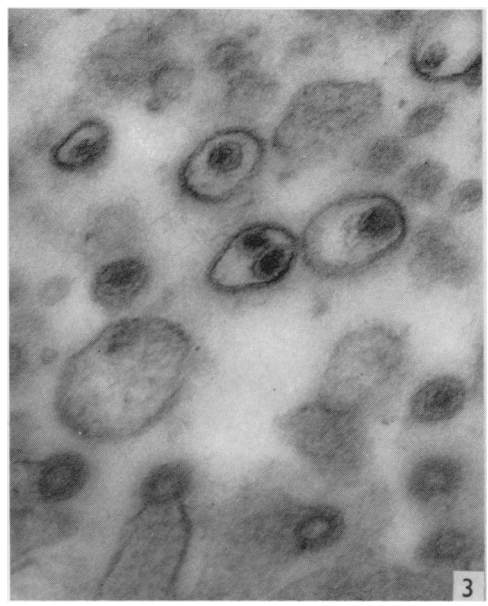
Fig. 10. Of the many PTA-penetrated Bittner particles studied no forms corresponding to the mature type B particle of thin sectioning were found. However, several of the internal bodies did appear to be in a state of disintegration, as is illustrated in the right-hand particle. No further structure was revealed when this occurred and it is suggested that although a disintegrating central body appears larger by negative staining it might shrink on fixation and dehydration and so appear smaller in thin sections. × 300,000.

Fig. 11. A Bittner virus particle containing two central bodies. Multiple internal components were found in both Bittner and influenza viruses. × 300,000.

Fig. 12. An influenza virus particle containing several internal components for comparison with fig. 11. × 300,000.







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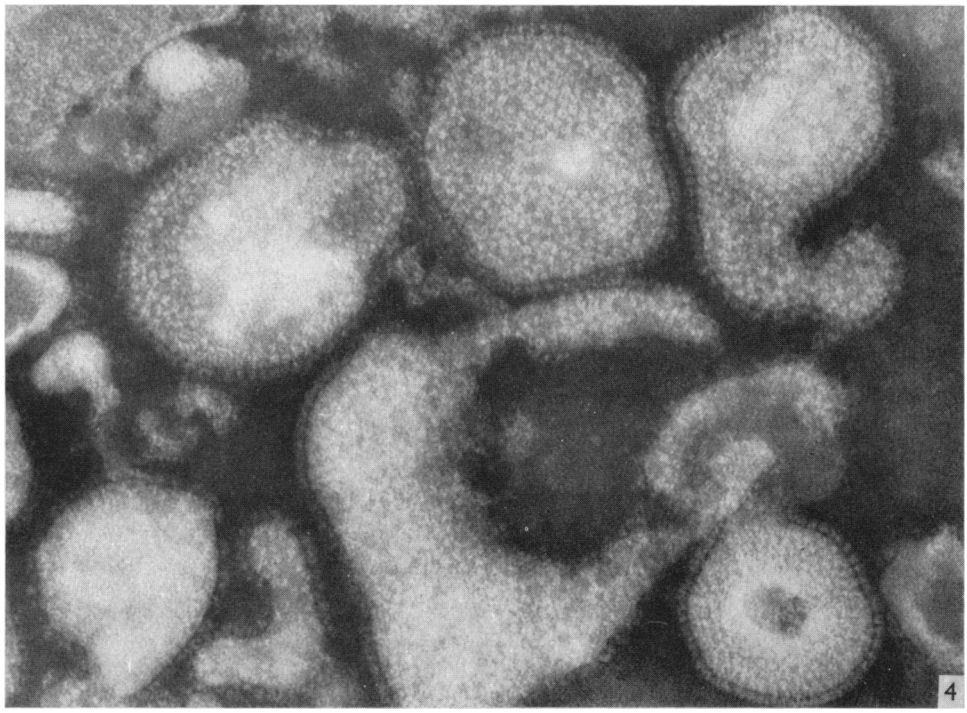
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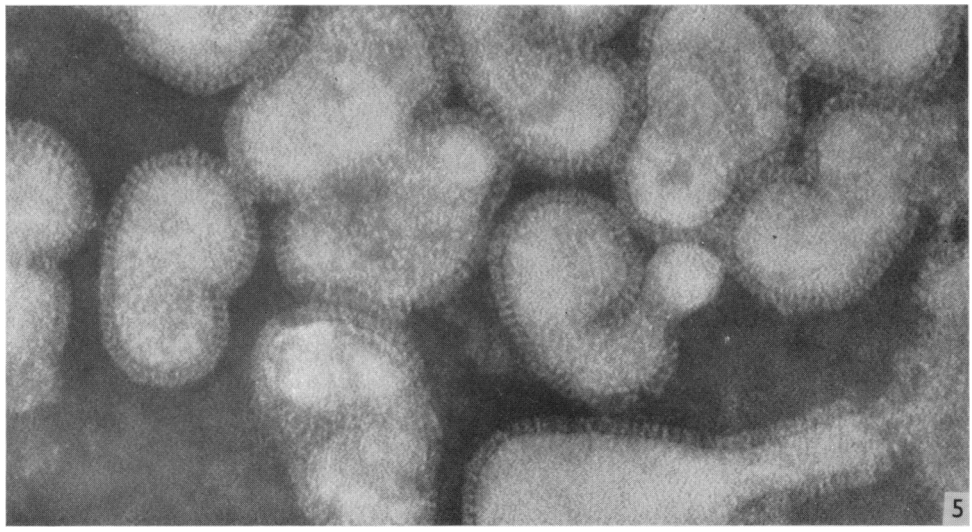
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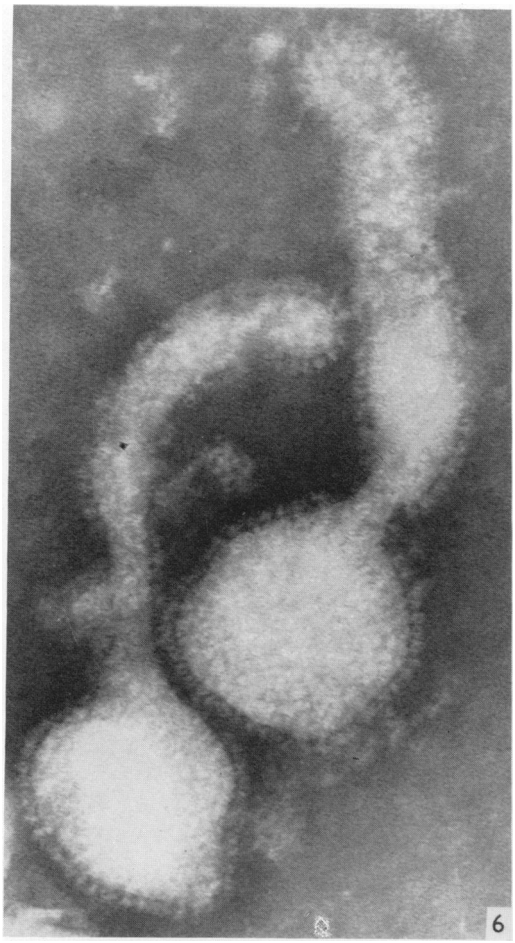
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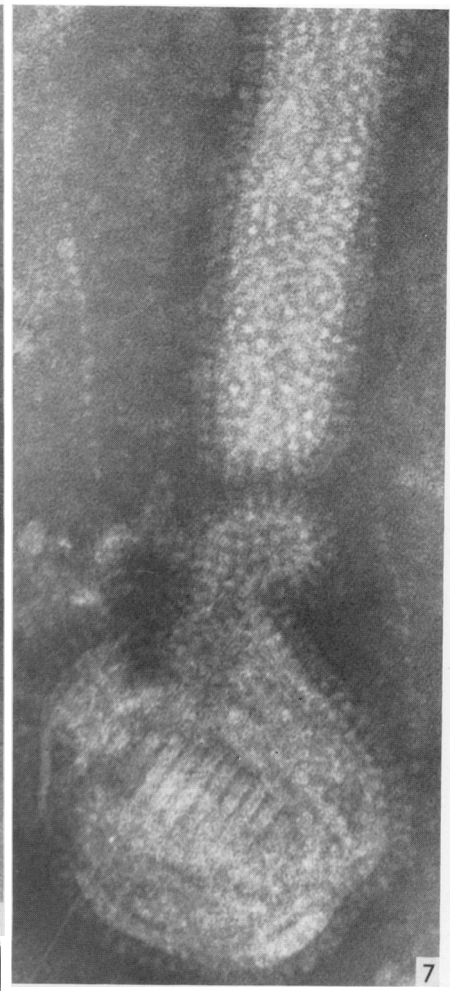
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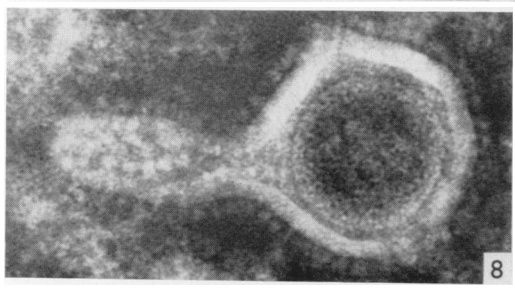
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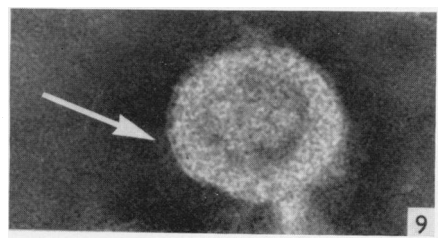
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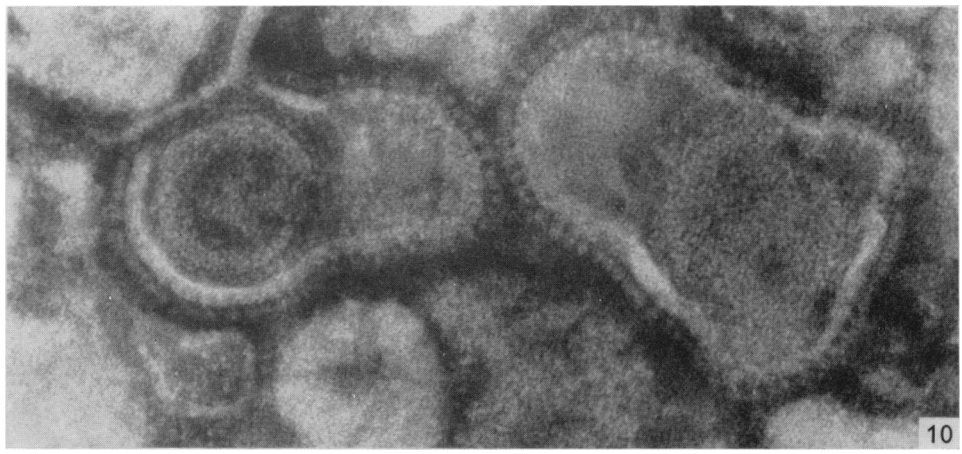
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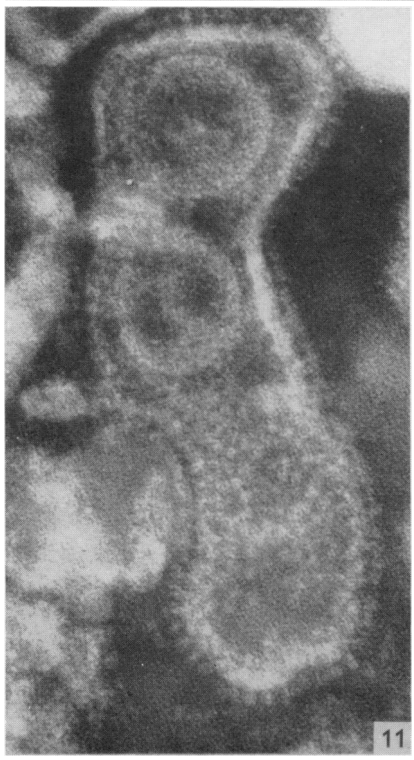
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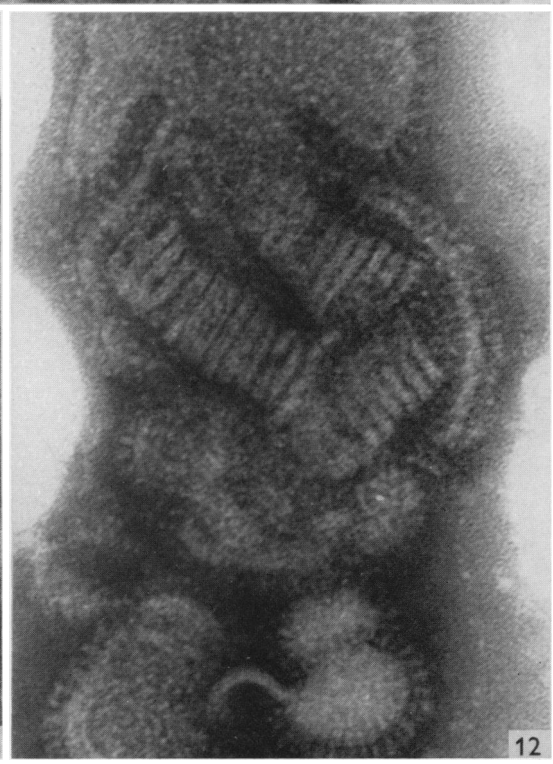
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