BIOLOGICAL AND ULTRASTRUCTURAL STUDIES ON HERPES SIMPLEX VIRUS TYPES 1 AND 2 IN EGGS

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SUMMARY

The relevant published literature concerning the nature of herpes simplex virus and its growth in fertile hens' eggs was reviewed.

Laboratory strains and fresh isolates of types 1 and 2 were grown on the chorioallantoic membrane of fertile hens' eggs and examined by biological, histolcgical and ultrastructural techniques. The type 1 strains induced small discreet pocks, gave no haemorrhage of the chorioallantoic membrane, or embryo, embryos did not die and virus was recovered only from the inoculated membrane. Similar inoculation with the type 2 strains induced large necrotic pocks and haemorrhage of the chorioallantoic membrane as well as haemorrhage and death of the embryo; virus was recovered from the inoculation site, allantoic fluid, amniotic fluid and various selected organs of the embryo. Inoculation of either virus type into the allantoic cavity did not result in spread to the embryo. The effects of adaptation to growth in eggs were examined.

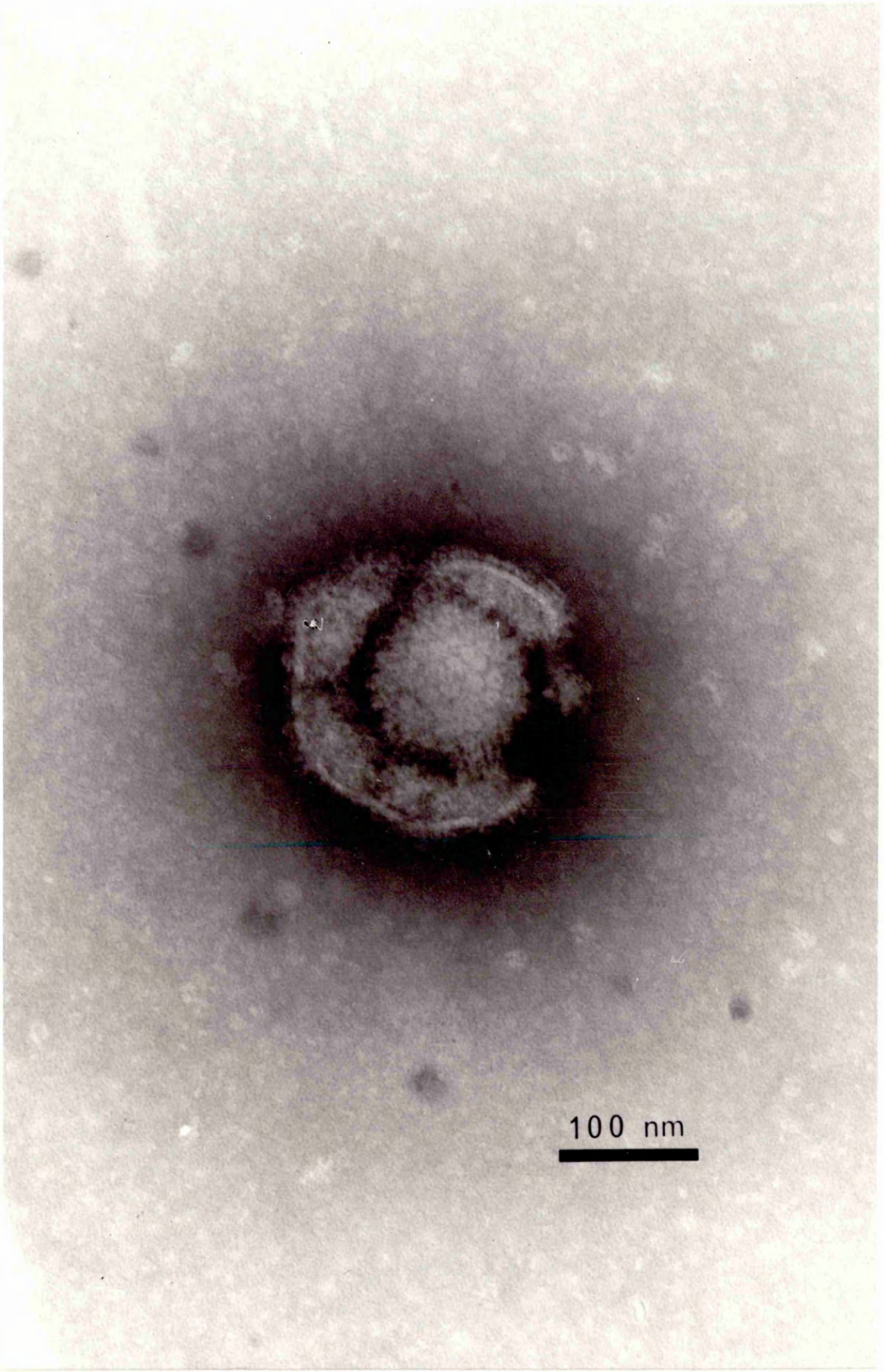
Temperature marker tests in eggs showed that fresh isolates of type 1 grew less readily on the chorioallantoic membrane at elevated temperatures than those of type 2. There was no difference in the capacity of laboratory strains of either type to grow in eggs at these temperatures.

Primary chick embryo fibroblasts and other egg-derived cell cultures were used to examine the growth characteristics of a strain of each virus type; the results obtained *in vitro* could not be entirely predicted from those *in ovo*.

The structure of the chorioallantoic membrane and the lesions produced by each virus type following inoculation were examined by optical and electron microscopy. The type 1 induced lesion was basically proliferative and confined primarily to the chorion with some inflammatory cell infiltration into the mesoderm, particularly following prolonged incubation. With the type 2 lesion, reaction occurred throughout the entire thickness of the membrane and haemorrhages, necroses, ulceration and cellular infiltration of the mesoderm were the most prominent features. The fine structure of the herpesvirus lesions and of viral morphogenesis was examined. Inoculation with type 2 virus resulted in many more infected chorion cells compared with type 1, whilst the cells of the mesoderm and the blood vessels also became infected with type 2 virus but not type 1. Features specific to type 2 virus infected cells were the presence of two types of intra-nuclear granules and lattice structures in both nuclei and cytoplasm. Cores with various structured forms were also found in type 2 capsids but not in those of type 1.

The results of this study were discussed in relation to other published work.

C O N T E N T S



Herpes simplex virus type 1 strain 22101/71 negatively stained with 3% phosphotungstic acid (x240,000)

PLATE 1

I N T R O D U C T I O N

1 Historical aspects of the human disease "herpes"

The word "herpes" (Greek = to creep) has been in use since early Greek medicine to describe spreading lesions of varied etiology. Hippocrates used it to describe eruptions resembling shingles. About 100 AD Herodotus, a Roman physician, first described oral herpetic eruptions during fevers and this association was stressed by Morton in 1694. In 1736 the French physician Astruc described similar eruptions on the male and female genitalia. The name "herpes simplex" was first applied by Plenck (1783) to describe vesicular eruptions of the face. At this time the cause of herpes was believed to be excretion of harmful waste material by the skin, a view which was held until Vidal (1873) demonstrated by human inoculation that herpes was infectious. Unna (1896) histologically examined the lesions of herpes and recorded differences in cellular patterns to distinguish herpes infections from those of smallpox. The first isolation of herpesvirus was from a human eye infection onto the rabbit cornea. From there the virus was successfully passaged onto the cornea of a blind man (Grüter 1920). Baum (1920) used the same system to isolate virus from cases of genital herpes infections. Subsequently Luger and Lauda (1921) demonstrated the filterability of the agent of herpes infections. Lipschütz (1921) described intranuclear inclusion bodies in infected cells which were characteristic of herpesvirus infections and Baumgartner (1935)

demonstrated that these inclusions contained infectious virus, whilst Cowdry (1934) critically reviewed the types of inclusions formed by various viruses including herpes. He classified as Cowdry type A the inclusions produced by herpesvirus which were pleomorphic, resistant to organic solvents and contained little or no thymonucleic acid. Nuclear involvement was complete with eventual destruction and the inclusion was separated by a clear halo from the chromatin which eventually became marginated (i.e. condensed at the periphery of the nucleus).

Lipschütz (1921 and 1932) suggested that herpes simplex infections should be divided into two groups (herpes febrilis and venereal herpes). On the basis of epidemiological, clinical, cytopathological and animal transmission studies he made a number of interesting observations. Outbreaks were caused by viruses of either group and did not appear to be interrelated. Herpes febrilis affected persons of all ages, usually resulting in facial or oral infections whilst venereal herpes affected only the sexually mature, usually on the genitalia. Venereal herpes frequently caused secondary complications including neurological symptoms but febrilis rarely did, tending secondarily to affect only the eye rather than the complete central nervous system. The lesions of venereal herpes penetrated deeper into tissues and were characterised by greater necrosis than those of herpes febrilis. Prior infection of the cornea with venereal herpes did not protect against re-infection with herpes febrilis but did protect against re-infection with venereal herpes. The lesions on the rabbit cornea infected with material

from human lesions differed with respect to appearance and time of development according to the site of origin of the inoculum: lesions caused by venereal herpes developed later and had a more destructive effect and generally did not progress to encephalitis, as was commonly the case when herpes febrilis material was inoculated into rabbits. These observations were ignored at the time and the term "herpes simplex virus" was applied to the causal agents of both infections, whilst the term "herpes" was used to describe both herpes simplex and herpes zoster syndromes.

2 Nomenclature of herpes simplex virus

With improvements in methods for virus culture, especially the introduction of cell cultures, there has been an increasing amount of work performed on herpes simplex virus and the other members of the rapidly expanding herpesvirus group (*Herpetoviridae*). This has led to the need for an efficient system of classification and nomenclature (Wildy 1971, Roizman *et al.* 1973, Melnick 1974, Fenner 1976). No one form of nomenclature for the virus of herpes simplex has been adopted by all the workers in the field. For example the names herpesvirus, human herpesvirus 1, herpes simplex virus type 1, *Herpesvirus hominis* type 1, *Herpesvirus primatis 1:1* and the cryptogram D/2, 68/7, S/S, V/O, are all synonyms for the virus isolated from non-genital lesions of herpes simplex. The latinised binomial, *Herpesvirus hominis*, has been recommended (Wilner 1966, Lwoff and Tournier 1966) but this gives rise to some misconceptions

suggesting that there is an accepted classification for the herpesvirus group and that the virus is the only member of the herpesvirus group infecting man or that it will infect man and no other species (Roizman *et al.* 1970). The two remaining systems of nomenclature, neither of which assumes a particular classification scheme, are the use of cryptograms (Wildy 1971) or trivial names. Cryptograms would be unacceptable for normal use because of their unwieldiness, whilst their precise nature rapidly renders them outdated. On the other hand, trivial names give little information about the properties or the inter-relationship of viruses but usually have historical precedence. Gibbs and colleagues (1966) suggested the use of the trivial name together with the cryptogram. The trivial name, herpes simplex virus, seems at present the least problematic one and should be the name of choice until an acceptable classification for the group has been developed when a more permanent nomenclature can be devised.

In this study the name herpes simplex virus (HSV) is used and the term 'type' is used to distinguish between non-genital (type 1) and genital (type 2) isolates of the virus.

3 Properties of the *Herpetoviridae*

Herpes simplex virus is a typical member of the *Herpetoviridae* (Fenner 1976). All members of the group have identical morphology: a capsid with icosahedral symmetry, 100 nm. in diameter, composed of 162 apparently hollow capsomeres and surrounded by an outer envelope

(plate 1). All the herpesviruses contain double-stranded deoxyribonucleic acid (DNA) which comprises approximately 7% of the particle by weight, whilst the molecular weight of the DNA, which varies according to method of measurement, is approximately 54-100 x 10^6 daltons. The guanine + cytosine (g+c) content ranges from 54-74% and the buoyant density in caesium chloride of the complete particle varies between 1.27 - 1.29 gm. per $cm.^3$, of the naked nucleocapsid 1.29 - 1.32 gm. per $cm.^3$, and of the viral DNA 1.692 - 1.731 gm. per $cm.^3$. Herpesviruses are relatively heat stable and because the envelopes contain lipid, they are sensitive to lipid solvents such as ether. Viral capsids are assembled in the nucleus of infected cells and maturation is usually completed by the acquisition of a virus specified lipo-protein envelope prior to release from the cells (Morgan *et al.* 1954b, 1959). This envelope is thought to be essential for infectivity (Holmes and Watson 1963, Darlington and Moss 1969, Becker *et al.* 1971).

Melnick and McCombs (1966) suggested that the *Herpetoviridae* should be divided in two: group A to include HSV, pseudorabies, B virus and equine abortion virus, all viruses which are readily released from infected cells into the culture fluid in an infectious form, and group B to include varicella zoster, EB virus, cytomegaloviruses and Marek's disease virus, which are all strongly cell-associated.

4 Properties of herpes simplex virus

4.1 Subdivision of herpes simplex virus into two types

On the basis of clinical, epidemiological, antigenic, serological and biological differences occurring between strains of HSV it is clear that the virus is not homogeneous although the criteria for dividing strains into types are not entirely clear. Dowdle and co-workers (1967) used kinetic neutralisation tests and demonstrated that some strains of HSV caused oral infections whilst other strains were responsible for genital infections, and they called these strains types 1 and 2 respectively. Other groups of viral agents, for example adenoviruses, had been divided into types using three criteria: the display of antigenic differences in cross-neutralisation tests with established prototype strains; the establishment of at least two instances of higher homotypic antibody responses compared with already established types, and the occurrence of at least two strains within the type proposed (Rowe *et al.* 1955). If these same criteria are applied to HSV then division into two types is valid. Plummer and colleagues (1970) pointed out that if the serological differences between strains of HSV were insufficient to warrant their division into two types, then herpes B virus must be regarded as a sub-type of HSV since the serological differences between these were of the same order. Roizman and co-workers (1970) suggested that strains of HSV existed within a bi-polar spectrum rather than in two distinct types, but this may only be the case for laboratory strains after numerous *in vitro* passages and ample opportunity for mutation and selection.

That there are two types of HSV is indicated by laboratory and clinical data which correlate well with the site of virus origin. Those properties related to type differentiation of HSV together with some of the more general properties relevant to both types of the virus are elaborated in the following sections, and these are summarised in table 1 (pp. 28-31).

4.2 Physico-chemical properties of herpes simplex virus

Many of the physico-chemical properties of HSV have been shown to be similar for types 1 and 2. The density of the complete particle of each of the two virus types was found to be of the same order (Schiek 1967), whilst the molecular weight of their DNA was also observed to be similar (Kieff *et al.* 1971, Graham *et al.* 1972). Some significant differences between the two types have been found with regard to the buoyant density in caesium chloride and the G+C ratio of their DNA (Plummer *et al.* 1969, Kieff *et al.* 1971, Graham *et al.* 1972, Halliburton *et al.* 1975). HSV type 2 was found to be more sensitive to ultra-violet irradiation (Smith *et al.* 1971) and to some chemical agents (Schneweis *et al.* 1972, Shinkai 1975) but not to others (Plummer *et al.* 1968, Vahlne *et al.* 1975, Schneweis *et al.* 1972, Lerner and Bailey 1972).

The major physico-chemical properties of HSV types 1 and 2 are listed in table 1.

4.3 Antigenic properties of herpes simplex virus

The value of dividing HSV isolates into two different types depends upon the use such a division would have in revealing the nature of the virus or the relationship of isolates to each other and to the disease produced. The

division of HSV into two types could be considered of academic importance only, but in the light of recent knowledge concerning latency and persistent infections and connecting HSV with carcinoma and neurological disorders, the need to distinguish type-specific isolates and type-specific antibody responses becomes of increasing medical importance.

Early attempts to differentiate strains of HSV on antigenicity produced divergent results. Burnet and Lush (1939) observed no antigenic differences between a laboratory strain and a fresh isolate, both from non-genital infections, whilst Armstrong (1943) could not differentiate a spinal fluid isolate from a laboratory strain. Similar results were found by a variety of neutralisation techniques with strains isolated from recurrent infections (Hayward 1950, Garabedian and Syverton 1955) and with strains isolated from throat lesions (Kilbourne and Horsfall 1951). The strains used in these studies were all non-genital in origin.

Significant antigenic differences between isolates of HSV were first observed by Slavin and Gavett (1946a). They compared a laboratory strain from a non-genital infection with genital isolates from primary vulvo-vaginitis by mouse neutralisation tests and showed antigenic differences between these strains. These differences were confirmed using strain-specific rabbit antisera in cross-neutralisation tests (Slavin and Gavett 1946b). Womack and Hunt (1954) divided six strains of unknown history into three groups using cross-complement fixation tests, but differences were slight and may have

resulted from variation within the test. Jawetz and co-workers (1955) examined numerous strains by cross-neutralisation tests in mice and eggs and found that strain differences could be correlated with site of virus origin. Chu and Warren (1960) and Schneweis (1967) differentiated their strains into two groups by neutralisation tests in cell culture. Plummer (1964) could separate strains into two groups with type-specific antisera to either type, but Schneweis (1962a) could do so only with type 1 antiserum, indicating that the members of the type 1 group were closely related, whilst those of type 2 were more heterogeneous. Shubladze and co-workers (1960) divided their strains into three groups with mouse neutralisation tests, but Plummer (1964) showed that only two of these groups were in fact HSV, and the third was Japanese B encephalitis virus. Pauls and Dowdle (1967) and Dowdle and co-workers (1967) found by the micro-quantal neutralisation technique that Shubladze's two HSV groups were prototypes for two antigenically distinct groups of HSV, and all other strains fitted into one of these two groups, which correlated well with site of virus isolation. Hampar and co-workers (1970, 1971) showed that late IgM rabbit antibodies to types 1 and 2 were very sensitive for differentiating strains. Smith and colleagues (1971) using cloned preparations found that strains bred true to type for at least 20 *in vitro* passages.

Numerous serological tests have been devised for the differentiation of the two types of HSV and include micro-indirect haemagglutination (Fuccillo *et al.* 1970),

immuno-fluorescence of fixed cells (Nahmias *et al.* 1969), indirect fluorescence (Leinikki 1973, Doerr *et al.* 1974), membrane fluorescence (Géder and Skinner 1971, Nahmias *et al.* 1971), *in vitro* stimulation of immune lymphocytes (Rosenberg *et al.* 1972), ^{51}Cr release (Smith *et al.* 1972), immuno-electrophoresis (Jeansson 1972, Vestergaard 1973) and immuno-diffusion and inhibition-passive haemagglutination (Schneweis and Nahmias 1971). These type-specific tests have been carried out with varying degrees of success, and their value depends upon the specificity of the serological reagents and the ease of performing the tests.

4.4 Morphology and morphogenesis of herpes simplex virus

Electron microscopic methods were first applied to HSV by Evans and Melnick (1949) and later by Munk and Ackermann (1953). These workers used metal shadowing and found that particles were 116 nm. in diameter and each had a central core.

The technique of negatively staining virus particles with heavy metal stains such as phosphotungstic acid to study the surface morphology was introduced by Brenner and Horne (1959) and was a simple reproducible procedure for the examination of virus particles at high resolution. HSV was one of the first animal viruses studied by this technique (Horne *et al.* 1959) and it was found that the virus surface was composed of regular sub-units (capsomeres), each of which was ring shaped. Wildy, Russell and Horne (1960) examined HSV and found that most particles consisted of a core surrounded by this characteristic shell of capsomeres termed the capsid. This structure was enclosed within a loose membrane envelope bearing a surface fringe

of projections. Depending upon whether or not the negative stain had penetrated the capsids, preparations of virus contained four particle types: enveloped 'full' particles, enveloped 'empty' particles, naked 'full' capsids and naked 'empty' capsids. The inner region of the 'empty' or penetrated particles was polyhedral in shape and measured 70-80 nm. in diameter, within which lay the 30 nm. diameter nucleic acid and protein core. Horne and colleagues (1959) suggested that the herpes capsid was icosahedral in form. This was confirmed by Wildy and co-workers (1960), who also noted that the capsid had an average diameter of 105 nm., and calculated that it was composed of 162 capsomeres, each approximately 10 nm. in diameter, arranged in a 5:3:2 axial symmetry. Each capsomere was tubular in shape with a centrally placed 4 nm. hole down the long axis. Capsomeres appeared hexagonal in end-on views, although some pentagonal capsomeres were present. Casper and Klug (1962) postulated that 150 capsomeres were hexamers and 12 vertical capsomeres were pentamers. Vernon and co-workers (1974) suggested that individual capsomeres exhibit a threefold symmetry and are held together by inter-capsomeric fibrils.

The enveloped particles were 180 nm. in diameter and were frequently covered with spikes 8-10 nm. long and 5 nm. apart. By immune electron microscopy Watson and Wildy (1963) showed that the envelope was host cell derived and could be agglutinated by antiserum to the host cell in which the virus was grown, whilst the capsid was virus-specific and no cross-reactions occurred.

There is no morphological difference between HSV type 1

and type 2 particles (plate 1).

Most of the information available on the surface structure of HSV particles has been derived by negative staining methods, and even with the limit on resolution of 2 nm. imposed by the technique, the particle morphology has been well described.

The morphogenesis of HSV is well documented and appears to be similar for both types but there are still some unknown features. The earliest work using sectioning techniques was performed in the chorioallantoic membrane of the fertile hen's egg by Morgan and co-workers (1953), but as the method used involved removal of the embedding matrix followed by metal shadowing little useful information was obtained. Pollard (1953) suggested a theoretical structure for HSV of a core with an outer envelope, which was confirmed by Morgan and colleagues (1954a, b). These workers observed that HSV particles developed in the nucleus of infected cells where they acquired a single membrane, and that a second membrane was obtained on passing into the cytoplasm. They described three structures in infected nuclei: small dense primary bodies 30-40 nm. in diameter, larger less dense bodies 40-50 nm. in diameter, which were frequently surrounded with a membrane making a particle 70-100 nm. in diameter. In the cytoplasm the larger less dense objects were present but these were surrounded by a double membrane and were 120-130 nm. in diameter. Morgan and co-workers (1958) described crystal-like formations of virus particles in the infected nuclei of HeLa cells. The arrangement of particles within these crystals has been described as

hexagonal (Morgan *et al.* 1959, Friedrich *et al.* 1969, Miyamoto 1971, Nii and Ono 1971) or pentagonal dipyramidal (Melnick *et al.* 1968).

An in-depth study of the replication of HSV by thin sectioning of infected cells was conducted by Morgan and co-workers (1959) who showed that the morphogenesis of the virus was similar in the chorioallantoic membrane, HeLa and human amnion cells. They noted dense granules near the site of viral synthesis and suggested that the two may be interrelated. Enveloped virus was present in the nucleus, whilst in the cytoplasm particles occurred in vacuoles by which means it was suggested they left the cell. These workers also noted the phenomenon of nuclear membrane reduplication which presumably prevented rupture of the nucleus due to extensive budding of virus particles into the cytoplasm. These observations were confirmed by various workers (Falke *et al.* 1959, Epstein 1962, Watson *et al.* 1964, Nii *et al.* 1968a). Holmes and Watson (1963) using BHK 21 cells examined HSV replication and noted that virus disappeared for the first three hours after inoculation, but this was followed by a sharp increase in the number of virus particles in the cells and culture medium.

Two hypotheses have been suggested for the mode of entry of HSV into cells. Holmes and Watson (1963) suggested a method of pinocytosis or "viropexis", after which the envelope was digested leaving naked capsids in the cytoplasm. Similar features were observed by Epstein and co-workers (1964), Siegert and Falke (1966), Dales and Silverberg (1969), and Hummeler and colleagues (1969). These latter workers used autoradiographic techniques to

demonstrate isolated cores in the cytoplasm of infected cells with complete breakdown of virus particles in the cytoplasm prior to the appearance of new particles in the nucleus. On the other hand, Morgan and co-workers (1968) suggested that enveloped virus entered cells by fusion of the virus envelope with the cell plasma membrane. This would involve digestion of the membranes in apposition followed by passage of the capsid directly into the cytoplasm. Irrespective of mode of entry, capsids would be broken down in the cytoplasm and the core released. This would be followed by an eclipse period after which new virus appeared in the nucleus. Miyamoto and co-workers (1971) used ferritin label immune electron microscopy and noted that HSV antigens entered the nucleus by the nuclear pores.

Numerous changes occur in the cytoplasm and nuclei of infected cells. Dense granules of variable size, often associated with virus capsids, have been noted (Watson *et al.* 1964) and these have been shown to contain virus antigens by immuno-ferritin techniques (Nii *et al.* 1968c, Miyamoto 1971). The production of particles with electron-dense, less electron-dense and hollow cores have been described (Watson *et al.* 1964, Nii *et al.* 1968a, Nii 1971). Nii and co-workers (1968b, a) indicated that hollow cored particles did not contain DNA and may be particles at an early stage of infection. Furlong, Swift and Roizman (1972) indicated that the core of HSV is composed of DNA and protein arranged in the form of a toroid or doughnut, and similar results were found for HSV and other herpesviruses (Nii and Yasuda 1975). Schwartz and Roizman (1969a)

have reported that the cores of HSV type 1 appeared spherical and electron translucent in section, whilst those of HSV type 2 were often ribbon shaped and electron-dense. These workers also noted that the frequency of intranuclear crystal formations of virus particles was greater with a fresh isolate of HSV type 1 than with HSV type 2 strains. Murphy and colleagues (1967) recorded the presence of intranuclear filaments in mouse brain infected with HSV and they suggested that if the hexamere sub-units were packed together at an angle to each other then a tubular structure would result. The virus used by these workers was later found to be a type 2 strain (Schwartz and Roizman 1969a). Following HSV type 2 inoculation, similar filaments and lattice structures 22 nm. in diameter were observed in HEp2 cells (Schwartz and Roizman 1969a), in ectodermal and mesodermal cells of chorioallantoic membranes (Couch and Nahmias 1969, Baroni *et al.* 1970), in BHK 21 cells (Smith *et al.* 1973) and in Vero cells (Mori *et al.* 1973). These filaments are thought to be protein in nature and associated with HSV type 2 capsid formation. Oda and Mori (1976) examined these structures by negative staining and goniometer specimen tilting of their sections of infected Vero cells. They noted a sharp increase in the number of infected cells showing these structures at 10-12 hours post-inoculation, and that the lattice structures were cross-sectional views of filaments. By negative staining, isolated tubules showed a periodicity of 10-12 nm. and were composed of globular sub-units arranged in pairs as regular arrays of ring shaped elements. Similar filaments

have not been observed in HSV type 1 preparations in thin sectioned or negatively stained material.

Falke and colleagues (1959) observed herpesvirus particle envelopment at the inner lamella of the nuclear membrane as particles budded into the perinuclear space. This was confirmed by Nii and co-workers (1968a), who also found that virus could pass directly from one cell to another after cell fusion had occurred, and they suggested (1968b) that the envelopment process was selective because enveloped capsids tended to have a nucleic acid core. Darlington and Moss (1969) detected thickening of the nuclear membrane in apposition to enveloping virus particles, and this thickened membrane was shown to contain virus-specific antigen (Miyamoto *et al.* 1971). Envelopment of virus has also been shown at golgi membranes and endoplasmic reticulum (Schwartz and Roizman 1969a, b, Darlington and Moss 1969). Schwartz and Roizman (1969a) noted many more non-enveloped nucleo-capsids in the cytoplasm of HSV type 2 infected cells compared with type 1.

Two methods of release of virus particles from infected cells have been postulated. Morgan and colleagues (1959) suggested a process of 'reverse phagocytosis' in which vacuoles containing enveloped virus particles rupture through the cell plasma membrane so releasing virus particles. Schwartz and Roizman (1969b) observed channels 65 nm. in diameter which were frequently continuous with the outer lamella of the nuclear membrane and with an opening in the plasma membrane, and these often contained enveloped virus particles. Stackpole (1969) suggested that the herpesvirus of Lucké tumour of frogs was enveloped at the inner

lamella of the nuclear membrane, de-enveloped at the outer lamella and re-enveloped in the cytoplasm. Darlington and Moss (1969) have pointed out that the envelopment process was non-specific and occurred at any cellular membrane, and they suggested that the inner lamella of the nuclear membrane was the major site of envelopment solely because this was the first membrane virus particles encountered.

4.5 Replication of herpes simplex virus in fertile hens' eggs

Goodpasture, Woodruff and Buddingh (1931) demonstrated that many viruses including HSV grew on the chorioallantoic membrane (CAM) of the fertile hen's egg with the production of discreet lesions or pocks. This technique was evaluated and shown to be as sensitive as rabbit corneal inoculation for virus isolation (Coriell *et al*. 1949). The CAM is a three-layered structure, each layer being at least two cells thick. The outer layer is the chorion (ectoderm), the middle layer is the mesoderm and the inner layer is the allantois (endoderm). Formation of intranuclear inclusions and giant cells in the ectodermal layer of the CAM following HSV inoculation was reported by Dawson (1933), Coriell and co-workers (1949) and Nii and Kamahora (1963). The latter workers also demonstrated that certain HSV strains caused inflammation in the mesoderm. Anderson (1940) found an increase in virulence after CAM passage when the virus showed a tendency to attack the mesodermal tissues and chorion more severely. She also showed cytological changes in organs of the chick embryo. Heath and co-workers (1956) showed that avian embryos inoculated directly developed microcephaly, axial flexion and damaged auditory vesicles.

Anderson (1940) suggested three modes of pathogenesis for HSV in chick embryos: spread by direct cell-to-cell contact; spread by the blood stream; and spread by the neural route.

Variation in the size of pocks produced on the CAM by different strains of HSV has been observed (Jawetz *et al.* 1955, Hutfield *et al.* 1967). Similar results were noted for virus strains after multiple egg passage (Wildy 1955, Schneweis 1962b) and for plaque selected variants (Rapp 1963). Using fresh isolates, Hutfield (1967) and Parker and Banatvala (1967) demonstrated that, after incubation of inoculated eggs for seven days, non-genital strains of HSV produced small pocks and genital strains produced large pocks on the CAM. Nahmias and co-workers (1968) correlated the size and the histology of virus-induced pocks with the site of origin of the inoculum showing that strains freshly isolated from non-genital lesions (type 1) produced pocks smaller than 0.5 mm. in diameter, whilst those from genital lesions (type 2) were larger than 0.5 mm. in diameter. The cellular changes induced in type 1 pocks were restricted to the outer ectodermal layer of the three-layered CAM structure, whereas those in the type 2 pocks spread throughout all three layers of the CAM. Nahmias and Dowdle (1968) pointed out that differentiation of strains of HSV by the pock size induced on the CAM was only valid when freshly isolated strains were used. It has been noted that the pocks resulting from inoculation of non-genital strains onto the CAM could be picked off the membrane, whilst this was not possible with pocks induced by genital strains (Macrae pers. commun.). This correlated with Nahmias' observation that pocks induced by genital strains were

deep-seated in the membrane. Gologan, Lupu and Surdan (1972) have reported even greater differences for their fresh isolates of HSV. These workers found that whilst both types of virus grew on the CAM producing infectious virus, only genital strains induced pock formation .

Nahmias and Dowdle (1968) urged caution when differentiating strains of HSV from those of poxviruses on the basis of the appearance of pocks on the CAM. They observed that the lesions produced by type 1 HSV resembled those of ectromelia virus, whilst the lesions produced by type 2 HSV resembled those of vaccinia virus. Histologically there are parallels between the pocks induced by variola virus and HSV type 1, which both affected the ectoderm of the CAM, and between pocks induced by vaccinia virus and HSV type 2 which both affected the entire thickness of the CAM.

It is necessary to ensure that lesions on the CAM following virus inoculation are truly virus induced. As has been pointed out by D'Anoy and Evans (1937) the nature of the fluid in which the virus is suspended may be an important factor. They also noted changes in the CAM due to inoculation trauma resulting in: proliferation and vacuolation of the cells of the ectoderm (chorion), oedema of the mesoderm, and occasional proliferation of the endoderm (allantois). Traumatic changes induced in the CAM have been observed following drilling of the egg, dropping of the membrane and the addition of distilled water and antibiotics (Wyler and van Tongeren 1957), inoculation of uninfected HeLa culture cells (MacDonald, pers. commun.), and inoculation of sub-cellular extracts (Rich *et al.* 1965).

The replication of HSV in CAM cells *in situ* was examined with the 'HF' strain (Scott *et al.* 1953). These workers assayed the production of infectious virus for 48 hours after CAM inoculation and found that adsorption of virus onto the cells of the CAM occurred rapidly (99% within one hour), followed by a latent period when no virus was detectable. Virus yields increased dramatically even when low dose inocula were used, and by 48 hours virus formation within cells equilibrated with loss of virus from infected cells. This particular strain of HSV, although an oral isolate (Flexner and Amos 1925), has never been conclusively typed but it was probably type 1 (Dowdle *et al.* 1967). Yoshino and co-workers (1961) observed that fresh isolates of HSV inoculated onto the CAM replicated for the first two days after which virus titres decreased, although cell proliferation of the chorion continued. Virus titres did not decline so rapidly when egg-passaged strains were used. Burnet (1955) suggested that infected CAM cells produced a substance which stimulated adjacent cells to proliferate, but so far no such substance has been identified. Using a fresh isolate, presumably a type 1 strain, from eczema herpeticum, Taniguchi (1966) correlated the interruption of virus replication with the production of interferon in CAM cells. She also observed that egg-adapted strains were less susceptible to interferon. As interferon was shown to be more efficient as an antiviral substance under low oxygen tension (Baron *et al.* 1961, Isaacs *et al.* 1961) Taniguchi suggested that pock formation on the CAM was a host-induced cellular proliferation which reduced the oxygen tension in the infected area and so favoured the

action of interferon.

The reason why genital strains of HSV should produce large necrotic pocks, whilst those produced by non-genital strains remain small, has not been clarified.

4.6 Replication of herpes simplex virus in cell cultures

Cultivation of HSV *in vitro* was first performed in pieces of rabbit testicular tissue by Parker and Nye (1925) and later in HeLa cell monolayers by Scherer and Syverton (1954). Gray and co-workers (1958) evaluated differences in cytopathic effects in HeLa cells produced by a strain of HSV isolated from stomatitis lesions. They described three types of cytopathic effect: proliferative, where cells piled up to form foci; non-proliferative, where cells became rounded and swollen at the periphery of small holes in the cell sheet; and those in which giant cells predominated. It was suggested that one type of cytopathic effect may eventually predominate in a cell sheet. By various methods it was possible to select from any strain variants giving a particular kind of cytopathic effect.

Schneweis (1962b) recorded consistent differences in the cytopathic effects produced by fresh isolates of HSV from different sites in a number of cell types (HeLa, mouse embryo, FL amnion, rabbit kidney and chick embryo cells). Non-genital (type 1) strains usually induced proliferative cellular changes, whilst those induced by genital (type 2) isolates occurred rapidly and consisted of rounded cells and giant cells. This close connection between type of cytopathic effect and site of virus origin was confirmed by Munk and Donner (1963), Kleger and Prier (1969), Smith and co-workers (1971) and many others. Hale and co-workers

(1963) noted that isolates from herpetic stomatitis (usually type 1) consistently produced proliferation in HeLa cells. Gologan and colleagues (1974) showed that unlike fresh isolates, laboratory strains failed to give reproducible kinds of cytopathic effect in cell cultures. Passage history of viruses affected the cytopathic effects produced in cell cultures: type 1 strains, after multiple egg passage, produced changes in cell culture resembling those of type 2, possibly as a result of mutation or selection (Nahmias and Dowdle 1968). Ejercito and colleagues (1968) classified HSV strains into four groups on the basis of the cytopathic effects they produced in HEp2 cells. Some strains caused rounding of cells but no fusion, whilst others stimulated the formation of giant cells. These two groups comprised laboratory strains only. Some laboratory strains and fresh genital isolates caused rounding-up of cells which formed into loose aggregates, whilst the fresh non-genital isolates induced tight aggregates of rounded cells. This has been confirmed by Smith and co-workers (1971) who also found that after 20 passages on rabbit corneal cells, the cytopathic effects produced by type 1 strains were unaffected, but the type 2 strains caused cellular changes intermediate between the loose aggregation of rounded cells and the giant cell type.

Figueroa and Rawls (1969) and Lowery and co-workers (1971a) found that type 2 strains grew and produced plaques in chick embryo fibroblast cells, whilst type 1 strains did not, or did so abortively, but only after many cell culture passages (Lowery *et al.* 1971b). Yang and co-workers (1975) made similar observations with a chick embryo cell

microtest, finding a 96% correlation with type. Figueroa and Rawls (1969) also showed that type 2 strains replicated more rapidly in chick embryo fibroblast cell cultures, attaining higher titres and producing a tenfold greater number of particles than the type 1 strains, which was contrary to the results found in other cell culture types (Plummer *et al.* 1968).

Incubation of infected cultures at high temperatures has been suggested as a method to differentiate strains of HSV. Wheeler (1964) noted that a type 2 laboratory strain replicated better at 40°C than a type 1 variant derived from it. Similarly Yoshino, Taniguchi and Takeuchi (1968) found that a laboratory strain of type 1 grew poorly in cell cultures at 40°C, and these workers showed that this could not be fully explained by thermal inactivation of virus. On the other hand, Longson (1971) observed that non-genital strains grew producing infectious virus at 40°C whilst genital strains did not, and Ratcliffe (1971) also noted that type 1 strains produced cytopathic effects but no infectious virus in Vero cell cultures incubated at 40.3°C, whilst type 2 strains failed to do either. Géder, Váczi and Boldogh (1973) described the development of cell lines from chick embryo fibroblast cell cultures exposed at 40°C to strains of HSV type 2, but not type 1. The high temperature of incubation inhibited the growth of both virus types, although the cells infected with type 2 strains rapidly degenerated. Crouch and Rapp (1972) showed that the inhibition of HSV type 2 strains in cell culture at supra-optimal temperature was cell dependent. Szántō and Lešťšo (1974) found that the production of infectious

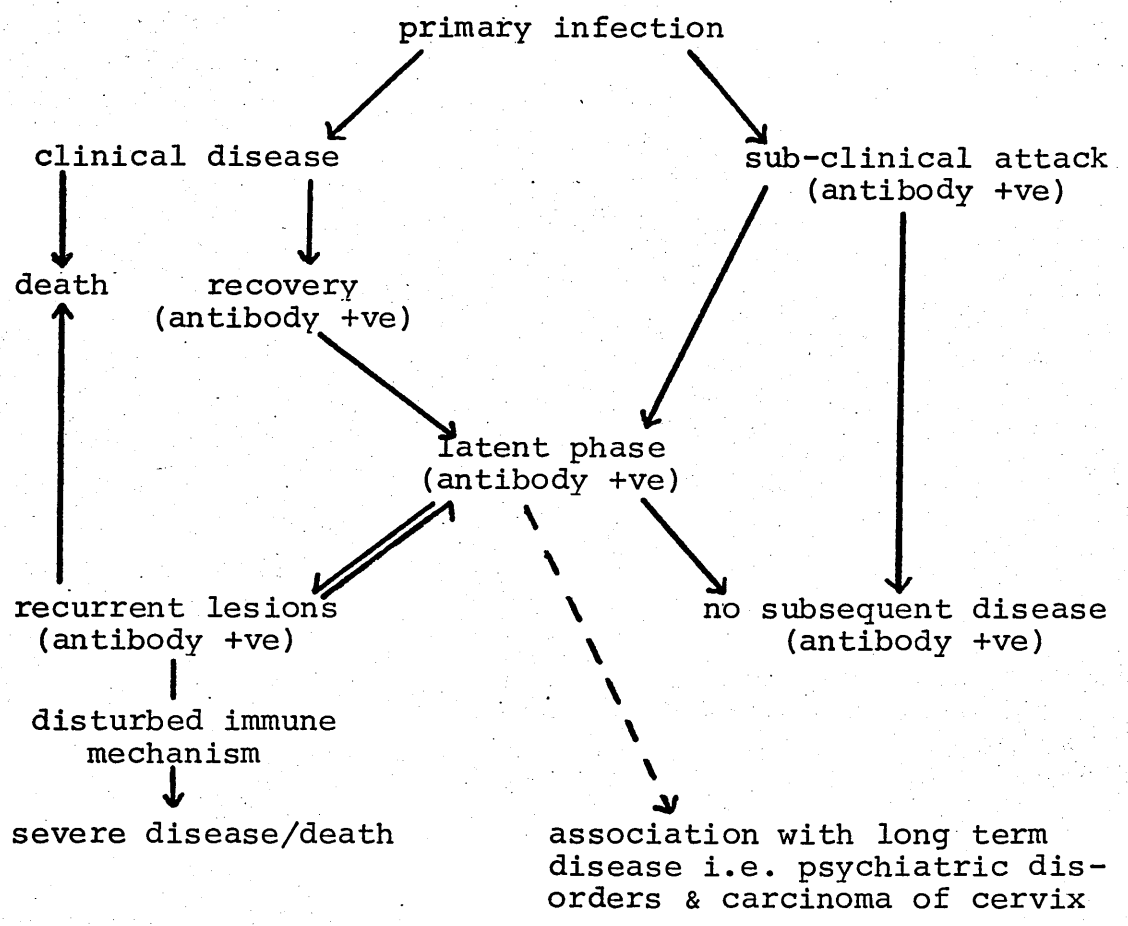
type 1 virus in cell culture was inhibited at 40°C, but strains could be adapted to grow at high temperatures.

Roizman (1968) and Ejercito, Kieff and Roizman (1968) suggested that by specifying components necessary for envelopment, HSV induced changes in the cell plasma membrane, and this altered membrane with bound viral products produced in excess brought about cytopathic alterations in infected cells. From this it would follow that the cytopathic effects induced in cells would be determined by the genetic effect of the virus, and that selection of virus clones producing different cytopathic effects would be a genetic selection indicating that each virus type may include a range of genetic variants. This may explain the variations in results with HSV types 1 and 2 in different cell systems observed in various laboratories.

4.7 Human infection with herpes simplex virus

The clinical syndrome in man caused by HSV is by no means simple. Several observations led to the suggestion of two kinds of infection, a primary attack which led to recovery followed by recurrent attacks in the presence of antibody, from which arose the notion of virus latency. Primary disease with a probable incubation period of four to five days (Ramsay 1974) is the result of initial viral infection via minor abrasions of the skin or mucous membranes of the oral cavity, eyes or genitalia. The recurrent infection follows stimulation of the latent form of the virus to become infectious by various traumas such as fever, menstruation or emotional or physical disturbances (figure 1).

Figure 1 Herpes simplex in man



Virus tends to be restricted to tissues derived from embryonic ectoderm: the mucous membranes, oral cavity, anal cavity, vagina, conjunctiva, nervous system and skin, and it rarely invades the blood stream except in neonates and those with defective immune mechanisms. Histological examination of HSV vesicular lesions shows proliferation of the cells deep in the epidermis followed by degenerative changes including ballooning of cells, formation of multi-nucleate giant cells and the development of intranuclear Cowdry type A inclusion bodies. Vesicles quickly rupture, forming shallow ulcers from which virus can be readily isolated. Non-genital lesions are usually caused by type 1

strains, but type 2 strains have been isolated from non-genital sites (Friedrich *et al.* 1969, Josey *et al.* 1972), especially following oro-genital contact (Kaufman and Rawls 1972). Conversely, genital lesions particularly in chronic or recurrent infections are usually caused by type 2 virus, but type 1 strains have been isolated from genital infections following oro-genital contact (Evrard 1974) and from babies born to mothers with HSV type 1 genital infections (Nahmias *et al.* 1969b). As many as 30% of all genital isolates, particularly from acute infections, prove to be type 1 virus (Smith *et al.* 1973, Kawana *et al.* 1974).

Type 1 virus is spread by the oro-respiratory route and causes a number of well described clinical syndromes ranging in severity from sub-clinical attacks through acute gingivo stomatitis, skin infections, eczema herpeticum, whitlows, eye infections, hepatitis in neonates to generalised infections and acute necrotising encephalitis (Juel-Jensen 1969, Dudgeon 1970, Rawls 1973). Meningitis occurs more frequently with HSV type 2 than with type 1 (Rawls 1973). Apart from disseminated neonatal infections, virus is rarely isolated from cerebro-spinal fluid in either encephalitis or meningitis (Rawls 1973, Morrison *et al.* 1974).

Infections with HSV type 2 are usually associated with the sexually mature and in males lesions occur as vesicular eruptions on the glans, prepuce, or shaft of the penis, whilst in females lesions occur on the vulva, vagina, cervix and perineum, although the thighs, mons pubis and perianal regions often become infected. On moist membrane-

ous tissues, lesions in association with diffuse inflammation appear as thin mucous-like patches which usually become necrotic (Gardner and Kaufman 1972). HSV type 2 accounts for 80% of neonatal herpes infections which vary in severity (Rawls 1973). Foetal infection which is acquired from the infected birth canal occurs more frequently with maternal primary infections than with recurrent attacks, although maternal antibody does not protect against infection (Nahmias *et al.* 1969b).

Sero-epidemiological studies have implicated HSV type 1 in psychiatric disorders (Rimon and Halonen 1969, Cleobury *et al.* 1971, Halonen and Rimon 1975), and HSV type 2 in cervical carcinoma (Naib *et al.* 1966, Rawls 1973).

4.8 Herpes simplex virus in animals

Most animals are susceptible to infection with HSV by one or more of the inoculation routes, but Blanc and Caminopetros (1921) observed that adult chickens, rhesus monkeys, sheep, pigeons, and toads were resistant irrespective of infective route.

Investigations have been conducted with laboratory animals to differentiate strains of HSV. In rabbits, type 2 strains were noted to be more neuro-virulent than those of type 1 (Plummer *et al.* 1968, 1970), whilst with hamsters type 2 strains induced sarcomas and type 1 strains did not (Nahmias *et al.* 1970). Duff and co-workers (1974) transformed hamster embryo cells with ultra-violet light irradiated type 1 virus and produced an epithelioid cell line inducing adenocarcinoma in hamsters, whilst hamster embryo cells transformed by ultra-violet irradiated type 2 virus formed a fibroblastoid cell line inducing fibro-

sarcoma in hamsters. A greater neurovirulence for type 2 strains has been observed in mice inoculated by a variety of routes (Wheeler 1964, Nahmias *et al.* 1967, Plummer *et al.* 1968 and 1970), whilst Mogensen and co-workers (1974) induced local necrotic liver lesions in intra-peritoneally inoculated mice with HSV type 2 strains, but not with those of type 1.

Significant differences have been observed in infections due to HSV types 1 and 2 in numerous animal species, and some of these have been suggested for type characterisation.

Table 1 Summary of properties of herpes simplex virus types 1 and 2

Property	HSV type 1	HSV type 2	Reference or review paper
1. Physico-chemical			
a) nucleic acid	double stranded DNA	double stranded DNA	Ben-Porat & Kaplan (1962) Epstein 1962 Smith & Melnick (1962) Russell & Crawford (1963)
b) buoyant density of particles ($gm.per\ cm.^3$)	1.253 - 1.271	1.267	Schiek (1967)
c) molecular weight of DNA ($\times 10^6$ daltons)	99±5 87.7	99±5 87.7	Kieff *et al.* (1971) Graham *et al.* (1972)
d) buoyant density of DNA ($gm.\ per\ cm.^3$)	1.727 1.726 1.725 1.725	1.729 1.728 1.727 1.727	Plummer *et al.* (1969) Kieff *et al.* (1971) Graham *et al.* (1972) Halliburton *et al.* (1975)

contd ...

Property	HSV type 1	HSV type 2	Reference or review paper
e) guanine + cytosine (g+c) content of DNA (%) (from buoyant density)	68 67 66 67	70 69 68 69	Plummer *et al.* (1969) Kieff *et al.* (1971) Graham *et al.* (1972) Halliburton *et al.* (1975)
f) stability at 45°C (in PBS) 37°C 4°C in $AgNO_3$ in PBS in heparin	less stable stable stable sensitive less stable more sensitive	stable less stable less stable stable more stable less sensitive	Smith *et al.* (1971) Plummer *et al.* (1968) Figueroa & Rawls (1969) Schneweis (1962a) Vahlne *et al.* (1975) Schneweis *et al.* (1972) Plummer *et al.* (1968)
g) inactivation by u.v.	inactivated	inactivated more readily	Smith *et al.* (1971)
h) inactivation by chemo-therapeutic agents	sensitive	less sensitive	Lerner & Bailey (1972)
2. Morphology & morphogenesis			
a) capsid size (nm.)	105	105	)
b) capsid morphology	icosahedral symmetry 162 capsomeres	icosahedral symmetry 162 capsomeres	) Wildy *et al.* (1960)
c) % enveloped particles	large	small	Figueroa & Rawls (1969)
d) intranuclear 22 nm. dia. tubules	no	yes	Couch & Nahmias (1969) Smith *et al.* (1973) Oda & Mori (1976)
e) virus crystals	common common	rare common	Schwartz & Roizman (1969a) Nii & Ono (1971)
f) ribbon or bizarre shaped cores	no	yes	)
g) capsids with dense cores in cytoplasm	no	yes	) Schwartz & Roizman (1969a)

contd ...

Property	HSV type 1	HSV type 2	Reference or review paper
h) frequency of non-enveloped cytoplasmic virus	rare	common	Schwartz & Roizman (1969a)
3. Growth in eggs			
a) pocks on CAM	small	large	Hutfield (1967) Parker & Banatvala (1967)
b) histological findings in CAM:			
proliferation	ectodermal	all three layers	)
hyperplasia	ectodermal	fibro-blastic	)
congestion	mild	marked	)
haemorrhages	rare	common	)
necrosis	rare	common	)
erosion	mild	marked	)
inflammatory cells	mild (monocytes)	marked (mixed monocytes, polymorpho-nuclear leucocytes)	) Nahmias *et al.* (1968)
multi-nucleate giant cells	rare	common	)
intranuclear inclusions	occasional	common	)
4. Growth in cell culture			
a) CPE	tight adhesion of rounded cells	loose aggregates of rounded cells + giant cells	Munk & Donner (1963)
b) growth at high temper-atures	yes	no	Longson (1971) Ratcliffe (1971)
c) plaques	small	large	Munk & Donner (1963)
d) plaques in chick embryo cells	no	yes	Figueroa & Rawls (1969) Lowery *et al.* (1971a&b)
e) virus titres	higher	lower	Plummer *et al.* (1968)

contd ...

Property	HSV type 1	HSV type 2	Reference or review paper
5. Clinical			
a) site of infection	non-genital	genital	Dowdle *et al.* (1967)
b) age distribution	all ages	sexually mature + neonates	Lipschütz (1921 & 1932) Rawls (1973)
c) neurological disorders	severe encephalitis	ass. with meningitis	)
d) association with other disorders	psychiatric disorders	cervical carcinoma	) Rawls (1973)))
6. Laboratory animals			
a) mouse inoculation:			
genital	low mortality	higher mortality	Nahmias *et al.* (1967)
intramuscular	less neurotropic	more neurotropic	Plummer *et al.* (1968 & 1970)
intracerebral	less neurotropic	more neurotropic	Wheeler (1964)
intraperitoneal	no lesions	local liver lesions	Mogensen *et al.* (1974)
b) rabbit inoculation:			
intramuscular	less neurovirulent	more neurovirulent	Plummer *et al.* (1968 & 1970)
corneal	less destructive	more destructive	Jawetz (1973)

MATERIALS AND METHODS

1 Origin and passage history of selected strains of herpes simplex virus (HSV)

Sixty-five strains of HSV were selected: 35 were HSV type 1 and 30 were HSV type 2. Of the 35 strains of HSV type 1, 14 had received multiple laboratory passages and the remaining 21 were fresh isolates. Of the 30 strains of HSV type 2, seven had received multiple laboratory passages and 23 were fresh isolates. Laboratory strains had received ten or more passages in cell culture, hens' eggs or laboratory animals. Fresh isolates had received less than ten laboratory *in vitro* or *in vivo* passes and usually only one or two.

The essential details of the strains used, together with the site of the lesion from which the strain was isolated, the laboratory where the isolation was done, the passage history, the methods used to type the strains and the experiments in which the strains were used in the present study are summarised in tables 2 and 3.

Of the 21 freshly isolated strains of HSV type 1, 13 were isolated from non-genital sites (including two from the central nervous system), two from urine, and six from female genitalia. All laboratory strains of HSV type 1 had been originally isolated from oral lesions. Of the 23 freshly isolated strains of HSV type 2, three were isolated from non-genital sites (wrist, mouth and face), one from urine, and 19 from genital lesions. No HSV type

2 strains were isolated from the central nervous system. All laboratory strains of HSV type 2 were originally derived from genital lesions.

All strains were characterised by the appearance of the cytopathic effects induced in cell cultures and by the gross appearance of pocks after inoculation onto the CAMs of 11 day old fertile hens' eggs. Twelve of the 14 laboratory strains of HSV type 1 and six of the seven laboratory strains of HSV type 2 were further typed by the kinetic neutralisation test (Department of Virology, University of Birmingham). In addition 22 of the HSV type 1 strains and 15 of the HSV type 2 strains were typed with temperature marker tests (Dr. H. Appleton, Virus Reference Laboratory, Colindale). The results of both these tests were made available for this study.

Large stocks of virus strains were prepared in cell cultures and were frozen in 1 ml. aliquots at -70^{o}C until required, at which point they were rapidly thawed in a water bath at 37^{o}C.

Table 2 Source and history of the selected strains of herpes simplex virus type 1

Strain	Fresh / laboratory isolate	Site from which strain isolated	Laboratory of isolation	Passage history[1]	Methods used to type[2]	Tests in present study[3]
10711/53	lab	face lesion (female)	VRL	eggs, mice, Vero, and many others	1 2 - 4	S T - -
Candeias	lab	throat swab, acute gingivostomatitis	"	HeLa 3, HA 1, Vero 1, and others	1 2 - 4	S - - -
1716	lab	oral lesion	Birmingham	HEL 1, Vero 3	1 2 3 4	- T - -
61446	lab	" "	"	HEL 1, Vero 2	1 2 3 4	S - - -
Duffy	lab	" "	"	Vero 3, HEL 1	1 2 3 4	S T - -
M5	lab	" "	"	mouse brain, HEL 2	1 2 3 4	S T - -
64179	lab	" "	"	HEL 1, Vero 3	1 2 3 4	S T - -
KIR	lab	" "	"	Vero 2, HEL 1	1 2 3 4	- T - -
HIL	lab	" "	"	HK 3, HEL 1, Vero 1, HEL 1	1 2 3 4	S T - -
HFEM	lab	" "	"	eggs, mice, HK, HEL 1, Vero 1, HEL 1	1 2 3 4	S T - EM
WAL	lab	" "	"	HK 4, HEL 2	1 2 3 4	S T - EM
5516	lab	" "	"	HEL 1, Vero 2	1 2 3 4	S T - -
1156	lab	" "	"	Vero 1, HEL 1	1 2 3 4	- T - -
2913	lab	" "	"	HEL 1, Vero 3	1 2 3 4	S T - -

contd ...

Strain	Fresh / laboratory isolate	Site from which strain isolated	Laboratory of isolation	Passage history[1]	Methods used to type[2]	Tests in present study[3]
409	fresh	oral lesion	VRL	HEL 2	1 2 - -	S - - -
24126/70	fresh	mouth swab (male), lymph-adenopathy	"	HEL 2, Vero 2	1 2 - 4	S - - -
1065/71	fresh	genital swab	Guildford	HEL 1, Vero 3	1 2 - 4	- T - -
1067/71	fresh	throat swab	"	HEL 1, Vero 1, HEL 1, Vero 2	1 2 - 4	S T - -
1068/71	fresh	throat swab	"	HEL 1, Vero 1, HEL 1, Vero 2	1 2 - 4	S T - -
2330/71	fresh	lip and tongue lesions	Neasden	HEK 1, WI-38 1, Vero 2	1 2 - 4	S T - -
17599/71	fresh	vaginal swab, vulval ulcers (female, pregnant)	VRL	HEL 1	1 2 - 4	S - - -
17962/71	fresh	urine (baby 5/12, infected from mother)	"	WI-38 1, Vero 1, HEL 1	1 2 - 4	S T - -
22101/71	fresh	vesicle fluid, recurrent lip lesion (male)	VRL	HEL 2	1 2 - 4	S T G -
14958/72	fresh	P.M. liver and kidney, liver failure, kidney transplant	"	HEL 1	1 2 - -	S - - -
15694/72	fresh	P.M. brain, encephalitis	"	HEL 1	1 2 - -	S - - -
15811/72	fresh	vulval swab, herpetiform vulval lesions	"	HEL 1	1 2 - -	S - - -
15815/72	fresh	oral lesion	"	HEL 1	1 2 - -	S - - -

contd ...

Strain	Fresh / laboratory isolate	Site from which strain isolated	Laboratory of isolation	Passage history[1]	Methods used to type[2]	Tests in present study[3]
20860/72	fresh	oral lesion	Neasden	HEL 2	1 2 - -	S - - -
7677/73	fresh	ulcer swab, herpetiform ulcers of labia	VRL	HEL 1	1 2 - -	S T - -
7747/73	fresh	generalised herpes (baby 10 days old)	Exeter	HEL 2	1 2 - -	S T - -
2104/74	fresh	ulcer swab, herpetiform ulcers of the labia	VRL	HEL 2	1 2 - -	S T - -
8037/74	fresh	labia swab	"	HEL 1	1 2 - -	S T - -
8802/74	fresh	vesicle fluid (male)	"	HEL 1	1 2 - -	- T - -
9892/74	fresh	urine, thrombocytophenia abnormal lymphocytes P.B. -ve (male)	"	HEL 1	1 2 - -	S T - -
2160/75	fresh	bladder, kidneys and brain, generalised herpes, P.M.	"	HEL 1	1 2 - -	S - - -

(for footnotes see bottom of table 3 page 39)

Table 3 Source and history of the selected strains of herpes simplex virus type 2

Strain	Fresh / laboratory isolate	Site from which strain isolated	Laboratory of isolation	Passage history[1]	Methods used to type[2]	Tests in present study[3]
25766	lab	genital swab	VRL	RK_{13} 1, Vero 3, HEL 1	1 2 - 4	S - - -
2248	lab	genital swab	Birmingham	Vero 2, HEL 2	1 2 3 4	S T - -
2219	lab	genital swab	"	HEL 2, Vero 4, HEL 1	1 2 3 4	S T - -
T514	lab	genital swab	"	HEL 2, Vero 3	1 2 3 4	S T - -
LOV	lab	genital swab	"	RK_{13} 2, HK 5, HEL 3	1 2 3 4	S T - EM
PAR	lab	genital swab	"	HK 4, HEL 3	1 2 3 4	S T - EM
3345	lab	genital swab	"	HEL 1, Vero 4, HEL 1	1 2 3 4	S T - -
669/67	fresh	vesicle fluid from wrist lesion (child, 5 years)	VRL	HEL 1, HA 1, Vero 1	1 2 - 4	S - - -
HSV2/69	fresh	cervical smear	"	HEL 1	1 2 - -	S - - -
5502/69	fresh	oral swab, generalised mouth lesions(female)	"	HEL 1, Vero 4	1 2 - 4	S T - -
9889/70	fresh	urine (female)	"	HEL 2	1 2 - 4	S T G EM
24055/70	fresh	cervical swab	"	HEL 1, Vero 3	1 2 - 4	S T - -
1066/71	fresh	genital swab	Guildford	HEL 1, Vero 1, HEL 1	1 2 - 4	S T - -
17152/71	fresh	cervical swab	VRL	HEL 2	1 2 - 4	S T - EM

contd ...

Strain	Fresh / laboratory isolate	Site from which strain isolated	Laboratory of isolation	Passage history[1]	Methods used to type[2]	Tests in present study[3]
17189/71	fresh	vesicle fluid, secondary herpes lesions on face (male)	VRL	CAM 1, WI-38 1, Vero 2	1 2 - 4	S T - -
24061/71	fresh	cervical swab (female)	"	HEL 2, Vero 3	1 2 - 4	S T - -
2304/72	fresh	cervical smear, genital warts (female)	"	HEL 1	1 2 - -	S - - -
7447/72	fresh	cervical smear, genital herpes (female)	"	HEL 1	1 2 - -	S T - -
8087/72	fresh	cervical smear, vaginal discharge (female)	"	HEL 1	1 2 - -	S T - -
9965/72	fresh	cervical swab, vulval lesions	"	HEL 1	1 2 - -	S T - -
12260/72	fresh	cervical swab	"	HEL 1	1 2 - -	S - - -
15286/72	fresh	cervical smear	"	HEL 1	1 2 - -	S - - -
18464/72	fresh	genital smear, herpes genitalis (female)	"	HEL 1	1 2 - -	S - - -
19630/72	fresh	vulval swab of lesions	"	HEL 1	1 2 - -	S - - -
10331/73	fresh	labial and cervical swab of lesions	"	HEL 1	1 2 - -	S T - -
20661/73	fresh	cervical swab, herpes cervicitis	"	HEL 1	1 2 - -	S - - -
294/74	fresh	swab of vaginal and cervical ulceration	"	HEL 1	1 2 - -	S T - -

contd ...

Strain	Fresh / laboratory isolate	Site from which strain isolated	Laboratory of isolation	Passage history[1]	Methods used to type[2]	Tests in present study[3]
1699/74	fresh	cervical smear, cervicitis	VRL	HEL 1	1 2 - -	S - - -
10089/74	fresh	swab of cervical lesions	"	HEL 1	1 2 - -	S T - -
15031/74	fresh	genital smear, herpes genitalis	"	HEL 1	1 2 - -	S - - -

Key to tables 2 and 3

[1] passage history which either forms part of this study, or was known at the time of receiving strains

HA = primary human amnion cell cultures
HEL = human embryo lung cell cultures (as described in section 3.1)
HK = primary hamster kidney cell cultures
WI-38 = Wistar Institute (diploid embryo lung) cell cultures
RK_{13} = rabbit kidney cell cultures
CAM = chorioallantoic membrane

[2] methods used to type strains:

1 = appearance of cytopathic effects in cell cultures
2 = appearance of pocks formed on the CAM 4 or 5 days post-inoculation
3 = kinetic neutralisation tests
4 = temperature marker tests

[3] tests in which the strains were employed in the present study:

S = spread studies (as described in sections 2.6 to 2.9)
T = temperature studies (as described in section 5.2)
G = growth studies (as described in sections 5.1 and 5.3)
EM = electron microscopy studies (as described in section 6.2)

2 Incubation, inoculation and harvest of eggs

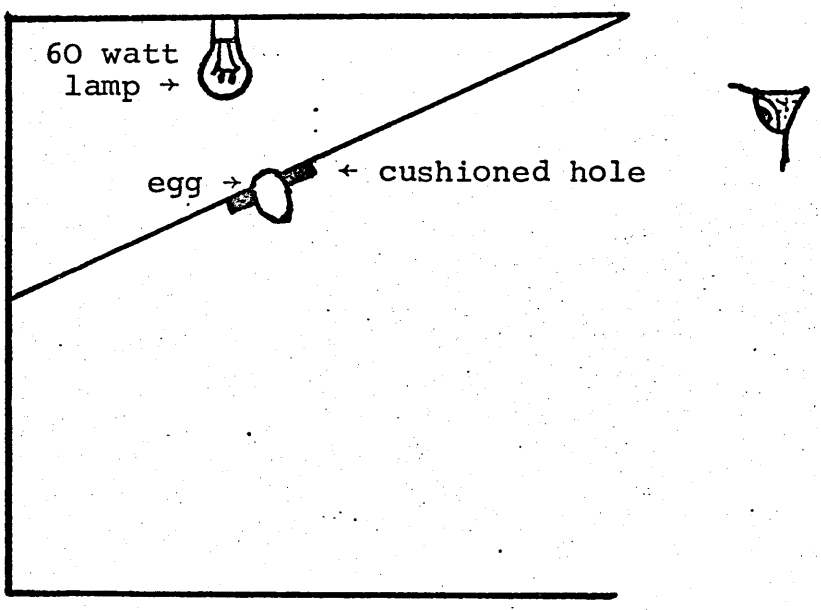
2.1 Egg type, source and incubation prior to inoculation

Fertile white Leghorn hens' eggs (Appleby Farms, Kent) were incubated at 37°C in a Hearson's egg incubator with air circulation and humidifier. Eggs were automatically rotated through 90° every four hours for the first 11 days of incubation, and thereafter transferred to another incubator and maintained stationary.

2.2 Inoculation onto the CAM

After 11 days' incubation eggs were removed from the incubator and the CAMs were exposed by a modification of the false air sac technique as described by Busby, House and MacDonald (1964). The blunt end of the egg was inserted into a cushioned hole in a black box equipped with a 60 watt bulb, so that the egg was illuminated from above (figure 2). At this time infertile eggs or eggs showing signs of embryo damage or death were discarded and only healthy fertile eggs were used in this study. Embryos were judged as dead or unsuitable by the presence of a stunted embryo floating at the top of the egg fluids, the presence of a distinct blood clot in the *sinus terminalis* around the inside of the egg, or failure of the embryo to respond to repeated light tapping of the egg with a pencil. The major blood vessels and the position of the air sac at the blunt end of the egg were marked on the shell surface with a soft pencil and in a similar way a small equilateral triangle approximately 5 mm. across was marked at the point where the membranes were to be exposed, taking care to ensure that the marked triangle avoided the major blood vessels. Shells were cut using a drill

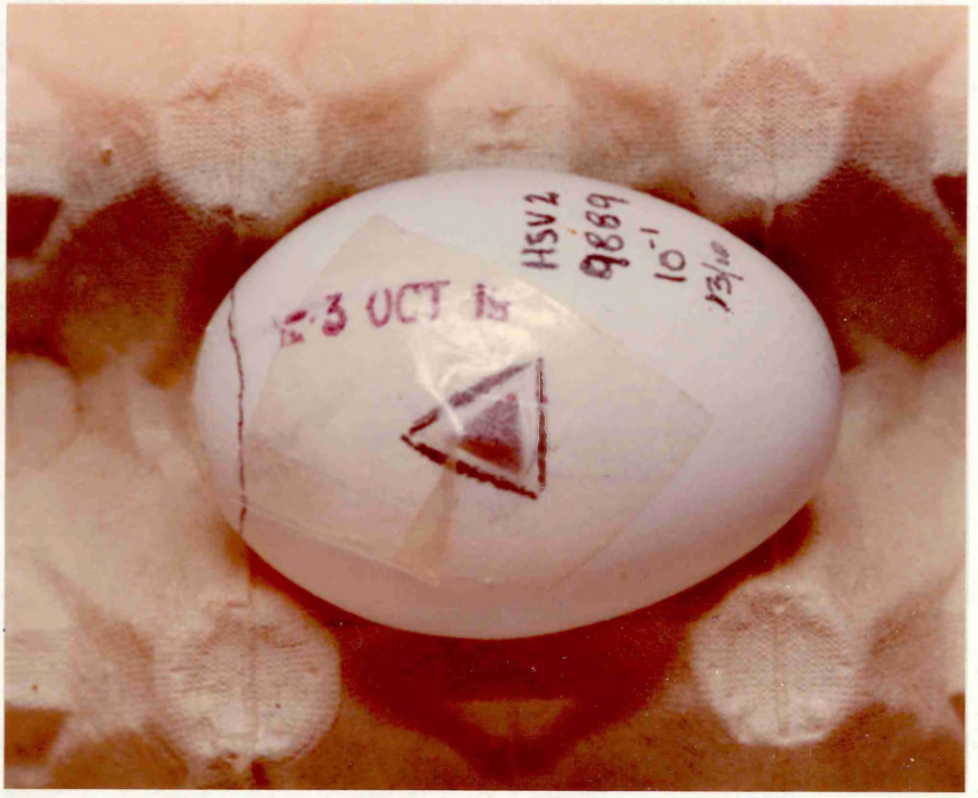
to candle eggs. All surfaces painted matt black to reduce reflections.



type 520 (Kavo, West Germany) fitted with a dental end piece (W and H, Austria), on which was mounted a diamond impregnated cutting wheel. The speed of the drill was controlled by means of a foot-operated switch. A small hole was opened in the air sac end of the egg after which the small marked triangle was cut, taking care only to penetrate the shell and not to damage the underlying shell membrane. Eggs which had their CAMs damaged at this point as shown by bleeding were discarded. The drilled area was smeared lightly with a small amount of vaseline (Appendix 1) to trap loose egg shell dust from the drilling process. This was wiped off with cotton wool and the area of the shell was de-greased by wiping with cotton wool soaked in methanol. Using the point of a sterile scalpel the shell membrane under the hole in the air sac end of the egg was pierced, and the small triangle of shell was removed by

gently lifting one of the corners so preventing any of the points of the triangle from piercing the underlying shell and chorioallantoic membranes. The shell membrane under the triangle was then pierced with a sterile dissecting probe, the tip of which was ground flat on one edge to give greater control of the piercing action. At this stage a drop of penicillin streptomycin broth saline (PSBS) (Appendix 1) was placed on the exposed shell membrane, and with a 2 ml. laboratory rubber teat negative pressure was applied to the hole in the blunt end of the egg. This resulted in the CAM parting from the shell membrane under the cut out triangle and this was indicated by the drop of PSBS disappearing through the hole in the shell membrane. The shell membrane was then peeled away using sterile curved forceps and the CAM, which was then clearly visible through the triangle, was examined for damage. The triangle and blunt end of the egg were sealed with two inch wide "Sellotape". The whole process was performed as rapidly and carefully as possible and eggs were quickly returned to the incubator in order to minimise heat loss which might cause embryo damage. When the eggs had been re-incubated for a few hours and normal temperature resumed they were ready for inoculation with virus (plate 2).

The "Sellotape" covering the triangle cut in the shell was peeled back and 0.1 ml. virus inoculum introduced directly onto the CAM by pasteur pipette fitted with a set volume automatic syringe. After resealing with the "Sellotape" the eggs were gently rocked by hand from end to end, side to side and in a circular motion, keeping the dropped region of the CAM uppermost at all times.



Egg after exposing the CAM. Labelled, sealed and incubated ready for virus inoculation. (x2)

PLATE 2

This was done to ensure even spreading of the inoculum over the membrane. Eggs were returned to the incubator and maintained stationary at 36°C with the inoculated area of the CAM facing upwards.

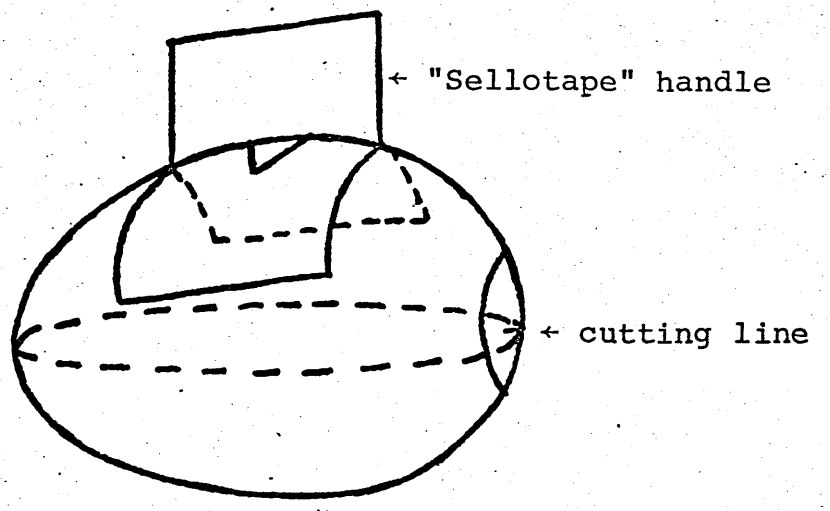
2.3 Inoculation of the allantoic cavity

Eggs were candled and selected as described earlier. A small area on one side of the egg was cleaned with methanol-soaked cotton wool, allowed to dry, and a small hole was made in the shell in this area with a 26G half inch needle fitted to a 1 ml. sterile syringe containing the virus inoculum. The needle was introduced four to five mm. into the egg and a small amount of allantoic fluid was withdrawn into the syringe to ensure that the tip of the needle was correctly sited in the allantoic cavity. The allantoic fluid was then injected back into the allantoic cavity together with 0.1 ml. of the virus inoculum. After removing the syringe the small hole was sealed with a piece of "Sellotape".

2.4 Removal of the CAM

A piece of doubled-over two inch "Sellotape" was fixed to the surface of the egg over the dropped region of the CAM. This formed a 'handle' for safe manipulation of infected eggs and using this handle the egg was held horizontally in a harvesting tray and cut in half with curved scissors. The cut was along the long axis, starting at the small hole already drilled prior to inoculation over the air sac at the blunt end of the egg (figure 3). The selected region of the CAM was then left attached to the upper half of the shell held by the "Sellotape" handle, and the egg contents were left in the lower half of the

Figure 3 Diagrammatic representation of egg with "Sellotape" handle in place



shell in the tray. After any pieces of membrane holding the two halves of the egg together had been cut away, the CAM was carefully peeled from the shell with curved forceps and placed in a six inch glass petri dish. The CAM was carefully washed in either PBS solution A (Appendix 1) or cacodylate buffer (Appendix 2), spread out on a glass plate, examined and photographed with reflected flash light against a black velvet background.

2.5 Serial passage of virus on the CAM

For passage, each harvested CAM was placed in a 25 ml. glass universal container with 10 ml. sterile distilled water. Using a rotary blade grinder fitted to the container, the contents were homogenised for four minutes. Grinders with macerated CAMs were then placed at $4^{o}C$ for ten minutes to allow aerosols and tissues to settle. The contents were then diluted in sterile distilled water and inoculated

onto further CAMs. Details of those strains passaged in eggs are given in Appendix 3.

If CAMs were to be stored before processing, they were placed directly into bijou bottles and stored at -70°C without additives.

2.6 Collection of allantoic fluid

Allantoic fluid was harvested at intervals of one to six days after inoculation of the eggs using a syringe fitted with a fine gauge needle (26G, half inch) inserted four to five mm. into the blunt end of the egg (well away from the inoculation site). Allantoic fluid was slowly withdrawn. Such fluid was withdrawn from eggs inoculated on the CAM or directly into the allantoic cavity. Harvests were stored in 2.5 ml. capacity glass bottles at -70°C without additives.

2.7 Collection of amniotic fluid

After harvesting allantoic fluids and carefully cutting away the dropped region of the CAM with the shell still attached (as described in 2.4), the amniotic sac surrounding the embryo was lifted carefully with blunt sterile forceps and the amniotic fluid removed with a syringe. Harvests were stored in 2.5 ml. capacity glass bottles at -70°C without additives.

2.8 Collection of embryos and individual organs

Embryo heart, gut and brain were harvested at intervals of one to six days after inoculation of the eggs. Great care was taken to prevent cross-contamination from one area of the egg to another. After allantoic fluids had been harvested and eggs had been opened and amniotic fluids collected, the embryo was removed from the amniotic sac

by carefully cutting the amniotic membranes so as to prevent contact between the embryo and allantoic fluid. The embryo was washed free of amniotic fluid in three changes each of 150 ml. PBS solution A and cut longitudinally to expose the heart which was removed together with any heart blood. Samples of liver and intestine were removed at this stage and pooled. The brain was aspirated by syringe with a coarse gauge needle (18G, one inch) inserted into the embryonic head between the eyes after which negative pressure was applied. Embryo harvests were stored in 2.5 ml. capacity glass bottles at -70°C without additives.

2.9 Detection of virus in egg harvests

The presence of virus in harvested suspensions was demonstrated by inoculation of these onto further CAMs. The CAMs were observed for the development of pocks three or four days post-inoculation. Undiluted allantoic and amniotic fluids were inoculated separately. Heart, brain and gut material were treated separately. Each was homogenised in PSBS with a syringe and coarse gauge needle (18G, one inch). Heart and gut were prepared as 40% (v/v) suspensions in PSBS and brains were prepared as 20% (v/v) suspensions in PSBS, and all inocula were 0.1 ml. volumes.

3 Preparation, inoculation and harvest of cell cultures

Cell culture media are described in detail in Appendix 1. All fluids were warmed to 37°C in a water bath prior to use.

The cell cultures used in this study were human embryo lung (HEL) cells, a continuous line of green monkey kidney

cells (Vero), HeLa cells, and a variety of primary egg and chick embryo derived cells. HEL or Vero cells were used to passage virus strains before egg inoculation and to perform virus titrations, whilst HeLa cells were used to check the infectivity of allantoic fluid harvests. The primary cells were used in growth studies.

3.1 Human embryo lung (HEL) cell cultures

HEL cell cultures were prepared by the method of Hayflick and Moorhead (1961) whereby lungs from human embryos two to five months post conception were removed and minced under sterile conditions with scissors and forceps. The resultant suspension was washed three times in PBS solution A by centrifugation at 500 r.p.m. for ten minutes in an MSE minor centrifuge, and the supernatants were removed by pipette and discarded. The fragments of tissue remaining were transferred to a sterile glass universal screw-capped bottle containing a small sterile teflon-coated magnet. To this was added 15 ml. of 0.2% trypsin in Hanks' Balanced Salt Solution (Hanks' BSS) and the universal bottle was incubated on a slowly rotating magnetic stirrer for five minutes at 36°C. The suspending fluid was removed by pipette and discarded, leaving the small tissue pieces in the bottle. To this was added a further 15 ml. of fresh 0.2% trypsin in Hanks' BSS and the bottle re-incubated on the stirrer at 36°C for 20 minutes, after which time the cell suspension was removed and centrifuged as described above. The supernatant was discarded. This process was repeated three or four times, the cell suspension being removed each time and replaced with fresh trypsin solution. After centrifuging, the

cell pellet was re-suspended in growth medium based on Eagle's Minimal Essential Medium (Eagle's MEM) containing 10% foetal calf serum (Appendix 1) and seeded into 4 oz. medical flat bottles, each containing 10-15 ml. growth medium, at a rate of 10^5 cells per ml. Bottles were incubated at 36°C for three or four days at which time the cell monolayers were confluent and ready for sub-culture.

The growth medium was removed and the cell sheet washed once with 10 ml. washing fluid which was discarded, and then 10 ml. of 0.2% trypsin in Hanks' BSS was added. This was allowed to overlay the cell sheet for two or three minutes on the bench at room temperature. Then most of the trypsin solution was decanted leaving 0.25 - 0.5 ml. in the bottle, which was incubated for 15 minutes at 36°C with occasional rotation. This ensured that the trypsin made good contact with the cell sheet. At this stage bottles were examined under the low power of an optical microscope, to ensure that trypsinisation was taking place. When the cells were released from the glass, 10 ml. Eagle's MEM growth medium was added. The cell suspension was thoroughly mixed by pipette, then seeded into fresh bottles in the proportion always of one into two. Eagle's MEM growth medium was added to bring the total fluid to 10-15 ml. per 4 oz. bottle. Sub-culture in this manner was performed every three and four days, until 14 sub-cultures had been done. At this stage the cells were considered to be stable and suitable for virus inoculation or for liquid nitrogen storage. For titration purposes one 4 oz. medical flat bottle with a confluent monolayer yielded

enough cells to seed 25 tubes (4 x ½ inch) each containing 1 ml. of medium. When the cell sheets in the tubes were confluent, the growth medium was replaced with Eagle's MEM maintenance medium and the tubes were ready for inoculation.

3.2 Vero cell cultures

Vero cells (Yasamura and Kawakita 1963), a continuous line of epithelioid cells originally derived from the kidneys of an African green monkey (*Cercopithecus aethiops*), were grown on one side of 6 oz. medical flat bottles. The cells were sub-cultured weekly: cell sheets were washed once with 10 ml. washing fluid, then 10 ml. of trypsin-versene mixture was allowed to remain in contact with the cell sheet for one minute at room temperature. The trypsin-versene solution was discarded and the bottles were left drained at room temperature until the cells began to detach from the glass. The cells were re-suspended in a growth medium based on Synthetic Medium 199 (SM 199) containing 2% foetal calf serum (Appendix 1). One 6 oz. bottle provided enough cells to seed six new 6 oz. bottles or 90 tubes (4 x ½ inch) at a rate of 5×10^4 cells per ml., and these were incubated for five days at 36°C at which time those bottles containing confluent cell monolayers had the growth medium removed and replaced by SM 199 maintenance medium with 1% foetal calf serum. Tubes were treated in the same way.

3.3 HeLa cell cultures

HeLa cells (Gey, Coffman and Kubicek 1952), a continuous line of epithelioid cells originally derived from a carcinoma of the cervix, were treated in a similar fashion to Vero cell cultures with the exception that Eagle's MEM

growth and maintenance media were used. One 6 oz. bottle provided enough cells to seed three new 6 oz. bottles or 40 tubes (4 x ½ inch) at a rate of 10^5 cells per ml.

3.4 Chick embryo fibroblast cell cultures (CEFC)

Fertile hens' eggs were used after nine to eleven days' incubation for all egg and embryo derived cell cultures and Eagle's MEM media were used.

Chick embryo fibroblasts were prepared according to the method of Waterson (1958). Embryos were extracted from the eggs and the legs, wings and heads were discarded. The torsos were finely divided with scissors and forceps under aseptic technique and trypsinisation of tissue was done in the manner described for HEL cells. After primary culture and one passage in medical flat bottles, cells were sub-cultured into tubes.

3.5 Chick embryo allantoic cell cultures (CEAC)

Eggs were cut in half along the long axis and the egg contents removed. The two halves of the shell with the adhering chorioallantoic and shell membranes were rinsed three times with washing fluid to remove all traces of egg contents. The shell halves with membranes attached were placed in sterile pots with the membranes uppermost, and 5 ml. of 0.2% trypsin in Hanks' BSS was added to the exposed allantoic layer of the CAMs. This released the cells of the allantois. To encourage cell detachment the membranes were stroked occasionally and very carefully with a smooth ended glass rod. After three or four minutes at room temperature, the cells suspended in trypsin were removed by pipette and centrifuged as described in 3.1. The cell pellet was re-suspended in

growth medium and seeded directly into 4 x ½ inch tubes at a rate of 10^5 cells per ml. After four or five days' incubation at 36°C cell monolayers were confluent and the growth medium was replaced with maintenance medium. Cultures were used as primary cells.

3.6 Chick embryo brain cell cultures (CEBC)

The brains of chick embryos were aspirated by syringe fitted with an 18G one inch needle inserted between the eyes. Brain materials harvested from numerous embryos were pooled and treated as in the preparation of HEL cell cultures. Cell harvests were re-suspended in growth medium and seeded directly into 4 x ½ inch tubes, at a rate of 10^5 cells per ml. The cultures were then treated in the same way as CEAC cultures above.

3.7 Chick embryo CAM cell cultures (CECC)

Eggs were cut in half along the long axis and the egg contents removed. The CAMs were removed as described in 2.4 and rinsed three times in washing fluid to remove all traces of egg contents. The CAMs were trypsinised as described for HEL cell cultures. Cell harvests were re-suspended in growth medium, seeded directly into 4 x ½ inch tubes at a rate of 10^5 cells per ml. and subsequently treated in the same way as CEBC cultures.

3.8 Storage of cells in liquid nitrogen

Cells were stored for future use in a gas phase liquid nitrogen Linde flask (Union Carbide Company). Cell monolayers were trypsinised as described in 3.1 and the cells from two 4 oz. medical flat bottles pooled, centrifuged at 500 r.p.m. for ten minutes and re-suspended in 0.9 ml. Eagle's MEM growth medium, and 0.1 ml. di-methyl sulphoxide

was slowly added as a cryo-protective agent. These 1 ml. seed pools were placed in hard glass ampoules, heat-sealed and frozen slowly at a temperature reduction rate of 1°C per minute until below the critical point of -65°C (Nagington and Lawrence 1962). To do this the ampoules were placed in a hollow polystyrene plug in the open neck of the liquid nitrogen Linde flask. Then the ampoules were stored in clips in the gas phase liquid nitrogen flask. When the cells were required an ampoule was thawed rapidly in a 37°C water bath. The ampoules were opened by scratching the neck with a diamond and heat-fracturing them, after which the cell suspension was transferred to a sterile screw-capped bottle. Growth medium was added dropwise to bring the total volume to 3 ml., the resultant cell suspension was mixed and transferred to a 4 oz. medical flat bottle. The volume was made up to 10-15 ml. with growth medium and bottles were ready for sub-culturing after three or four days' incubation at 36°C.

4 Virus assay methods

Serial tenfold dilutions of virus (0.1 ml. volumes) prepared in sterile distilled water were inoculated into each of four tubes of HEL cell cultures. The cultures were examined for cytopathic effects at three, four and seven days post-inoculation and the titre of infectious virus was calculated by the method of Reed and Münch (1938) and expressed as TCD_{50} per 0.1 ml. of suspension.

5 Growth studies on viruses

5.1 Assay of virus from CAMs inoculated with different concentrations of virus

Eggs were inoculated with 0.1 ml. of 10^{-2} or 10^{-4} dilutions of stock virus pools of HSV type 1 strain 22101/71 (3.0×10^5 TCD_{50} per 0.1 ml.) and HSV type 2 strain 9889/70 (3.0×10^4 TCD_{50} per 0.1 ml.) after these had been stored at -70°C for five years (table 5).

The CAMs were harvested exactly 72 and 144 hours post-inoculation, in order to compare the production of infectious virus in these tissues after inoculation with the different amounts of virus of each type. One batch of eggs was used for all tests with two eggs per dilution of each strain for each time of harvest. Only the exposed area of each membrane was harvested, measuring about 30 mm. in diameter. Harvests were stored at -70°C until experiments were completed, at which point all harvested membranes were thawed. Each was suspended in 10 ml. sterile distilled water and homogenised as described in 2.9. Tenfold dilutions of these suspensions were made in sterile distilled water and assayed for infectious virus by inoculating 0.1 ml. volumes of suspension into each of four tubes of HEL cell cultures per dilution. The TCD_{50} per 0.1 ml. of inoculum was calculated by the method of Reed and Münch (1938).

5.2 Growth of virus on CAMs incubated at high temperatures

A total of 42 strains of HSV was used to study the growth of the two virus types on CAMs at temperatures above 36°C. Twenty-four strains of HSV type 1 and 18 strains of HSV type 2 were inoculated onto CAMs and the

eggs were incubated at 36°C, 38.5°C, 39°C and 39.5°C. These temperatures were accurately maintained in an incubator (Laboratory Thermal Equipment) with air circulation and regional temperature differential.

Prepared eggs were re-incubated at 36°C for a minimum of six hours pre-inoculation. Inoculated eggs were immediately transferred to the region of the incubator corresponding to the temperature under study. Three eggs were used for each strain at each temperature and all were incubated undisturbed for four days.

The effect of the amount of virus in the inoculum on the growth of HSV strains at temperatures above 36°C was also examined with the HSV type 1 strain HIL and the type 2 strains PAR and 17152/71. The HSV type 1 strain HIL was studied over the temperature range at dilutions of the stock pool in sterile distilled water from 10^{-1} to 10^{-4}, the HSV type 2 strain PAR at dilutions of 10^{-2} and 10^{-4} of the stock virus pool, and the HSV type 2 strain 17152/71 at dilutions of 10^{-1} and 10^{-3} of the stock virus pool. The pools of all strains were examined undiluted.

5.3 Growth of virus in primary egg and chick embryo derived cells

Primary egg and chick embryo derived cell cultures were drained of maintenance medium and cell counts were performed (Paul 1965). Tubes were inoculated with 0.1 ml. virus suspension of HSV type 1 strain 22101/71 and HSV type 2 strain 9889/70 to give a multiplicity of 1 TCD_{50} per cell (Géder *et al.* 1973). Cultures were incubated at room temperature for two hours to allow virus adsorption,

after which the inocula were removed and the cultures were washed twice with maintenance medium (Figueroa and Rawls 1969). One ml. of maintenance medium was added to each culture and the tubes were incubated on a roller apparatus at 36°C. At time intervals 0 to 200 hours post-inoculation three tubes of each of the three cell types CEFC, CEBC and CECC, and one tube of the cell type CEAC were removed and immediately frozen at -70°C. After the final samples were frozen all the tubes were thawed. Identical harvests (cell type and time of harvest) were pooled. Each pool was assayed for infectious virus by making serial tenfold dilutions in distilled water and 0.1 ml. of each dilution was inoculated into each of four tubes of Vero cell cultures. These were incubated at 36°C and examined for cytopathic changes after three, four and seven days. The infectious virus titre was calculated by the method of Reed and Münch (1938) and expressed as TCD_{50} per 0.1 ml. of each of the harvested pools.

6 Electron microscopy

The reagents used for electron microscopy are described in detail in Appendix 2.

6.1 Negative staining of virus preparations

Negatively stained preparations of HSV were made by a modification of the methods described by Horne and co-workers (1959) and Wildy and colleagues (1960). After inoculation with HSV, cell cultures were incubated at 36°C until 75 to 100% of the cells demonstrated cytopathic changes typical of HSV infections. Cells were scraped off

the glass into the maintenance medium with a silicone rubber 'policeman' (glass rod with a small piece of silicone tubing around the tip). The suspension was centrifuged at approximately 1,000 g for ten minutes in a conical centrifuge tube, after which the fluid was decanted and retained. The pellet was re-suspended in PBS solution A and re-centrifuged. The supernatant was tipped from the tube and the tube containing the cell pellet was allowed to drain onto absorbent tissue for about 20 minutes. Small samples of the cell pellet were removed by aspiration with a pasteur pipette and the cells in these samples were lysed in a drop of distilled water on a microscope slide. The volume of distilled water necessary was found to be fairly critical to ensure even, clean preparations. Dilution was judged by assessing the turbidity of the lysate: this was correct when, on progressive dilution, the 'milkiness' appeared minimal when the suspension was held in the capillary of a pasteur pipette and viewed against a black tile background. Equal amounts of lysed cellular material and phosphotungstic acid (PTA) 3% at pH 6.5 were mixed on a glass slide and a drop of the resultant mixture was placed onto a formvar-carbon-coated grid held in fine forceps. The mixture was allowed to remain on the grid for about 10-15 seconds before being drained off by touching the edge of the grid with a piece of Whatman No. 1 filter paper. The rate at which the virus-stain mixture drained from the grid into the filter paper gave an indication of the suitability of the preparation. Drops which appeared to run off the grid quickly usually

had insufficient material present, and those which appeared to come off the grid very sluggishly were usually too thick for examination. In addition 2.5 ml. of the cell culture fluid retained from the first low speed centrifugation was centrifuged for one hour at 39,100 g. The supernatant was decanted and the tube containing the pellet was allowed to drain for 20 minutes in a glass container with absorbent tissue beneath the tube. The pellet was re-suspended in approximately 0.05 ml. distilled water, mixed with an equal volume of 0.05% bovine plasma albumin to ensure even distribution of virus suspension on the grid and stained by mixing with an equal volume of 3% PTA and mounted on a grid as described previously.

Grids were examined and photographed in an AEI EM6B, EM801 or Corinth 500 electron microscope at an accelerating voltage of 60kV. using instrumental magnifications from 25,000 to 63,000.

6.2 Resin embedding and thin sectioning of CAMs

CAMs were harvested as described in 2.4 and rinsed three times in cacodylate buffer to remove all adhering blood cells and egg materials. Whole membranes were fixed in 3% glutaraldehyde in cacodylate buffer for 30 minutes and then rinsed thoroughly in ten changes of cacodylate buffer for three or four minutes per rinse to remove all traces of glutaraldehyde. Post-fixation was effected with 1% osmium tetroxide (OsO_4) in cacodylate buffer for 25 minutes. The membranes were then rinsed twice in cacodylate buffer followed by a dehydration procedure of immersion for five minutes in each of 50%, 70%, 90% and 95% ethanol in cacodylate buffer. Dehydration

of CAMs was completed by two rinses of ten minutes each in absolute ethanol. Ethanol was removed by pipette and the CAMs rinsed twice in epoxy propane (propylene oxide) for ten minutes each rinse. At no stage in this procedure was any part of the specimen allowed to dry. Resin-infiltration was done by placing the CAMs in a 2:1 mixture of epoxy propane and complete resin for one hour, and this mixture was then replaced by a 1:2 mixture of epoxy propane and complete resin. CAMs were cut up at this stage: segments approximately one mm. square of normal uninfected CAMs and of infected CAMs containing pocks produced by HSV were taken with a fresh scalpel blade for each CAM. This was done with transmitted fluorescent illumination from a light box and a stereo microscope at x20 magnification to ensure accurate removal of pocks from infected CAMs. The small pieces of CAM tissue were then placed in complete resin for one hour after which the resin was changed for fresh complete resin which had been degassed for 20 minutes in a vacuum desiccator. Tissue pieces were allowed to remain in the degassed resin for one hour. After this they were removed and located either individually in Reichert flat embedding blocks, each trough being filled with degassed complete resin, or five or six tissue pieces were located in hollowed polypropylene discs, 50 mm. in diameter and 5 mm. deep. These discs were topped up with degassed complete resin. Tissues were manipulated using wooden swab sticks sharpened at one end to form a flexible spatula which was pre-treated by dipping in degassed complete resin. These treated spatulas were placed at 60°C for one hour prior to use to allow the resin end to

dry and so prevent the introduction of air bubbles around the specimen. Blocks were labelled with 5 mm. squares of hardened paper written on with a sharp hard pencil and placed in the resin adjacent to the tissue. The embeddings were polymerised in a 60°C oven for 24 hours. After removal from the oven, blocks were left at room temperature for two or three days to allow complete hardening of the resin prior to their removal from the moulds.

Resin mixtures were made by mixing the components listed in Appendix 2 in a glass medical flat bottle and shaking vigorously by hand for at least ten minutes. After thorough mixing, aliquots of the resin were removed for use at the various stages in the procedure. When the resin was required the accelerator (DMP-30) was added at the appropriate concentration to the aliquot and the mixture was shaken vigorously for three to five minutes. Embedding of tissue was done during one day, followed by 24 hours' polymerisation, the whole procedure being completed in approximately 30 hours.

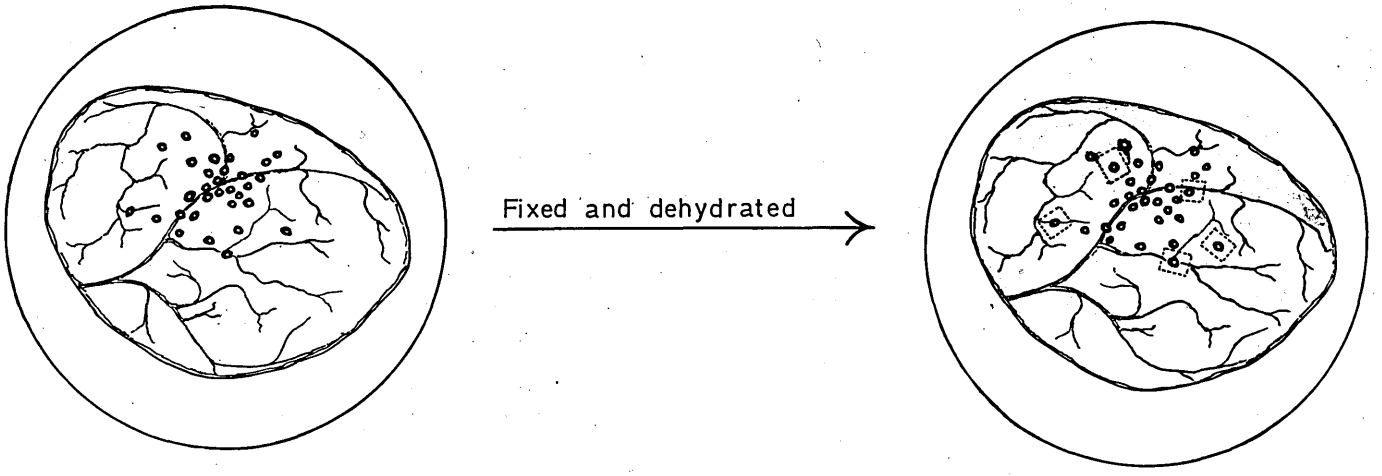
The embedding procedure and the times required are summarised in table 4.

Polymerised blocks were placed in the flat embedding chuck of the Reichert OMU-2 ultra microtome, so as to present a cross-section of the three layers of the CAM to the knife, and pyramidal faces were produced by hand trimming using a fresh razor blade for each block (figure 4). Block faces were finally trimmed with a glass knife. Fresh glass knives were made on the LKB knife maker and these were equipped with a collecting trough or boat made of insulation tape sealed with dental wax.

Table 4 Embedding of CAMs for electron microscopy

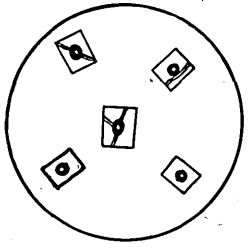
	Procedure	Time
1	Wash in cacodylate buffer	15 minutes
2	Fix in 3% glutaraldehyde in cacodylate buffer	30 minutes
3	Wash in cacodylate buffer (ten changes)	30-40 minutes
4	Post-fix in 1% OsO_4 in cacodylate buffer	25 minutes
5	Wash in cacodylate buffer	5 minutes
6	Dehydrate in ethanol series in cacodylate buffer	20 minutes
7	Complete dehydration in absolute ethanol and epoxy propane	40 minutes
8	Resin / epoxy propane mixtures	120 minutes
9	Resin (two changes)	120 minutes
10	Polymerisation of embeddings	24 hours (at 60°C)
	TOTAL TIME REQUIRED	approximately 30 hours

Figure 4 FLAT EMBEDDING of C.A.M. for THIN SECTIONING

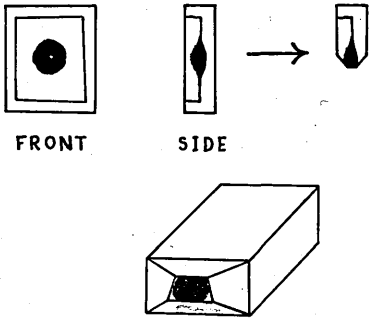


① Infected C.A.M.

② Pocks cut out



③ Embedded in resin



④ Trimmed for sectioning

Optical survey sections one micron thick were cut using the mechanical advance on the ultra microtome and these were floated onto fresh glass-distilled water in the knife boat. These sections were collected from the boat using an eyelash attached to a wooden swab stick and placed in a drop of glass-distilled water on a slide. Sections were then dried down onto the slide at 60°C on a hot plate and left for a few minutes at this temperature to ensure adhesion between the section and slide. Sections were then stained at 60°C on the hotplate for one minute using either 1% toluidine blue in 1% borax (modified from Trump *et al.* 1961) or 0.1% azure II in 1% borax (Jeon 1965). The stain was washed off with glass-distilled water and the sections allowed to dry. The slides were examined and photographed in the light microscope using x10, x25 and x40 objectives.

These stains were used for two reasons: firstly, the appearance of stained sections resembled ultra thin sections in the electron microscope, and secondly, great difficulty was encountered in using other histological stains, as these would not penetrate the resin-embedded material. Toluidine blue and azure II could only be used at pH values approaching 11.0 in borax solutions and temperatures of 60°C were required to allow penetration of the tissue by the stains.

When blocks had been appropriately trimmed, as ascertained from the optical sections, ultra thin sections were cut using the thermal advance on the ultra microtome and using a DuPont diamond knife filled with clean glass-distilled water. The diamond knife edge was cleaned with

a clean soft wooden tooth pick. Sections giving grey or silver interference colours (50-90 nm. in thickness) were chosen. Individual sections and small ribbons were picked up on polybutene-coated copper grids (Appendix 2). Grids were submerged in the boat and sections located above them with a mounted eyelash, then sections were removed by lifting the grid out of the water. Grids with sections on were blotted dry by touching the edge of the grid to a piece of filter paper and then allowed to dry completely in a small glass petri dish on fluffless Whatman No. 50 filter paper for five to ten minutes.

Ultra thin sections were stained with 5% aqueous uranyl acetate for 60 seconds by floating grids, sections downwards, on a drop of stain placed on parafilm and covered by a glass petri dish lid. Grids were removed and sections washed thoroughly in a running stream of glass-distilled water using approximately 15 ml. per grid, after which they were blotted and allowed to dry. The grids were then held in fine forceps and a drop of lead citrate placed on them for 20 seconds, after which they were washed as before using approximately 25-30 ml. distilled water per grid. Grids were blotted and allowed to dry as before. Stain was removed from the stock bottles using a fine pasteur pipette with the tip located about 5 mm. below the meniscus and the first drop in the pipette was always discarded, thus ensuring minimal stain deposit. The pasteur pipettes were freshly rinsed in glass-distilled water before use.

Thin sectioning was performed under strictly dust free conditions and all materials coming into contact with sections, knife or boat water were stringently cleaned

and rinsed under running glass-distilled water.

Sections were examined and photographed in an AEI EM801 or a Corinth 500 electron microscope, at an accelerating voltage of 60kV., using various instrumental magnifications.

7 Photographic techniques

7.1 CAMs and embryos

CAMs and embryos were photographed using flash light illumination with a Micro Precision Products Technical 4 x 5 inch sheet film camera loaded with Ektacolour type S sheet colour negative film. Prints were prepared by Mr. J.R. Gibson, Photographic Department, Central Public Health Laboratory, Colindale, on a DeVere 504 colour enlarger using Kodak Aktaprint 3 photographic paper and chemicals.

7.2 Optical sections

Optical sections were photographed with a Leitz Orthoplan photomicroscope equipped with an Orthomat photographic system at instrumental magnifications of x25, x62.5 and x100. The film used was Kodak photomicrography 2483 Ektachrome colour reversal film. Prints were prepared by Mr. J.R. Gibson, using Cibachrome materials.

7.3 Electron micrographs

Electron micrographs were taken on 6.5 x 9 cm. Ilford EM5 glass plates in an AEI EM 801 electron microscope or on Ilford N7E50 70 mm. roll film in an AEI Corinth 500 electron microscope. All negatives were developed in Kodak D19 developer at half recommended strength, fixed in a one plus three solution of Kodafix, and washed in running filtered tap water for 30 minutes. A Durst A600 enlarger equipped with a 105 mm. Schneider Componon lens was used

to prepare prints on Ilford resin-coated papers using the Ilfospeed system.

R E S U L T S

1 Infectivity and purity of virus stock pools

The infectivity titres of the stock herpes simplex virus pools are shown in table 5. These titres remained constant over a two year period of storage at -70°C. After five years' storage at -70°C the titre of the 22101/71 strain of HSV type 1 was reduced by one log, whilst that of the 9889/70 strain of HSV type 2 was reduced by approximately two logs of infectivity.

Stock virus pools were examined by electron microscopy of negatively stained preparations to exclude the presence of any viral contaminants. No other viruses were detected.

The possibility that pock formation on the CAM was due to bacterial contamination of the stock virus pools was investigated by drop inoculation of the CAM homogenates in sterile PBS solution A onto sterile blood agar plates and into sterile nutrient broths. No bacterial growth was observed.

Of the HSV type 1 strains used, the laboratory adapted strain WAL and the fresh isolate 22101/71 were negative for mycoplasmas. Both the remaining laboratory strains were found to contain mycoplasmas: the stock pool of the strain HFEM had *Mycoplasma arginini* and strain HIL had *Mycoplasma hominis*. None of the HSV type 2 strains nor the HEL cell cultures used to grow up the stock pools had mycoplasmal contaminants. All tests for the presence of mycoplasma species were done by the Mycoplasma Reference

Laboratory, Colindale. Due to the unknown effects of mycoplasmas on the systems under study, those strains free of mycoplasmas were selected for use in further detailed studies.

Table 5 Infectivity titres of stock pools of herpes simplex virus

Type	Strain	Passage history	Infectious virus titre* after storage at -70°C for 2 years	after storage at -70°C for 5 years
1	HIL	high passage in cell culture	2.1×10^5	nt
	WAL	high passage in cell culture	4.7×10^6	nt
	HFEM	multiple passage in eggs, mice and cell culture	5.6×10^5	nt
	22101/71	2 passes in cell culture (HEL)	3.2×10^6	3.0×10^5
2	LOV	high passage in cell culture	3.2×10^5	nt
	PAR	high passage in cell culture	3.2×10^6	nt
	17152/71	high passage in cell culture	2.1×10^4	nt
	9889/70	2 passes in cell culture (HEL)	1.0×10^6	3.0×10^4

* TCD_{50} per 0.1 ml. of stock virus pool inoculated into HEL cell cultures

nt = not tested

2 Gross effects of virus on CAM and embryo of fertile hens' eggs following CAM inoculation

Control uninoculated CAMs and embryos showed no lesions, haemorrhages or death (plates 3 and 12a). The gross appearances of the chick embryos and the lesions induced in the CAM following inoculation of the dropped region of the CAM with 0.1 ml. of HSV type 1 strain 22101/71 or HSV type 2 strain 9889/70 are illustrated in plates 4a to 11b and 12b, and tabulated in tables 6 and 7. Observations were made one to seven days after inoculation.

One day after HSV type 1 inoculation tiny pin-point lesions, less than 0.1 mm. in diameter, developed on the CAM. They were white and circular with a heaped-up appearance. The inoculation site often showed an opalescent region as well as tiny pocks, particularly in those CAMs inoculated with high concentrations of virus (more than 10^4 TCD_{50} per 0.1 ml.). There was no evidence of haemorrhage or necrosis in the CAM (plate 4a). The embryo appeared normal and indistinguishable from controls.

Two days after HSV type 1 inoculation the CAM lesions appeared similar to those of day one, with the exception that they were slightly larger, up to 0.5 mm. in diameter. The opalescent regions seen on day one in eggs inoculated with high concentrations of virus developed into lesions that consisted of pocks which had coalesced to form confluent areas of virus infection. Pocks maintained a superficial appearance and there was no evidence of haemorrhage or necrosis in the CAM (plate 5a). Embryos appeared normal and indistinguishable from controls.

On the third day lesions were similar to those of day two, measuring approximately 0.5 mm. in diameter, with a bright, crisp appearance. They gave the impression of being on the surface of the CAM rather than infecting it, resembling small bacterial colonies. There was no evidence of haemorrhage or necrosis in the CAM (plate 6a). The embryos appeared normal.

Four, five and six days post-inoculation the HSV type 1 lesions on the CAM were similar to those of day three and usually measured up to 0.5 mm. in diameter, but on rare occasions measured up to 1.0 mm. in diameter. CAMs inoculated with the higher concentrations of virus produced confluent infections. Tiny secondary pocks of approximately 0.1 mm. in diameter often developed. There was never any evidence of haemorrhage or necrosis in the CAM (plates 8a, 9a and 10a). Embryos appeared normal and indistinguishable from controls.

Seven days after inoculation with HSV type 1, lesions frequently began to resolve and dry up, whilst those pocks which were present were small, never larger than 1.0 mm. in diameter (plate 11a). As with shorter incubation periods, the embryos were normal.

On the first day after HSV type 2 inoculation small lesions developed on the CAM and were usually in the region of 0.1 mm. in diameter. They appeared off-white or creamy in colour and were circular (plate 4b). Like HSV type 1, high concentrations of HSV type 2 inoculated onto CAMs produced an opalescent area, but unlike HSV type 1 small haemorrhages of blood vessels frequently occurred.

There was no evidence of necrosis in the CAM. Pocks appeared to be somewhat less superficial than with HSV type 1, though the lesions of either type of the virus were difficult to differentiate at this stage. The embryos appeared normal.

Two days after HSV type 2 inoculation lesions on the CAM continued to develop and usually measured up to 1.0 mm. in diameter. In heavily infected membranes showing confluent lesions, haemorrhages occurred frequently, but no necrosis was evident. Lesions had a slightly flatter appearance and were more off-white in colour than the HSV type 1, and they seemed much less superficial (plate 5b). CAMs inoculated with low doses of virus showed no signs of haemorrhage and the embryos under these conditions appeared normal, whilst following inoculation of high doses of virus CAMs showed haemorrhages and the embryos were often haemorrhagic and occasionally dead.

Three days after HSV type 2 inoculation CAM lesions continued to increase in size and appeared to be deep-seated in the membrane and frequently affected the blood vessels. Haemorrhages were very common and lesions with necrotic centres often occurred. Lesions were normally flatter than those produced by HSV type 1 and frequently measured up to 3.0 mm. in diameter (plate 6b). Often membranes inoculated with high concentrations of virus showed signs of breakdown and degeneration across the whole membrane and not only at sites of pocks (plate 7). This breakdown was always associated with, and possibly secondary to, death of the embryo. The

embryo frequently showed signs of virus infection and this was always associated with the presence of haemorrhages in the CAM. Commonly occurring signs of infection of the embryo were: retardation of embryonic development; haemorrhage of the skin which showed as blotchy, reddened areas often spreading to cover most of the embryo; development of diffuse lesions on the cornea, and ultimately embryo death. These manifestations were similar from day three to day seven after CAM inoculation (plate 12b).

Four to seven days after HSV type 2 inoculation the CAM lesions appeared similar to those of day three but gradually increased in size over the days up to 7.0 to 8.0 mm. or more in diameter. The frequency of haemorrhages and necrotic lesions increased (plates 8b, 9b, 10b and 11b). The effects noted on embryos resembled those observed at day three with the exception that virus spread to the embryo was more common as shown by more frequent signs of infection and death.

Oedematous swelling of the inoculation site which appeared as a jelly-like mass together with pocks occurred equally when either virus type was inoculated onto the CAM. The frequency of this reaction reduced with prolonged incubation.

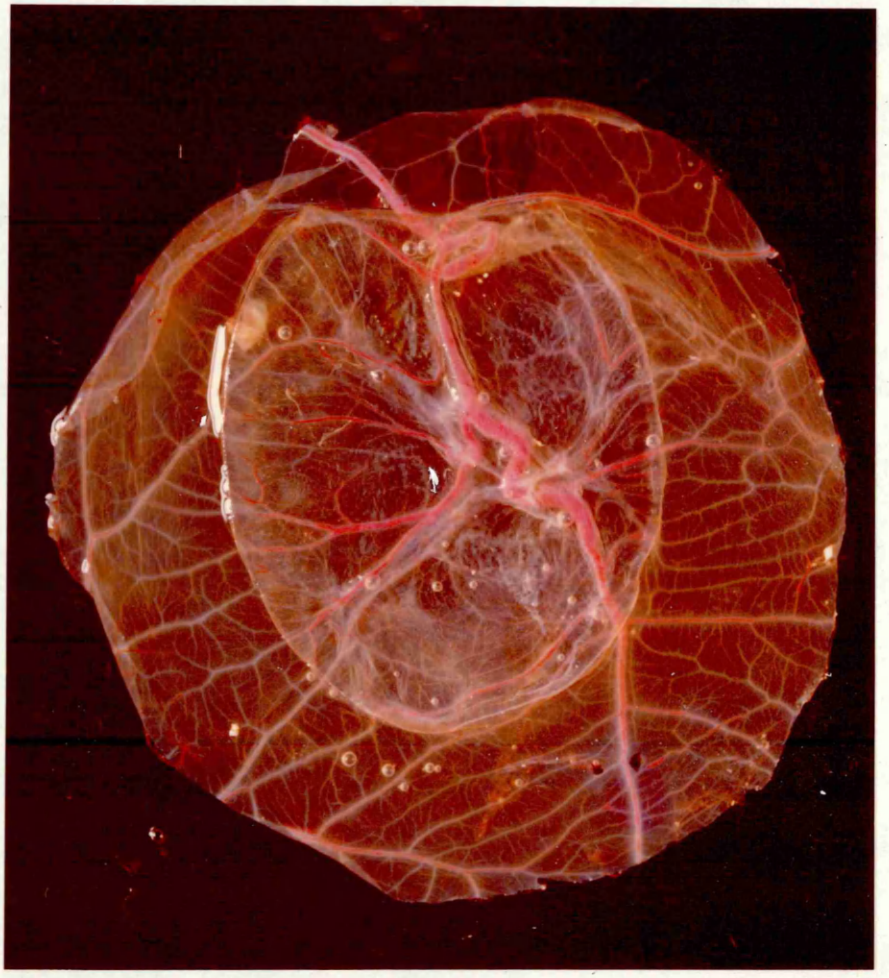
Table 6 Gross effects of HSV types 1 and 2 on the CAM

Days after inoculation of HSV onto CAM	HSV type 1 strains		HSV type 2 strains	
	Low titred virus inoculum ($\leqslant 10^2$ TCD_{50}/0.1 ml.)	High titred virus inoculum ($\geqslant 10^3$ TCD_{50}/0.1 ml.)	Low titred virus inoculum ($\leqslant 10^2$ TCD_{50}/0.1 ml.)	High titred virus inoculum ($\geqslant 10^3$ TCD_{50}/0.1 ml.)
1	white circular pocks < 0.1 mm. diameter	white opalescent area	white circular pocks 0.1 mm. diameter	white opalescent area occasionally with haemorrhages
2	white circular pocks up to 0.5 mm. dia., pocks superficial	white opalescent area, some individual pocks present	off-white flattened pocks up to 1.0 mm. dia., less superficial than HSV type 1	confluent area, haemorrhages present, no necrosis
3	as day 2	confluent superficial area	necrotic centred pocks up to 3.0 mm. dia., haemorrhages of blood vessels	confluent haemorrhages and necrosis present
4	as day 2, occasionally pocks 1.0 mm. diameter	confluent as day 3	as day 3, but pocks increased in size	as day 3
5	as day 4	confluent as day 3	as day 4, but pocks larger	as day 3, CAM often showed signs of breakdown
6	as day 4	confluent as day 3	as day 5, haemorrhages and necrosis evident	as day 5, haemorrhages and necrosis pronounced
7	as day 4, but sometimes pocks began to resolve	confluent as day 3	as day 6, pocks 7.0 - 8.0 mm. dia. or more	as day 6, CAM frequently broken down due to egg death

Table 7 Gross effects of HSV types 1 and 2 on embryos following CAM inoculation

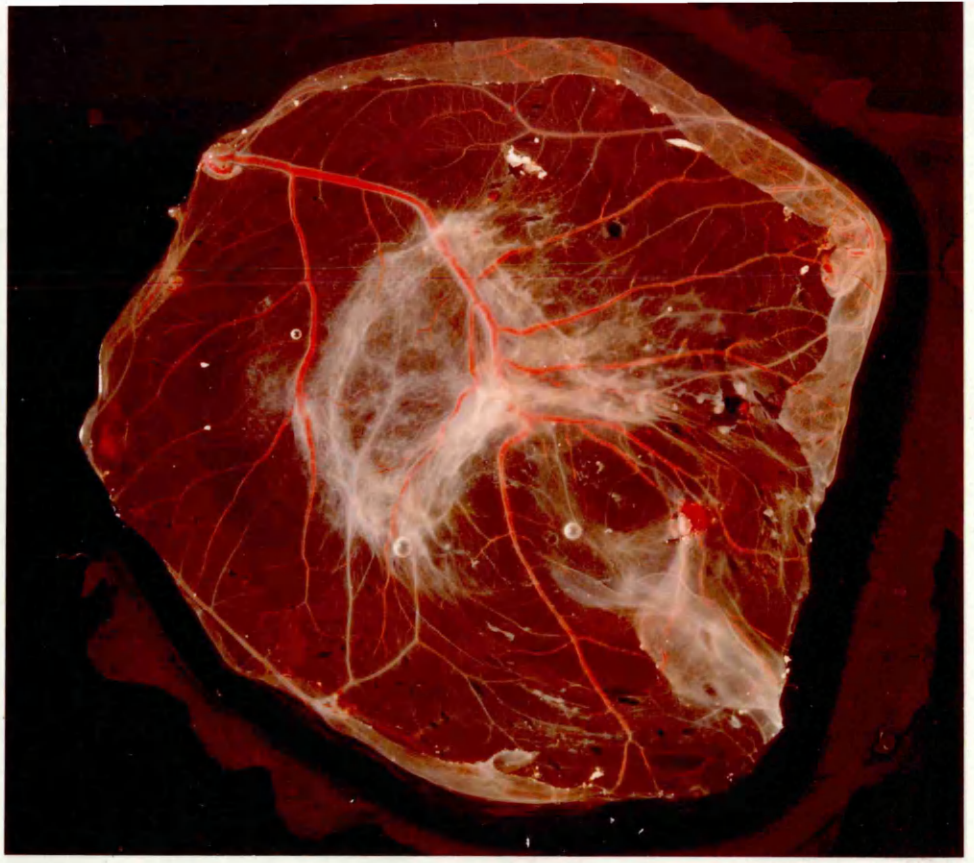
Virus strains	Infectivity of inocula (TCD_{50} per 0.1 ml.)	Condition of harvested embryo				
		Day 1	Day 2	Day 3	Day 6	Day 7
HSV type 1						
HIL	2.1×10^5)					
	2.1×10^2)					
WAL	4.7×10^6)					
	4.7×10^3)					
HFEM	5.6×10^5)	N, A	N, A	N, A	N, A	N, A
	5.6×10^2)					
22101/71	3.2×10^6)					
	3.2×10^3)					
HSV type 2						
LOV	3.2×10^5	N, A	N, A	H, S, D	H, S, D	H, S, D
	3.2×10^2	N, A	N, A	Often H, S, D	Often H, S, D	H, S, D
PAR	3.2×10^6	N, A	Often H, S&A	H, often S, A	H, S, D	H, S, D
	3.2×10^3	N, A	N, A	Often H, occasionally D	Often H, often D	H, S, D
9889/70	1.0×10^6	N, A	Often H, occasionally D	Often H, often D	H, S, D	H, S, D
	1.0×10^3	N, A	N, A	Often H&S, A	Often H&S, occasionally D	H, S, D
17152/71	2.1×10^4	N, A	N, A	Often H, A	Often H, often D	Often H, often D
	2.1×10^1	N, A	N, A	N, A	Occasionally H, A	Occasionally H, A

N = normal A = alive H = haemorrhagic S = stunted D = dead

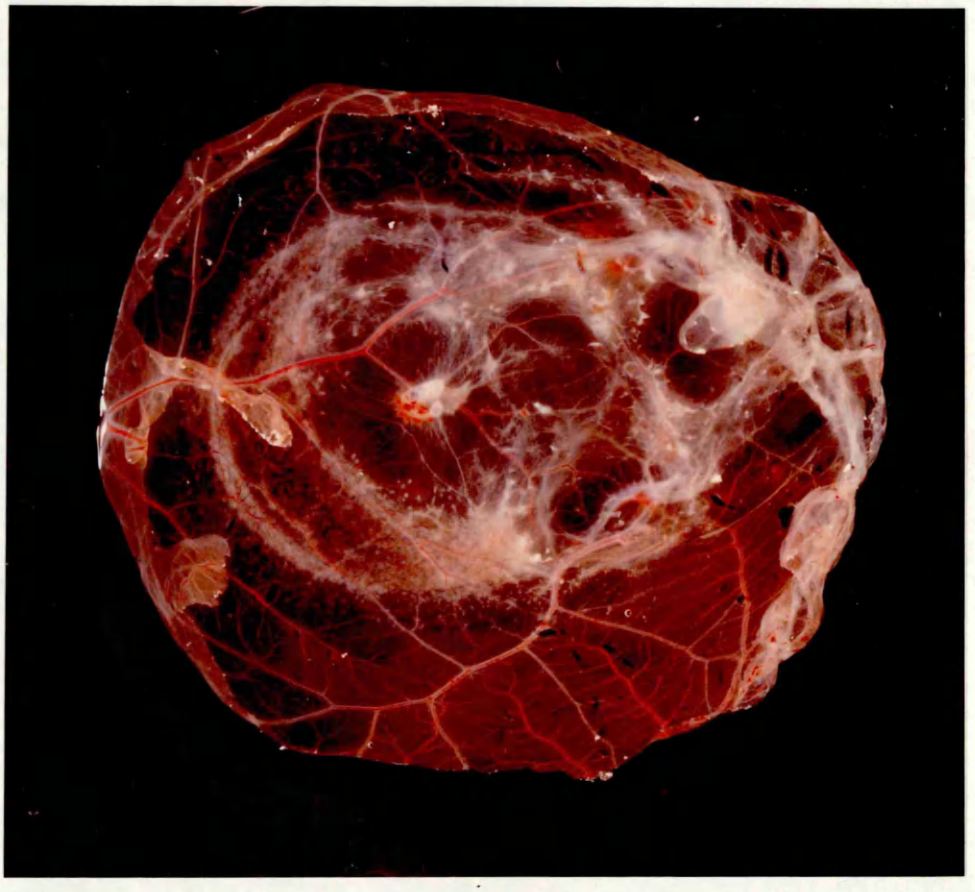


Normal CAM after 17 days' incubation, inoculated six days previously with control cell culture lysate. (x2)

PLATE 3

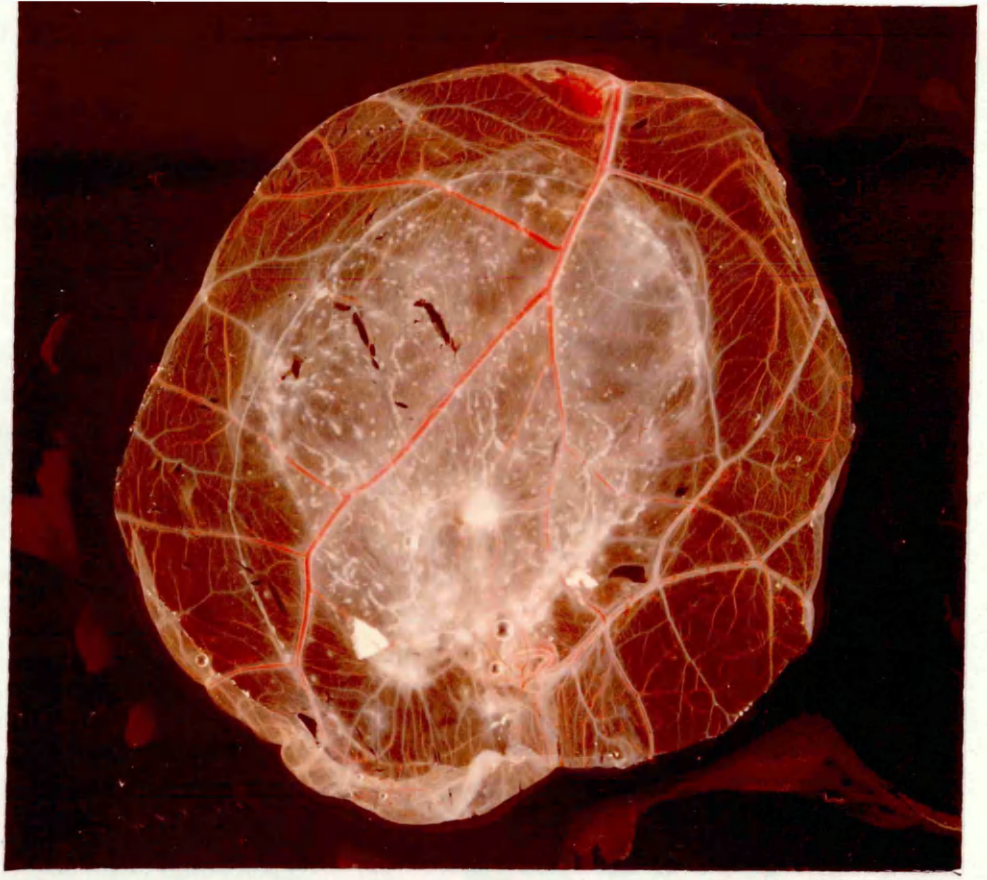


a) CAM after 12 days' incubation, inoculated one day previously with HSV type 1 strain 22101/71. (x2)

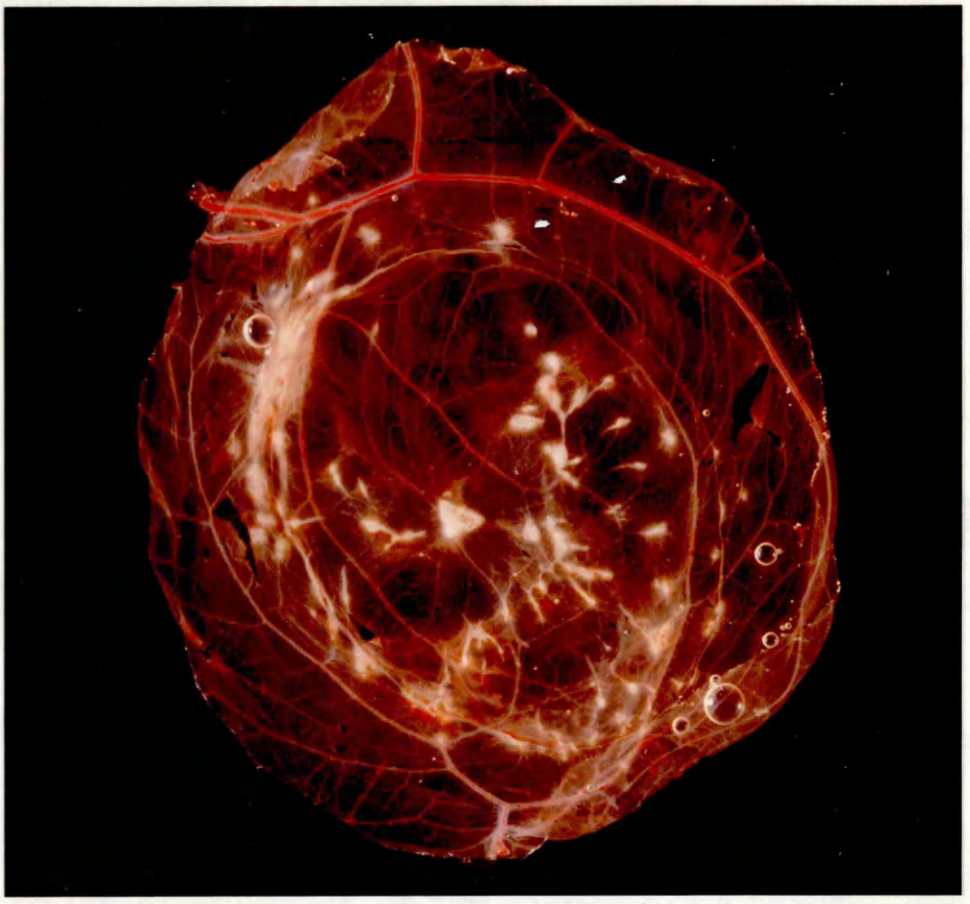


b) CAM after 12 days' incubation, inoculated one day previously with HSV type 2 strain 9889/70. (x2)

PLATE 4

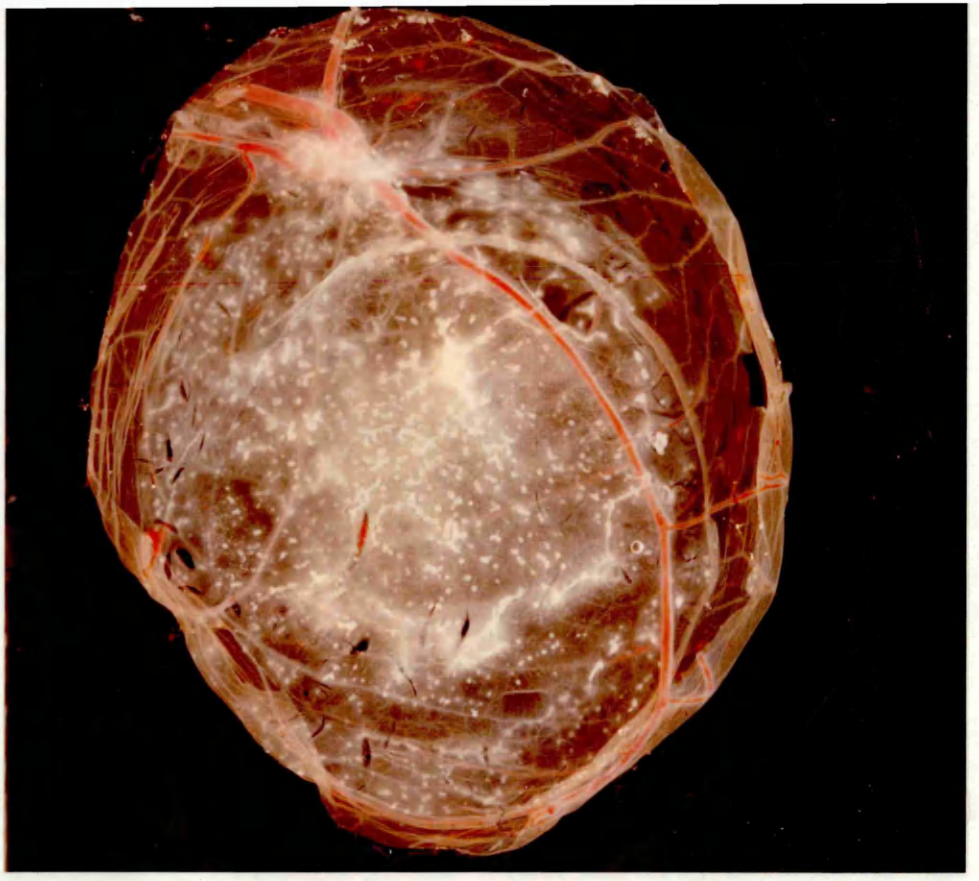


a) CAM after 13 days' incubation, inoculated two days previously with HSV type 1 strain 22101/71. (x2)

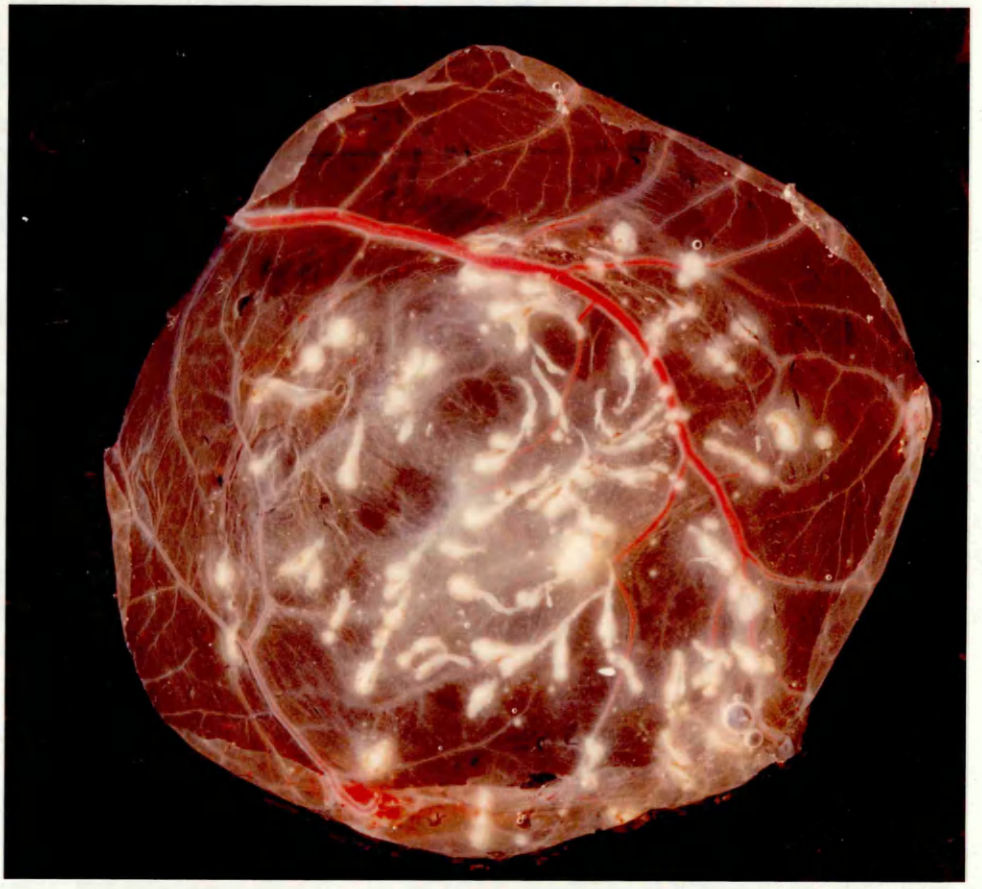


b) CAM after 13 days' incubation, inoculated two days previously with HSV type 2 strain 9889/70. (x2)

PLATE 5

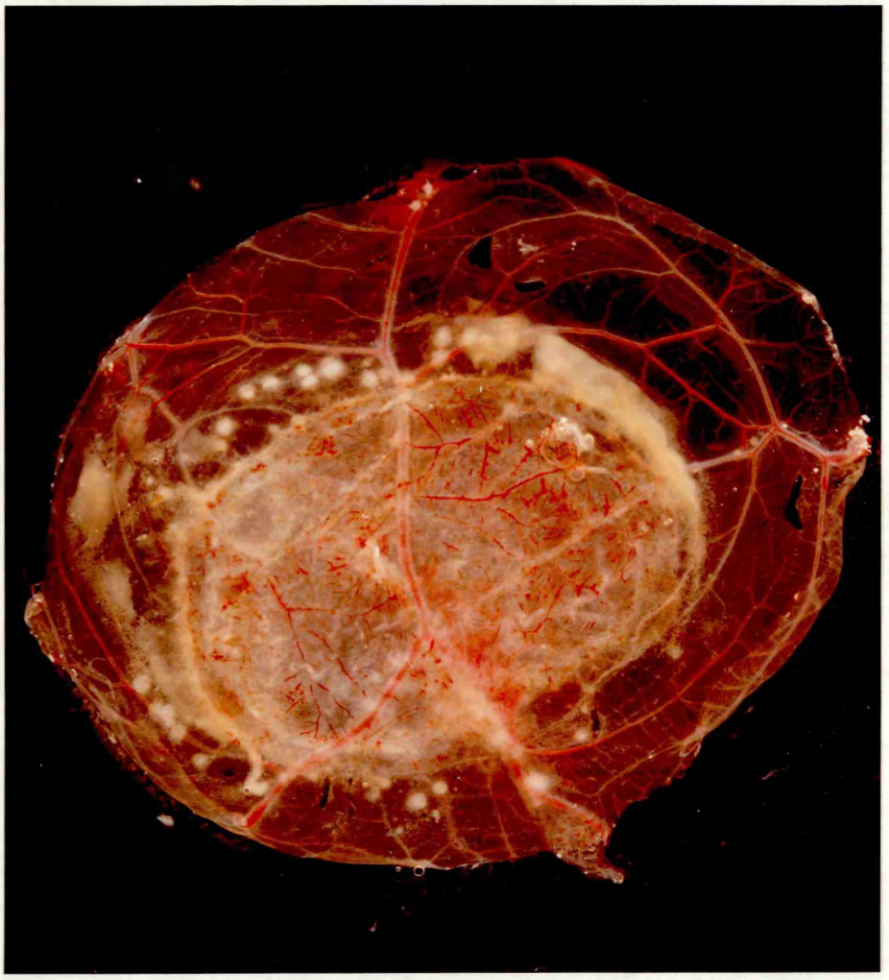


a) CAM after 14 days' incubation, inoculated three days previously with HSV type 1 strain 22101/71. (x2)

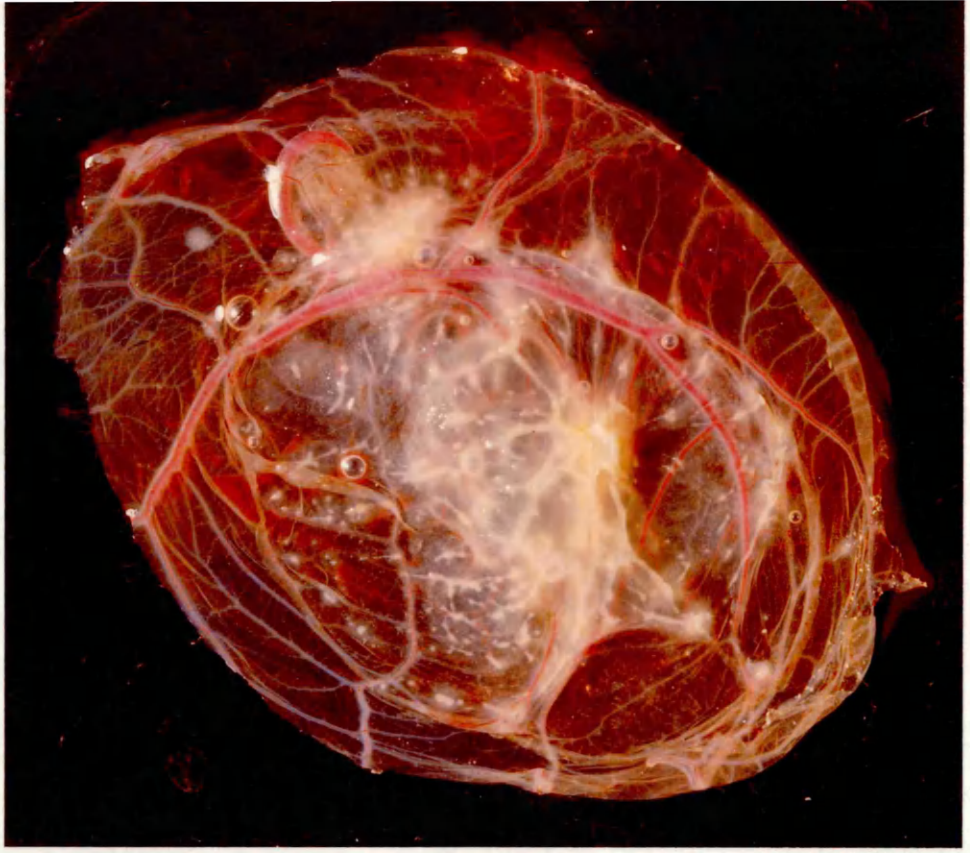


b) CAM after 14 days' incubation, inoculated three days previously with HSV type 2 strain 9889/70. (x2)

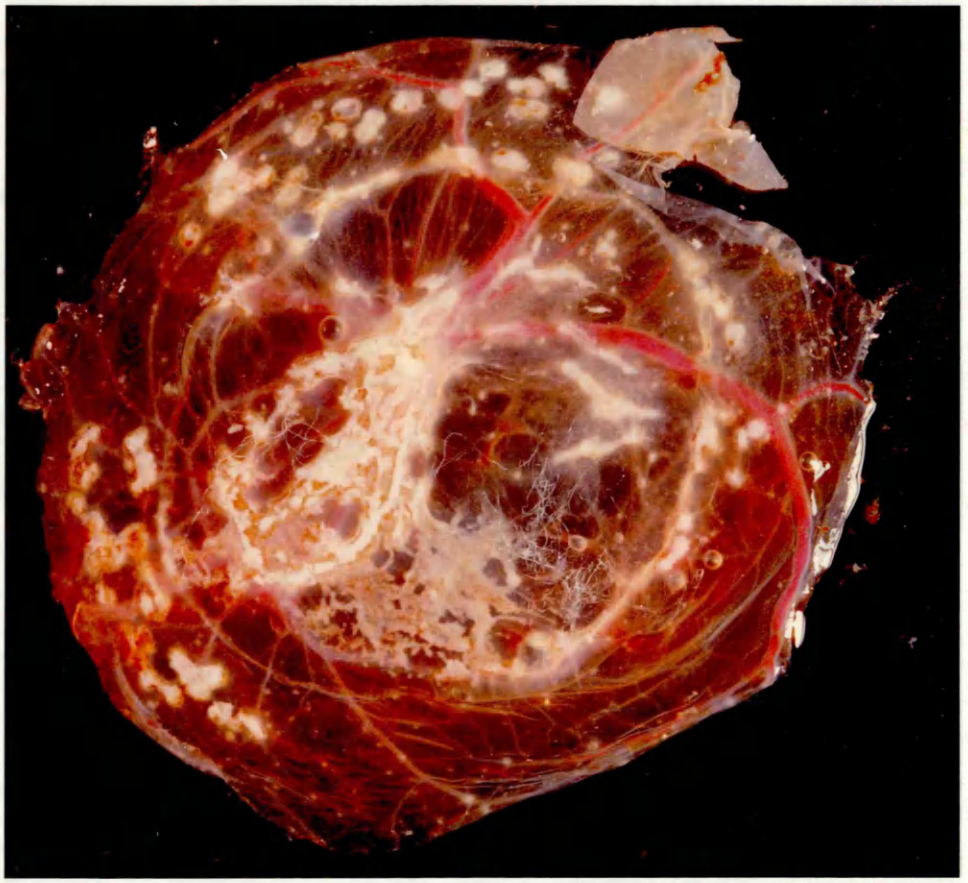
PLATE 6



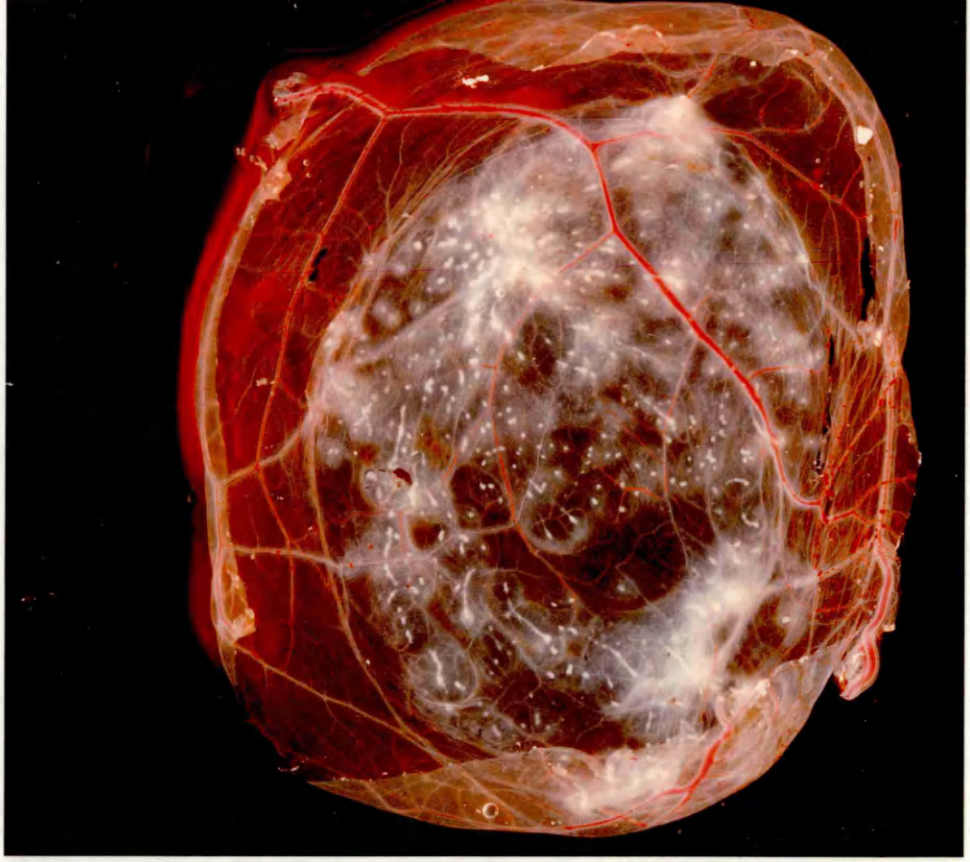
CAM after 14 days' incubation, inoculated three days previously with a high concentration of HSV type 2 strain 9889/70. (x2)
(Note haemorrhages and necrosis of pocks and membrane)



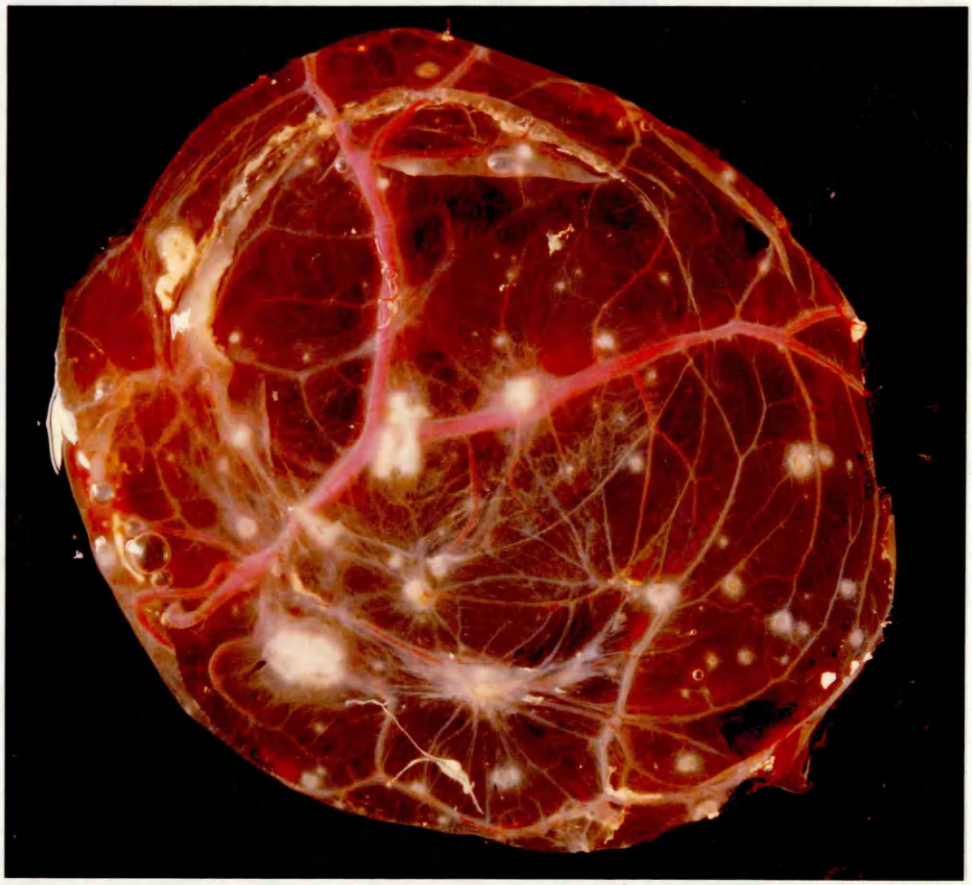
a) CAM after 15 days' incubation, inoculated four days previously with HSV type 1 strain 22101/71. (x2)



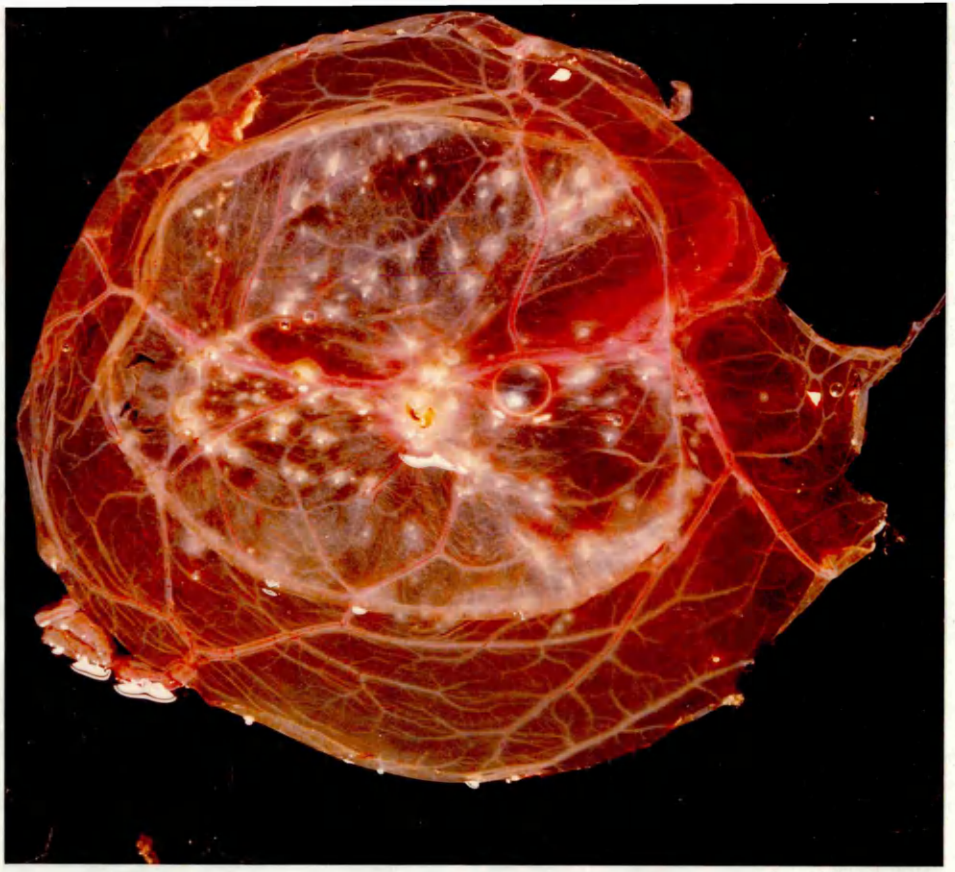
b) CAM after 15 days' incubation, inoculated four days previously with HSV type 2 strain 9889/70. (x2)



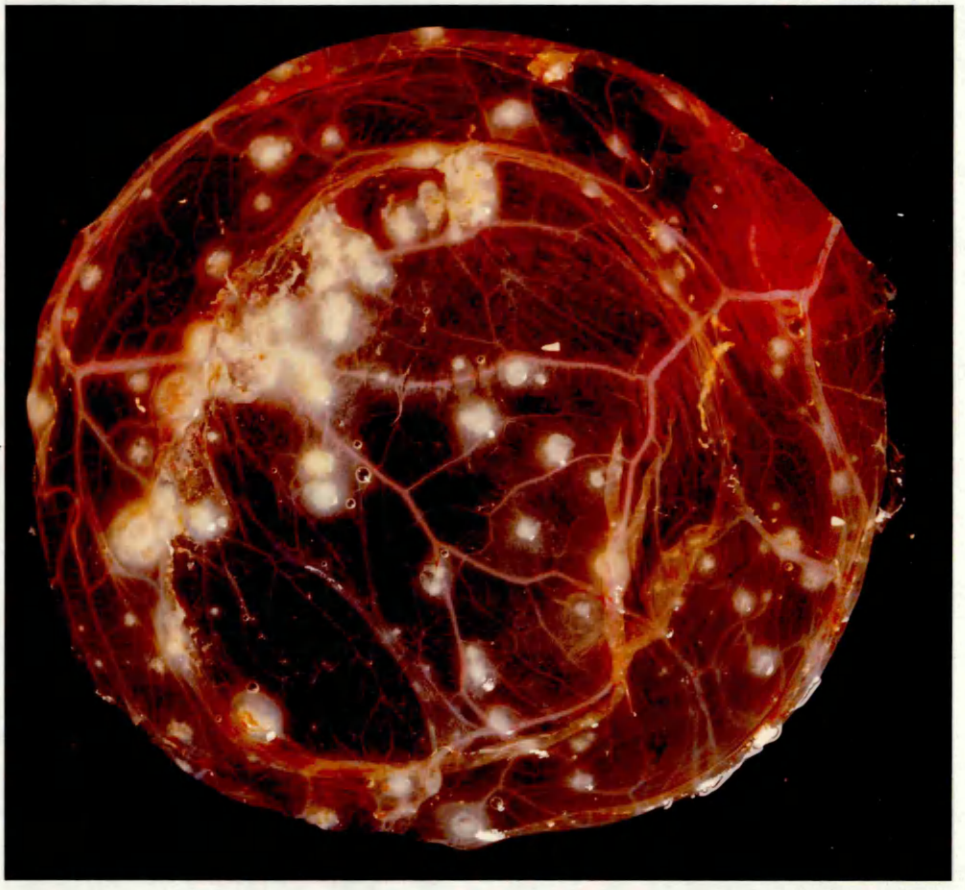
a) CAM after 16 days' incubation, inoculated five days previously with HSV type 1 strain 22101/71. (x2)



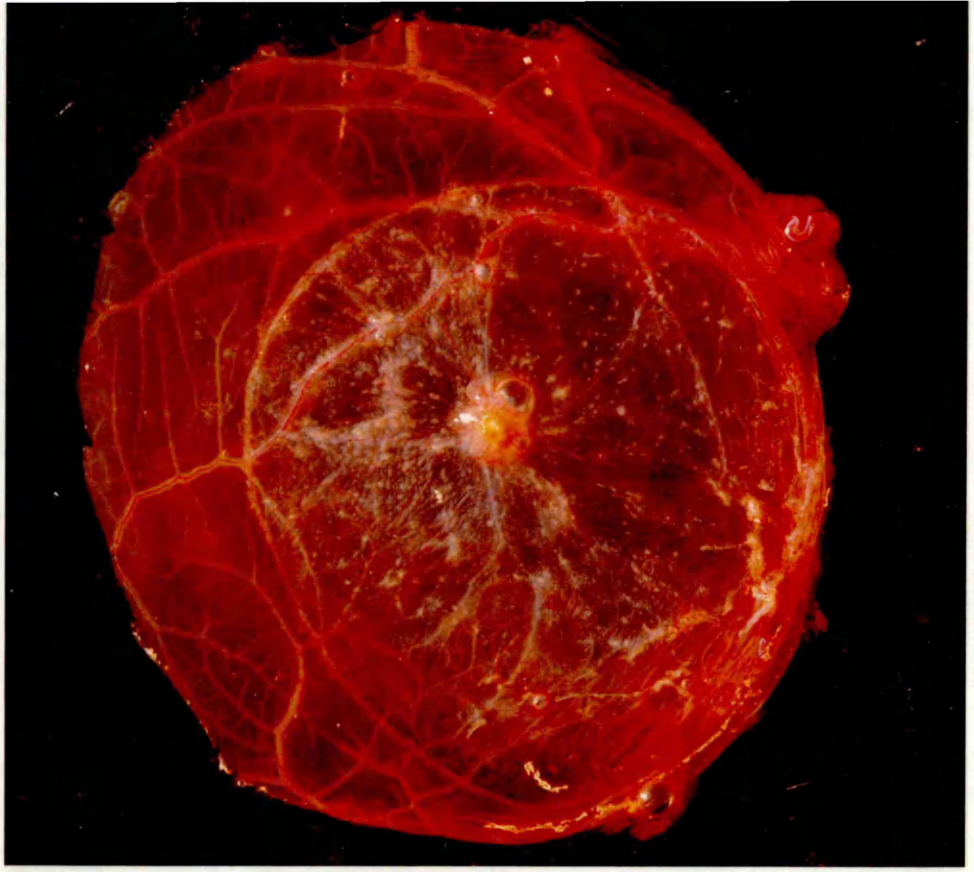
b) CAM after 16 days' incubation, inoculated five days previously with HSV type 2 strain 9889/70. (x2)



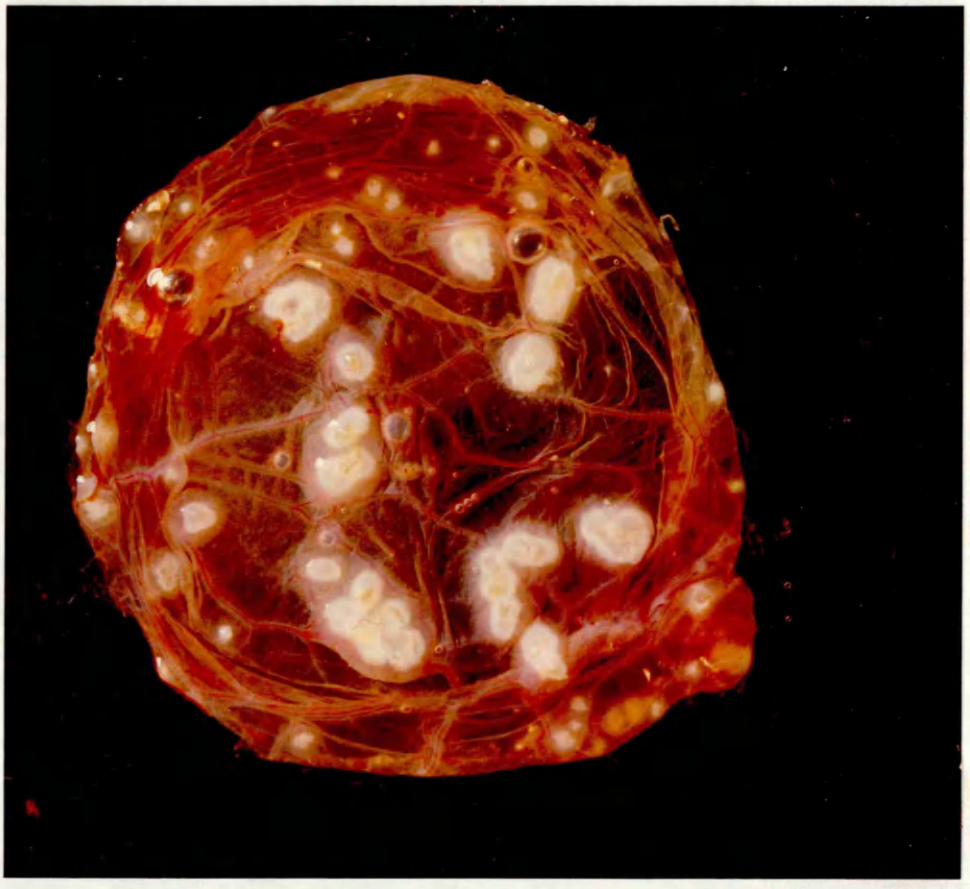
a) CAM after 17 days' incubation, inoculated six days previously with HSV type 1 strain 22101/71. (x2)



b) CAM after 17 days' incubation, inoculated six days previously with HSV type 2 strain 9889/70. (x2)

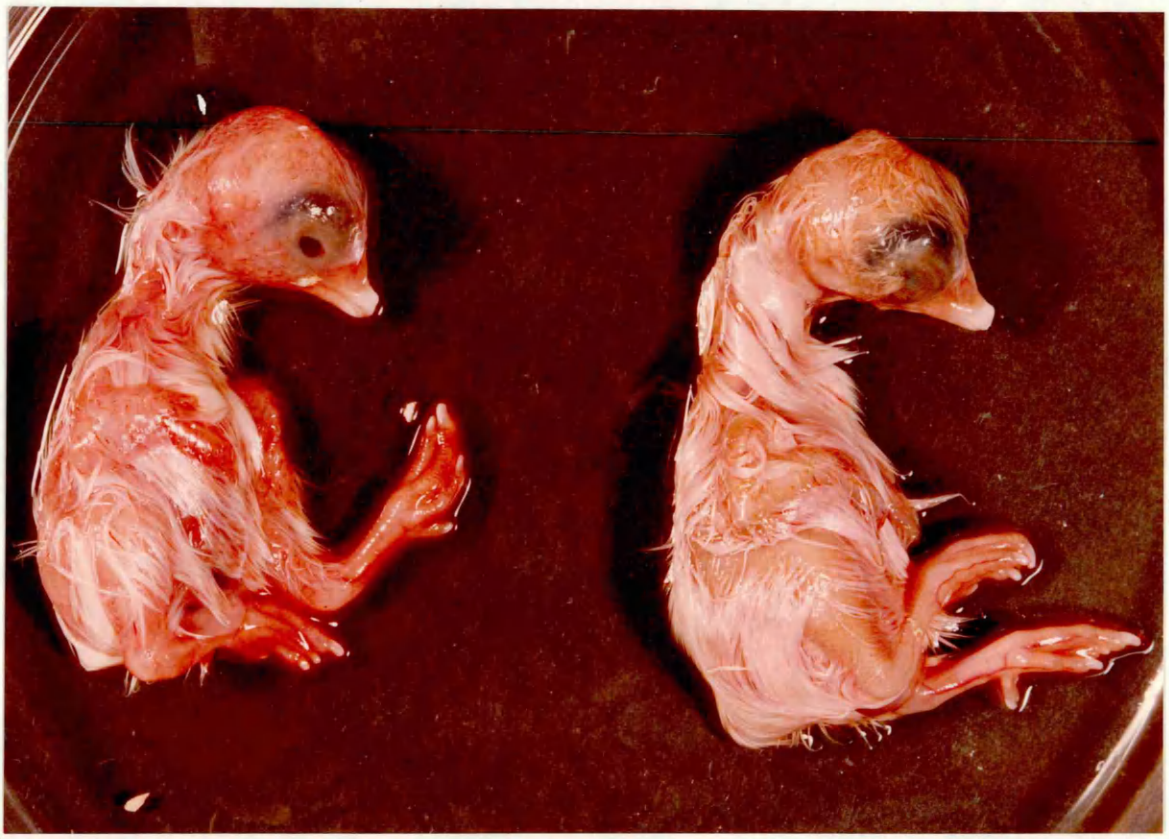


a) CAM after 18 days' incubation, inoculated seven days previously with HSV type 1 strain 22101/71. (x2)



b) CAM after 18 days' incubation, inoculated seven days previously with HSV type 2 strain 9889/70. (x2)

a) Normal chick embryo after 17 days' incubation. Equivalent to six days of inoculated eggs. (x1.2)



b) Infected chick embryos after 17 days' incubation. The CAMs from these eggs had been inoculated six days previously with HSV type 2 strain 9889/70. (x1.2)
(Note haemorrhagic condition of embryos and corneal lesions)

3 Yield of infectious virus from inoculated CAMs of fertile hens' eggs

The two strains used were the type 1 strain 22101/71 and the type 2 strain 9889/70, both fresh isolates, each of which had received only two passages in HEL cell cultures, and had been stored at $-70^{o}C$ for five years prior to use (table 5). CAMs were inoculated with 0.1 ml. of 10^{-2} and 10^{-4} dilutions of the stock virus pools, harvested and assayed for virus exactly three and six days later by titration in HEL cell cultures ('Materials and Methods' section 5.1). Virus was released from the CAMs as described in the 'Materials and Methods' section 2.4.

Production of infectious virus at three days after CAM inoculation appeared to be dose dependent. In the case of the type 1 virus there was little evidence of large scale viral replication unless a low virus concentration inoculum was given (figure 5), but with the type 2 strain, replication of virus was observed with both inocula, although to a lesser degree with the higher inoculum. The yield of infectious virus at six days after CAM inoculation declined with both virus types at high or low virus concentration inocula, but was more evident for the type 1 virus.

Titres of infectious virus in harvests are detailed in table 8.

Figure 5 Yield of infectious virus from CAMs 3 and 6 days post-inoculation with different concentrations of virus

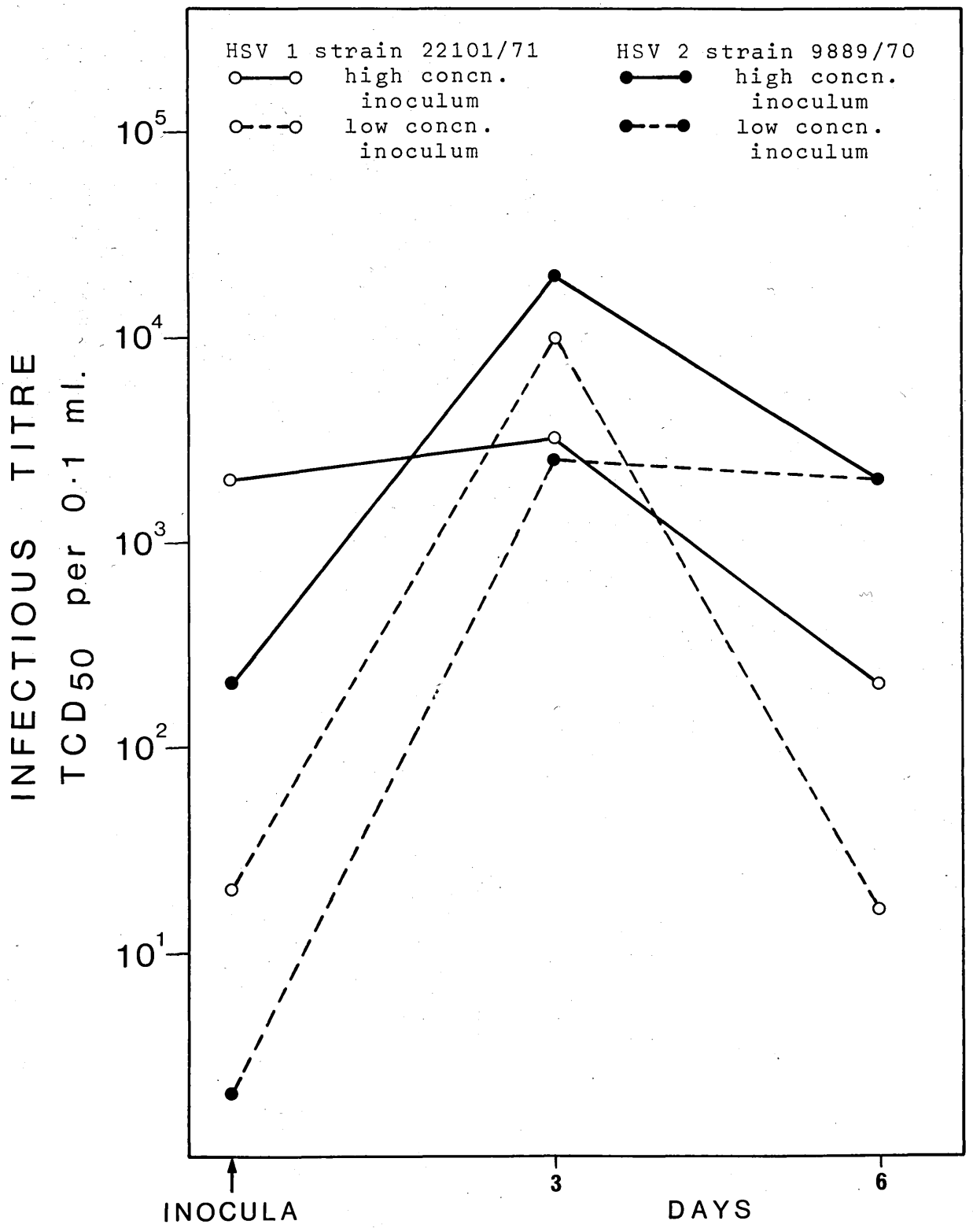


Table 8 Yield of infectious virus from CAMs *in situ*

HSV type 1 strain 22101/71			HSV type 2 strain 9889/70		
Titre of infectious virus in inoculum	Days of harvests	Titre of infectious virus in harvests*	Titre of infectious virus in inoculum	Days of harvests	Titre of infectious virus in harvests*
3.0×10^3	Day 3	5.0×10^3	3.0×10^2	Day 3	3.0×10^4
	Day 6	3.0×10^2		Day 6	3.0×10^3
3.0×10^1	Day 3	1.0×10^4	3.0	Day 3	4.0×10^3
	Day 6	2.0×10^1†		Day 6	3.0×10^3

All titres are expressed as TCD_{50} per 0.1 ml. as titrated in HEL cell cultures.

* titres are the average of the titrations of two CAM harvests

† titre is the result of the titration of one CAM harvest - the second harvest failed to yield infectious virus

4 Virus growth on CAMs of fertile hens' eggs inoculated at high temperatures

Of the 42 viruses examined in this experiment, 24 were HSV type 1 and 18 were HSV type 2 strains. All strains examined produced type-specific pocks and reactions on the CAM of 11 day old fertile hens' eggs (as described in section 2), when incubated at 36°C.

Of the 24 HSV type 1 strains 12 were laboratory adapted strains and 12 were fresh isolates. When inoculated eggs were incubated at 38.5°C, nine of the laboratory strains and three of the fresh isolates grew and produced pocks on the CAMs. The remaining three laboratory strains and nine fresh isolates failed to grow at incubation temperatures of 38.5°C or higher. Of the nine laboratory strains which grew at 38.5°C five also grew at 39°C and three at 39.5°C, whilst none of the fresh isolates which grew at 38.5°C produced pocks on CAMs when incubated at 39°C or 39.5°C.

Six of the 18 HSV type 2 strains examined were laboratory strains and 12 were fresh isolates. Sixteen of the type 2 strains tested grew on CAMs at 38.5°C, only the laboratory strains T514 and 2248 failing to grow at the higher temperature. The strain T514 produced only a small number of pocks on CAMs incubated at 36°C, whilst strain 2248, although it produced semi-confluent pocks at 36°C, failed to grow at temperatures higher than this. Three of the laboratory strains which grew at 38.5°C also grew at 39°C and two of these also grew at 39.5°C. The laboratory strain 3345, which did not grow at 39°C, produced only a small number of pocks on the CAM when incubated at 36°C or 38.5°C. Of the 12 fresh isolates all grew at

38.5°C, seven at 39°C and three at 39.5°C. No strain which grew at any given temperature failed to produce pocks on CAMs at the lower temperatures. The control eggs, inoculated on the CAM with control uninoculated cell culture fluids containing cell lysates, did not induce pocks on the CAM when incubated at any of the four temperatures tested. These results are listed in tables 9 and 10.

When expressed as percentages these results show only small differences between the laboratory strains of HSV type 1 and type 2. However, with fresh isolates major type-specific differences were observed: only 25% of HSV type 1 strains induced pocks even at 38.5°C, and none at temperatures higher than this; in contrast, 100% of HSV type 2 strains induced pock formation at 38.5°C, 58% at 39°C, and 25% even at 39.5°C. These results are summarised in table 11.

The laboratory strains HIL (HSV type 1) and PAR (HSV type 2) and the fresh isolate 17152/71 (HSV type 2) were used to test whether the titre of the inoculum influenced subsequent growth of virus. The infectious virus titres of the undiluted stock pools of these strains were 2.1 x 10^5, 3.2 x 10^6 and 2.1 x 10^4 TCD_{50} per 0.1 ml. virus inoculum respectively. All strains grew at 36°C on CAMs, producing pocks at all dilutions studied, although as expected a progressive dilution resulted in a decrease in the number of pocks produced on the CAM. The HSV type 1 strain HIL failed to grow and produce pocks on CAMs at any of the elevated temperatures studied and at any of the tenfold dilutions examined from 10^{-1} to 10^{-4}.

Table 9 Effect of temperature of incubation on growth of HSV type 1 strains on CAMs

Strain	History	Temperature of incubation (°C) 36.0	38.5	39.0	39.5
10711/53	lab.	++++	++++	-	-
1716	lab.	++++	++++	+++	-
Duffy	lab.	++++	++	-	-
M5	lab.	++++	+++	++	++
64179	lab.	++++	++	-	-
KIR	lab.	++++	++++	-	-
HIL	lab.	++++	-	-	-
HFEM	lab.	++++	+	+	+
WAL	lab.	++++	-	-	-
5516	lab.	++++	+++	+	-
1156	lab.	++++	-	-	-
2913	lab.	++++	++++	+++	+++
1065/71	fresh	+++	-	-	-
1067/71	fresh	++++	++	-	-
1068/71	fresh	++++	++	-	-
2330/71	fresh	++++	++++	-	-
17962/71	fresh	++++	-	-	-
22101/71	fresh	++++	-	-	-
7677/73	fresh	++	-	-	-
7747/73	fresh	++	-	-	-
2104/74	fresh	+++	-	-	-
8037/74	fresh	+++	-	-	-
8802/74	fresh	+++	-	-	-
9892/74	fresh	++++	-	-	-

++++ = confluent growth of HSV on CAM
+++ = semi-confluent growth of HSV on CAM
++ = 100+ pocks produced by HSV on CAM
+ = <100 pocks produced by HSV on CAM
- = no growth of HSV on CAM

Table 10 Effect of temperature of incubation on growth of HSV type 2 strains on CAMs

Strain	History	Temperature of incubation (°C) 36.0	38.5	39.0	39.5
2219	lab.	+++	+++	+	+
T514	lab.	+	-	-	-
2248	lab.	+++	-	-	-
LOV	lab.	++++	++++	++	+
PAR	lab.	++++	++++	++	-
3345	lab.	+	+	-	-
9889/70	fresh	++++	++++	+++	+
24055/70	fresh	++++	+	-	-
1066/71	fresh	++++	++++	-	-
17152/71	fresh	++++	++	+	-
17189/71	fresh	++	++	-	-
24061/71	fresh	++++	++++	+	-
7447/72	fresh	+++	++++	++	+
8087/72	fresh	++++	+++	+	-
9965/72	fresh	++++	++	-	-
10331/73	fresh	++++	++++	++	++
294/74	fresh	+++	++	++	-
10089/74	fresh	+++	+	-	-

++++ = confluent growth of HSV on CAM
+++ = semi-confluent growth of HSV on CAM
++ = 100+ pocks produced by HSV on CAM
+ = <100 pocks produced by HSV on CAM
- = no growth of HSV on CAM

Table 11 Summary of the effects of temperature of incubation on the growth of HSV types 1 and 2 on CAMs

Temperature	HSV type 1 strains			HSV type 2 strains		
	TOTAL	LAB.	FRESH	TOTAL	LAB.	FRESH
36°C	$\frac{24}{24}$* (100%)	$\frac{12}{12}$ (100%)	$\frac{12}{12}$ (100%)	$\frac{18}{18}$ (100%)	$\frac{6}{6}$ (100%)	$\frac{12}{12}$ (100%)
38.5°C	$\frac{12}{24}$ (50%)	$\frac{9}{12}$ (75%)	$\frac{3}{12}$ (25%)	$\frac{16}{18}$ (89%)	$\frac{4}{6}$ (67%)	$\frac{12}{12}$ (100%)
39.0°C	$\frac{5}{24}$ (21%)	$\frac{5}{12}$ (42%)	$\frac{0}{12}$ (0%)	$\frac{10}{18}$ (56%)	$\frac{3}{6}$ (50%)	$\frac{7}{12}$ (58%)
39.5°C	$\frac{3}{24}$ (13%)	$\frac{3}{12}$ (25%)	$\frac{0}{12}$ (0%)	$\frac{5}{18}$ (28%)	$\frac{2}{6}$ (33%)	$\frac{3}{12}$ (25%)

* numerator = number of strains showing growth on CAM
denominator = total number of strains tested at each temperature

Both HSV type 2 strains PAR and 17152/71 grew on CAMs at 38.5°C at all dilutions examined. The PAR strain grew on CAMs at 39°C when inoculated undiluted, but failed to grow at a dilution of 10^{-4}. The 17152/71 strain grew on CAMs at 39°C when inoculated undiluted or at a dilution of 10^{-1}, but failed to produce pocks when inoculated onto CAMs at 10^{-3} dilutions. Neither of the type 2 strains grew on CAMs at 39.5°C at any dilution examined (table 12).

Table 12 Effects of concentration of virus inoculum on growth of HSV types 1 and 2 on CAMs at elevated temperatures

Strains (TCD_{50} per 0.1 ml.)	Dilution of inoculum	Temperature of incubation (°C) 36.0	38.5	39.0	39.5
HSV type 1					
HIL (2.1×10^5)	undiluted	++++	-	-	-
	10^{-1}	++++	-	-	-
	10^{-2}	++	-	-	-
	10^{-3}	++	-	-	-
	10^{-4}	+	-	-	-
HSV type 2					
PAR (3.2×10^6)	undiluted	++++	+++	++	-
	10^{-2}	++	+	nt	-
	10^{-4}	++	+	-	-
17152/71 (2.1×10^4)	undiluted	++++	++	+	-
	10^{-1}	+++	+	+	-
	10^{-3}	++	+	-	-

++++ = confluent growth of HSV on CAM
+++ = semi-confluent growth of HSV on CAM
++ = 100+ pocks produced by HSV on CAM
+ = <100 pocks produced by HSV on CAM
- = no growth of HSV on CAM
nt = not tested

5 Spread of infectious virus from the inoculated CAM to other regions of the egg and embryo

The CAMs of two eggs were inoculated with strains of HSV types 1 and 2, and the following different regions of the egg and embryo were harvested: CAM, allantoic fluid, amniotic fluid, heart, gut, and brain. These harvests were taken using the precautions outlined in the 'Materials and Methods' sections 2.6 to 2.8 to prevent cross-contamination, at daily intervals from day one to day three and on day six. Harvests from each egg were tested for the presence of infectious HSV by passage of material onto the CAMs of two further eggs. This procedure was performed at least twice for each strain and with some strains three or four times, and gave identical results unless stated otherwise in the text.

Harvests made from eggs initially inoculated with six type 1 strains (HIL, WAL, HFEM, 22101/71, 15694/72, 15811/72) revealed virus only in the CAM. All other regions of the egg harvested after inoculation of type 1 virus strains onto the CAM failed to yield pocks on further passage in eggs, indicating that HSV type 1 had not spread beyond the CAM. Results were identical for all type 1 strains examined on all days tested, and are summarised in table 13.

In contrast, all five type 2 strains tested (LOV, PAR, 9889/70, 17152/71, 15286/72) spread from the CAM to other regions of the egg. Four of the five type 2 strains examined for spread to the allantoic fluid did so within one day of CAM inoculation. The exception, strain 17152/71, had however spread to the allantoic fluid by day two.

Table 13 Spread of HSV type 1 strains HIL, WAL, HFEM, 22101/71, 15694/72 and 15811/72 from the inoculated CAM to other regions of the egg and embryo

Strain	Harvests tested for virus	Recovery of infectious virus from harvests			
		Day 1 harvests	Day 2 harvests	Day 3 harvests	Day 6 harvests
HIL, WAL, HFEM, 22101/71	CAM	+	+	+	+
	FLUIDS:				
	allantoic	-	-	-	-
	amniotic	-	-	-	-
	EMBRYO:				
	heart	-	-	-	-
	gut	-	-	-	-
	brain	-	-	-	-
15694/72 and 15811/72	CAM	+	+	+	+
	FLUIDS:				
	allantoic	-	-	-	-
	amniotic	nt	nt	nt	nt
	EMBRYO:				
	heart	nt	nt	nt	nt
	gut	nt	nt	nt	nt
	brain	-	-	-	-

+ = herpesvirus recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests

- = herpesvirus not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests

nt = not tested

Spread to the amniotic fluid was achieved by day two after CAM inoculation with all four strains examined. Spread to the heart, gut, and brain was accomplished by day three with these strains except LOV which spread to all regions of the embryo tested within only two days and 9889/70 which spread to the heart by day two and to the gut and brain by day three. Additionally, strain 15286/72 spread to the brain three days post CAM inoculation. All selected parts of the embryo yielded virus and within the slight time differences outlined above, the results for all type 2 strains tested were identical, and are summarised in tables 14 and 15.

A further 49 strains of HSV (24 type 1 and 25 type 2) were examined for spread of infectious virus from the CAM of inoculated eggs to the allantoic fluid. Harvests of allantoic fluid collected three days post-inoculation were tested by passage onto either further CAMs which were examined for pock formation or into HeLa cell cultures which were examined for HSV cytopathic effects. No virus was detected in the allantoic fluid harvests of eggs inoculated onto the CAM with any of the laboratory strains or the fresh isolates of HSV type 1. Of the 25 HSV type 2 strains tested four failed to grow on inoculated CAMs and so allantoic fluid harvests of these were negative for virus growth. With the remaining 21 strains the allantoic fluid harvests produced pocks when inoculated onto CAMs, or typical HSV cytopathic effects in HeLa cell cultures, demonstrating spread of all the viable HSV type 2 strains from the CAM to the allantoic fluid. These results are summarised in table 16.

Table 14 Spread of HSV type 2 laboratory strains LOV, PAR and 17152/71 from the inoculated CAM to other regions of the egg and embryo

Strain	Harvests tested for virus	Recovery of infectious virus from harvests			
		Day 1 harvests	Day 2 harvests	Day 3 harvests	Day 6 harvests
LOV	CAM	+	+	+	+
	FLUIDS:				
	allantoic	+	+	+	+
	amniotic	-	+	+	+
	EMBRYO:				
	heart	-	+	+	+
	gut	-	+	+	+
	brain	-	+	+	+
PAR	CAM	+	+	+	+
	FLUIDS:				
	allantoic	+	+	+	+
	amniotic	-	+	+	+
	EMBRYO:				
	heart	-	-	+	+
	gut	-	-	+	+
	brain	-	-	+	+
17152/71	CAM	+	+	+	+
	FLUIDS:				
	allantoic	-	+	+	+
	amniotic	-	+	+	+
	EMBRYO:				
	heart	-	-	+	+
	gut	-	-	+	+
	brain	-	-	+	+

+ = herpesvirus recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests

- = herpesvirus not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests

Table 15 Spread of HSV type 2 fresh isolates 9889/70 and 15286/72 from the inoculated CAM to other regions of the egg and embryo

Strain	Harvests tested for virus	Recovery of infectious virus from harvests			
		Day 1 harvests	Day 2 harvests	Day 3 harvests	Day 6 harvests
9889/70	CAM	+	+	+	+
	FLUIDS:				
	allantoic	+	+	+	+
	amniotic	-	+	+	+
	EMBRYO:				
	heart	-	+	+	+
	gut	-	-	+	+
	brain	-	-	+	+
15286/72	CAM	+	+	+	+
	FLUIDS:				
	allantoic	+	+	+	+
	amniotic	nt	nt	nt	nt
	EMBRYO:				
	heart	nt	nt	nt	nt
	gut	nt	nt	nt	nt
	brain	-	-	+	+

+ = herpesvirus recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests

- = herpesvirus not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests.

nt = not tested

Table 16 Spread of HSV strains from the inoculated CAM into the allantoic cavity

HSV type 1 strains inoculated onto CAMs	Virus recovered from allantoic fluid (Day 3 harvests) Harvests checked by CAM inocn.	HeLa CC inocn.	HSV type 2 strains inoculated onto CAMs	Virus recovered from allantoic fluid (Day 3 harvests) Harvests checked by CAM inocn.	HeLa CC inocn.
10711/53 L	-		25766 L	+	
Candeias L	-		2248 L		+
61446 L	-		T514* L		-
Duffy L	-		2219* L		-
M5 L	-		3345* L		-
64179 L	-		669/67* F	-	
5516 L	-		HSV2/69 F		+
2913 L		-	5502/69 F	+	
409 F		-	24055/70 F	+	
24126/70 F	-		1066/71 F	+	
1067/71 F		-	17189/71 F	+	
1068/71 F		-	24061/71 F	+	
2330/71 F		-	2304/72 F	+	
17599/71 F	-		7447/72 F	+	
17962/71 F	-		8087/72 F	+	
14958/72 F	-		9965/72 F	+	
15815/72 F	-		12260/72 F	+	
20860/72 F	-		18464/72 F	+	
7677/73 F		-	19630/72 F	+	
7747/73 F		-	10331/73 F		+
2104/74 F		-	20661/73 F		+
8037/74 F		-	294/74 F		+
9892/74 F		-	1699/74 F		+
2160/75 F		-	10089/74 F		+
			15031/74 F		+

L = laboratory passaged strain

F = fresh isolate

* = no growth on CAM, i.e. original material not viable

+ = herpesvirus recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests, or by typical HSV cytopathic effects in HeLa cell cultures

- = herpesvirus not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests, or no cytopathic effects in HeLa cell cultures

Following inoculation of CAMs with HSV type 1 strain 22101/71 and HSV type 2 strain 9889/70 embryos were removed after six days. Embryos from eggs inoculated with the type 1 strain showed no gross signs of virus infection, whilst those from eggs inoculated with the type 2 strain showed signs of extensive virus infection, similar to those described in section 2 (table 7, plate 12b). Whole embryos were rinsed in three changes of 150 ml. PBS solution A, to remove contaminating amniotic fluid, and the eyes were scraped with a scalpel. The eyes of embryos from eggs which had been inoculated with the type 1 strain showed no signs of virus infection and appeared similar to those of uninoculated controls (plate 12a). The eyes of embryos from eggs which had been inoculated with the type 2 strain showed extensive signs of virus infection: the cornea had become clouded with virus lesions and small haemorrhages (plate 12b). The eye scrapings were re-suspended in 5 ml. PSBS and frozen at -70^{o}C and thawed at 37^{o}C to lyse potentially infected cells. The resultant fluid was inoculated onto further CAMs of eggs. Scrapings from embryo eyes harvested from eggs inoculated with the HSV type 1 strain yielded no virus, whilst those harvested from eggs inoculated with the HSV type 2 strain yielded virus which produced typical HSV type 2 pocks. These results were identical when repeated using two inoculated eggs per strain to harvest eye material and two eggs to test each harvest.

5.1 Effect of concentration of virus inoculum on subsequent invasiveness within the egg

Using 0.1 ml. inocula of undiluted stock HSV pools (table 5) all HSV type 2 strains spread rapidly to the embryo (tables 14 and 15). Similar results were obtained when the HSV type 2 strain 9889/70 pool was diluted 10^{-1} to 10^{-4}, although progressive dilution resulted in delayed spread to the embryo. Spread to the allantoic fluid occurred within one day when the stock virus strain was used diluted 10^{-1}, whilst 10^{-2} and 10^{-3} dilutions delayed the spread, virus not appearing in the allantoic fluid until after two days. The rate of spread of virus to the embryo brain was unaffected by such dilutions: inoculation of 10^{-1} to 10^{-3} dilutions was followed by repeated recovery of virus from the brain on the third day after CAM inoculation. But inoculation of a 10^{-4} dilution of the virus was followed by spread of the virus to the allantoic fluid after a delay of three days, and to the embryo brain after six days. When even higher dilutions of the stock virus pool were inoculated onto the CAM so that inocula contained only 5-20 pock forming units, no haemorrhages of the CAMs were observed and no virus was recovered from the embryo. Under these conditions embryos hatched normally. These tests were performed twice using a minimum of two eggs for each dilution of virus. Each harvest of allantoic fluid and embryo brain was treated separately and each was inoculated onto the CAMs of at least two eggs to check for the presence of virus. These results are summarised in table 17.

Table 17 Effect of virus concentration of inoculum on rate of spread of HSV type 2 strain 9889/70 from the inoculated CAM to other regions of the egg and embryo

Inoculum		Virus recovered from allantoic fluid harvests				Virus recovered from embryo brain harvests			
Dilution of stock pool (titre = 1.0 x 10^6 TCD_{50} / 0.1 ml.)	Pocks on inoculated CAM	Days of harvest 1	2	3	6	Days of harvest 1	2	3	6
undiluted	confluent	+	+	+	+	-	-	+	+
10^{-1}	confluent	+	+	+	+	-	-	+	+
10^{-2}	semi-confluent	-	+	+	+	-	-	+	+
10^{-3}	semi-confluent	-	+	+	+	-	-	+	+
10^{-4}	>100	-	-	+	+	-	-	-	+
10^{-5}	50	-	-	+	-	-	-	-	-
10^{-6}	3	-	-	-	-	-	-	-	-

+ = HSV recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests

- = HSV not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests

Spread of type 1 virus beyond the CAM was never demonstrated even when the stock virus pools were used undiluted (table 13). The titres of type 1 virus in these pools were comparable with those of the type 2 virus pools (table 5).

Some one day old chicks which hatched from eggs after CAM inoculation with HSV type 2 strain 9889/70 at dilutions of 10^{-5} and 10^{-6} were bled by heart puncture. There was no virus present in the blood which was checked by inoculation onto further CAMs.

5.2 Effect of multiple egg passage of virus strains on subsequent invasiveness within the egg

Herpes simplex virus type 1 strains HFEM and 22101/71 and HSV type 2 strain LOV were tested for spread from the CAM after 12 and 24 serial egg passages. HSV type 1 strain HFEM and HSV type 2 strain LOV were further tested after 36 serial egg passages (tables 18 and 19). Following 12 serial egg passages the nature and rate of spread of the virus from the inoculated CAM with the three strains tested were unaffected, compared with the stock pools: neither type 1 strain spread beyond the CAM by the sixth day post CAM inoculation and the type 2 strain spread as usual to the allantoic fluid after one day and to the embryo brain on the third day. Following 24 serial egg passages the type 1 strain HFEM spread to the allantoic fluid in some three day harvests and in all four, five and six day harvests, but had not spread to the embryo brain after six days, whilst the type 1 strain 22101/71 had spread into the allantoic fluid four days post CAM inoculation, but had not spread to the brain of the embryo after six days.

Table 18 Effect of multiple serial CAM passage on spread of HSV type 1 from the inoculated CAM to other regions of the egg and embryo

Strains		Harvests	Days of harvest					
			1	2	3	4	5	6
HFEM	CAM pass 12	allantoic fluid	-	-	-	nt	nt	-
		brain	-	-	-	nt	nt	-
	CAM pass 24	allantoic fluid	-	-	+/-	+	+	+
		brain	-	-	-	-	-	-
	CAM pass 36	allantoic fluid	-	-	-	nt	nt	+
		brain	-	-	-	nt	nt	-
22101/71	CAM pass 12	allantoic fluid	-	-	-	nt	nt	-
		brain	-	-	-	nt	nt	-
	CAM pass 24	allantoic fluid	-	-	-	+	+	+
		brain	-	-	-	-	-	-

+ = HSV recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests

- = HSV not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests

nt = not tested

Table 19 Effect of multiple serial CAM passage on spread of HSV type 2 from the inoculated CAM to other regions of the egg and embryo

Strain		Harvests	Days of harvest					
			1	2	3	4	5	6
LOV	CAM pass 12	allantoic fluid	+	+	+	nt	nt	+
		brain	-	-	+	nt	nt	+
	CAM pass 24	allantoic fluid	+	+	+	+	+	+
		brain	+	+	+	+	+	+
	CAM pass 36	allantoic fluid	+	+	+	nt	nt	+
		brain	-	+	+	nt	nt	+

+ = HSV recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests

- = HSV not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests

nt = not tested

After 24 serial egg passages the type 2 strain LOV spread more rapidly to the organs of the embryo compared with the stock pool and the 12 CAM passaged inocula, appearing in both the allantoic fluid and embryo brain only one day after CAM inoculation. Following 36 serial egg passages the type 1 strain HFEM spread to the allantoic fluid after six days (harvests taken four and five days post CAM inoculation were not available for testing), whilst the type 2 strain LOV appeared in the allantoic fluid after one day and in the embryo brain after two days. This spread a little more slowly than the CAM pass 24 material but still more rapidly than the stock pool and the CAM pass 12 inocula. The HSV type 1 strain HFEM was passaged on CAMs up to pass level 24 on two separate occasions. Tests on both HFEM CAM pass 12 strains were identical. With the two HFEM CAM pass 24 strains, one spread to the allantoic fluid from the CAM by day three, whilst the other required four days (see Appendix 3 for passage details). All these tests were performed twice using two eggs per test with the exception of the CAM pass 36 material which was examined once using two eggs per test. All harvests were made separately and tested separately on the CAMs of at least two eggs per harvest.

The type specificity of the apparent aberrant CAM pass 24 of the HSV type 1 strain HFEM was investigated. A total of eight pocks was picked individually from CAMs which had been inoculated four and seven days previously with strain HFEM CAM pass 24, and these materials were inoculated individually into HEL cell cultures and incubated for three days. The cytopathic effects produced

were typical of HSV type 1 strains, i.e. cytoplasmic granulation of cells which became rounded and refractile, either lysing or falling off the glass. Harvests from these infected cultures were then inoculated back onto CAMs of 11 day old eggs and incubated for five to seven days. The resultant pocks formed were small (< 1.0 mm. in diameter) and the membranes showed no signs of haemorrhage or necrosis, i.e. they were typical of type 1 pocks. Eighteen pocks were picked individually from these membranes seven days post-inoculation and inoculated back into further HEL cell cultures which were incubated for three days, after which the cell culture harvests were again inoculated onto 11 day CAMs and incubated for seven days to check for size of pocks. Of the 18 pocks originally picked off, one gave rise on one CAM to a pock slightly larger than all the others, measuring approximately 2.5 mm. in diameter. The rest of the pocks on this and all other membranes were typically small. This larger pock was picked off, inoculated into HEL cell cultures and incubated at 36^{o}C for three days. The cell culture harvest was inoculated onto further CAMs of 11 day old eggs to check if large pock formation would recur, indicating selection of a true variant. The resultant pocks were examined after seven days' incubation and all were small (< 1.0 mm. in diameter) and resembled those of HSV type 1 strains as described in section 2, and there was no haemorrhage or necrosis of the membrane. Following this, HFEM CAM pass 24 was considered a true HSV type 1 strain, and the large pock was thought to be a coalescence of two or more small pocks.

6 Spread of infectious virus from the inoculated allantoic cavity to other regions of the egg and embryo

It was observed that when harvesting HSV inoculated eggs, which exhibited CAMs with holes through the membrane into the allantoic fluid, the embryos were always alive and had no haemorrhages. Under these conditions very few or no pocks were present on the damaged membrane. This was the case whether the CAM had been inoculated with HSV type 1 or HSV type 2 strains. Virus had not spread to the embryo even though virus was in effect inoculated into the allantoic fluid.

Following inoculation of either HSV type 1 or type 2 strains directly into the allantoic fluid, no virus was recovered from the embryo brain harvested one, two, three and six days post-inoculation. Furthermore, the amount of virus recovered from the allantoic fluid, as judged by the number of pocks produced on further CAMs, slowly declined. The results were similar for fresh isolates and laboratory adapted strains as well as for egg adapted strains within each virus type, although the decline was somewhat less pronounced with the egg adapted strains (tables 20 and 21). These tests for each virus were carried out on two eggs for the allantoic cavity inoculation, and the CAMs of two further eggs were used to test each fluid harvest.

Table 20 Persistence and spread of strains of HSV type 1 after inoculation into allantoic fluid of fertile eggs

Inoculum	Virus recovered from allantoic fluid harvests Days of harvests 1	2	3	6	Virus recovered from brain harvests Days of harvests 1	2	3	6
Stock pools								
WAL	++++	++	++	-	-	-	-	-
22101/71	++++	+++	+++	-	-	-	-	-
HFEM	+++	+	-	-	-	-	-	-
Passaged pools								
HFEM CAM pass 12	++++	+++	+	-	-	-	-	-
HFEM CAM pass 24	+	+++	+++	nt	-	-	-	nt

++++ = confluent growth of HSV on CAM
+++ = semi-confluent growth of HSV on CAM
++ = 100+ pocks produced by HSV on CAM
+ = <100 pocks produced by HSV on CAM
- = no growth of HSV on CAM
nt = not tested

Table 21 Persistence and spread of strains of HSV type 2 after inoculation into allantoic fluid of fertile eggs

Inoculum	Virus recovered from allantoic fluid harvests Days of harvests 1	2	3	6	Virus recovered from brain harvests Days of harvests 1	2	3	6
Stock pools								
PAR	++++	+++	+++	+	-	-	-	-
9889/70	++++	+++	+++	-	-	-	-	-
LOV	++++	+++	+	-	-	-	-	-
Passaged pools								
LOV CAM pass 12	++++	+++	++++	-	-	-	-	-
LOV CAM pass 24	+++	++	+++	nt	-	-	-	nt

++++ = confluent growth of HSV on CAM

+++ = semi-confluent growth of HSV on CAM

++ = 100+ pocks produced by HSV on CAM

\+ = <100 pocks produced by HSV on CAM

\- = no growth of HSV on CAM

nt = not tested

7 Yield of infectious virus from inoculated cell cultures derived from egg and embryo tissues

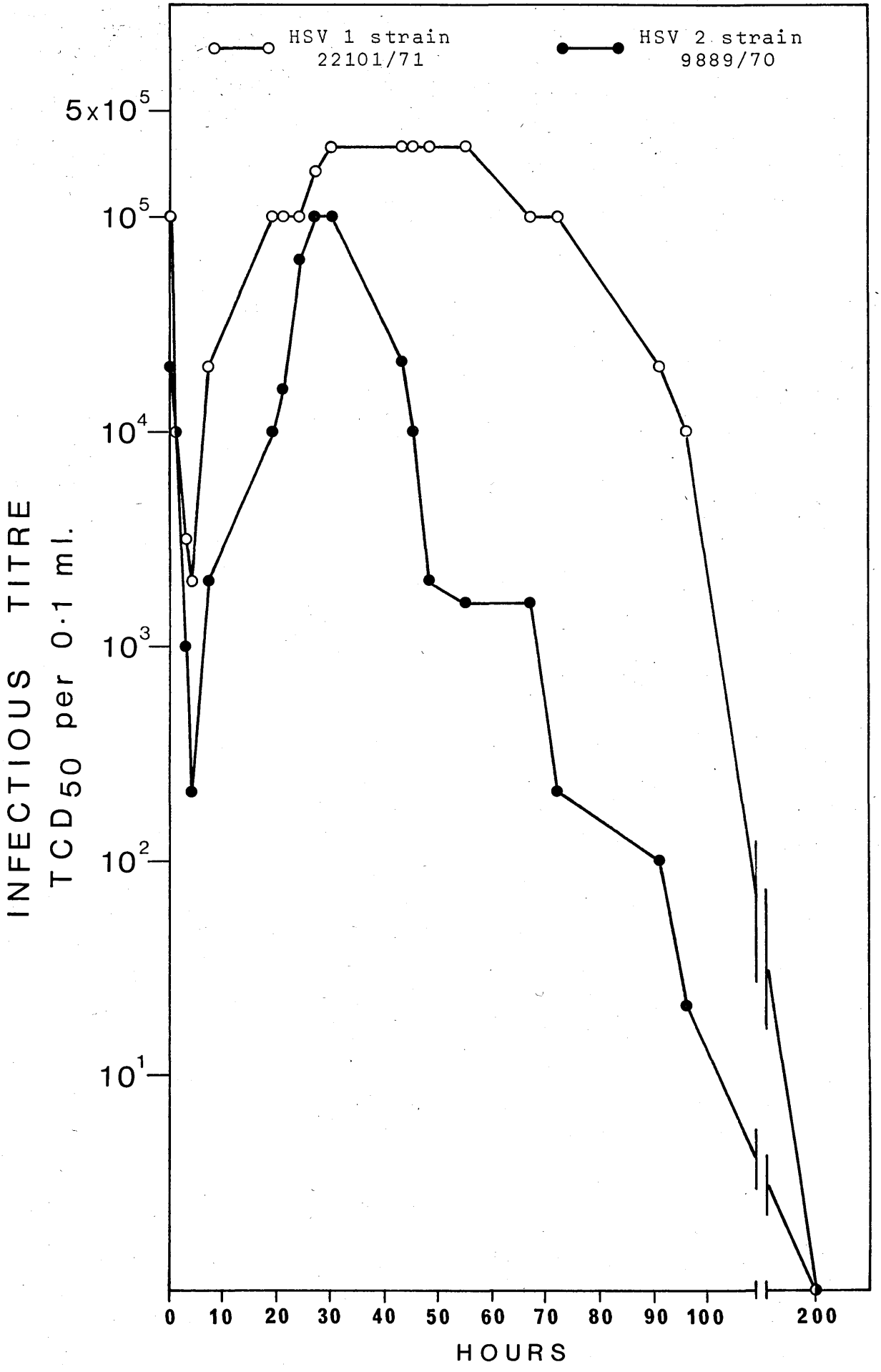
Details of preparation, inoculation and extraction of virus from these cell cultures are described in the 'Materials and Methods' sections 3.4 to 3.7.

After inoculation of the HSV types 1 or 2 strains into chick embryo fibroblast cell (CEFC) cultures, type-specific cytopathic changes were observed. The type 1 strain induced cells to round up in tight groups, whilst the type 2 strain caused cells to swell and round up in loose groups with giant cell production a common feature. Cell cultures rapidly degenerated and became detached from the glass.

The growth curves of the two strains in CEFC cultures were similar (figure 6). The type 1 strain reached the highest titre of 3.0×10^5 TCD_{50} per 0.1 ml. of harvest at 30 hours post infection and this level of infectivity was maintained until 54 hours after which time titres slowly decreased. With the type 2 strain, the highest titre of 1.0×10^5 TCD_{50} per 0.1 ml. of harvest was realised 27 hours post infection and was maintained for three hours before decreasing over the rest of the study period at a more rapid rate than the type 1 strain. No infectious virus was observed 200 hours post infection with either virus strain. At this stage no cells were attached to the glass. Details of titres are listed in Appendix 4.

The chick embryo allantoic cell (CEAC) cultures consisted of a mixture of epithelioid and fibroblastic cells. The cytopathic effects produced in these cultures following inoculation of the strains of each virus type were similar

cell cultures (CEFC)

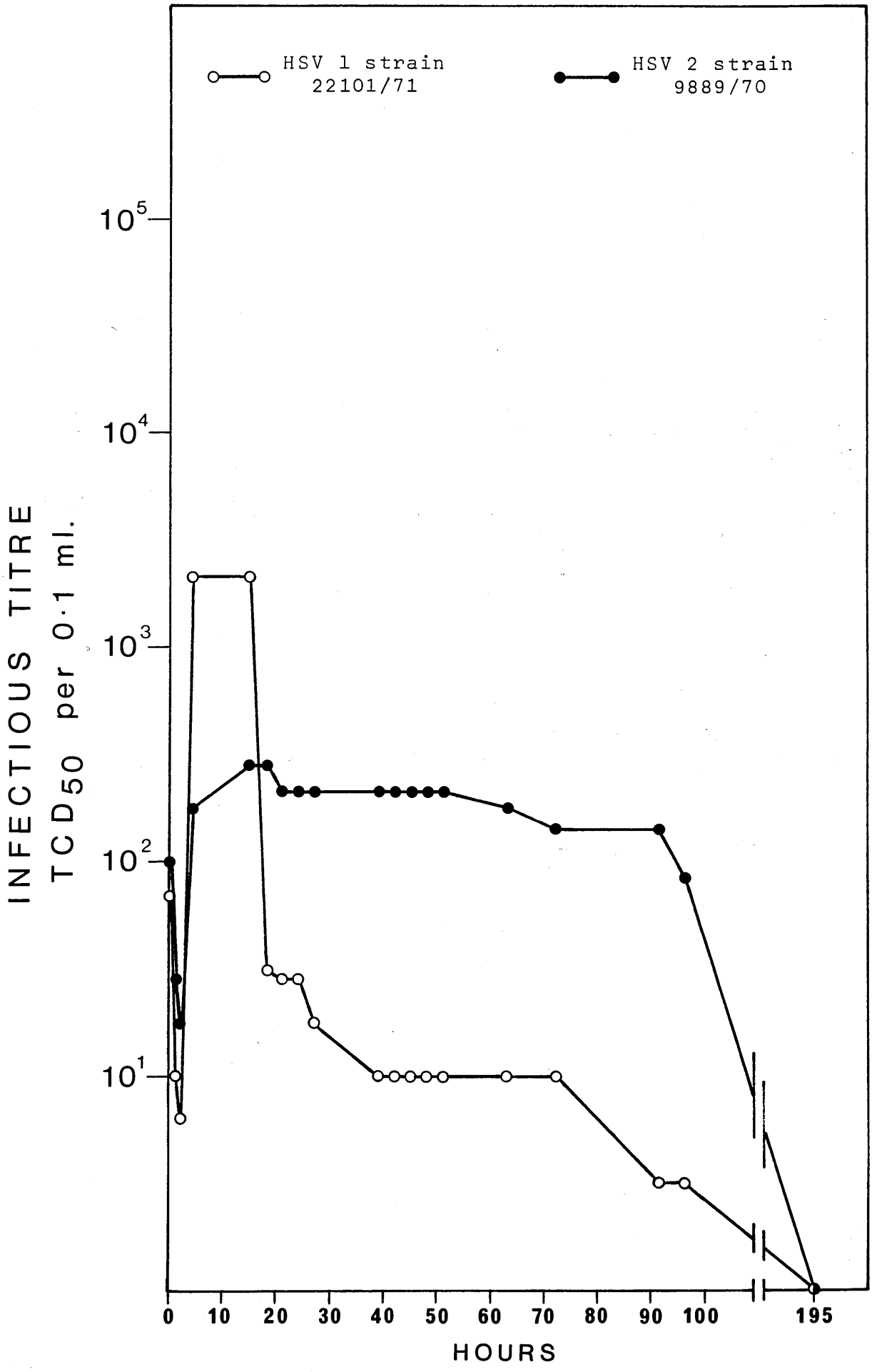


to those described for CEFC cultures.

With the type 1 strain the highest titre of 3.0×10^3 TCD_{50} per 0.1 ml. of harvest was obtained four hours post infection and was maintained for 11 hours, after which titres decreased rapidly over the period examined. The type 2 strain attained the highest titre of 4.0×10^2 TCD_{50} per 0.1 ml. of harvest at 15 and 18 hours after infection of the cultures. These titres were maintained until 91 hours post infection before slowly decreasing. No infectious virus was observed at 195 hours post infection with either virus strain (figure 7). At this stage no cells were attached to the glass. Details of titres are listed in Appendix 4.

The cultures of chick embryo brain cells (CEBC) showed the greatest variation of all the tissues examined. There were three cell types present: epithelioid, fibroblastic and neuronal. The epithelioid cells occurred in small clumps and these formed about 10% of the cells in the cultures, fibroblast cells about 40% and the rest consisted of clumps of flat granular cells, each of which had a long straight cytoplasmic projection more than twice the cell length, and looked like neuronal cells. The cytopathic changes induced by inoculation of the type 1 or type 2 virus strains into these cell cultures were similar to those described for CEFC cultures, with the exception that the type 1 strain caused less cell destruction. With both strains the fibroblastic cells degenerated more rapidly than the epithelioid cells and the neuronal cell extensions developed refractile nodules before detaching from the glass.

cell cultures (CEAC)

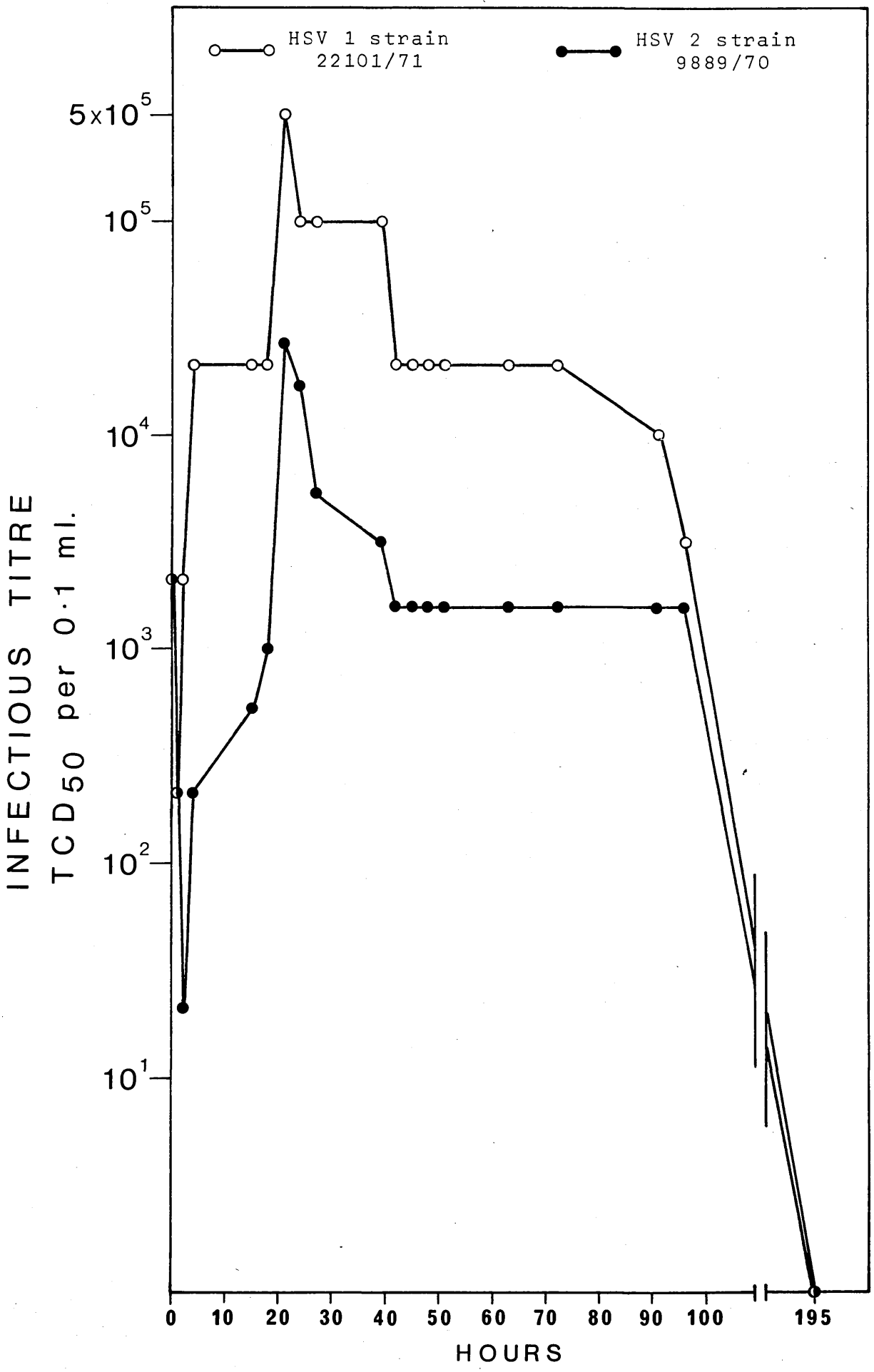


The shape of the growth curves was similar for the strains of both virus types in these cells, but titres were consistently higher with the type 1 strain. Production of infectious virus was initiated more rapidly with the type 1 virus strain than with the type 2. The highest titres were obtained at 21 hours after infection. These titres were 5.0×10^5 and 4.0×10^4 TCD_{50} per 0.1 ml. of harvest for the type 1 and type 2 strains respectively (figure 8). No infectious virus was detected at 195 hours post infection with either virus type and at this stage no cells were attached to the glass. Details of titres are listed in Appendix 4.

The chick embryo CAM cell (CECC) cultures consisted of fibroblasts with occasional small clumps of epithelioid cells intermixed. The cytopathic changes produced by inoculation with virus of each type were similar to those described for CEFC cultures. As with CEBC cultures, the type 1 strain caused less severe cytopathic changes than the type 2 strain which affected the entire cell sheet within 36-48 hours after inoculation. Seven days post inoculation (with a culture medium change at four days) the amount of cell destruction remained between 25-50% of the cell sheet with the type 1 strain.

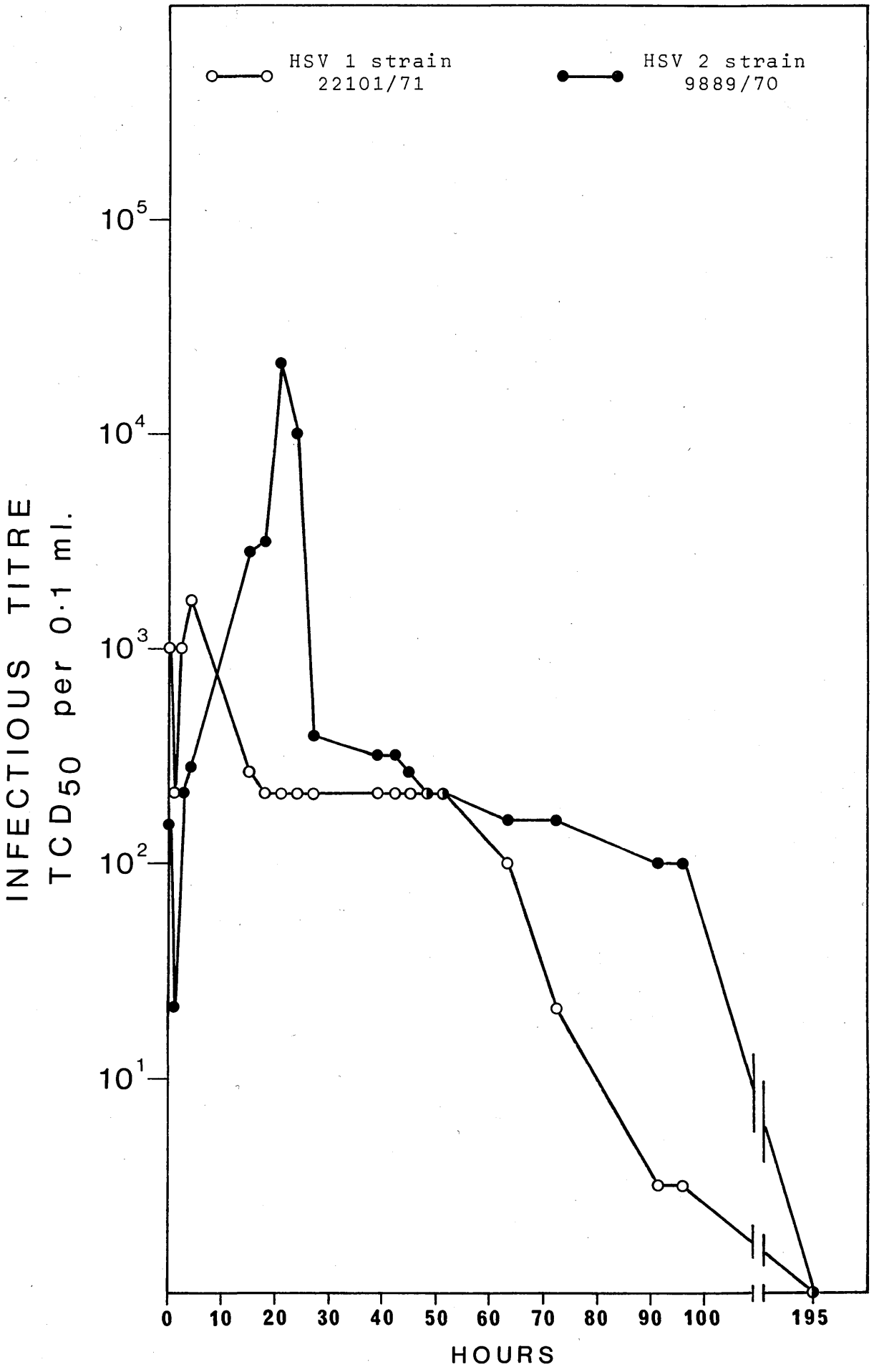
For the type 1 strain the maximum titre of 2.0×10^3 TCD_{50} per 0.1 ml. of harvest was obtained four hours post infection, after which titres declined and remained at 3.0×10^2 TCD_{50} per 0.1 ml. of harvest until 51 hours post culture infection. Titres of infectious virus declined rapidly from 51 to 96 hours. The type 2 strain

cultures (CEBC)



attained the greatest titre of 3.0×10^4 TCD_{50} per 0.1 ml. of harvest at 21 hours post infection, and titres declined slowly thereafter reaching 1.0×10^2 TCD_{50} per 0.1 ml. of harvest at 96 hours post infection (figure 9). No infectious virus was detectable in cultures at 195 hours post infection with either virus strain. At this stage no cells were attached to the glass. Details of titres are given in Appendix 4.

cultures (CECC)



8 Structure of normal and inoculated CAMs by optical microscopy

The CAMs embedded in resin and cut in sections one micron thick were stained at 60°C with azure II or toluidine blue in borax solution (Appendix 2). The choice of stains was limited by the resin embedding material and such stains as haematoxylin and eosin which would normally show herpesvirus inclusions extremely well could not be used with this resin. Removal of resin in either alcoholic potassium hydroxide or xylene followed by staining also proved unsatisfactory.

8.1 Normal CAM

Sections of uninfected CAM were examined at daily intervals from 11 to 18 days of incubation, intervals which were equivalent to 0 to 7 days after virus inoculation of 11 day old eggs in infection experiments.

The CAM consisted of three clearly distinguishable layers: the chorion or ectoderm which was the layer nearest the shell, the mesoderm, and the allantois or endoderm which bordered the allantoic cavity (plate 13a,b,c). All the layers were in direct contact with each other and the chorion was closely connected to the overlying shell membrane. The shell membrane, which remained basically unaltered throughout the incubation period studied, consisted of densely staining material which appeared globular in form showing little or no structure.

At 11 days the chorion consisted of two or more layers of flattened cells with prominent chorionic blood sinuses in the upper part of the chorion below the shell membrane. The appearance of the layer was suggestive of stratified

squamous epithelium. With continued incubation changes in the chorion were minor but included an increase in the number of cells present and an apparent flattening of these cells. The cells in the upper part of the chorion, and particularly their nuclei, stained more densely than those cells deeper in the layer. After the preparation of an artificial air sac with separation of the CAM from the shell membrane, the chorionic blood sinuses in this area underwent degenerative changes but with very little apparent damage to underlying cells.

The mesoderm, which consisted largely of intercellular space composed of apparently non-structured material, had bizarre shaped, mainly stellate, fibroblastic cells scattered throughout the entire thickness of the layer. These stellate cells had numerous cytoplasmic protuberances giving the appearance that the mesoderm was held together by the cytoplasmic bridges between these cells. Fine fibrillar material was also present in this layer as well as highly developed blood vessels of varying size containing nucleated erythrocytes. Apart from an increase in both the cellular and fibre content with an associated increase in the overall granularity, shown by an increase in the background staining, little change occurred in the mesoderm during incubation of the eggs up to 18 days. The thickness of the CAM varied over a wide range at all periods of incubation and this was due to differences in the thickness of the mesoderm resulting from variation in vascularity and general cell content.

At 11 days the allantois consisted of a single layer of epithelioid cells with large oval shaped nuclei. As

the incubation period progressed the allantois became more undulated on the side in contact with the allantoic cavity. The allantoic cells became much flatter and more numerous and by 18 days of incubation the layer was at least two cells thick and consisted of elongated cells with darkly stained nuclei. The cytoplasm of these cells was granular and often highly vacuolated.

8.2 Herpes simplex virus type 1 infected CAM

The three layers of the CAM: the chorion, mesoderm, and allantois remained intact and readily distinguishable from one another throughout the seven days of examination.

On the first day after inoculation with virus the early signs of infection were manifest as areas of cellular proliferation of the chorion containing an increased number of vacuolated cells as well as an increased number of mitotic figures (plate 14a). There was no change in either the mesoderm or allantois, compared with uninoculated controls.

Two days after infection the chorionic cell proliferation had increased considerably as had the number of vacuolated cells in this layer. The chorionic hyperplastic tissue occasionally erupted through the chorionic epithelium and small numbers of inflammatory cells appeared in the mesoderm (plate 15a). Although the basal lamina between the chorion and mesoderm was not clearly visible, there was some suggestion of shedding of cells from the chorion into the mesoderm, but the major involvement of the mesoderm was of an infiltrative nature. The allantois was unaffected. Prominent nucleoli within the nuclei of the cells of the allantois were often associated with mitoses.

Three days after infection chorionic hyperplasia was very pronounced, and inflammatory cell infiltration from mesodermal blood vessels was present. Inflammatory cells at the base of the pock were a mixture of blood-borne leucocytes and macrophages of mesenchymal origin. They were sited within the mesodermal space up to and in contact with the basal lamina and chorion. There was no evidence of necrosis, erosion, haemorrhage or congestion in any of the three layers of the CAM (plate 16a).

Apart from a possible increase in the vascularity of the membranes, reactions induced in the CAM by the type 1 virus four and five days post-inoculation were basically similar to those described for day three (plates 18a, 19a).

Six days after inoculation the lesions were still essentially small although they tended to be ulcerative in appearance. The lesions particularly involved the chorion. Oedema occurred between the chorionic cells and necrosis was occasionally present in the centre of the pocks, staining as a homogeneous mass. There was a localised cellular infiltration of the mesoderm particularly around the base of the pock. Even at this late stage the lesions were well circumscribed and basically confined to the chorion, with little involvement in the mesoderm other than a mild infiltrative reaction, and no involvement of the allantois was seen (plate 20a).

Little change occurred upon further incubation and often pocks began to resolve.

8.3 Herpes simplex virus type 2 infected CAM

The early changes (day one) induced by the type 2 infection of the CAM were similar to those of type 1

infections. The three layers of the CAM were readily distinguishable and the reaction was limited to the chorion only, though there were early signs of infiltration of the mesoderm with small numbers of inflammatory cells. The cellular proliferation of the chorion was more advanced than in the equivalent early HSV type 1 infection, but there appeared to be slightly less vacuolation in the cells forming the type 2 pock (plate 14b).

Two days after infection cellular proliferation of the chorion had progressed and the pocks had a more florid appearance with abnormalities present in all three layers of the CAM. There was marked oedema in the hyperplastic tissue of the chorion which frequently gave the pocks a blister-like appearance. The mesoderm was infiltrated with inflammatory cells which were histiocytic or macrophagic in appearance with clear ovoid nuclei and stellate cytoplasm, the cells generally being rather large. The allantois in the region below the pock became thickened, and consisted of two or three layers of normal looking cells (plate 15b).

The third day pocks had a similar appearance, with proliferation of the chorion, necrosis and granulation tissue in the region of the pock. Oedema with increased density of staining was also present in the chorion. Mesodermal involvement was significantly greater than with the equivalent stage of type 1 lesions. A number of mesodermal cells had fused to form giant cells, some of which had a concentric whorl appearance. These were most numerous in the centre of the pock structure, and

their presence was more pronounced in the type 2 than in the type 1 virus infected CAMs. Inflammatory response was marked and there was some evidence of proliferation of the vascular channels below the chorion. The allantois had à similar appearance to that of day two (plate 16b). With day three haemorrhagic pocks, the three-layered structure of the CAM was less clear. The chorion showed extensive proliferation mixed with numerous erythrocytes leaked from blood vessels. A large degree of ulceration, erosion and oedema was most evident in the chorion. The mesoderm was heavily infiltrated with inflammatory cells and showed signs of oedema, whilst the blood vessels appeared distended and congested with erythrocytes and leucocytes. The allantois became thickened and consisted of normal looking cells with pale staining nuclei frequently containing two prominent nucleoli. In the region below the ulcerated chorion, the allantois was four or five cells thick (plate 17).

Four days after virus inoculation the reaction in the CAM had progressed so that cells from the highly proliferated chorion had sloughed and the pocks were ulcerated. The mesoderm was highly infiltrated with inflammatory cells and the mesodermal blood vessels were congested with erythrocytes and leucocytes (plate 18b).

The lesions progressed and by day five focal necrosis was present in both the chorion and mesoderm. Proliferation of the cells of the chorion and dense infiltration of the mesoderm with inflammatory cells were the two prominent features. Leucocytes were present in high numbers in the mesodermal blood vessels, especially around necrotic foci

near the pock (plate 19b).

By the sixth day of infection the chorion was highly necrotic and replaced with granulation tissue. The mesoderm had become highly infiltrated with mixed inflammatory cells, lymphocytic with large round nuclei, histiocytic with both monocytes and macrophages present and also plasma cells especially near necrotic foci. Proliferation of cells, some of which were fibroblastic in type, occurred between the capillary loops. There was granulation tissue in the mesoderm and the allantois was also affected in that it appeared thickened (plate 20b).

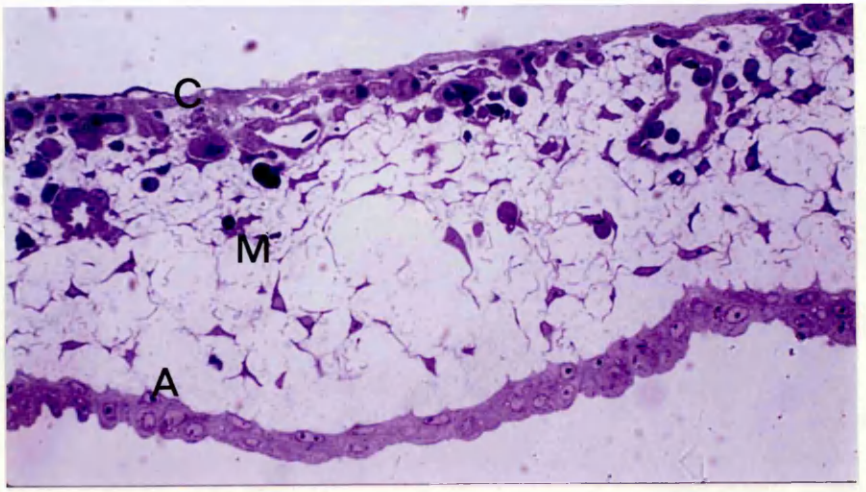
Seven days after inoculation the chorion was often severely ulcerated, eroded and replaced with granulation tissue and infiltrative cells. Necrosis occurred deep into the membranes particularly in the chorion with numerous vacuoles present between cells, and occasionally fatty degeneration developed in the chorions of these membranes (plate 21).

Three day pocks produced by HSV type 2 on CAMs incubated at 38.5^{o}C were similar to three day pocks produced on membranes at 36^{o}C. The chorion appeared more heaped-up than with the other type 2 pocks and showed necrosis deep in the layer. Vacuolation was also prominent. The mesoderm which appeared to have widened below the pock was highly infiltrated with inflammatory cells migrating towards the pock, whilst the allantois appeared thickened (plate 22).

In summary, the type 1 induced pocks showed cellular proliferation and small degrees of necrosis, whilst in the mesoderm oedema and infiltration were present but

only in small amounts. The allantois appeared relatively unaffected. In the type 2 induced pocks, the chorion showed cellular proliferation spreading downwards into the mesoderm. Necrosis, haemorrhages and erosion were frequently present. In the mesoderm massive infiltration occurred as well as congestion of the blood vessels. Shrinkage of the mesoderm, a common feature, resulted in the allantois being drawn up towards the chorion. The allantois appeared thickened particularly in the region below the pock.

(Many of the structures shown in plates 13 to 22 are best viewed with a hand lens)



Optical sections (1 µm. thick) of uninoculated chorioallantoic membrane

a) at 13 days of incubation, stained with azure II (x300)

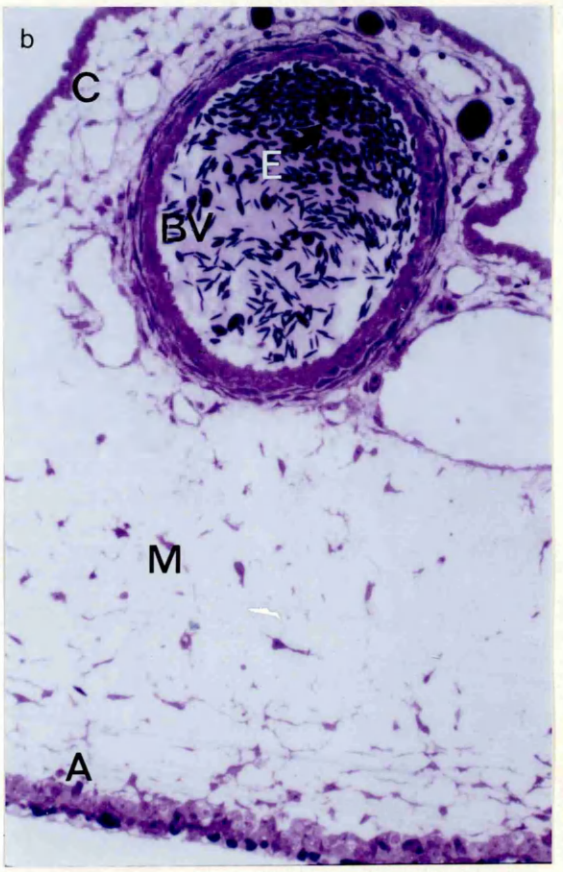
b) at 15 days of incubation, stained with toluidine blue (x187.5)

c) at 18 days of incubation, stained with azure II (x 187.5)

C = chorion E = erythrocytes

BV = blood vessels

M = mesoderm A = allantois



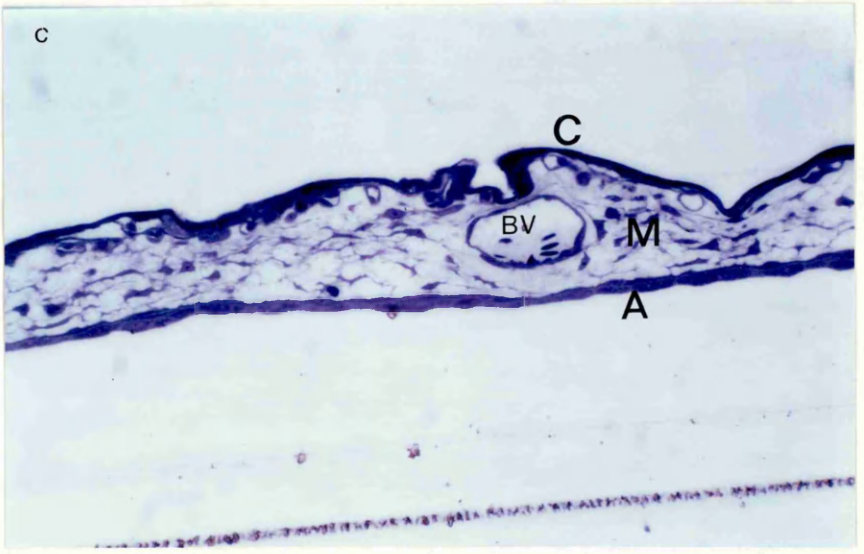
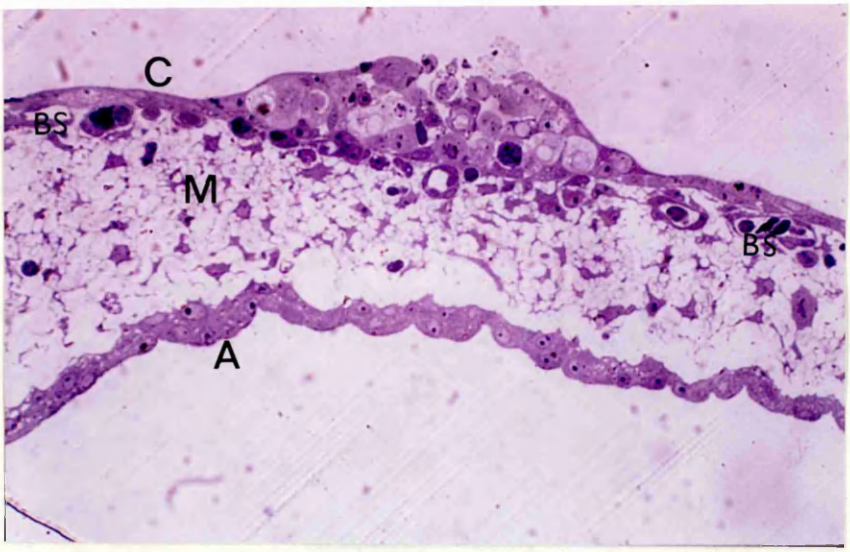
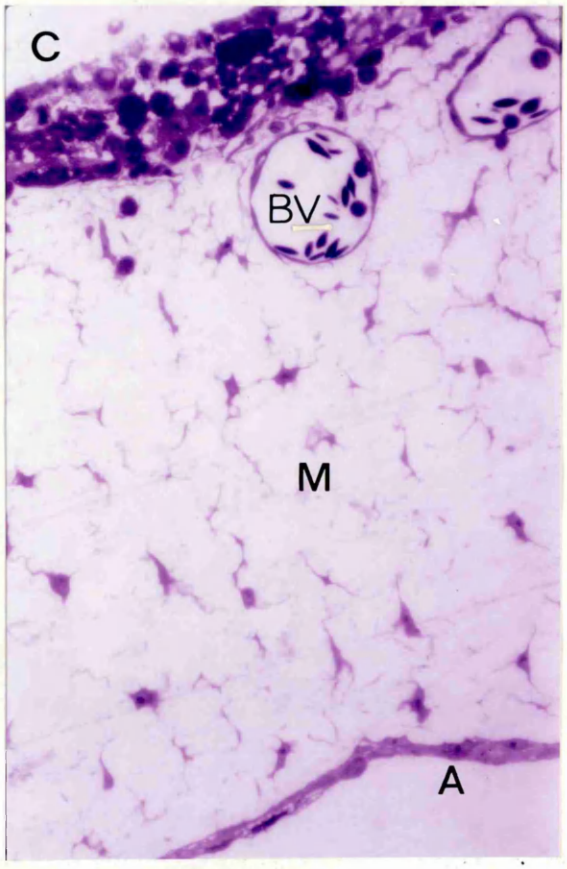


PLATE 13



a) Optical section (1 µm. thick) of HSV type 1 pock in the chorioallantoic membrane, one day after virus inoculation (12 days of incubation). Stained with toluidine blue (x300)

C = chorion M = mesoderm A = allantois
BS = blood sinuses

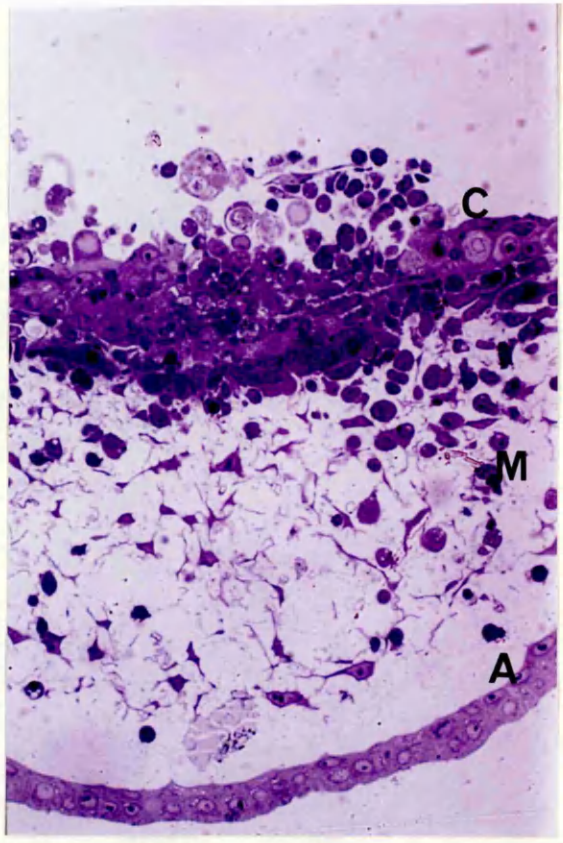


b) Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, one day after virus inoculation (12 days of incubation). Stained with toluidine blue (x300)

C = chorion M = mesoderm A = allantois

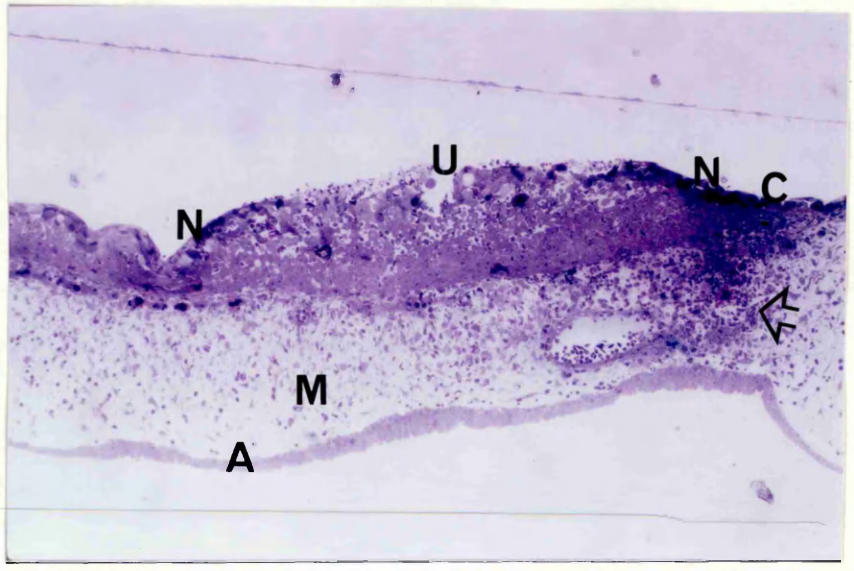
BV = blood vessel

PLATE 14



a) Optical section (1 µm. thick) of HSV type 1 pock in the chorioallantoic membrane, two days after virus inoculation (13 days of incubation). Stained with azure II (x300)

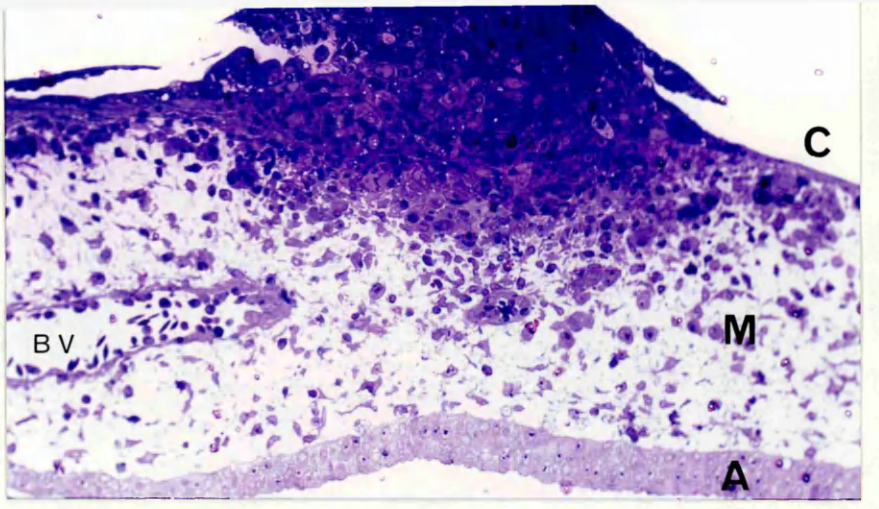
C = chorion M = mesoderm A = allantois



b) Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, two days after virus inoculation (13 days of incubation). Stained with azure II (x75)

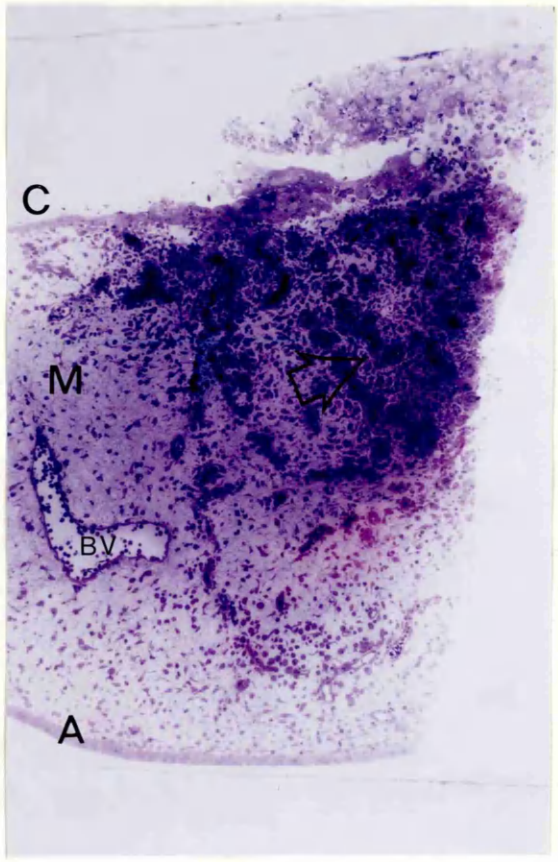
C = chorion M = mesoderm A = allantois
N = areas of necrosis U = ulceration
Note inflammatory response (open arrow)

PLATE 15



a) Optical section (1 µm. thick) of HSV type 1 pock in the chorioallantoic membrane, three days after virus inoculation (14 days of incubation) stained with toluidine blue (x187.5)

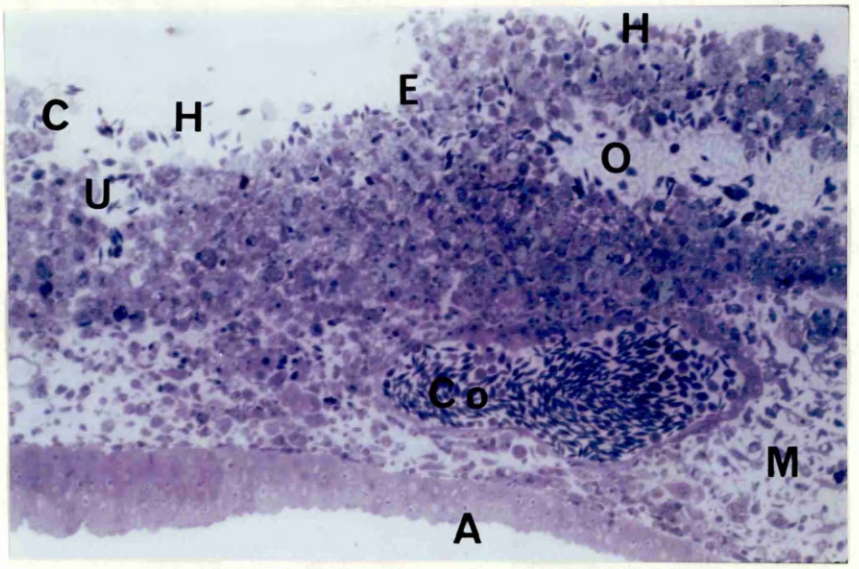
C = chorion M = mesoderm A = allantois
BV = blood vessel



b) Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, three days after virus inoculation (14 days of incubation) stained with toluidine blue (x75)

C = chorion M = mesoderm A = allantois
Note oedema and inflammatory response in mesoderm (open arrow).
BV = blood vessel

PLATE 16

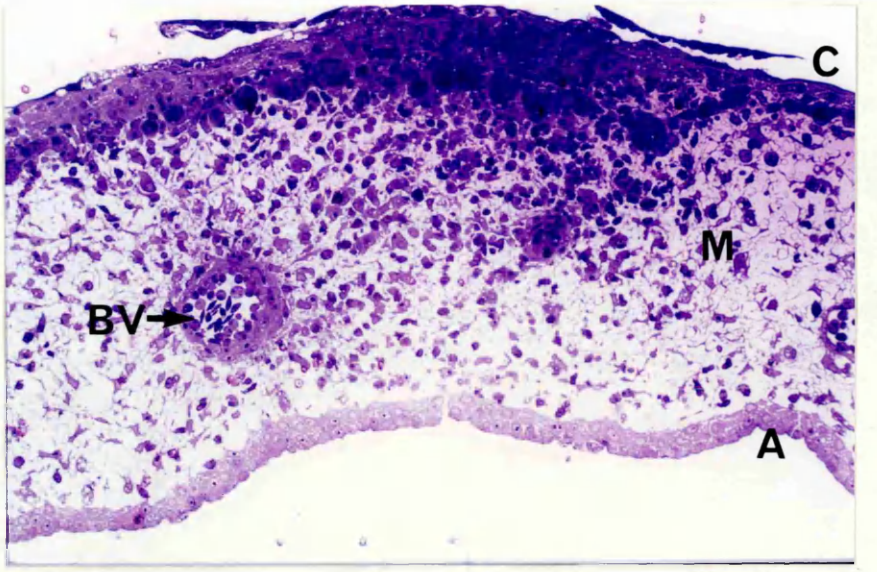


Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, three days after inoculation with a high concentration of virus (14 days of incubation). Stained with azure II (x187.5)

C = chorion M = mesoderm A = allantois
U = ulceration O = oedema E = erosion
H = haemorrhages Co = congestion

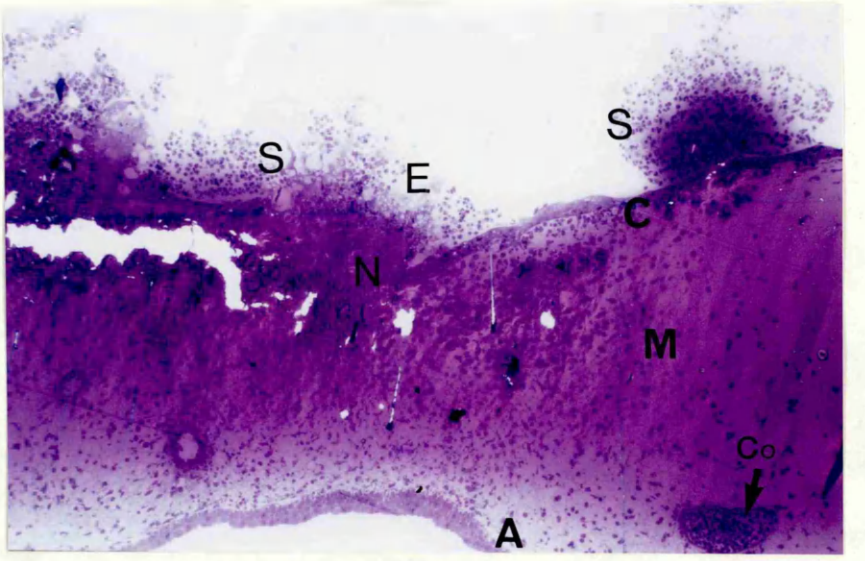
Note thickening of allantois and inflammatory response in mesoderm below region of chorion where erosion is most severe.

PLATE 17



a) Optical section (1 µm. thick) of HSV type 1 pock in the chorioallantoic membrane, four days after virus inoculation (15 days of incubation). Stained with azure II (x187.5)

C = chorion M = mesoderm A = allantois
BV = blood vessel

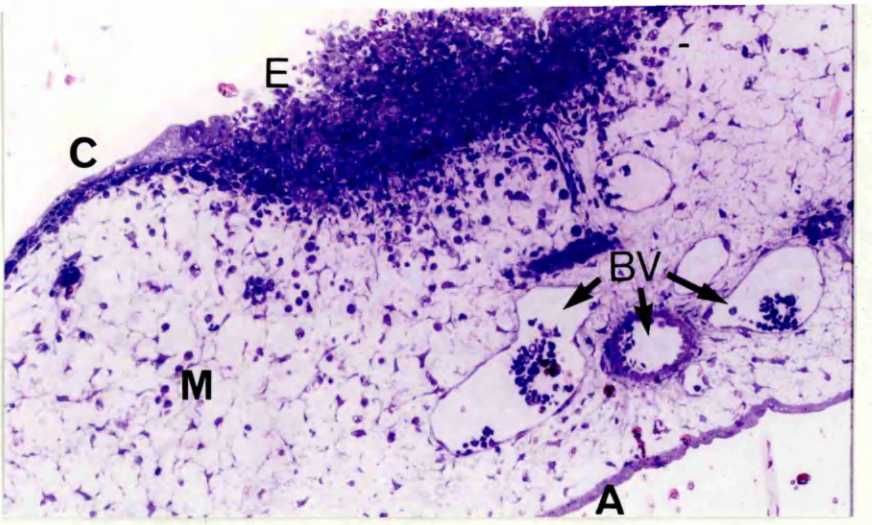


b) Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, four days after virus inoculation (15 days of incubation). Stained with azure II (x75)

C = chorion M = mesoderm A = allantois
N = necrosis E = erosion Co = congestion
S = sloughing of cells from the membrane

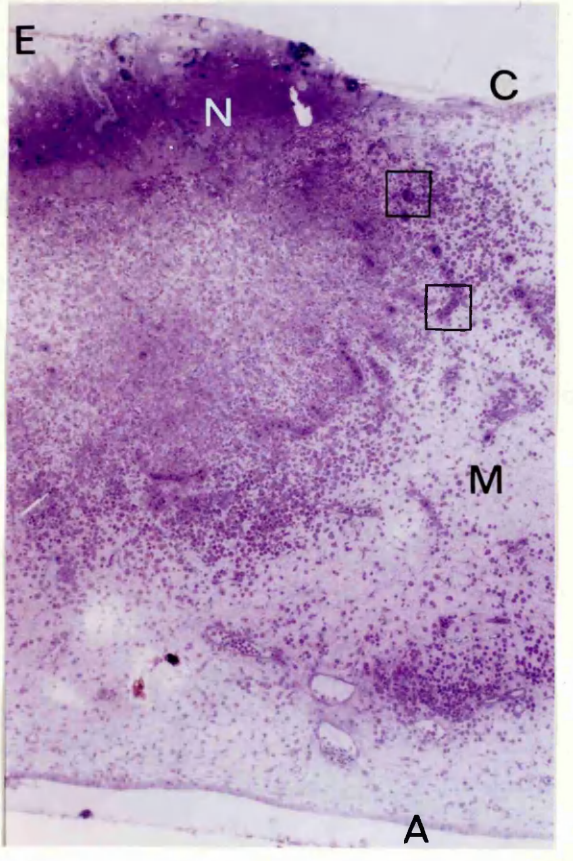
Note oedema and inflammatory response throughout the mesoderm, and the thickening of the allantois below the pock region.

PLATE 18



a) Optical section (1 µm. thick) of HSV type 1 pock in the chorioallantoic membrane, five days after virus inoculation (16 days of incubation).
Stained with azure II (x120)

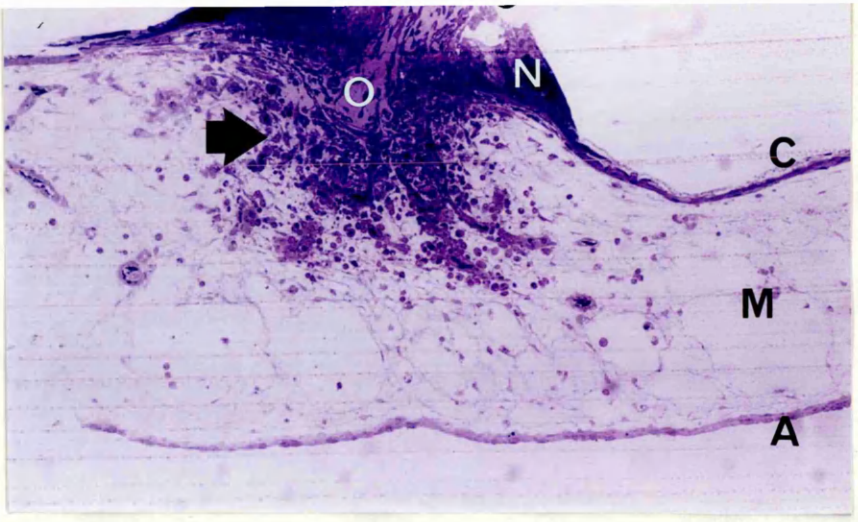
C = chorion M = mesoderm A = allantois
BV = blood vessel E = erosion



b) Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, five days after virus inoculation (16 days of incubation).
Stained with azure II (x75)

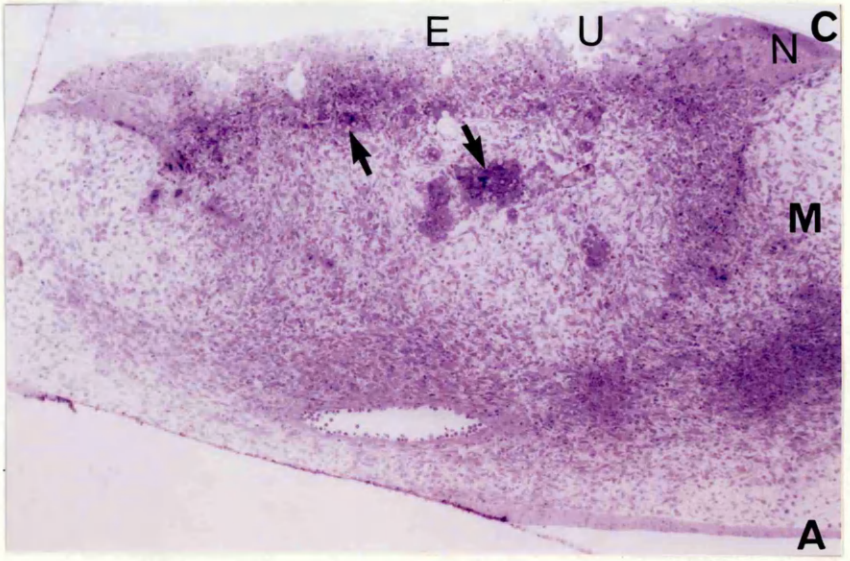
C = chorion M = mesoderm A = allantois
N = necrosis E = erosion
Note massive inflammatory response in mesoderm.
Multi-nucleate giant cell (boxed)

PLATE 19



a) Optical section (1 µm. thick) of HSV type 1 pock in the chorioallantoic membrane, six days after virus inoculation (17 days of incubation). Stained with azure II (x120)

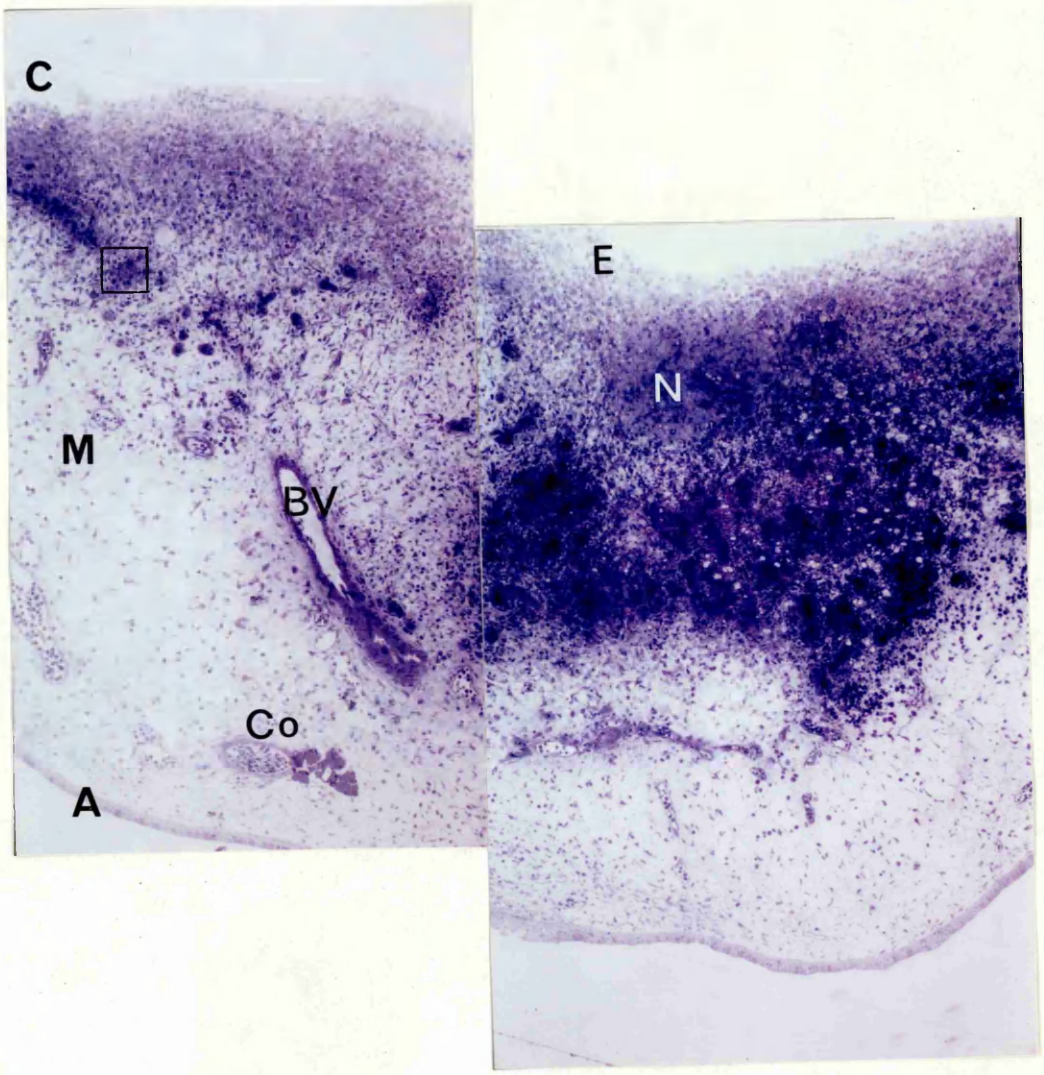
C = chorion M = mesoderm A = allantois
N = necrosis U = ulceration O = oedema
Note localised inflammatory response (arrowed) in mesoderm



b) Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, six days after virus inoculation (17 days of incubation). Stained with azure II (x75)

C = chorion M = mesoderm A = allantois
N = necrosis U = ulceration E = erosion
Note oedema and massive inflammatory response throughout mesoderm. Highly vacuolated cells (arrow)

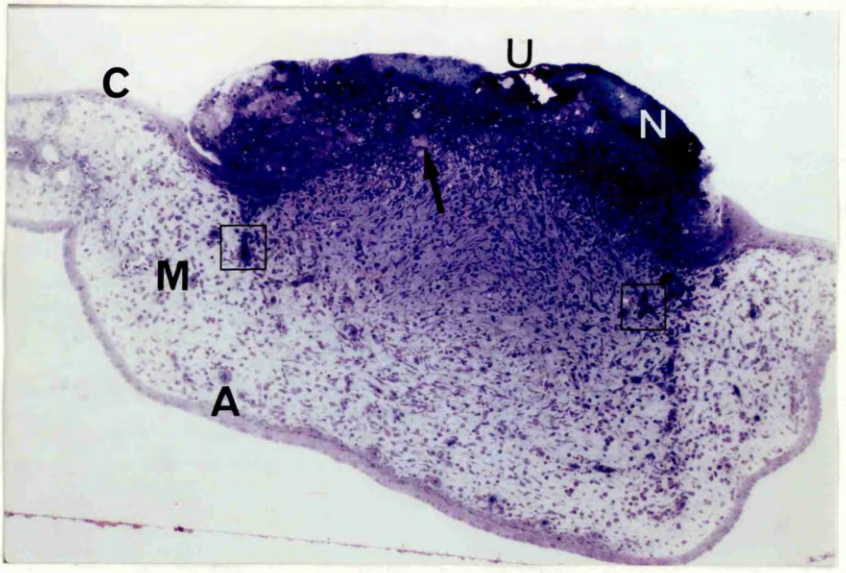
PLATE 20



Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, seven days after virus inoculation (18 days of incubation).
Stained azure II, composite (x75)

C = chorion M = mesoderm A = allantois
BV = blood vessel N = necrosis E = erosion
Co = congestion (particularly with leucocytes)
Multi-nucleate giant cell (boxed)
Note inflammatory response and highly vacuolated cells in mesoderm

PLATE 21



Optical section (1 µm. thick) of HSV type 2 pock in the chorioallantoic membrane, three days after virus inoculation and incubation at 38.5°C (14 days of incubation) stained with azure II (x75)

C = chorion M = mesoderm A = allantois
N = necrosis U = ulceration
Multi-nucleate giant cell (boxed)
Note oedema and inflammatory response in mesoderm, and thickening of allantois and whole membrane
Arrow indicates highly vacuolated cells

PLATE 22

9 Ultra structure of normal and inoculated CAMs by electron microscopy

Optical microscopy not only provided information about pocks, but was necessary for orientation of the specimen prior to thin sectioning. After the required area of the specimen had been identified the blocks were trimmed and thin sections were cut for examination in the electron microscope.

9.1 Normal CAM

Uninfected CAM was examined at the time specified for light microscopy. At the lowest magnifications in the electron microscope the triple-layered structure of the CAM with the closely associated shell membrane was clearly discernible (plate 23a,b).

The shell membrane consisted largely of a loose network of densely staining bodies suspended in extensive areas of ground substance. These bodies were lobulate and pleomorphic and varied in diameter from 0.2 to 4 μm. Apart from the presence of vacuoles, these bodies showed no discernible fine structure (plate 24). Surrounding each of these bodies, and separated from them by a gap which varied from 20 to 40 nm. in thickness, was a layer of densely staining granular material varying in thickness and showing no detailed structure. Where it contacted the underlying chorion, the shell membrane consisted of a densely stained basement membrane-like layer, approximately 130 nm. thick, from which arose spherical structures of granular material closely resembling those already present in the shell membrane (plate 24). The shell membrane was separated from underlying structures

by an irregular space, 6 to 18 nm. thick, containing finely granular, amorphous material (plate 25). Between this space and the blood sinuses was a layer of cell cytoplasm and a prominent basal lamina (plate 24). The blood sinuses contained densely staining nucleated erythrocytes. A less obvious basal lamina separated the sinuses from the underlying cells of the chorion. The cells lining the sinuses were thin with long strands of cytoplasm sometimes as narrow as 50 nm., lying between the vascular lumen and the shell membrane. Numerous small invaginations occurred in the plasma membrane of these cytoplasmic strands immediately below the shell membrane, and these thin cytoplasmic strands also had mitochondria present, suggesting these were functional extensions of the endothelial cells (plate 24).

At 11 days of incubation, the chorion consisted of two or more layers of well differentiated cells with mostly large oval nuclei, although spherical or irregular shaped nuclei were also seen, as were densely stained nucleoli (plates 24 and 26). Cells deeper in the chorion varied in shape from flat to cuboidal. The cytoplasm of chorionic cells was well supplied with mitochondria which were pleomorphic and varied in size. Chorion cells had long cytoplasmic projections which gave this layer a network structure made up of a complex of interlocking cytoplasmic bridges with frequent junctions of the macula adherens (desmosome) type (plate 26). Cytoplasmic vacuoles were also a common feature (plate 27). The amount of interlocked network of cytoplasm formed by the cells of this layer increased in extent with further incubation.

Ribosomes, which occurred singly and in small groups, were free in the cytoplasm and not generally associated with endoplasmic reticulum, although they occurred in high concentrations around lipid-like bodies. Small numbers of vesicles were present in the cytoplasm of chorionic cells. At the base of the chorionic layer, and dividing it from the mesodermal layer was a fine extracellular basal lamina which varied in thickness from 60 to 140 nm., with an average of 80 nm. With prolonged incubation the cells of the chorion became more electron dense and increasing numbers of vacuoles and lipid droplets of various sizes were present.

The mesoderm consisted of a loose matrix of fibroblasts and collagen fibres in featureless interstitial material. The blood vessels of the CAM were sited in this layer. Variation in thickness of the mesoderm was responsible for any variation in width of the total CAM. The mesoderm was separated from both the chorion and allantois by well developed basal lamina. Fibroblastic cells with stellate appearance were characteristic of the mesoderm. The nuclei of these cells were large and either oval or irregular in shape with prominent nucleoli and chromatin which was situated mostly at the nuclear membrane (plate 28). Many of these cells had prominent rough endoplasmic reticulum (plate 29). In contrast to chorionic cells, few free ribosomes were present. The mitochondria were large and spherical. Vacuoles were often present in the cytoplasm as well as sacs containing numerous vesicles (plate 28). Intracytoplasmic ribbon-like structures which have been described in the literature were not seen in the

fibroblasts. In close association with the fibroblasts were reticular fibres (narrow collagen fibres) measuring 40 to 45 nm. in cross-section. Occasionally collagen fibres passed through the cytoplasm of mesodermal cells which added to the collective strength of an otherwise loose layer (plate 30). Fibroblasts and collagen fibres were distributed throughout the mesoderm, but were present in higher concentrations around the blood vessels. Mylin whorls were observed only in these cells.

The inner surface of the blood vessels was lined with cuboidal endothelial cells containing large nuclei and prominent nucleoli. These cells were linked to each other by numerous macula and zonula adherentes and gap junctions. Peripheral to these cells were pericytes, and the fibroblastic cells and collagen fibres spread around the structure to form a sheath (plate 31). Glycogen granules were occasionally present in the cytoplasm of these cells (plate 32). Extra-vascular red blood cells and leucocytes were rarely seen. In the lumen of the vessels, the erythrocytes were surrounded by a fine granular network of serum proteins. Erythrocytes were tapered and each contained a large densely stained, centrally placed, oval nucleus. The cytoplasm, due to the high haemoglobin concentration, appeared electron dense and contained mitochondria, ribosomes and vesicles but no endoplasmic reticulum or other organelles. There was little difference in density between the nucleus and the cytoplasm of erythrocytes, and the distinct gap between them was presumably due to fixation artefact and shrinkage (plate 32). Leucocytes were occasionally seen in blood vessels. These often contained numerous

vacuoles, pinocytotic vesicles, centrioles and golgi regions (plate 33).

The allantoic layer of the CAM consisted of one to four (usually two) layers of well organised cells separated from the mesoderm by a basal lamina. These cells were of cuboidal epithelium type with oval or elongated nuclei which had prominent centrally placed nucleoli. The inter-cell borders were clearer in the allantois than in the chorion and the interstitial gaps measured 27 to 30 nm. (plate 34). The plasma membranes of these cells were convoluted, and at various points of cell-to-cell contact there were gap junctions, desmosomes and associated tonofilaments. The cell membranes on the two surfaces of the layer bordering the mesoderm and the allantoic fluid were less complex. On the mesoderm border the plasma membranes adjacent to the basal lamina were smoothly undulating with no apparent appendiges, although 'blebs' projected from the surface of these cells into the basal lamina (plate 35). On the allantoic surface, the cell membranes were straighter with numerous protruding, often balloon-shaped microvilli, 130 to 150 nm. in diameter and of variable length. Numerous pinocytotic vesicles occurred at this plasma membrane which separated the cells from the allantoic fluid (plates 36 and 37). The cytoplasm of the allantoic cells contained prominent mitochondria, some endoplasmic reticulum, golgi zones, free ribosomes and small amounts of glycogen (plate 34). The most striking feature of the allantoic cells was the presence of dense cytoplasmic granules in the cells adjacent to the allantoic cavity. These granules were

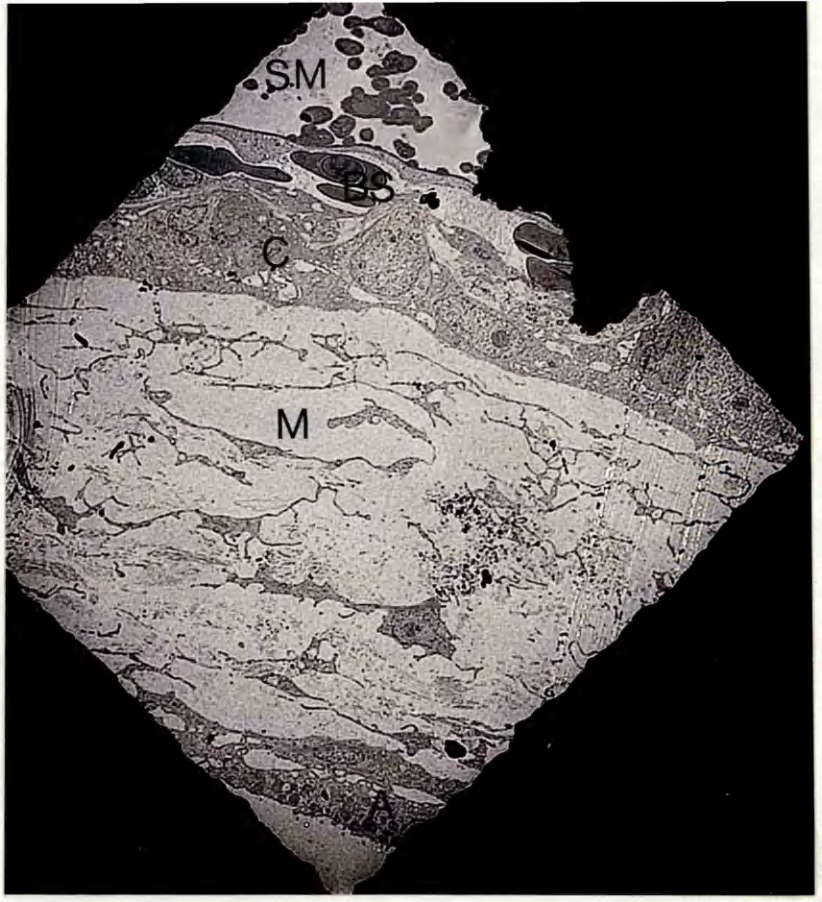
bounded by a unit membrane and ranged in diameter from 250 to 1000 nm. They were granular in appearance and often surrounded by a clear zone of variable thickness.

On further egg incubation, the thickness of the allantoic layer increased by deposition of more layers of cells. The plasma membranes on the mesodermal side became convoluted and the plasma membranes bordering the allantoic cavity showed more numerous microvilli and pinocytotic vesicles. The number of dense granules increased. There was a simultaneous increase of similar but less electron dense bodies which may have been lipid or pinocytosed material from the allantoic cavity, or a manifestation of some cellular response to toxic substances such as urates and uric acid which gradually accumulated in the allantoic cavity (plate 38).

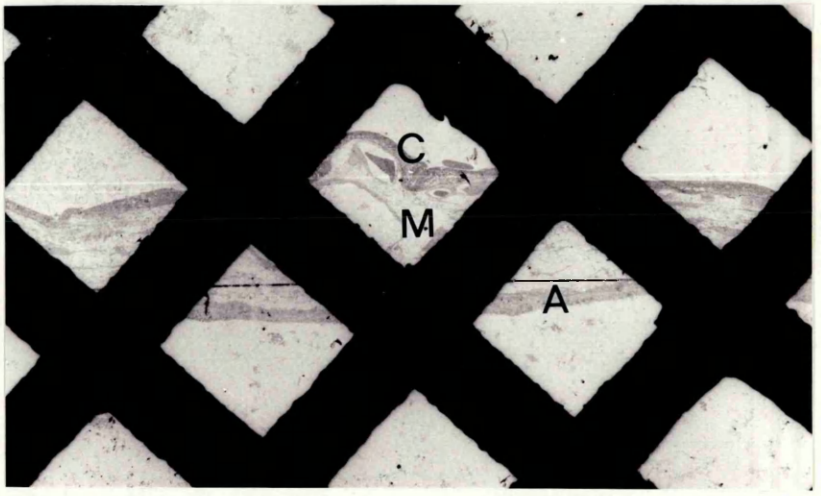
After exposure of the CAM by means of an artificial air sac followed by continued incubation, the chorion took on a thickened appearance presumably as a result of trauma. This was particularly evident in the region facing the shell membrane side of the blood sinuses. Some degenerative changes shown by ballooning of the cytoplasm occurred in this region and the number of vacuoles and vesicles increased. The electron density of the exposed surface cells increased with incubation and some loss of cellular sub-structure was observed. Occasionally erythrocytes were found on the surface of the chorion, presumably due to trauma. Microvilli were present at the surface of the chorion, but these became less apparent with increased incubation. Apart from the presence of occasional leucocytes immediately below the

chorion, the underlying mesoderm and allantois appeared unaffected by the manipulations (plates 39 and 40a,b).

In summary, the uninoculated CAM was a well organised structure divided into three morphologically distinct layers and richly supplied with blood vessels in the central layer. It provided an interesting variety of cells within the organised structure in which to study the activity of the herpesvirus infection.

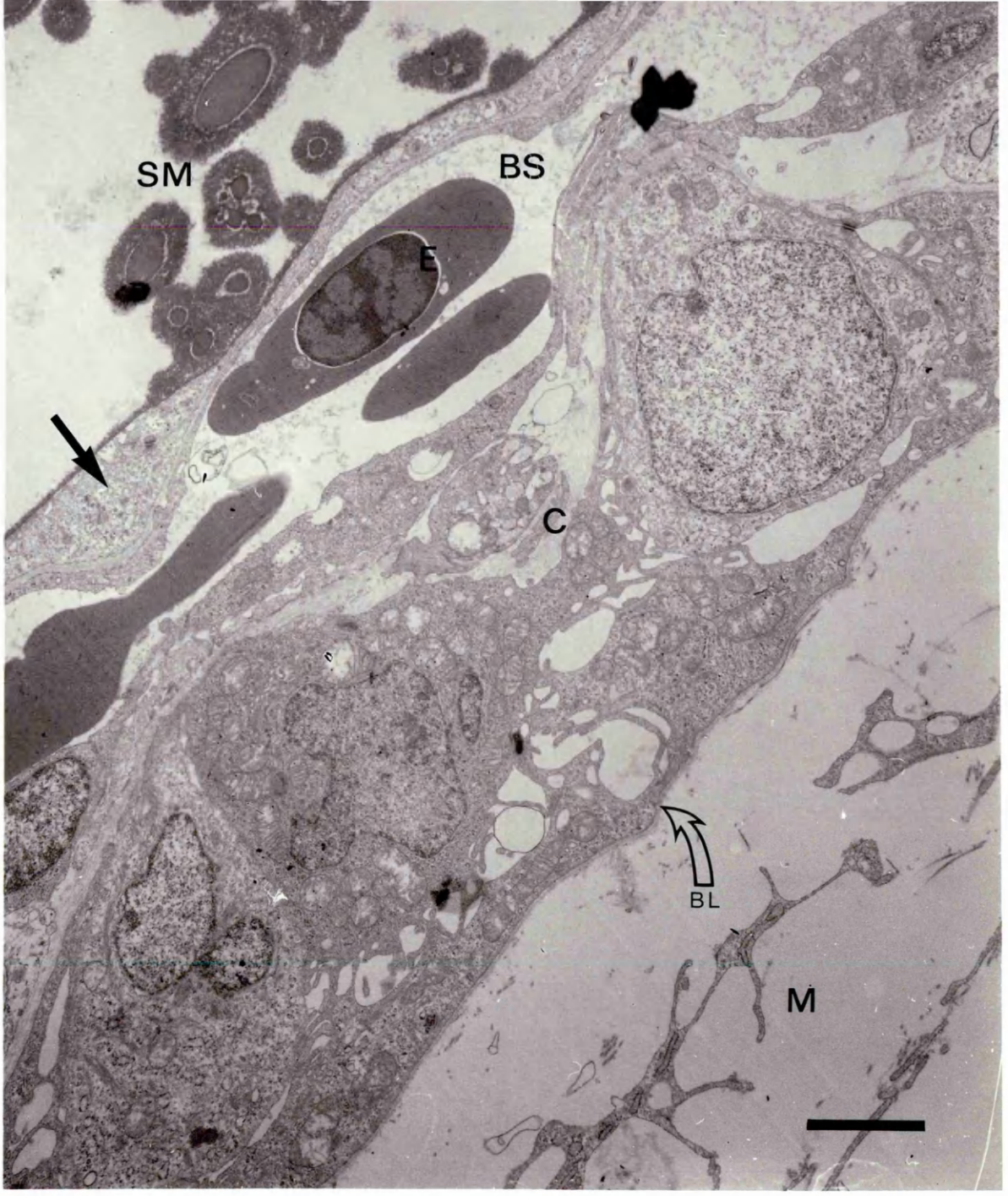


a) Low power electron micrograph of the chorioallantoic membrane at 11 days of incubation showing the shell membrane and the three layers of the chorioallantoic membrane
SM = shell membrane BS = blood sinus C = chorion
M = mesoderm A = allantois



b) Low power electron micrograph of the chorioallantoic membrane at 18 days of incubation
C = chorion M = mesoderm A = allantois

PLATE 23

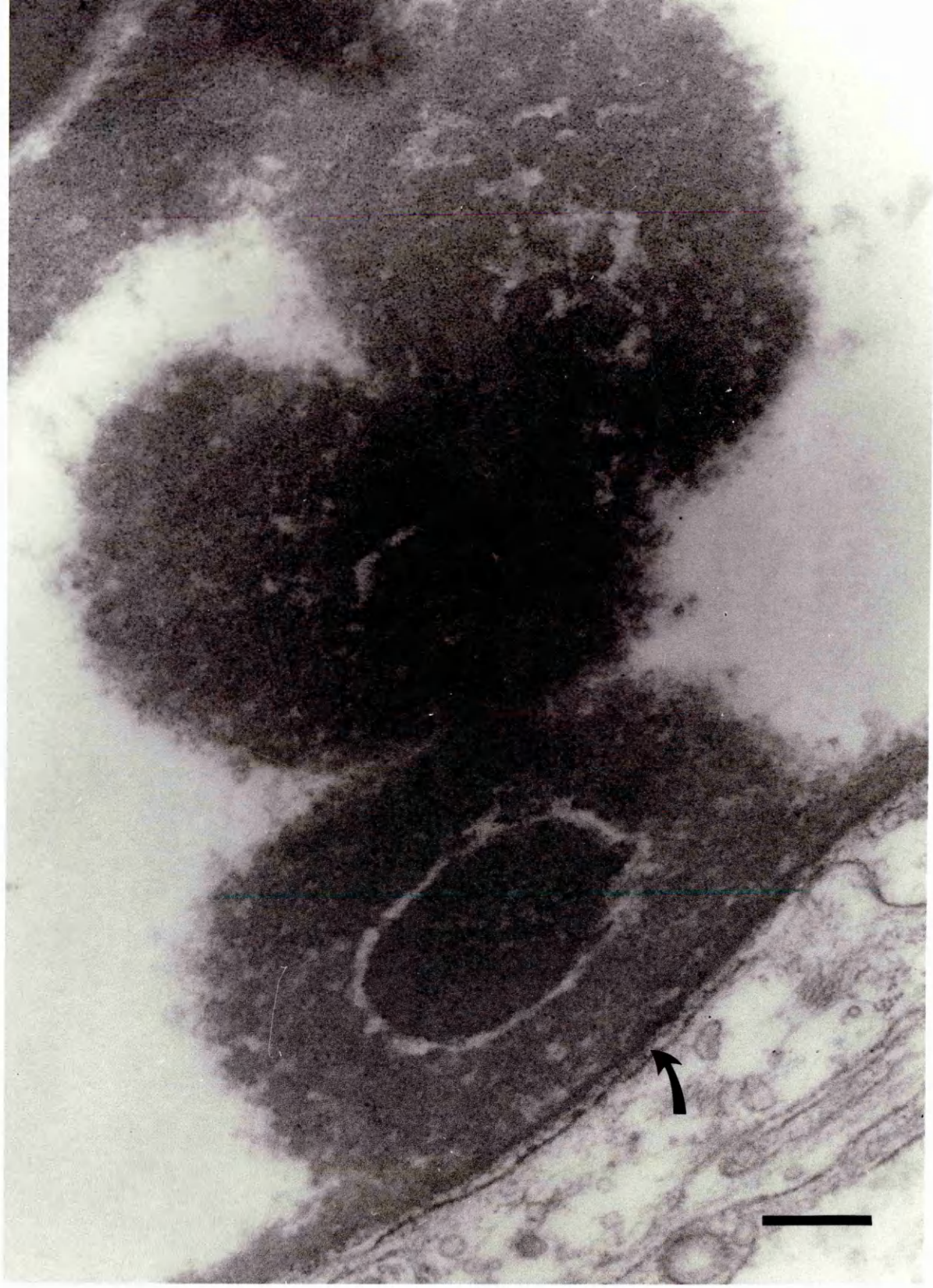


Chorioallantoic membrane with adhering shell membrane at 11 days of incubation (x9,000)

SM = shell membrane BS = blood sinus E = erythrocyte
C = chorion M = mesoderm BL = basal lamina (open arrow)

The cytoplasmic extension between the shell membrane and the blood sinuses is indicated by a solid arrow. (Bar = 2µm.)

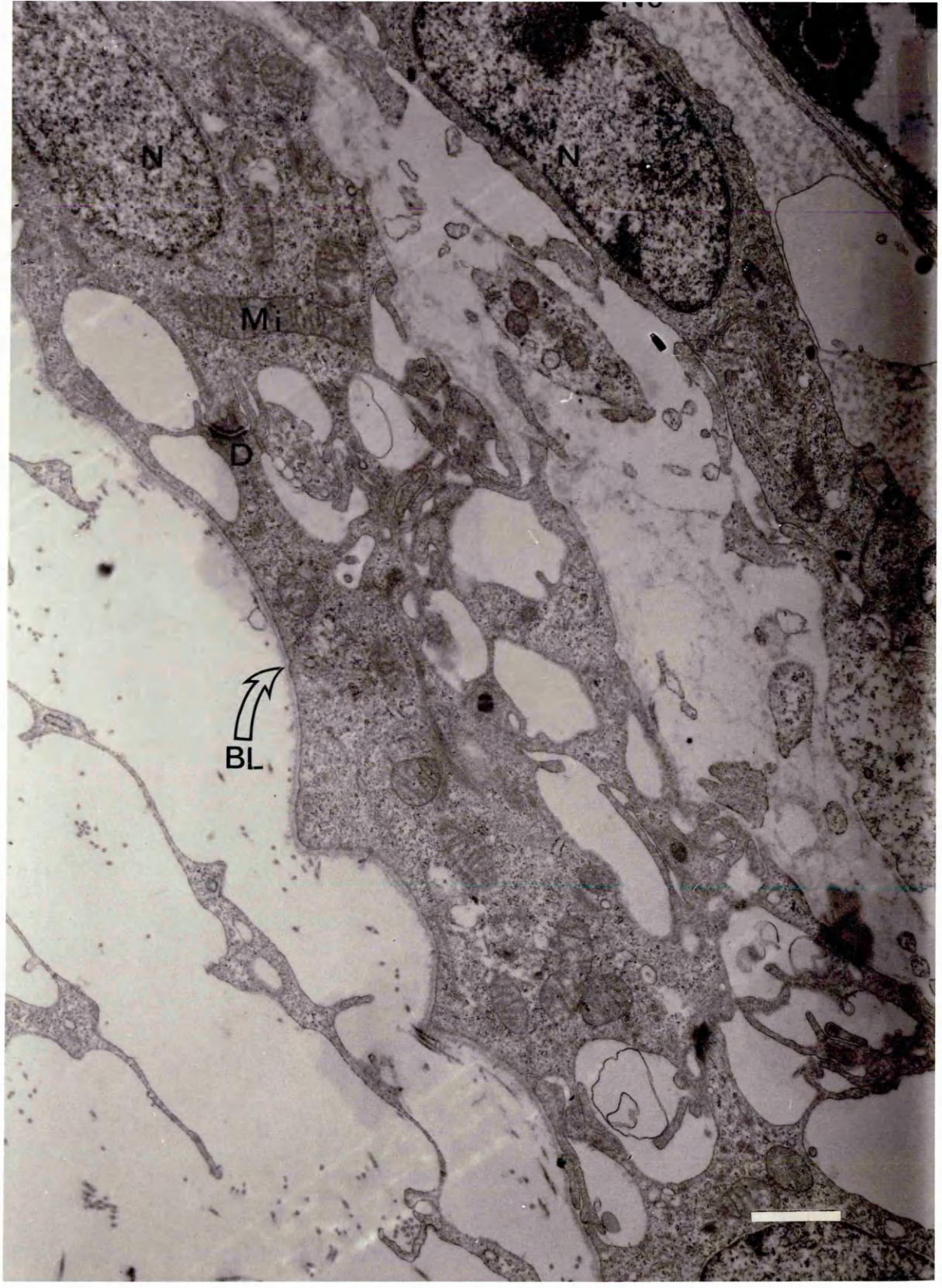
PLATE 24



Granular masses in the shell membrane at 11 days of incubation (x90,000)

The space between the cytoplasm of the blood sinus cells and the granular layer of the shell membrane is indicated by a solid arrow. (Bar = 200 nm.)

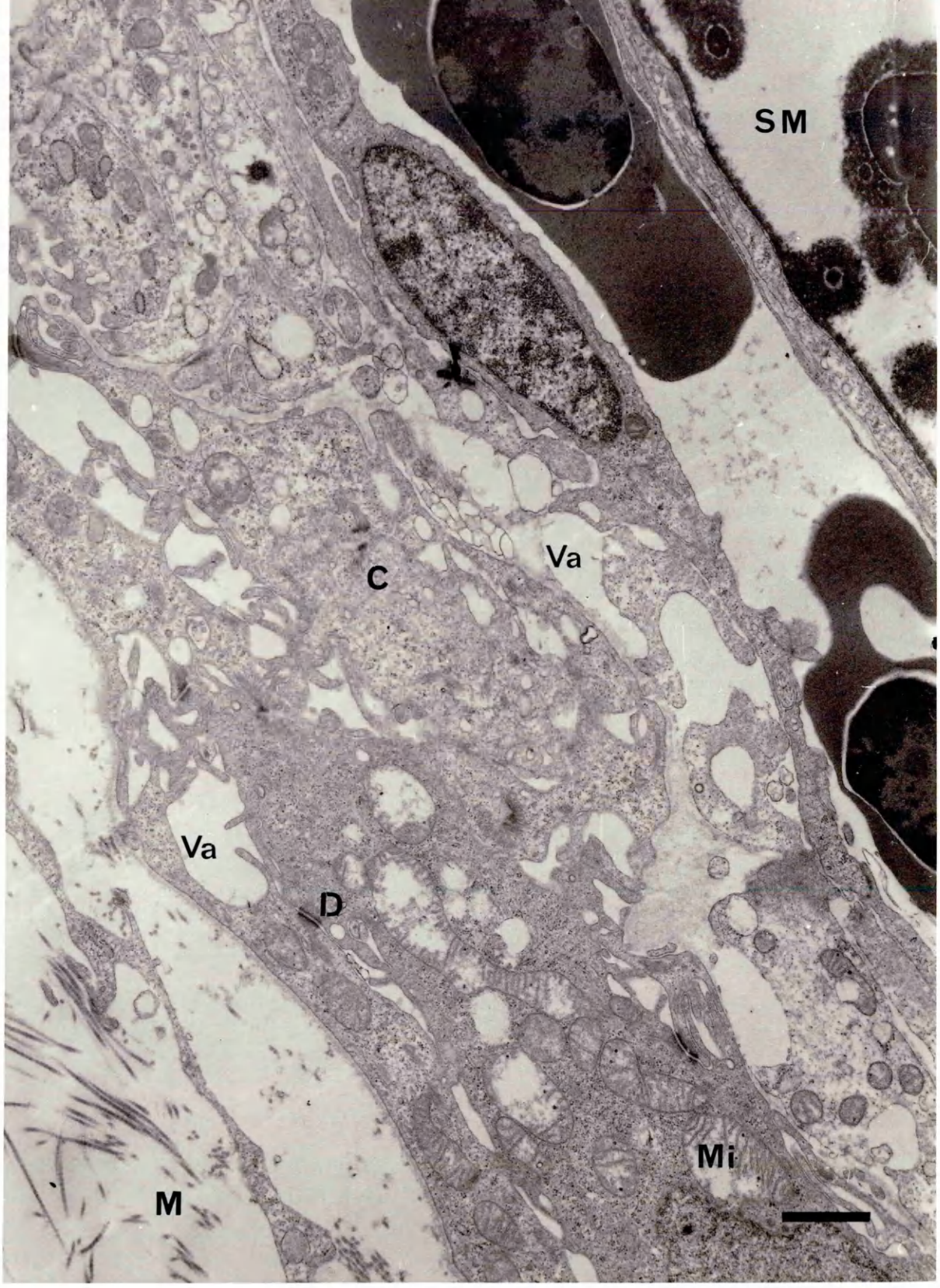
PLATE 25



Chorion at 11 days of incubation (x15,000)

N = nucleus No = nucleolus (solid arrow) Mi = mitochondria
D = desmosome BL = basal lamina (open arrow) (Bar = 1 µm.)

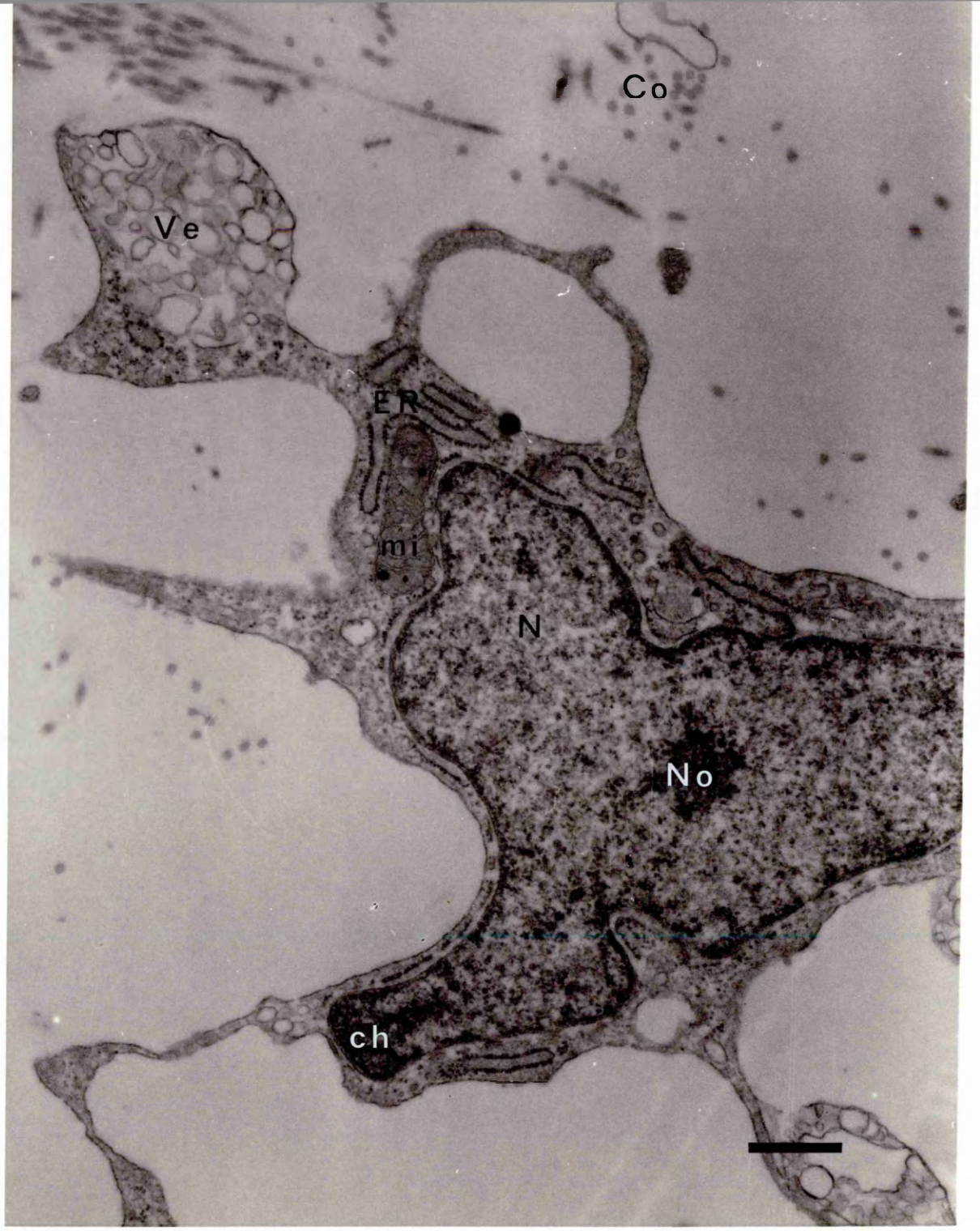
PLATE 26



Region of the chorion at 11 days of incubation (x15,000)

SM = shell membrane C = chorion Va = vacuoles M = mesoderm
D = desmosomes Mi = mitochondria (Bar = 1 µm.)

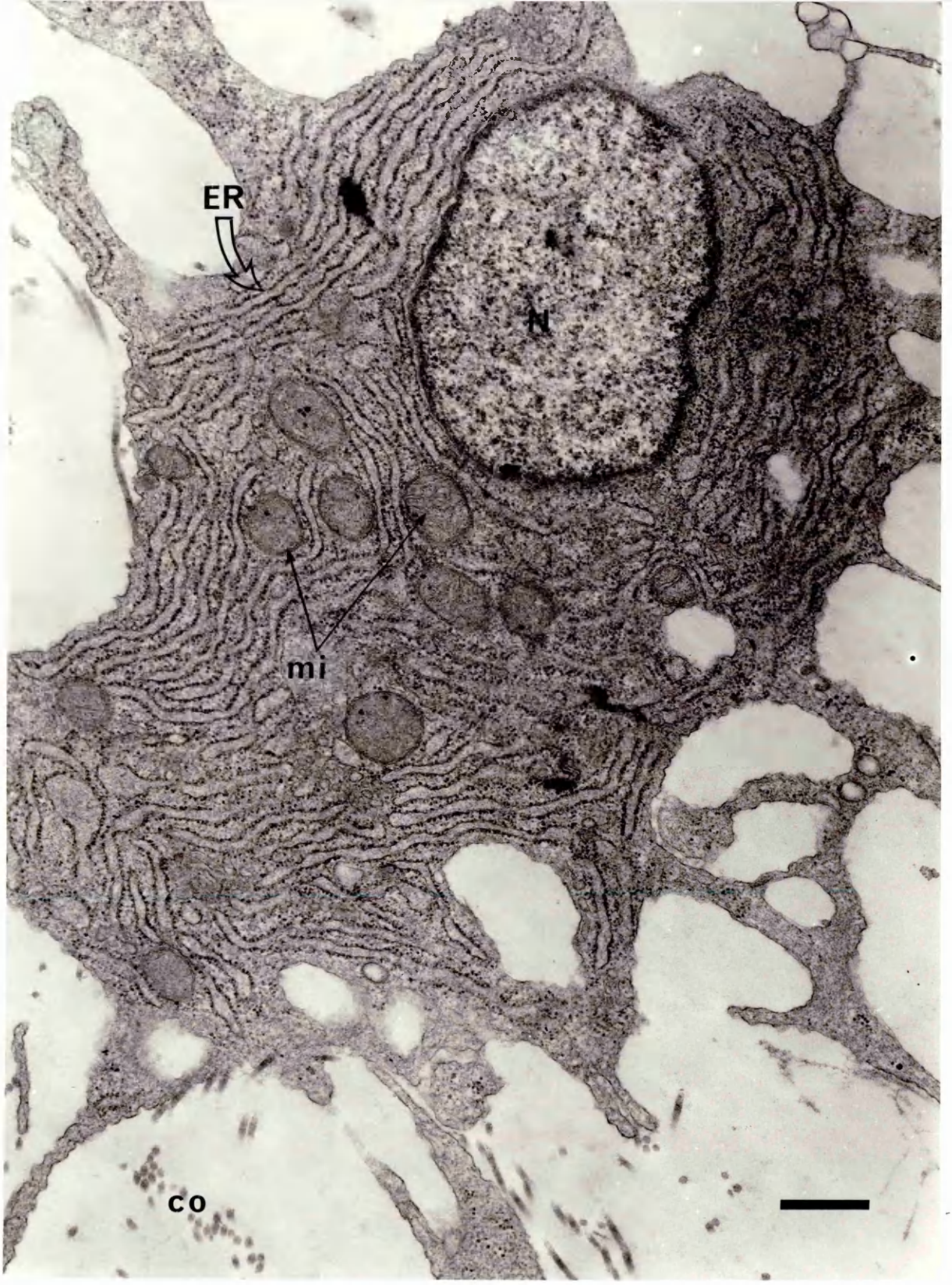
PLATE 27



Cell in the mesoderm at 11 days of incubation (x30,000)

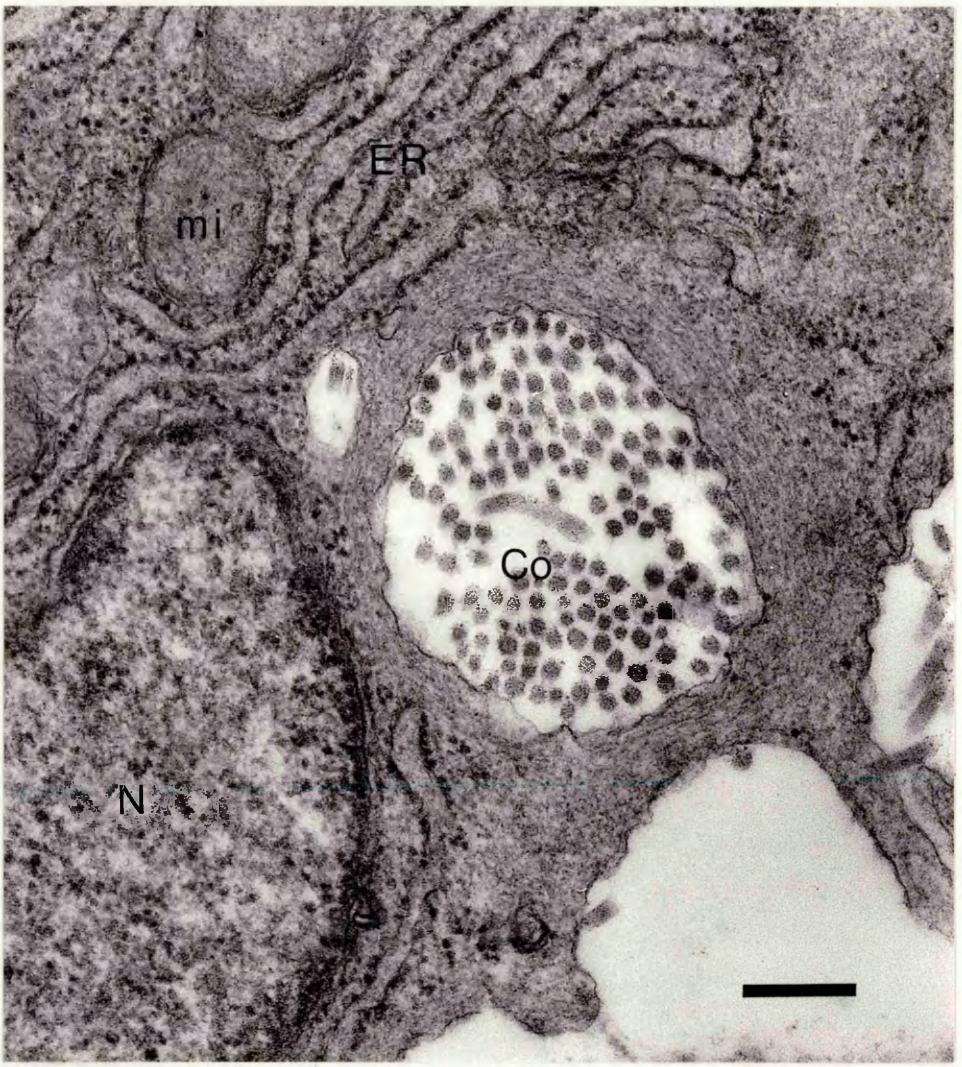
Co = collagen Ve = vesicles ER = endoplasmic reticulum
mi = mitochondria N = nucleus No = nucleolus
ch = chromatin (Bar = 500 nm.)

PLATE 28



Fibroblastic cell in the mesoderm at 14 days of incubation (x30,000)
ER = endoplasmic reticulum, arrowed N = nucleus
mi = mitochondria co = collagen (Bar = 500 nm.)

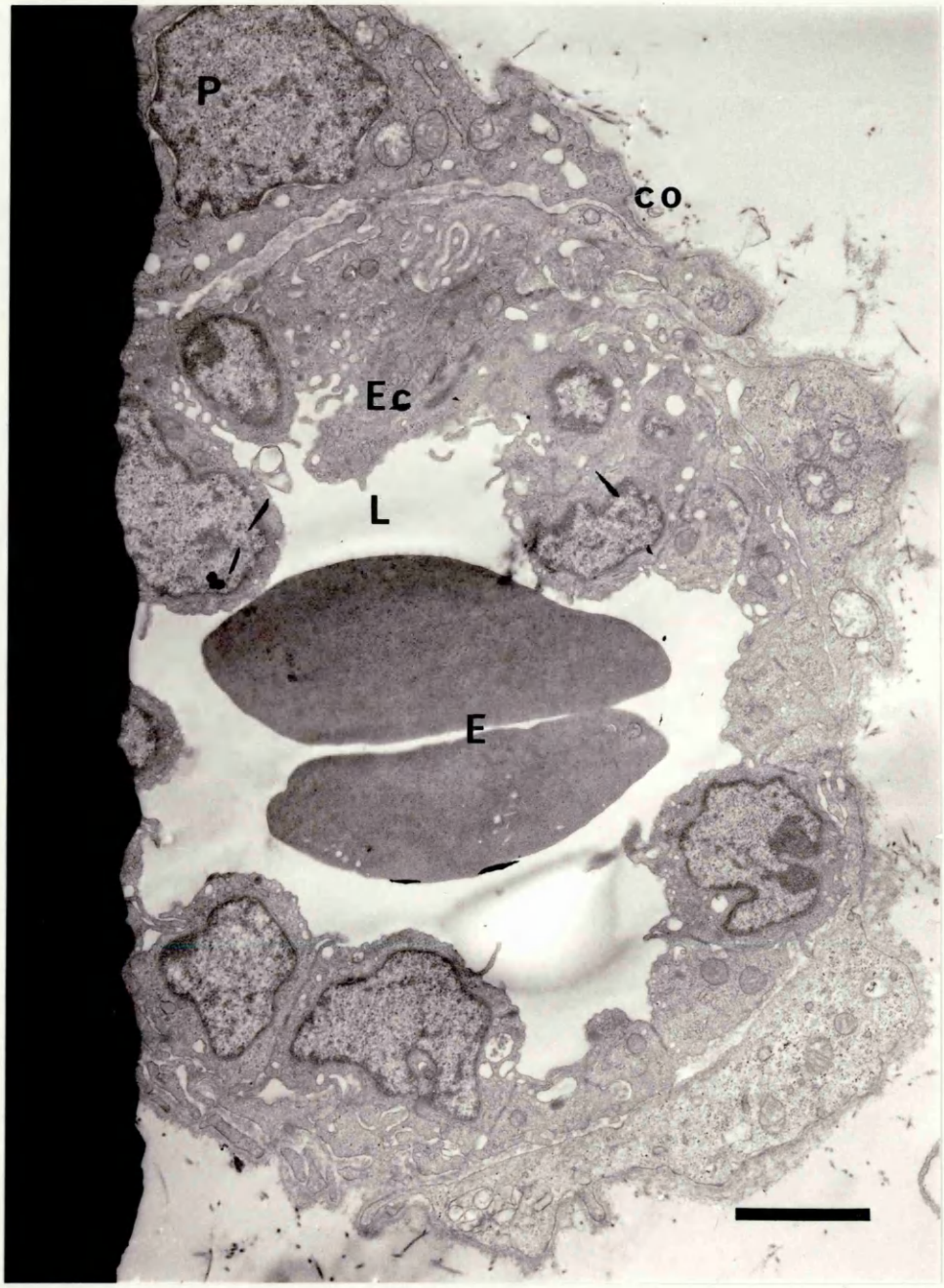
PLATE 29



Collagen fibres passing through the cytoplasm of a mesodermal cell at 14 days of incubation (x60,000)

ER = endoplasmic reticulum mi = mitochondria Co = collagen
N = nucleus (Bar = 250 nm.)

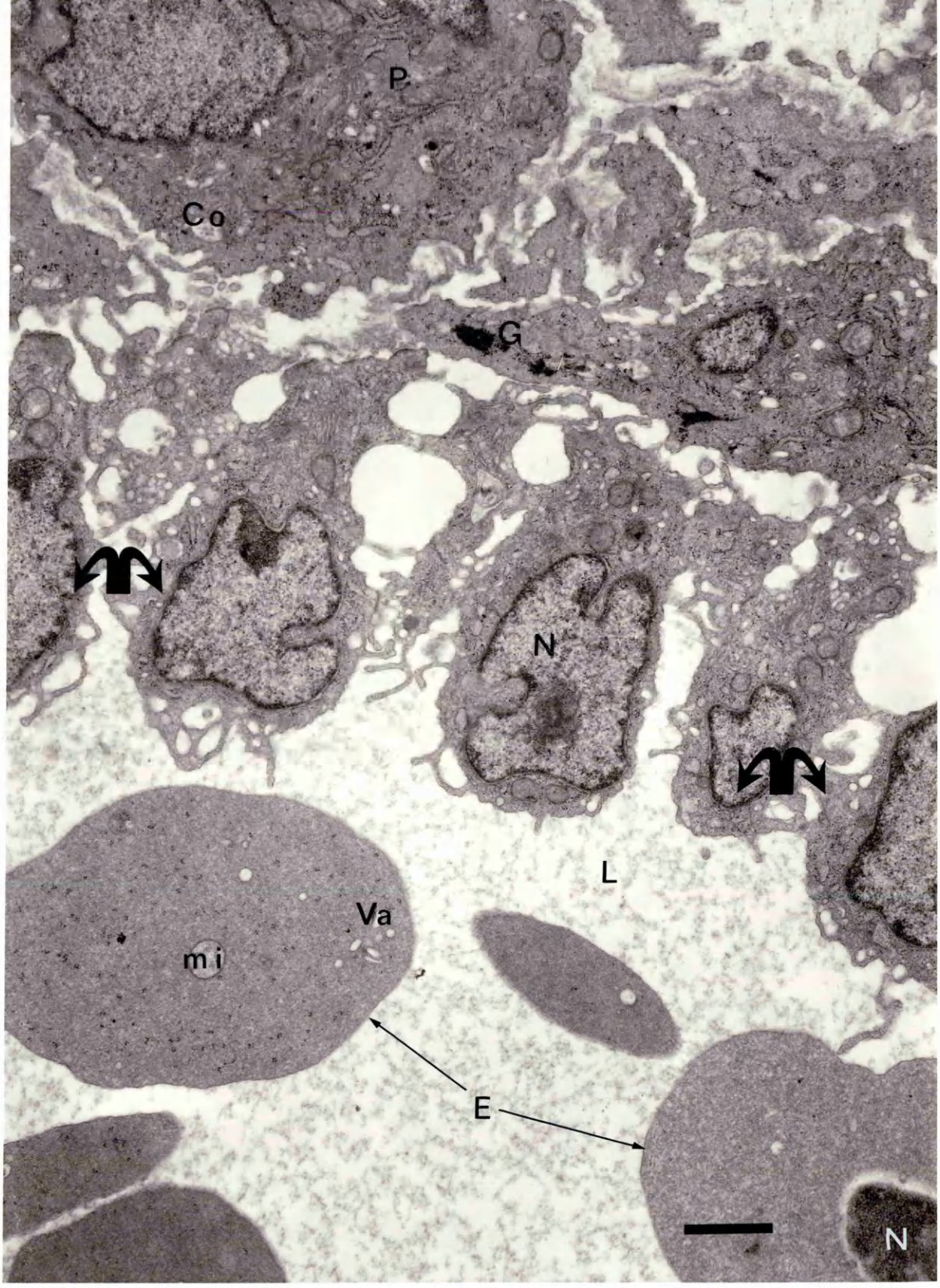
PLATE 30



Blood vessel in the mesoderm at 15 days of incubation (x9,000)

P = pericytes co = collagen Ec = endothelial cells
L = lumen of blood vessel E = erythrocytes (Bar = 2 µm.)

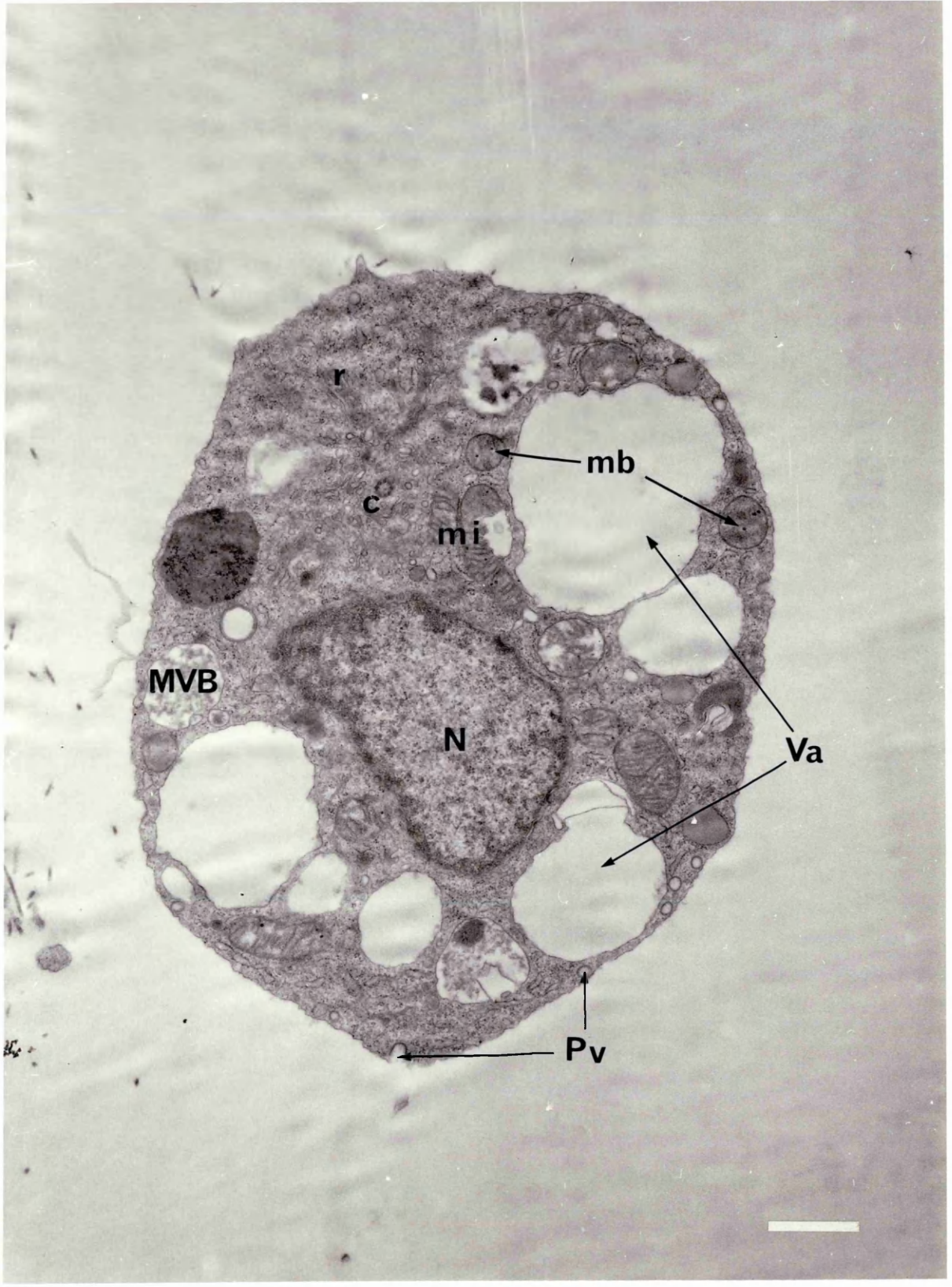
PLATE 31



Blood vessel in the mesoderm at 14 days of incubation (x15,000)

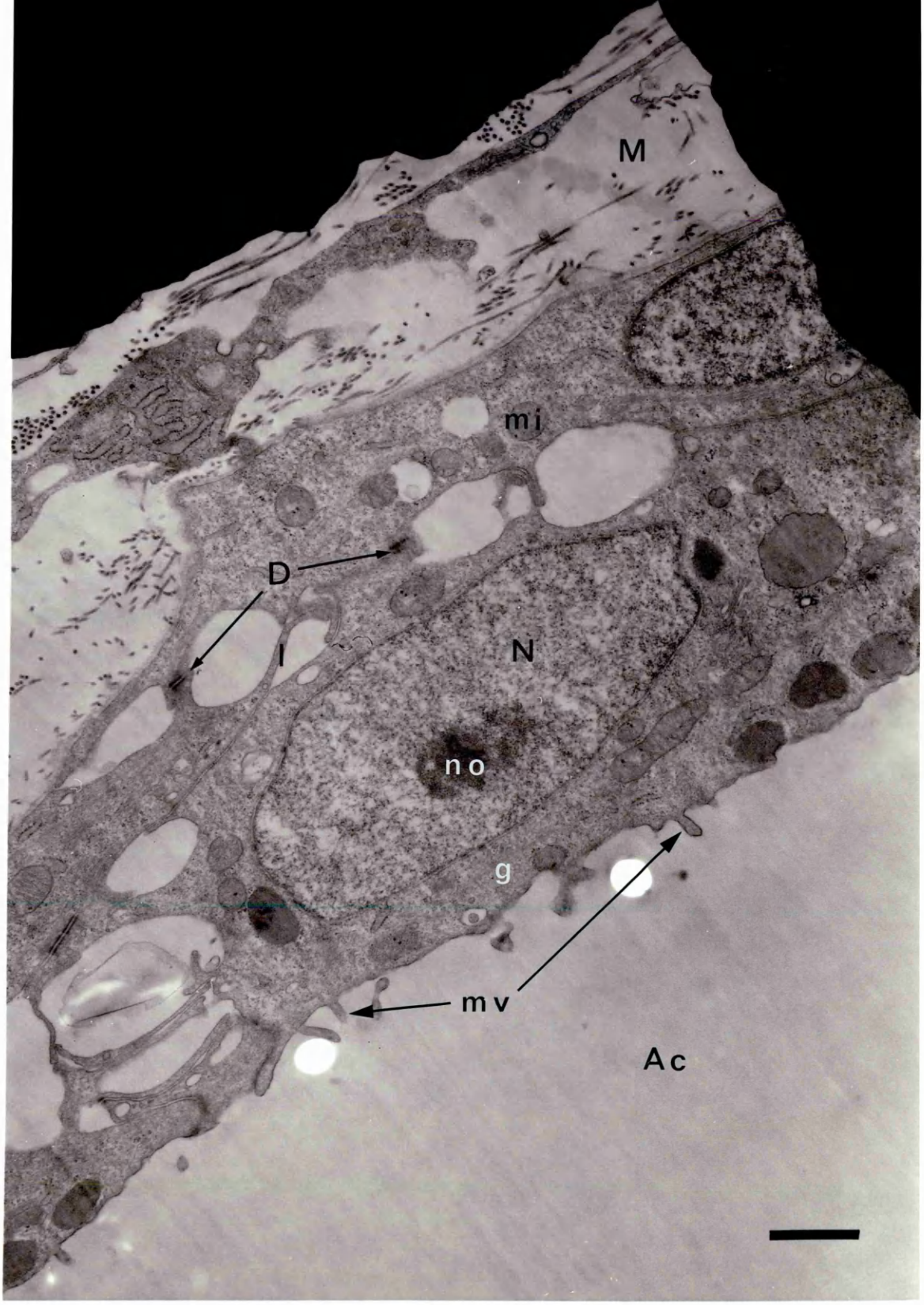
P = pericytes Co = collagen N = nucleus mi = mitochondria
Va = vacuoles L = lumen of blood vessel E = erythrocytes
Double arrows indicate the endothelial cells. (Bar = 1 µm.)
G = glycogen granules

PLATE 32



Monocyte in a blood vessel in the mesoderm at 14 days of incubation (x15,000)
r = ribosomes mb = microbodies in mitochondria
mi = mitochondria MVB = multi-vesiculate body
N = nucleus Va = vacuoles Pv = pinocytotic vesicles
c = centriole (Bar = 1 µm.)

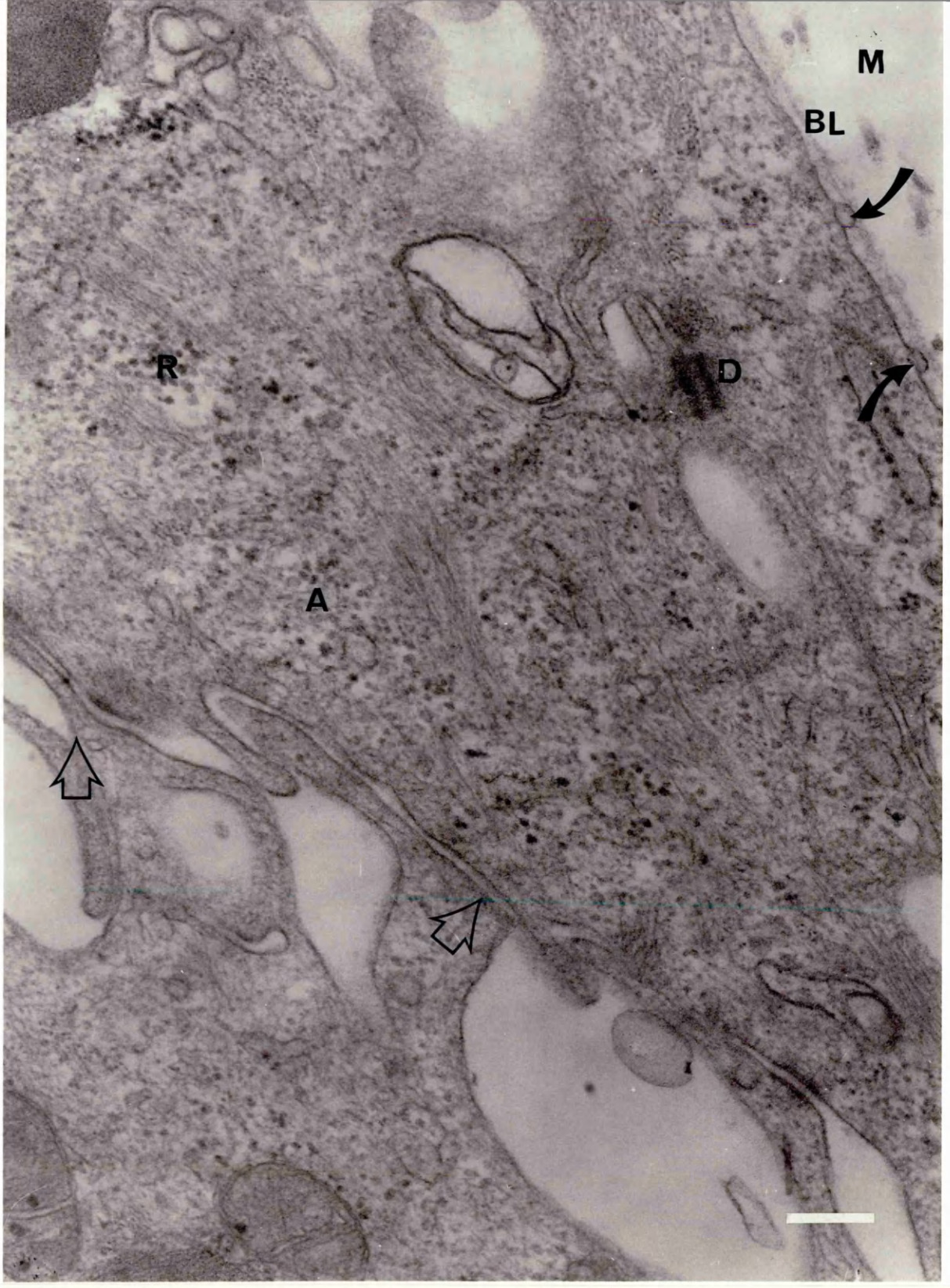
PLATE 33



Allantois at 11 days of incubation (x15,000)

M = mesoderm mi = mitochondria N = nucleus D = desmosomes
I = intercellular gaps no = nucleolus g = golgi complex
mv = microvilli Ac = allantoic cavity (Bar = 1 µm.)

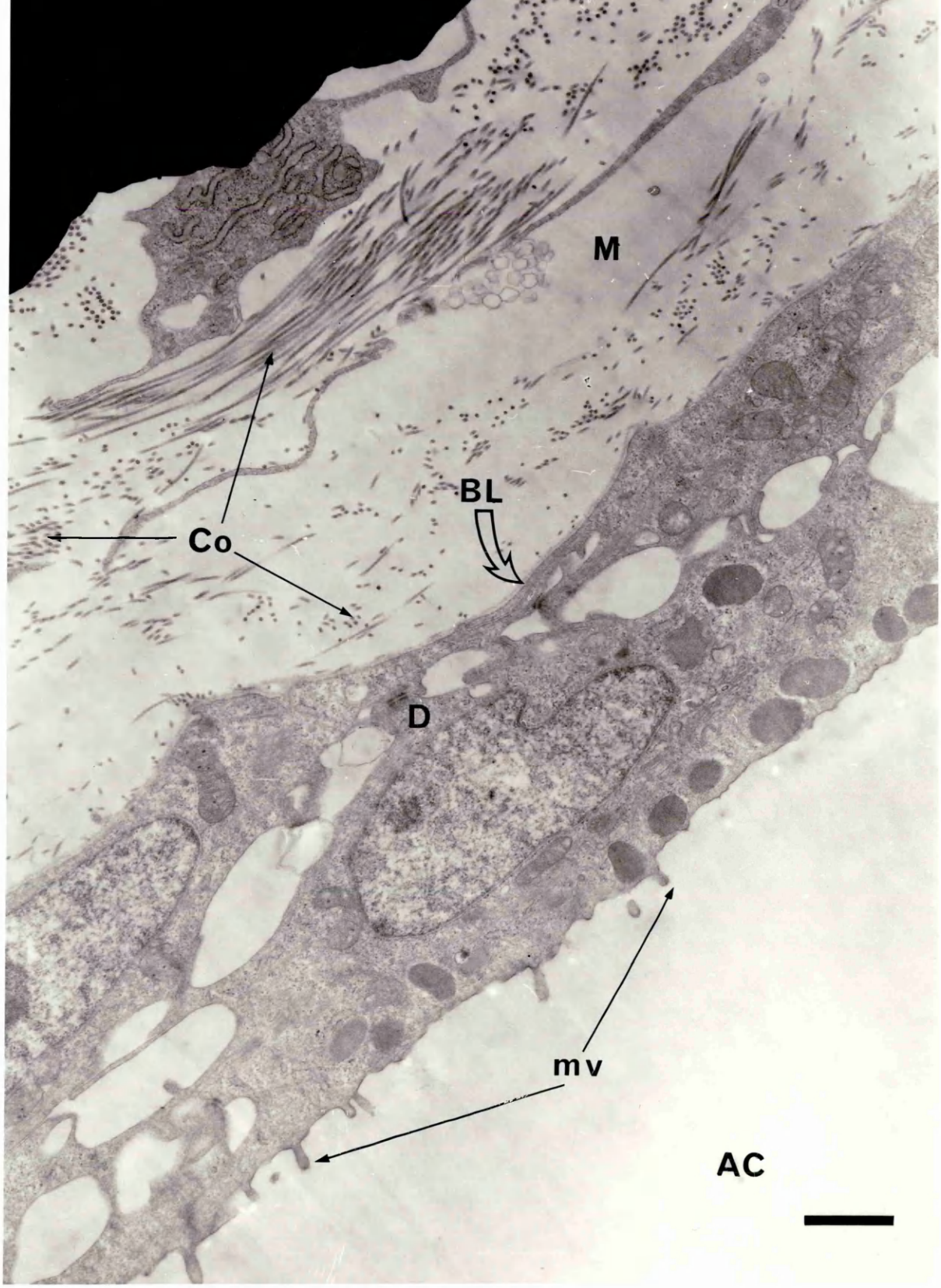
PLATE 34



Region in allantois at 11 days of incubation showing cytoplasmic bridging (open arrows), and allantoic cell projections ('blebs') into the mesoderm (closed arrows) (x60,000)

M = mesoderm BL = basal lamina R = ribosomes
D = desmosomes A = allantois (Bar = 250 nm.)

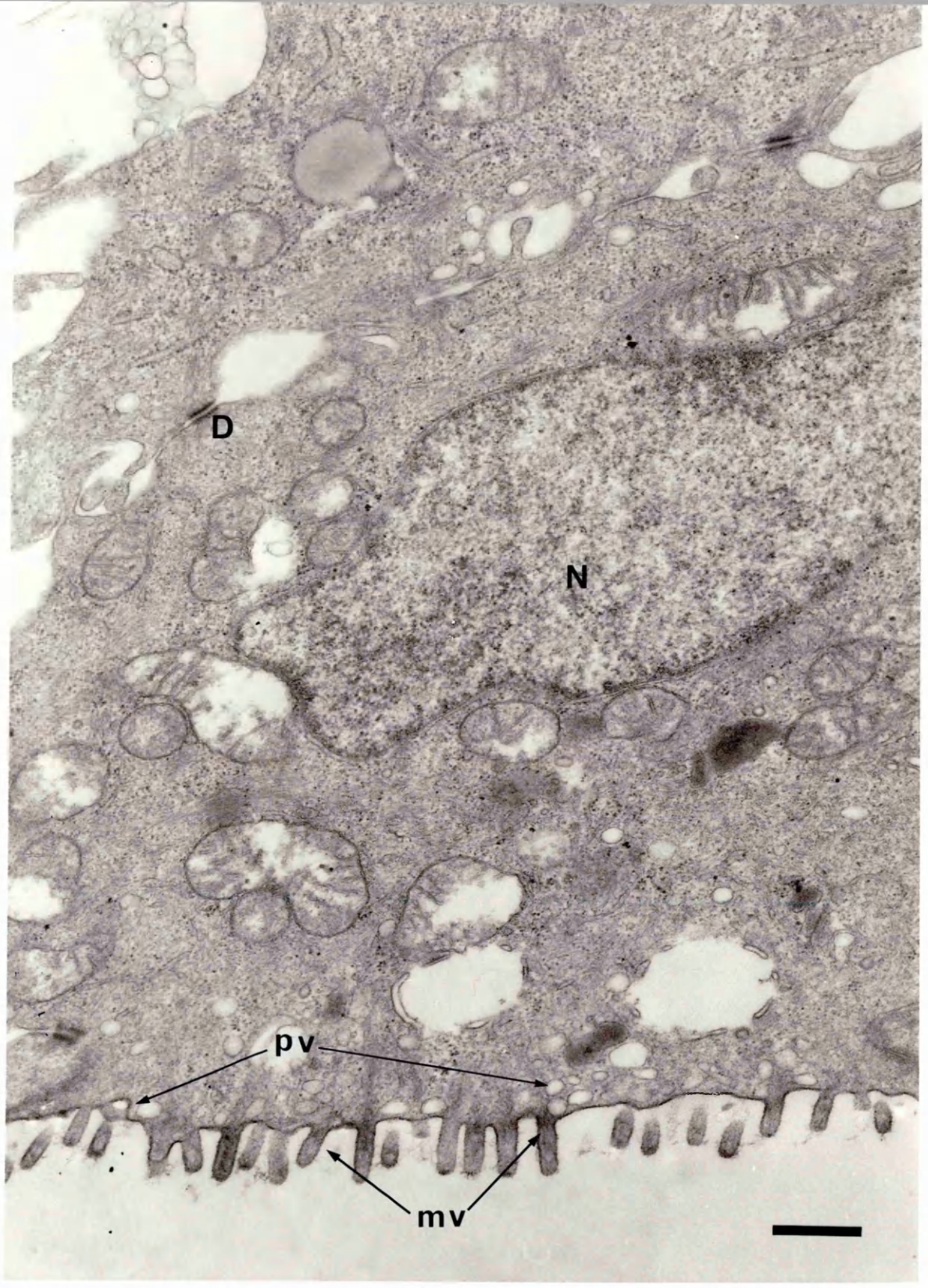
PLATE 35



Allantois at 11 days of incubation (x15,000)

M = mesoderm BL = basal lamina (arrowed) Co = collagen
D = desmosomes mv = microvilli AC = allantoic cavity
(Bar = 1 µm.)

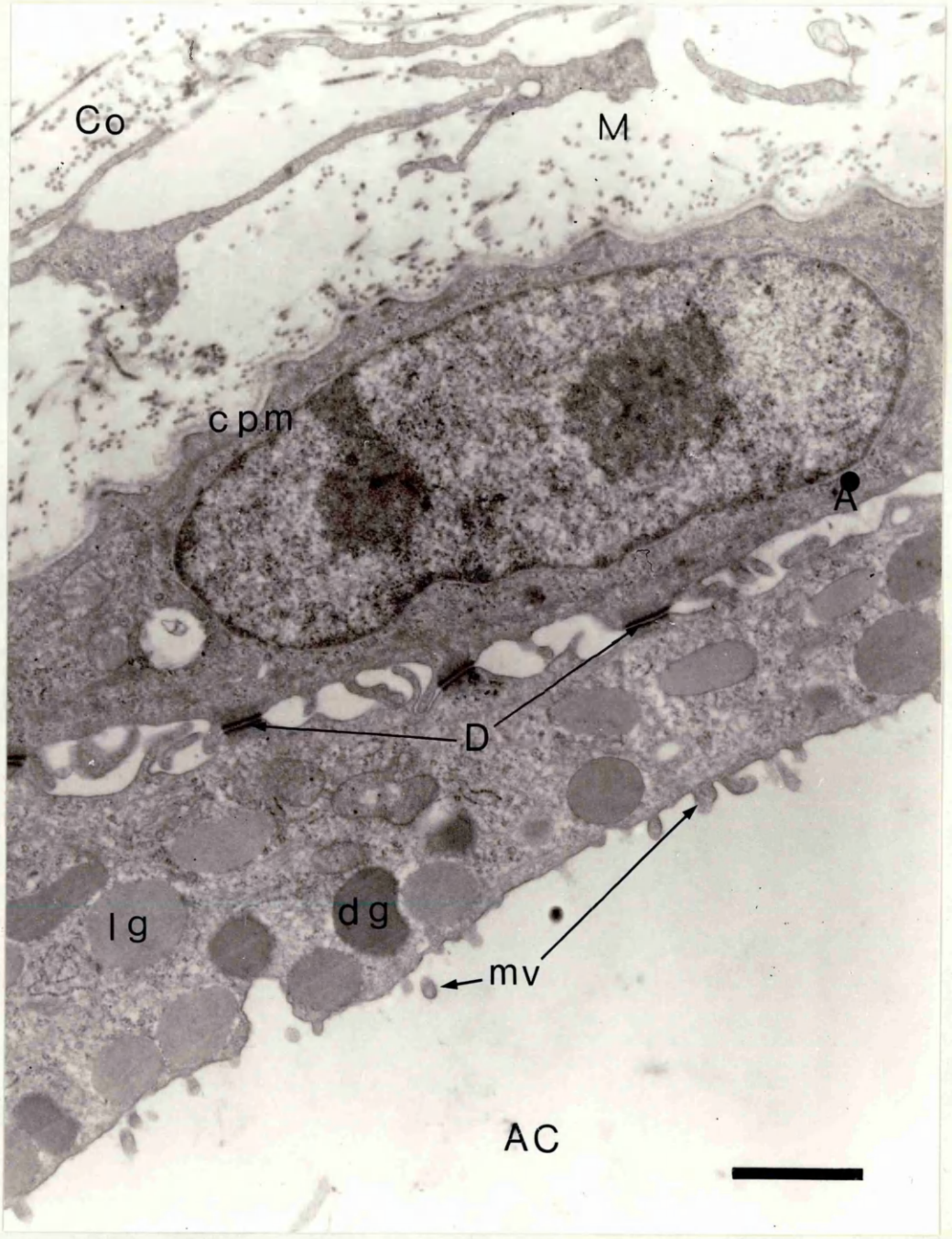
PLATE 36



Allantois at 14 days of incubation (x30,000)

D = desmosomes N = nucleus pv = pinocytotic vesicles
mv = microvilli (Bar = 500 nm.)

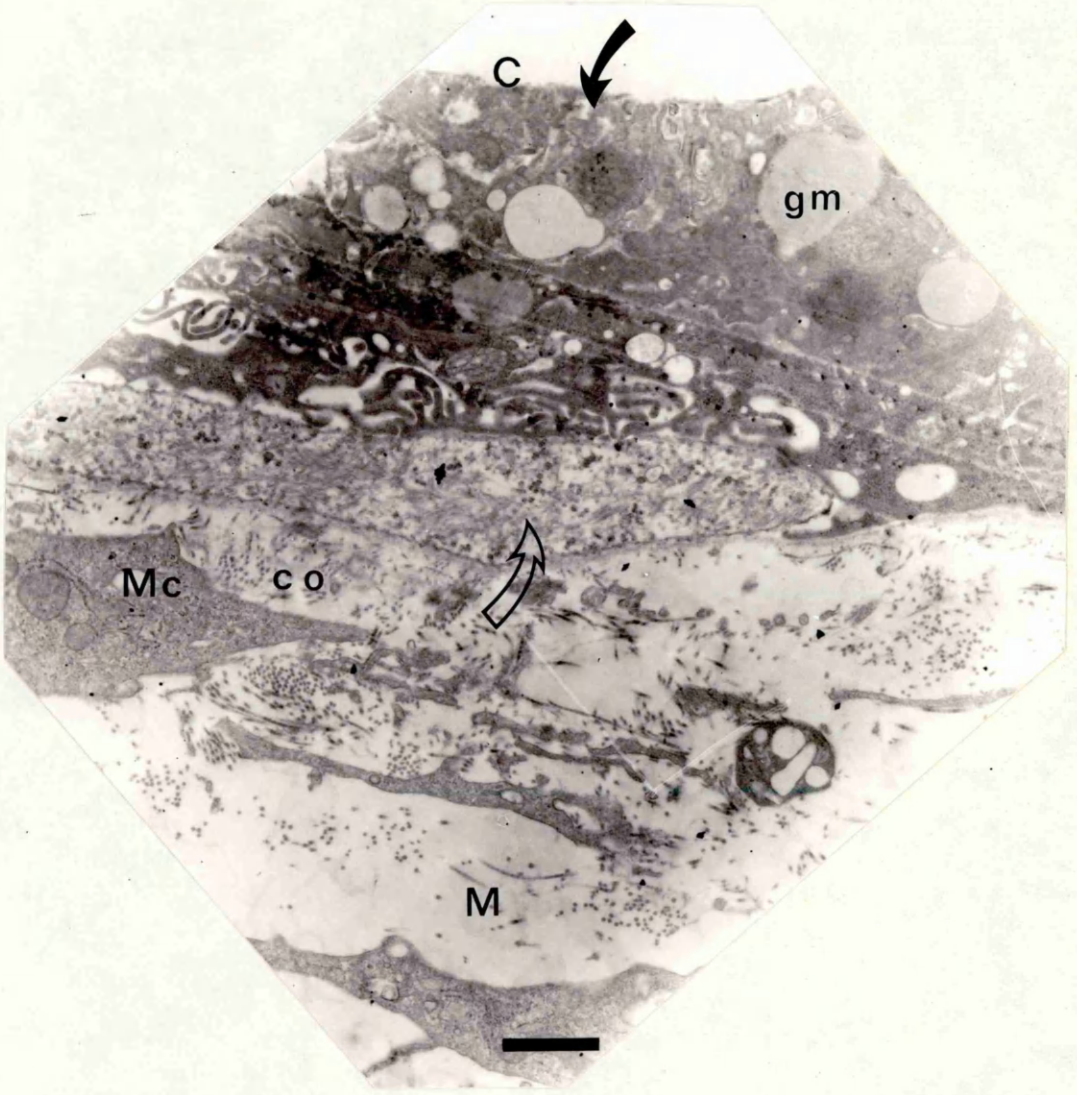
PLATE 37



Allantois at 18 days of incubation (x18,900)

Co = collagen M = mesoderm cpm = convoluted plasma membrane
A = allantois D = desmosomes lg = light staining granules
dg = dark staining granules mv = microvilli
AC = allantoic cavity (Bar = 1 µm.)

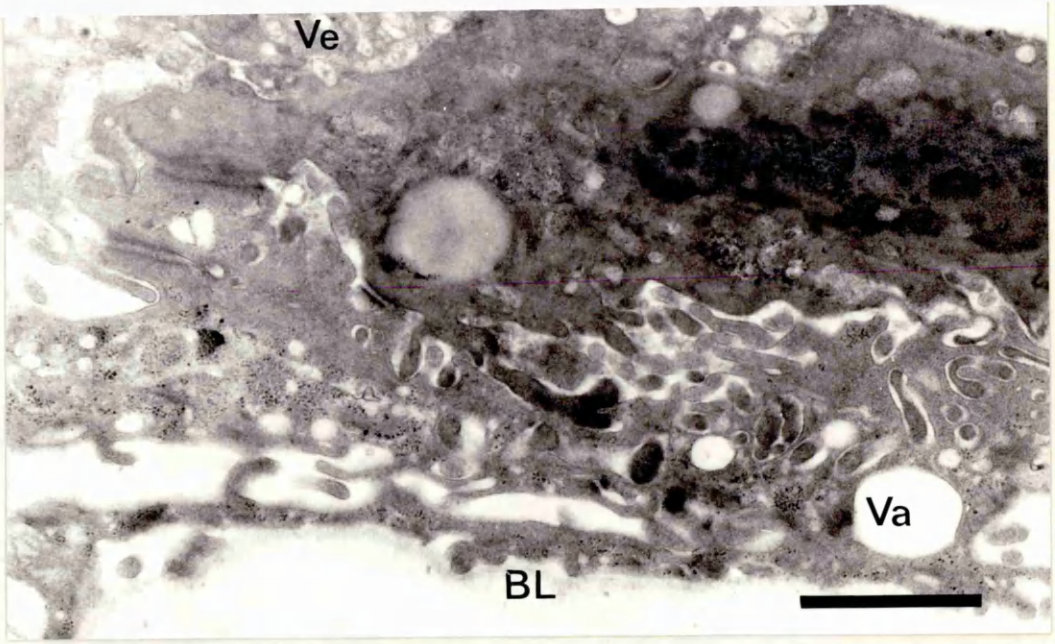
PLATE 38



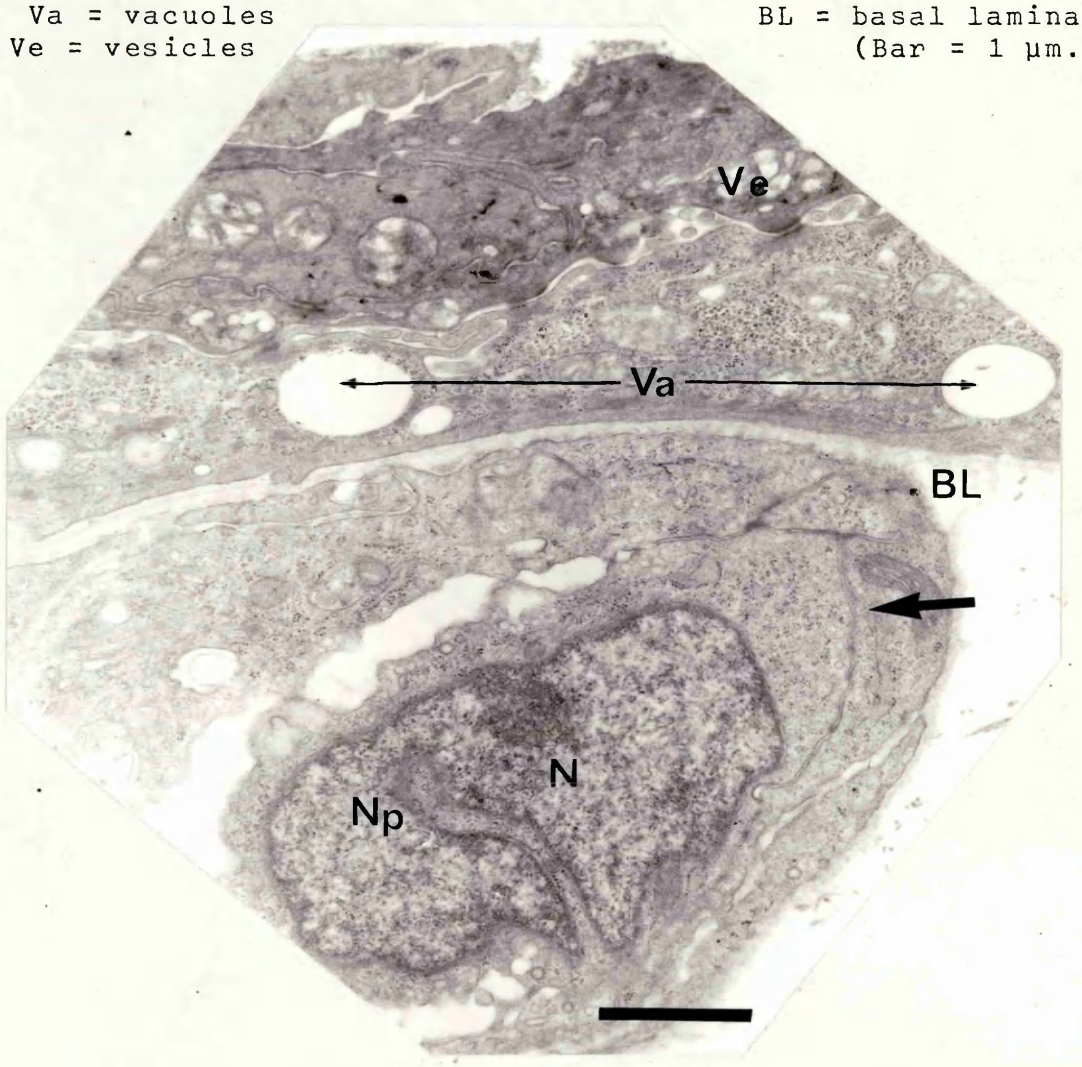
Chorion at 17 days of incubation showing a two-layered appearance (x12,500)

C = chorion gm = globular material Mc = mesodermal cells
co = collagen M = mesoderm (Bar = 1 µm.)
solid arrow = dark staining layer
open arrow = light staining layer

PLATE 39



a) Chorion at 14 days of incubation (x24,000). Note the increased electron density of the chorion cells. Trauma is apparent as a loss of cellular sub-structure (open arrow) and ballooning of the cytoplasm (b).
Va = vacuoles BL = basal lamina
Ve = vesicles (Bar = 1 µm.)



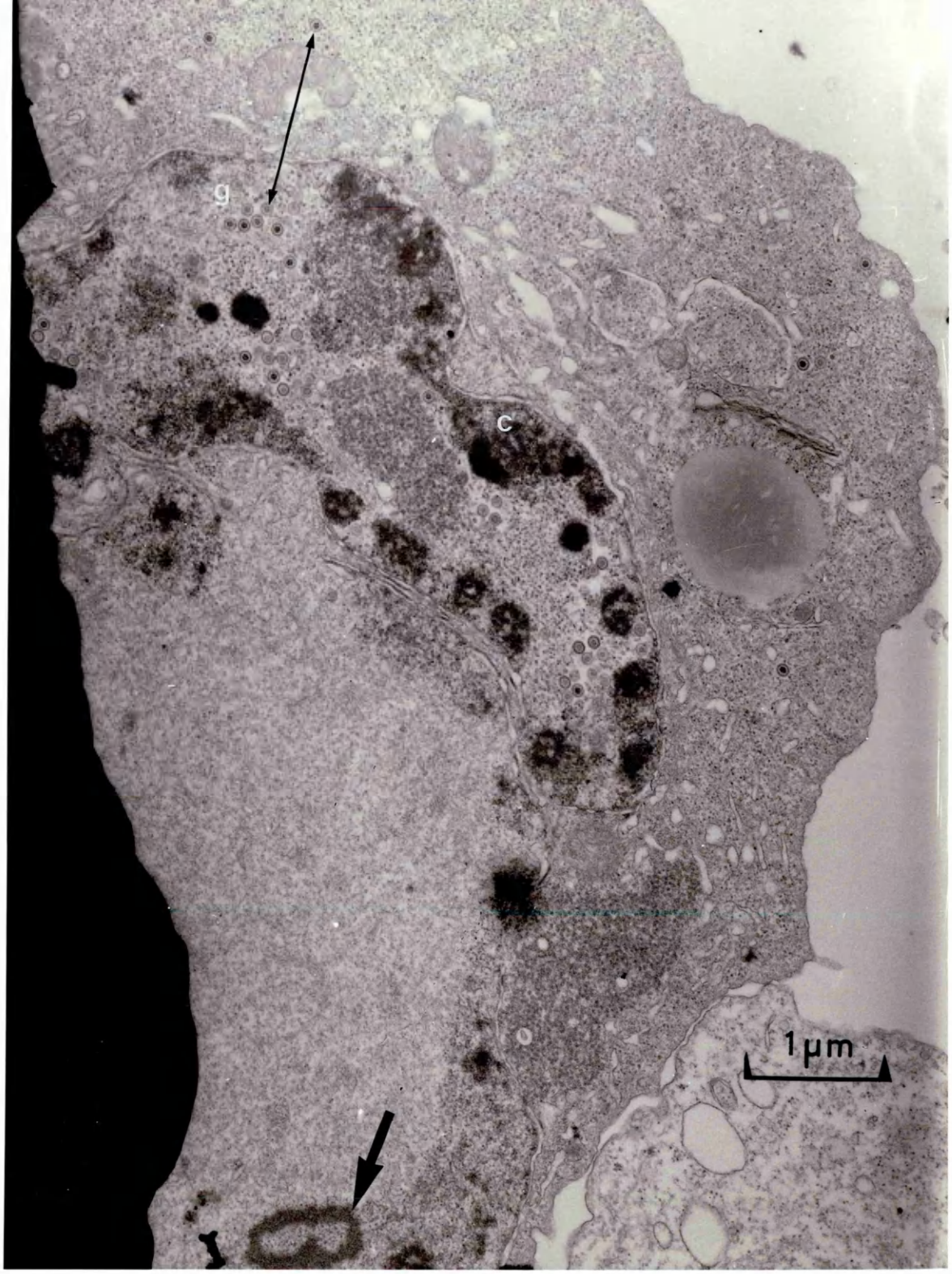
b) Chorion at 14 days of incubation (x20,000). A leucocyte (arrowed) is present in the mesoderm in contact with the basal lamina (BL). Va = vacuoles Ve = vesicles
N = nucleus Np = nuclear pores (Bar = 1 µm.)

PLATE 40

9.2 Herpes virus infected CAM

Thin sections of CAMs previously inoculated with HSV types 1 or 2 were examined daily from one to seven days post-inoculation. These days were equivalent to 12 to 18 days of egg incubation of the uninoculated controls. A series of electron micrographs of sections through pocks were taken at magnifications of x100 to x300 to prepare montages which were intermediate between optical micrographs and high magnification electron micrographs and so facilitated location of infected cells within the tissues.

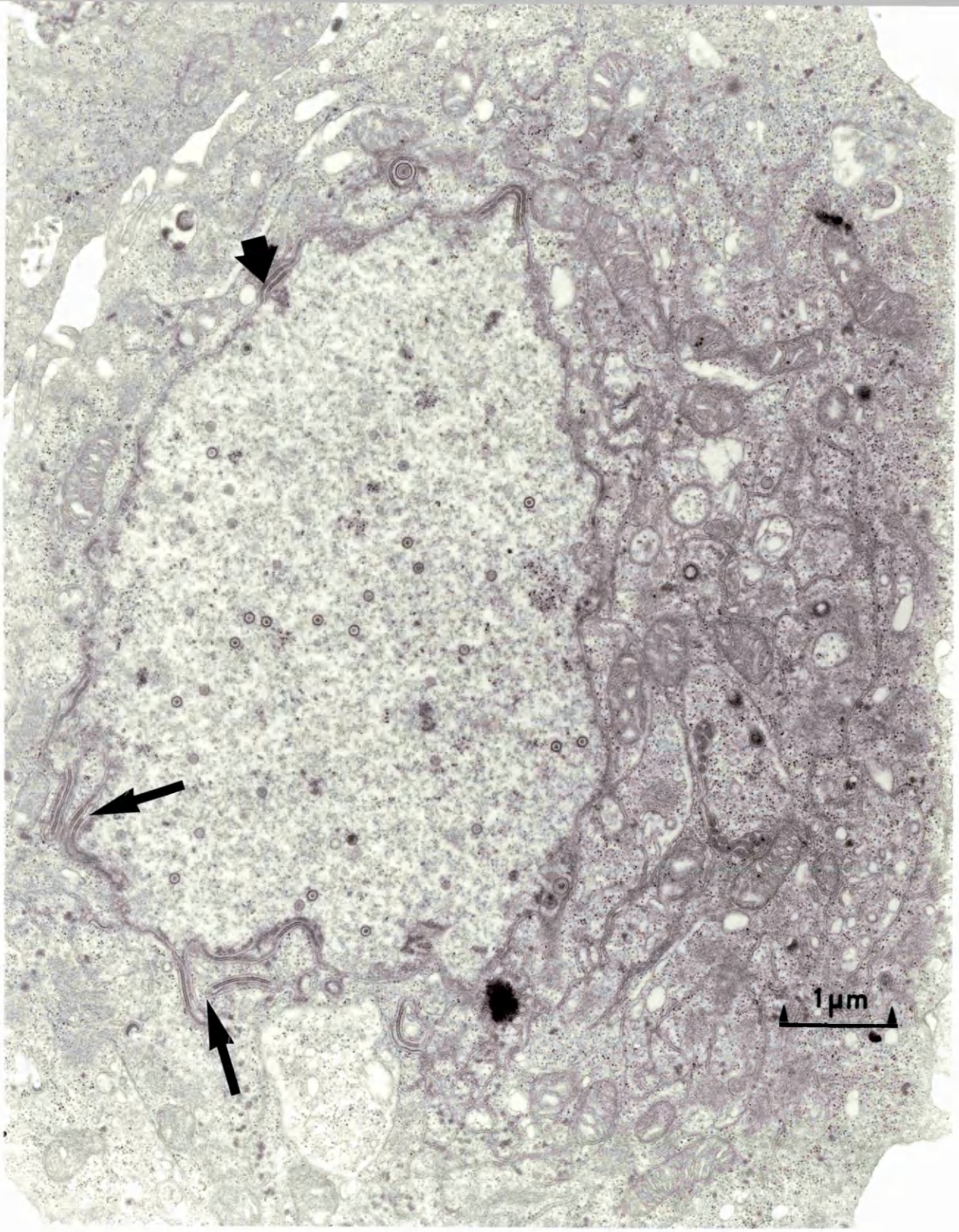
Many of the ultrastructural changes induced in individual cells of the CAM were common to infections with either HSV type 1 or type 2 strains. These changes included: clumping of chromatin which localised at the periphery of the nucleus (margination); disruption of nuclei; reduplication of nuclear membranes, and shrinkage of nuclei resulting in the development of irregular nuclear membranes and enlargement of the perinuclear space. Virus particles at various stages of maturation were present in the nuclei and cytoplasm, the nuclei frequently became more electron translucent compared with controls and also became disorganised, and the nuclear membranes eventually ruptured allowing mixing of nuclear and cytoplasmic contents including enveloped and non-enveloped virus particles. Cellular breakdown in the presence of lysosomes completed the virus-induced cellular lysis (plates 41 to 45).



HSV type 1 day two pock

Infected cell in chorion showing unenveloped virions in the nucleus and cytoplasm (arrowed) (x22,500)
Also present in the nucleus is an annular structure containing granular material (arrowed)
c = marginated chromatin g = 37 nm. granules in nucleus

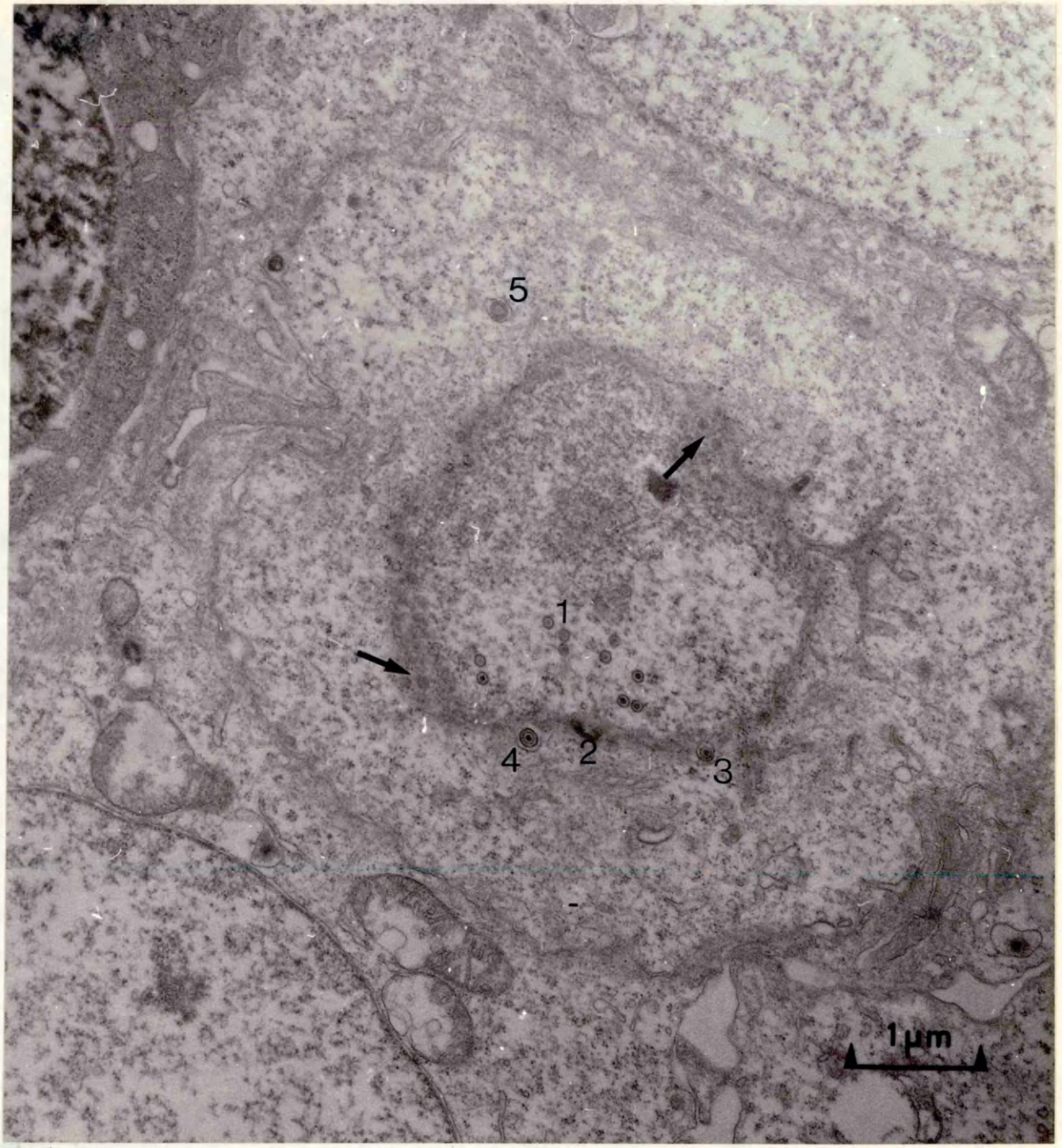
PLATE 41



HSV type 2 day two pock

Infected cell in chorion (x18,900)
Infected nucleus has irregular outline. Unenveloped nuclear and enveloped cytoplasmic virus particles are present. Reduplication of the nuclear membrane is evident (arrowed)

PLATE 42



HSV type 1 day two pock

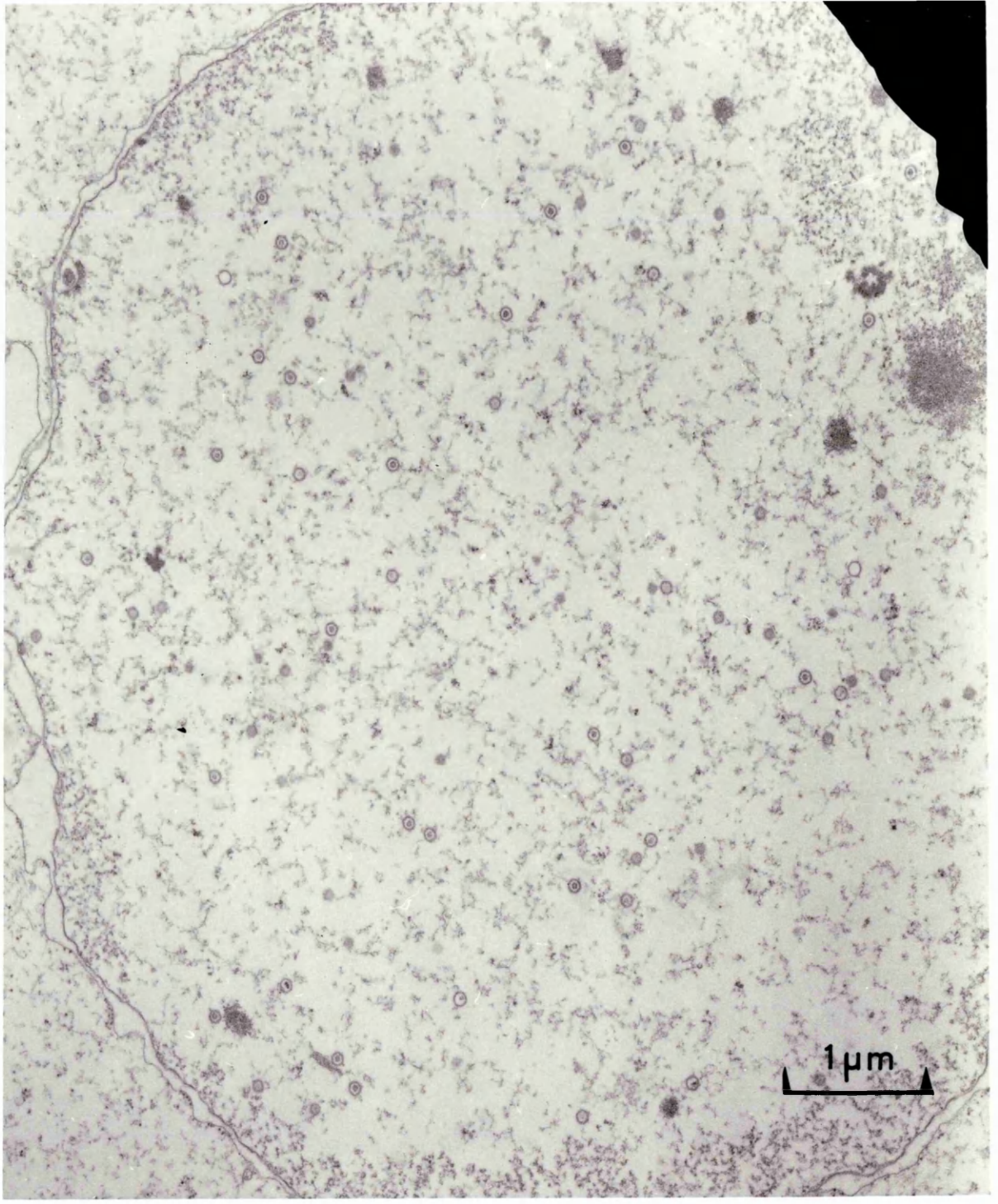
Infected cell in chorion (x20,000)

Virus particles are present at various stages of maturation:

1 = naked nuclear virions
2 = thickening of nuclear membranes prior to virus envelopment
3 = early virus envelopment
4 = late virus envelopment
5 = enveloped virus in the cytoplasm

nuclear pores are arrowed

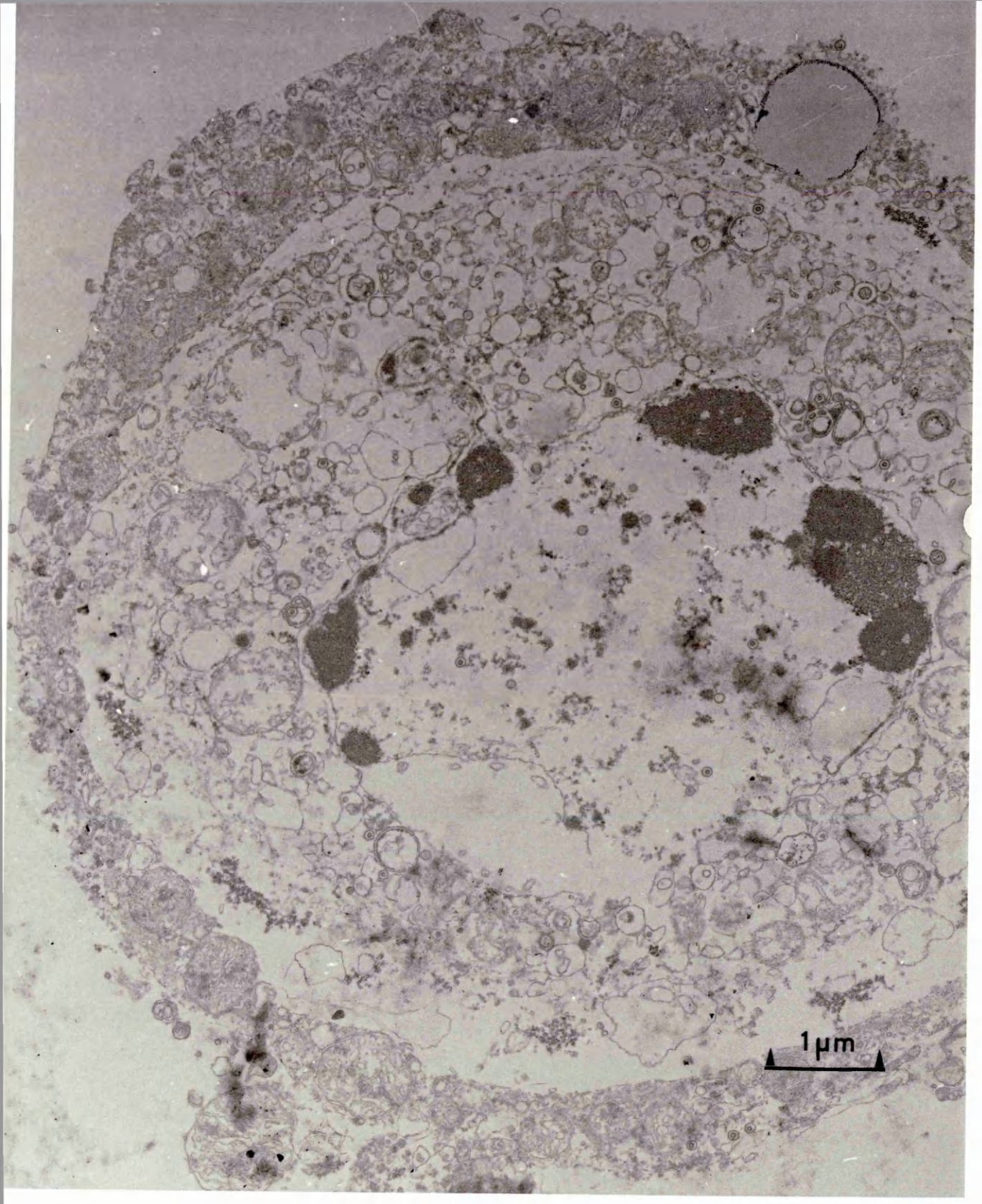
PLATE 43



HSV type 1 day two pock

Nucleus of infected cell (x22,500) containing naked virus particles with and without cores
The nucleus is highly electron translucent and irregular in outline and the perinuclear space is dilated

PLATE 44

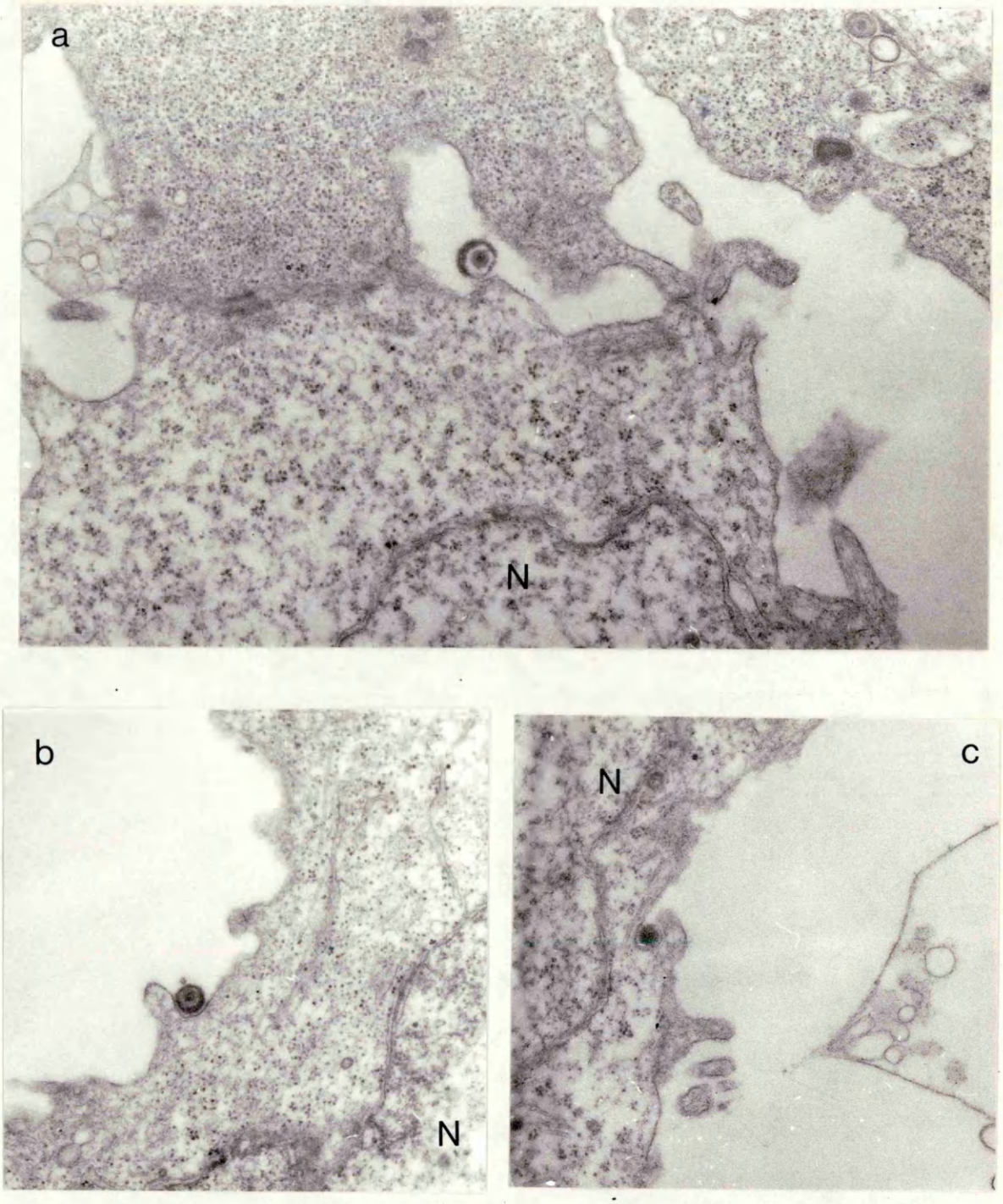


HSV type 1 day two pock

Cell late in infection (x20,000)
Note disruption of the nucleus and cytoplasm mixed with virions

PLATE 45

Characteristic features of herpesvirus morphogenesis were seen in CAMs infected with HSV type 1 or type 2 strains. Virus particle attachment and penetration of the plasma membrane appeared to be similar for either virus type. After attachment, penetration of virus particles into the CAM cells was observed through pinocytotic vesicles or "viropexis", as well as by fusion of the viral envelope with the plasma membrane, although viropexis (plates 46 and 47) was more frequently seen than fusion (plate 48). It was difficult to be sure that virus particles observed in the cytoplasm were entering rather than leaving the cells (plate 49). Virus capsids appeared in the nucleus, envelopment occurred at the inner lamella of the nuclear membrane and enveloped virus particles were frequently present in the perinuclear cisternae and in vesicles within the nucleus (plates 50 and 51). Egress from cells appeared to be through tubule-like structures which were extensions of the outer lamella of the nuclear membrane and enveloped virus particles were frequently seen in these tubules (plate 52). Some enveloped particles were present in vesicles in the cytoplasm (plate 53), but whether these were tubules in cross-section or particles in sacs ready for release by reverse pinocytosis was not obvious (figure 10).



HSV type 2 day two pock

Virus particles at various stages of penetration of cells by "viropexis" (x18,900)

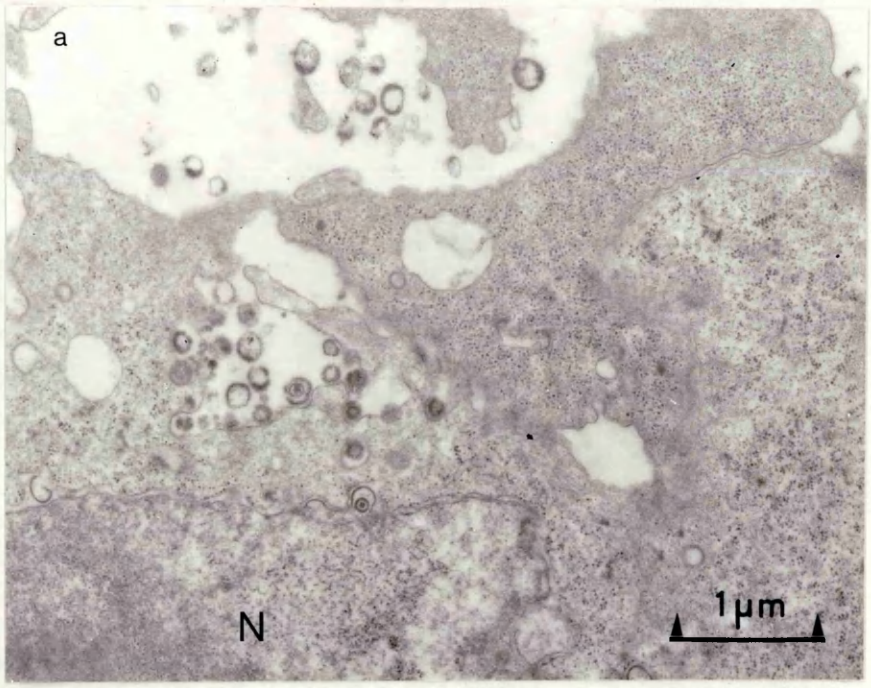
a) virus particle closely associated with the plasma membrane

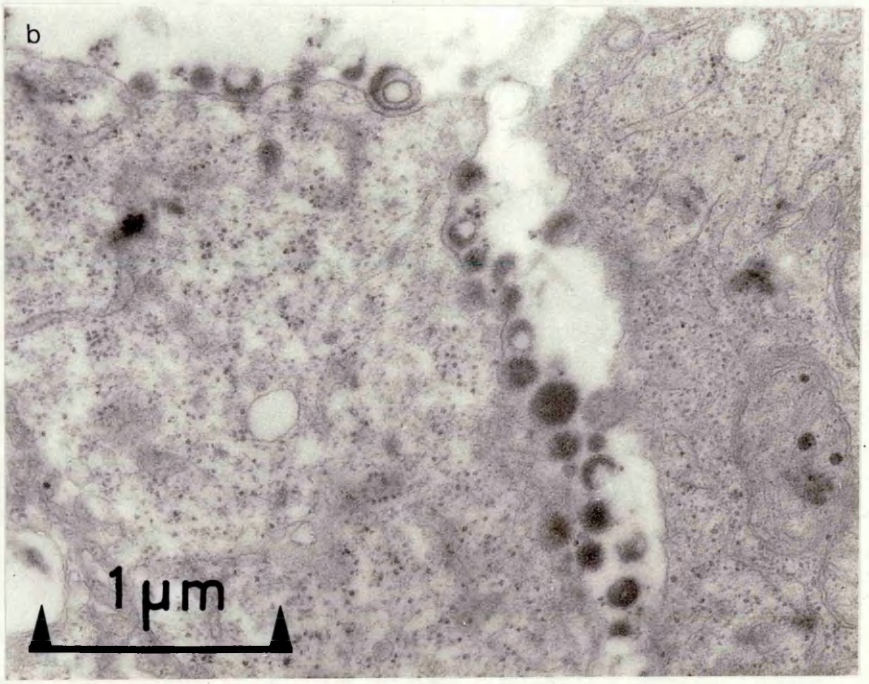
b) beginning of engulfment

c) engulfment almost complete

N = nucleus

PLATE 46





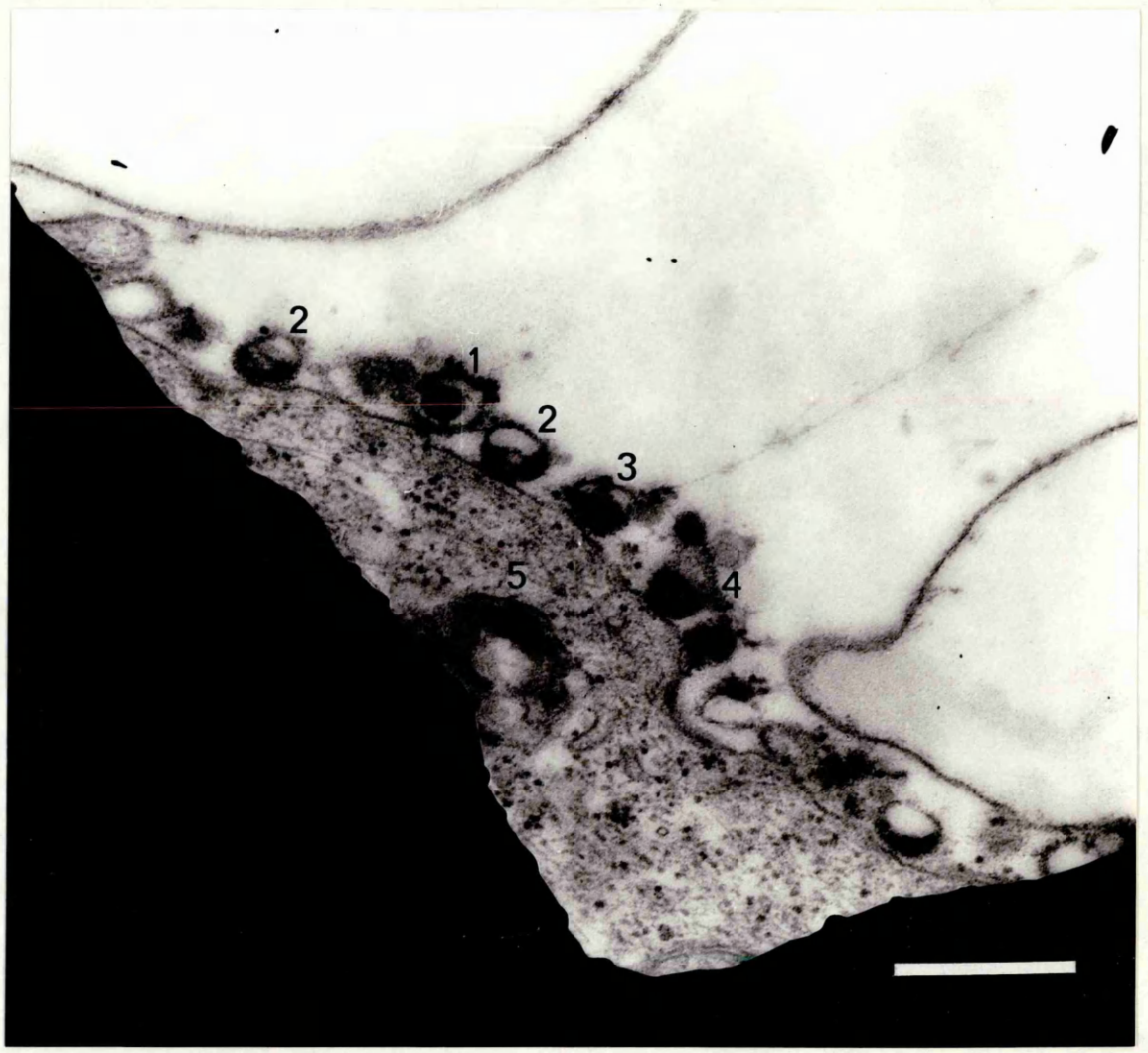
HSV type 2 day two pock

a) virus particles at various stages of engulfment by the plasma membrane of a cell in the process of infection (x18,900)

b) part of an infected cell showing virus particles entering the cell by "viropexis" or phagocytosis (x32,000)

N = nucleus

PLATE 47

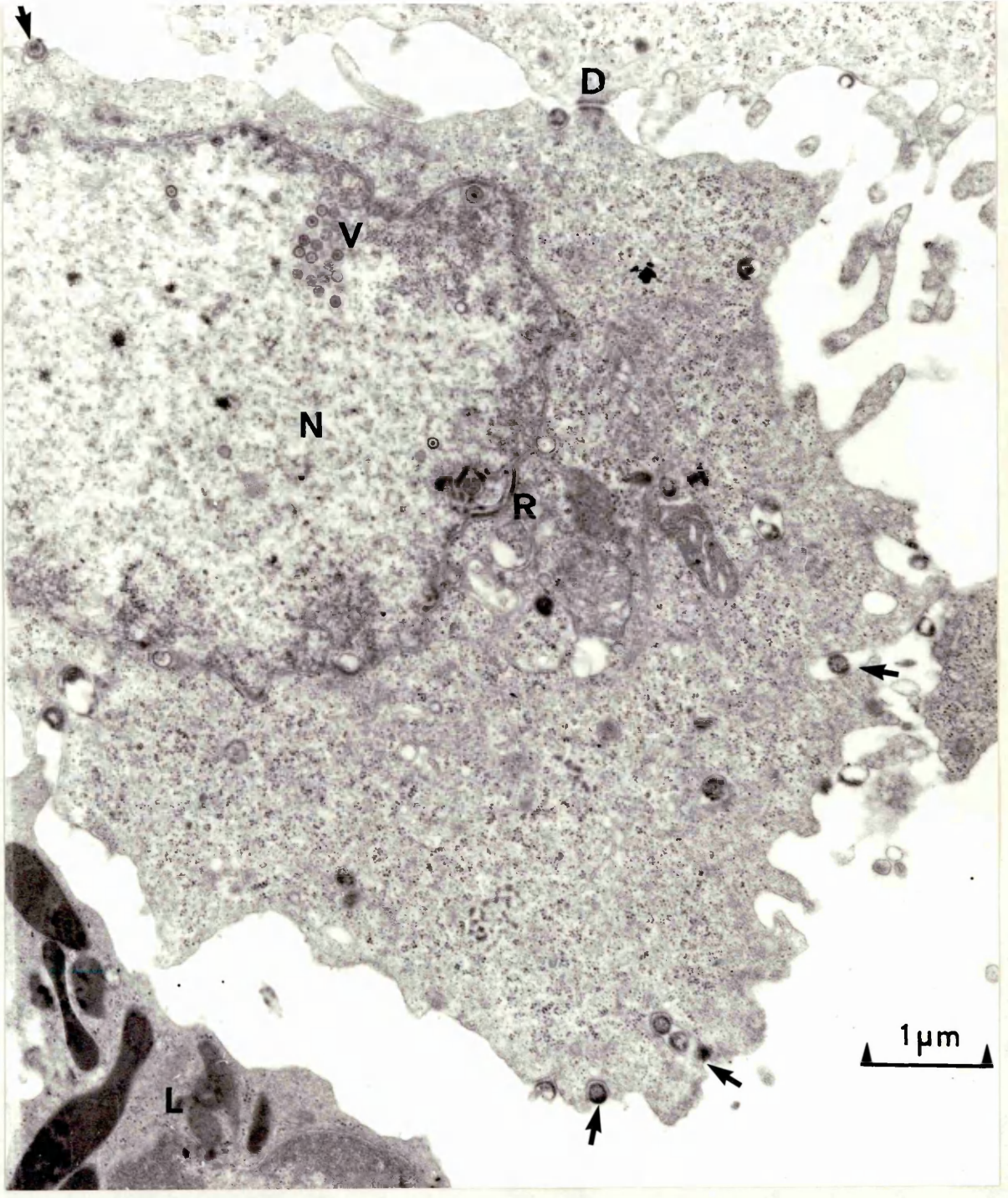


HSV type 2 day two pock

Numerous enveloped virus particles in the process of fusion with a cellular plasma membrane (x48,000)

1) virus in apposition to plasma membrane
2) virus and plasma membrane in close association; note fibrillar strands attaching the virus envelope to the cell membrane
3) digestion of membranes
4) fusion
5) entry of virus into cytoplasm

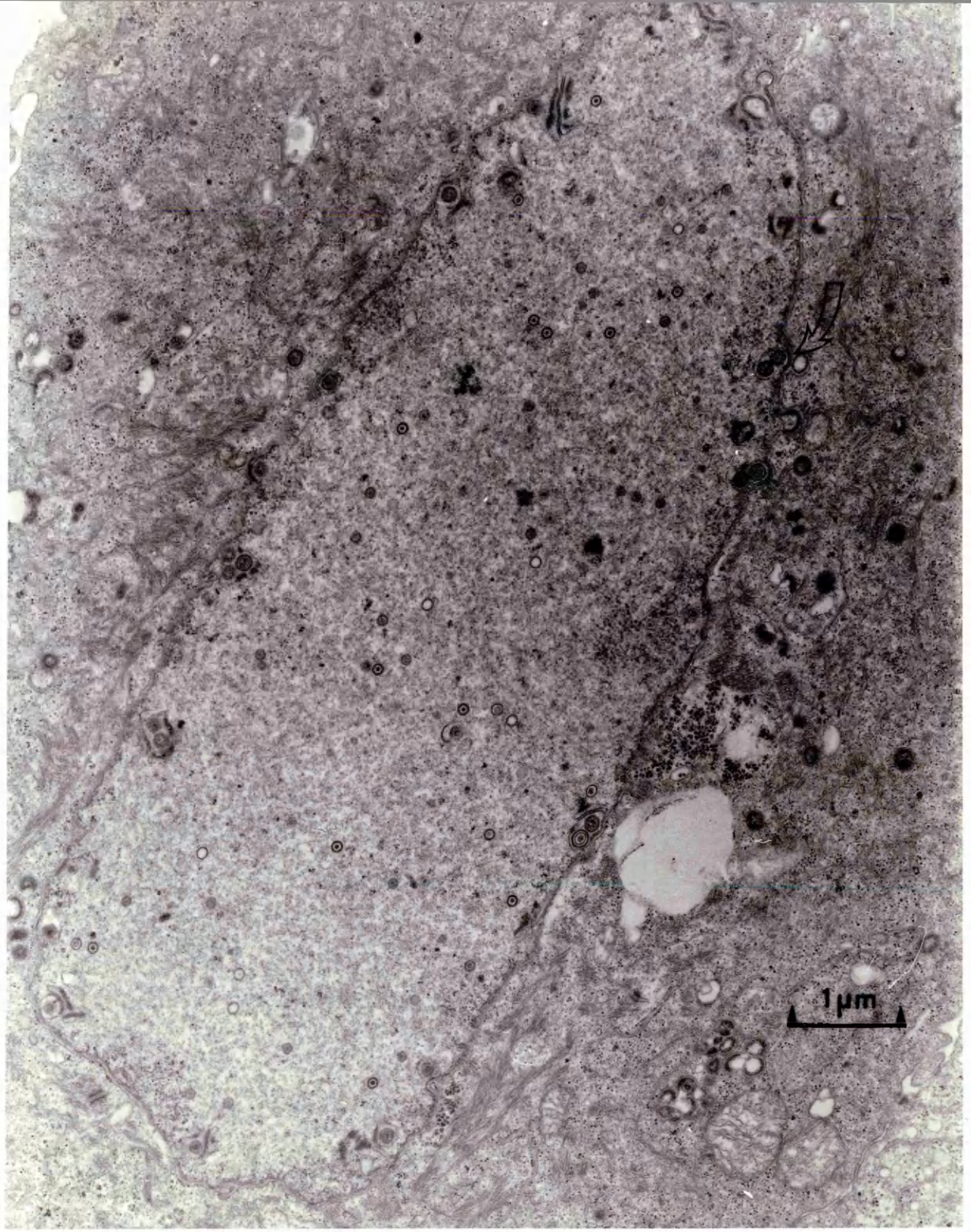
(Bar = 500 nm.)



HSV type 2 day two pock

Infected cell with numerous virus particles (x18,900)
Virions are present in pinocytotic vesicles either entering or leaving the cell

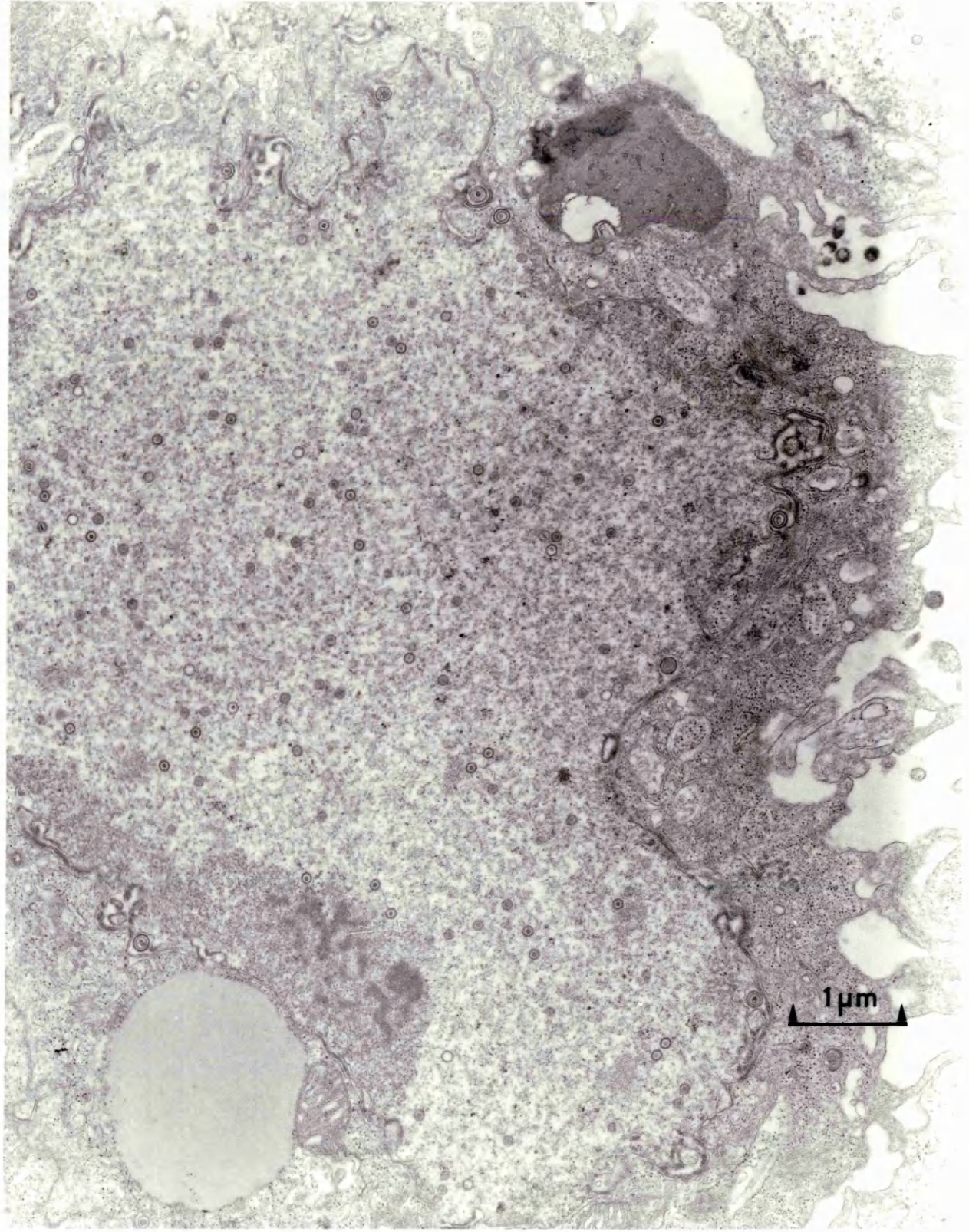
Virus particles involved with plasma membrane (arrows)
V = nuclear virus particles
N = nucleus D = desmosome
R = reduplication of nuclear membrane L = leucocyte



HSV type 2 day two pock

Infected cell showing virus particles in the nucleus and cytoplasm (x18,900)
Enveloped virus particles are present within vesicles in the nucleus and in the perinuclear space
Thickening of the nuclear membrane in apposition to virus particles is indicated (arrow)

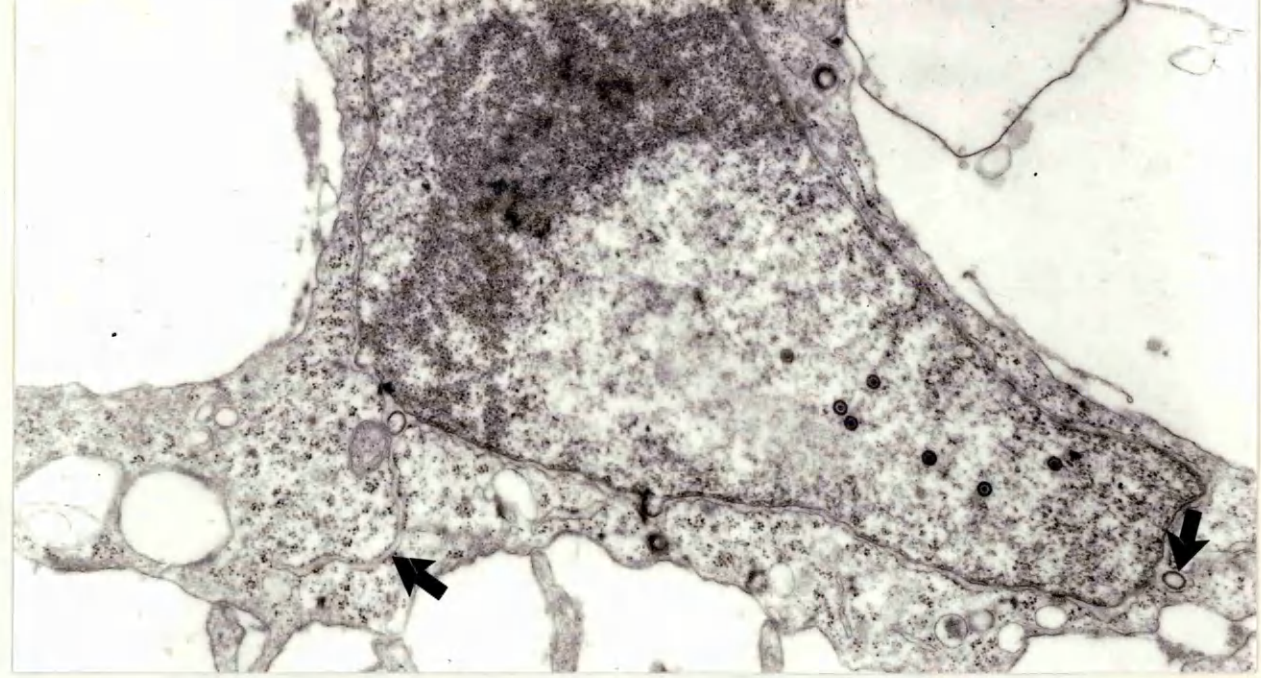
PLATE 50

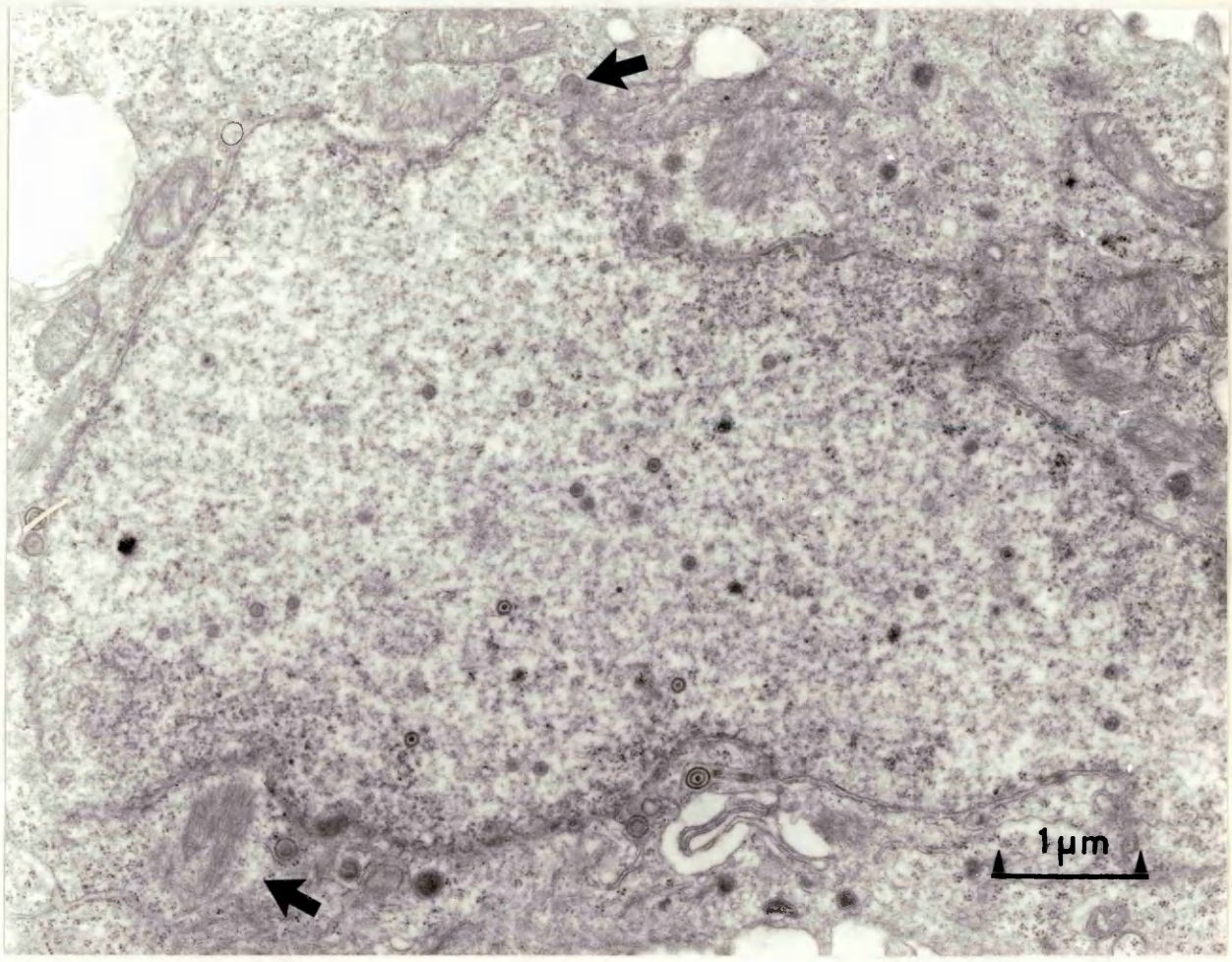


HSV type 2 day two pock

Infected nucleus showing envelopment of virions budding from the nuclear membrane (x18,900)

PLATE 51

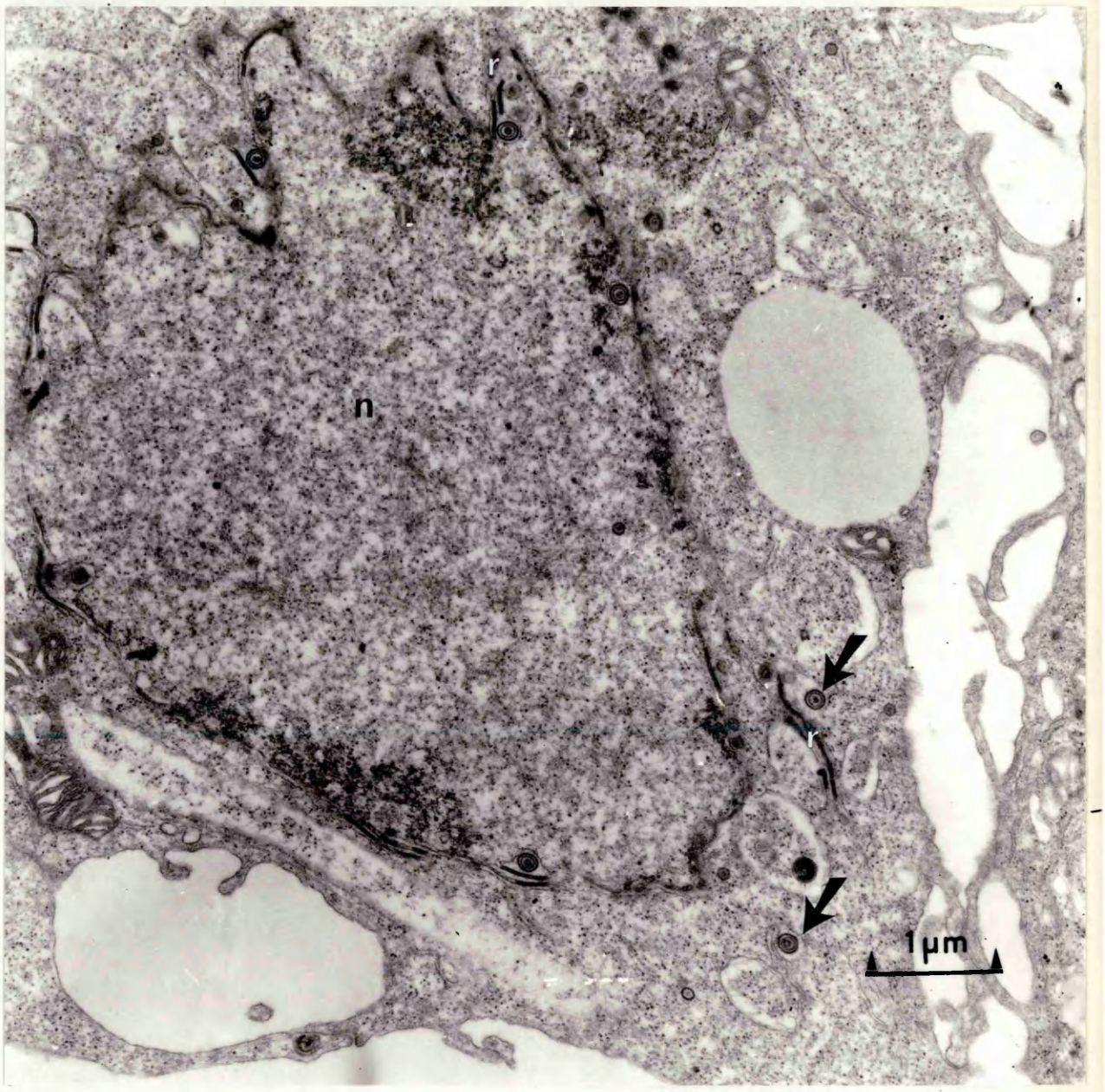




HSV type 2 day two pock

Infected cells with naked virus particles in the nuclei (x18,900)

Virus particles are present in tubules which are contiguous with the perinuclear space of the infected nuclei (arrowed)



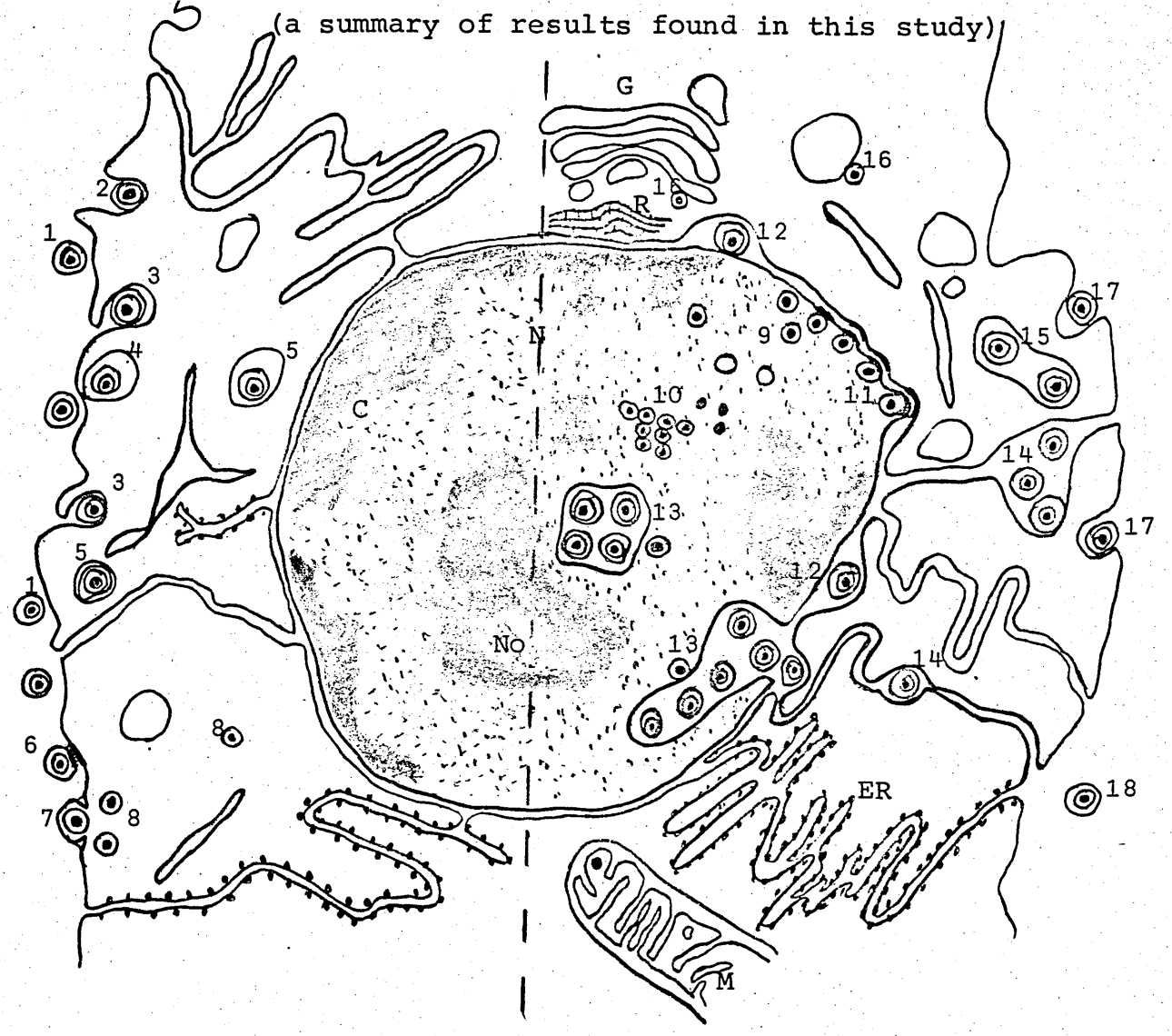
HSV type 2 day two pock

Enveloped virus particles present in vesicles (arrowed) in the cytoplasm (x18,900)

n = nucleus r = reduplication of nuclear membrane

PLATE 53

(a summary of results found in this study)



Virus penetration

1 extracellular enveloped particle

2 particle entering the cell by "viropexis"

3 later stage of "viropexis"

4 final stage of "viropexis"

5 particle in vesicle in cytoplasm

---

6 fusion of viral envelope and plasma membrane

7 late stages of fusion

8 naked capsids free in cytoplasm

Virus release

9 various stages of virus maturation in the nucleus

10 paracrystalline array of capsids in nucleus

11 virus budding into the inner lamella of nuclear membrane; note thickening of membrane

12 enveloped particles in the perinuclear space

13 enveloped particles in vesicles in the nucleus

14 enveloped particles in tubular structures leaving the cell

15 enveloped particles in vesicles in the cytoplasm

16 naked capsid in cytoplasm

17 mature particles leaving the cell

18 mature particles released into the extracellular space

N = nucleus No = nucleolus
C = chromatin G = golgi
M = mitochondrion complex
R = reduplication of nuclear membrane ER = endoplasmic reticulum

9.3 Herpes simplex virus type 1 infected CAM

One day after CAM inoculation with HSV type 1 pocks formed by cellular proliferation were present. Pocks consisted largely of heaped-up cells on the chorion and the proliferative rather than inflammatory nature of the lesions was suggested by the minimal amount of cellular infiltration of the mesoderm immediately below the pock (plate 54). Although the majority of cells within the pock area appeared uninfected, some cells contained numerous comparatively mature virus particles indicating that these cells had been infected at or shortly after CAM inoculation (plate 55). There was no virus discernible in either the mesoderm or allantois, but small numbers of inflammatory cells were occasionally present in the mesoderm below the pock region (plate 56). Cellular disruption was most evident at the apex of pocks (plate 57). Heavily infected cells, surrounded by normal apparently uninfected ones (plate 58), occurred both at the surface of the pock and deep in the chorion. Some cells showing advanced infection contained pieces of capsids and also empty or possibly defective virus particles with no internal nucleoprotein (plate 59). Other cells had non-enveloped virus within their cytoplasm (plate 55). The number of microvilli on the cells at the surface of pocks showed a dramatic increase compared with controls (plate 60), the number of cell-to-cell bridges within the chorion was reduced, and these cells appeared rounded and electron translucent (plate 61). In most infected cells the virus particles were found in the nucleus but were rarely present in

the cytoplasm. However some cells had cytoplasmic virus, but no nuclear virus particles, although virus-associated nuclear changes were apparent (plate 62). Membraneous material was also present in infected cells (plate 63). Naked virus particles in nuclei were occasionally arranged in small paracrystalline arrays (plate 64). Annular structures containing dense granular material were seen in some infected nuclei (plates 41 and 65). Virus particles were sometimes found positioned at the periphery of the nucleus adjacent to the nuclear membrane (plate 65) which thickened in apposition to virus particles prior to their passage into the cytoplasm (plate 66).

The most striking feature of day one HSV type 1 induced pocks was the lack of virus infected cells within pocks.

Two days after inoculation the pocks were essentially similar to those of day one except that cellular infiltration of the mesoderm had increased slightly. The allantois appeared unaffected and no virus particles were detected in either the mesoderm or allantois although infected cells were present throughout the chorion and in contact with the basal lamina between the chorion and mesoderm. Cells either side of this basal lamina were morphologically indistinguishable (plate 58). Both non-infected and infected cells were present in the chorion throughout the pock lesion which was basically confined to this layer. The number of microvilli on cells at the surface of pocks was increased compared with uninoculated controls. Some cells showed mixing

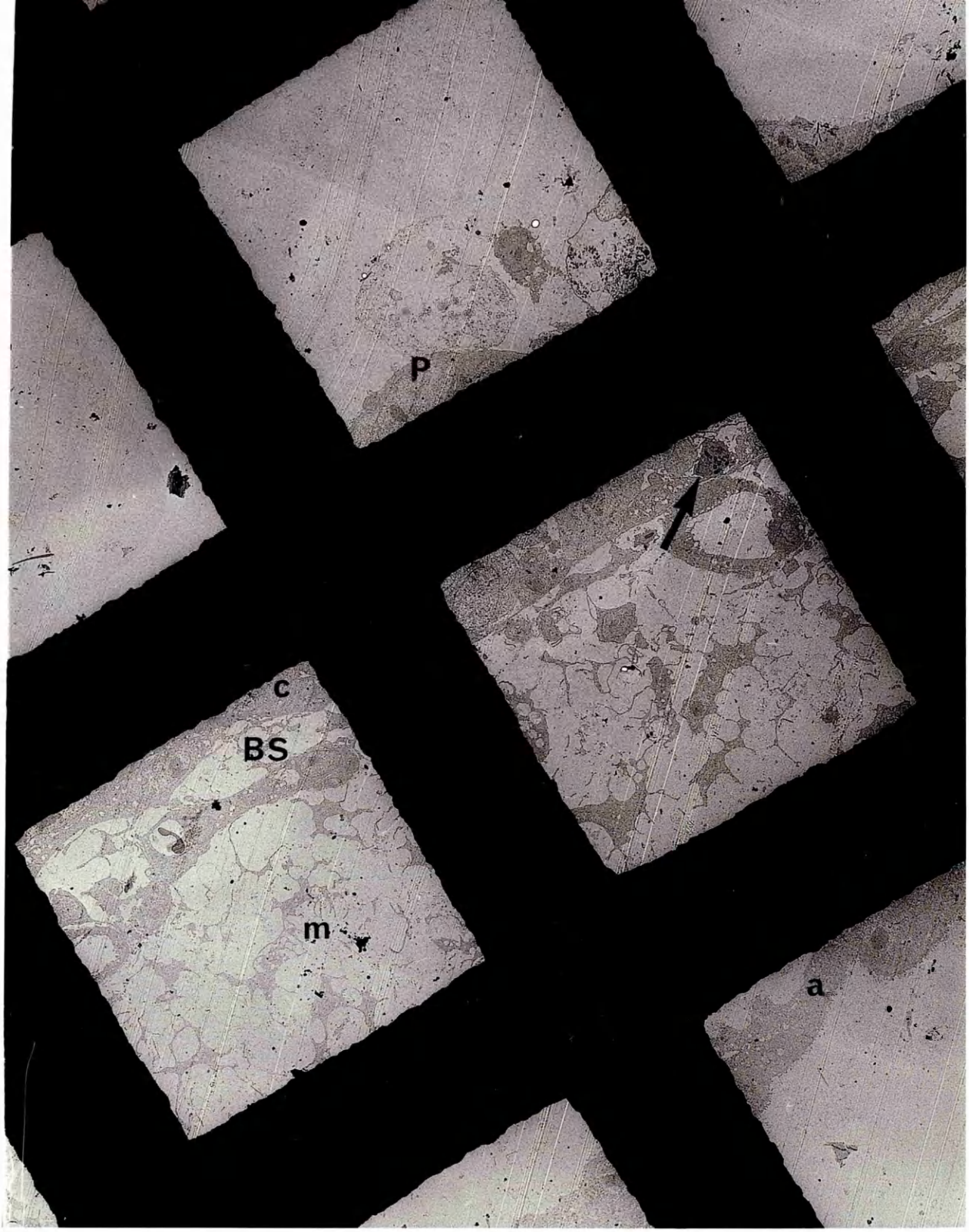
of cytoplasmic and nuclear material and had 22 nm. diameter microtubular structures in the nuclei. These changes in the nucleus may have been virus-specific, or alterations in preparation for cell division (plate 67). Numerous 'empty' capsids were present in some cells, particularly those in advanced degeneration (plate 59). Some infected cells at the pock surface contained densely staining spherical bodies 37 nm. in diameter in association with chromatin and virus capsids (plate 41). The infected cells contained comparatively few virus particles and these were mostly nuclear rather than cytoplasmic although unenveloped cytoplasmic virus was present in some cells (plate 41).

From the third to the seventh day, the pocks continued to enlarge slightly due to proliferation of the chorion, and in the pock region this layer was composed of two cell types. Those in the upper region of the chorion were electron dense, and those deeper in the layer were electron translucent (plate 68). These corresponded to the dense and pale staining areas in the optical sections. The dense cells had small nuclei and contorted cytoplasmic bridges and there were large intercellular gaps. The lower cells had large round nuclei, were less granular and appeared epithelioid with no intercellular gaps. Although virus infected cells occurred throughout the thickness of the chorion within the pock, the greater number were concentrated in the lower, electron translucent, part of the layer. The pocks consisted largely of piled-up chorion cells, although infiltration of the mesoderm below the pock was more evident in three to

seven day pocks than in those of days one or two (plate 69). Oedema was occasionally present in the centres of some pocks late in infection and these frequently appeared to ooze cellular material. Some infected nuclei contained small numbers of incomplete capsids, and empty vacuoles resembling virus envelopes were budded from the nuclear membrane into the cytoplasm of some infected cells (plate 70).

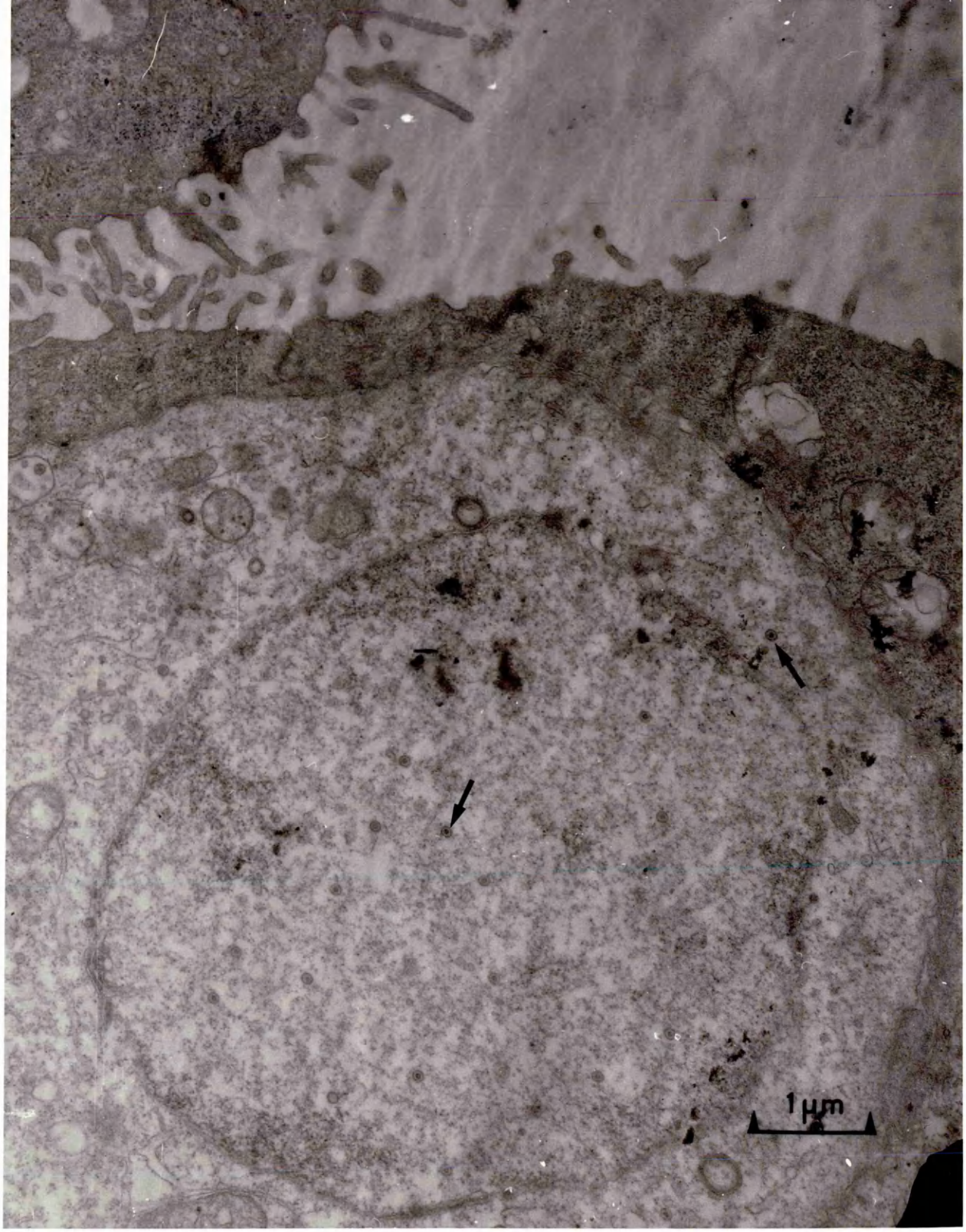
Features of the later stage pocks which were also seen in early stage pocks induced by HSV type 1 were: virus in association with 37 nm. dense granules in nuclei (plate 41); large numbers of microvilli at the pock surface (plate 60); no virus in the cells of the mesoderm or allantois, and few virus infected cells in the chorion compared with the extent of the reaction induced.

By the seventh day after inoculation, most lesions on membranes had dried up and pocks did not appear as discreet as on earlier days. The ultrastructure of those seven day pocks which were sectioned was similar in appearance to that of days three to six, except that in one pock 100 nm. structures similar in appearance to virus particles were observed in the interstitial spaces around a blood vessel in the mesoderm. These could not be identified positively as herpesvirus particles due to their fragile and slightly irregular appearance, and also they were never observed within the cytoplasm or nuclei of cells (plate 71). At all stages after inoculation nuclear pores (plate 72) present on the nuclear membranes also resembled virus particles, necessitating caution in interpretation.



Chorioallantoic membrane at 12 days of incubation inoculated one day previously with HSV type 1
P = pock formation c = chorion m = mesoderm a = allantois
BS = blood sinuses Leucocyte arrowed

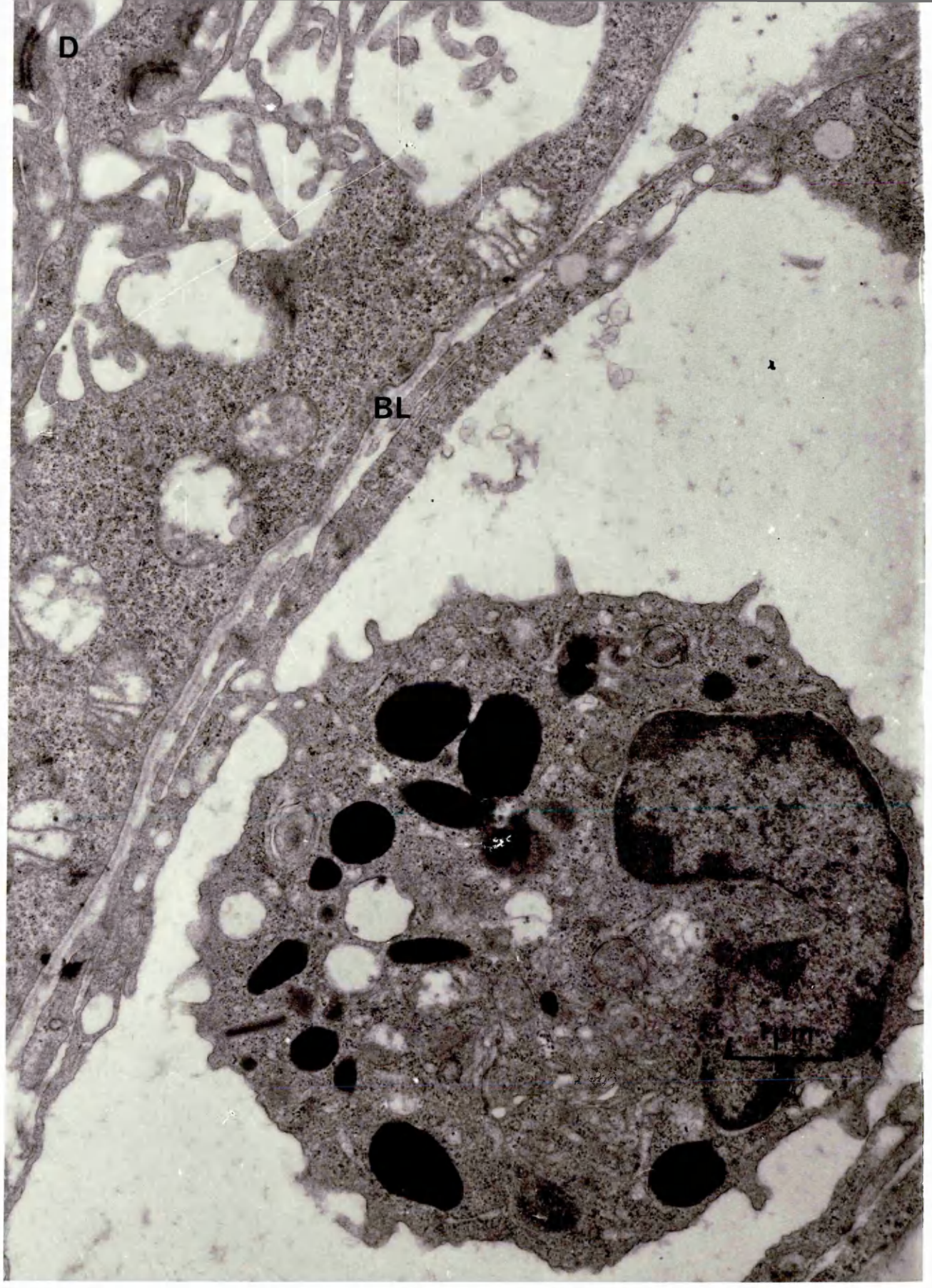
PLATE 54



HSV type 1 day one pock

Infected cell in chorion showing virus particles in the nucleus and cytoplasm (arrowed) (x20,000)

PLATE 55

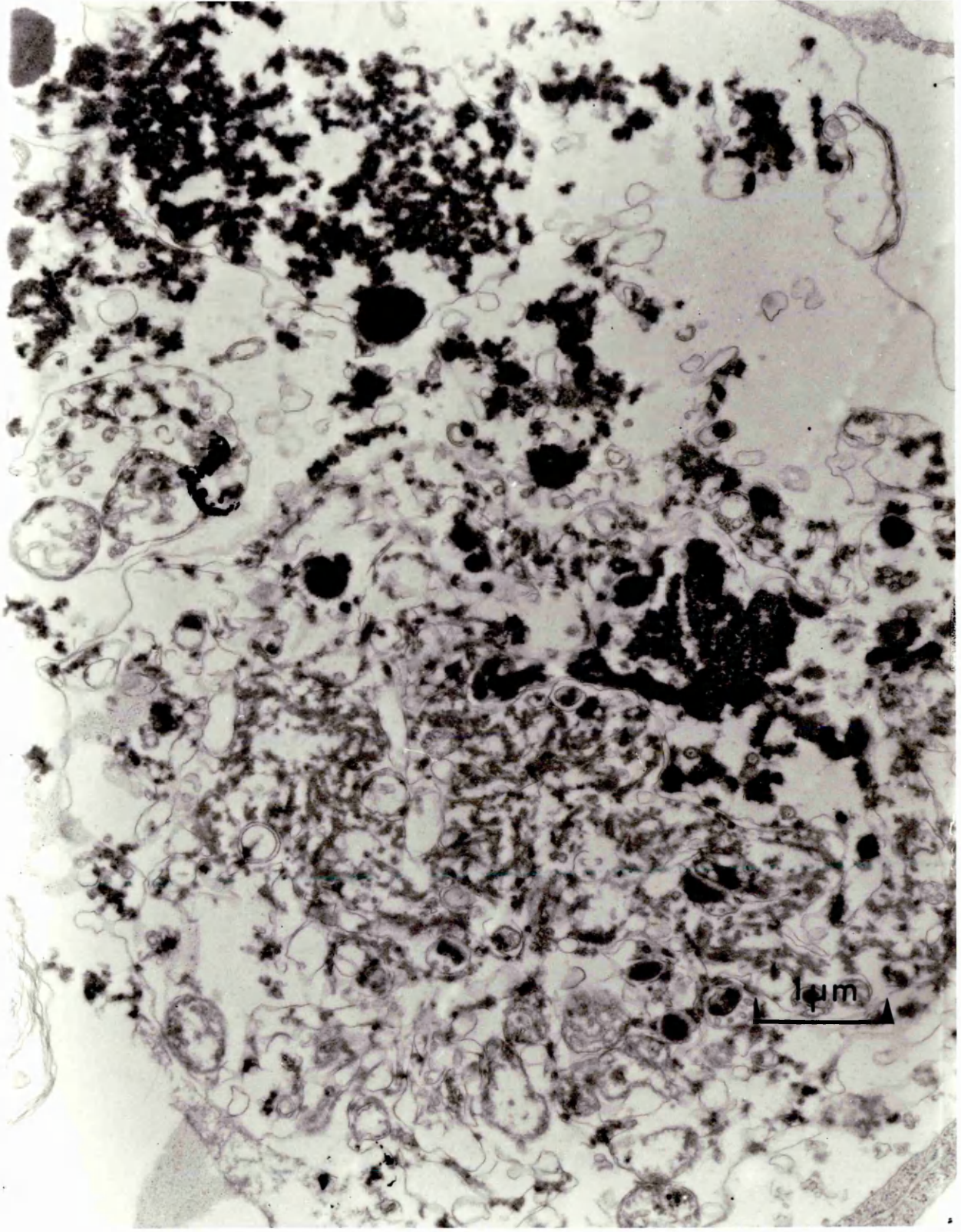


HSV type 1 day one pock

Inflammatory cell in the mesoderm

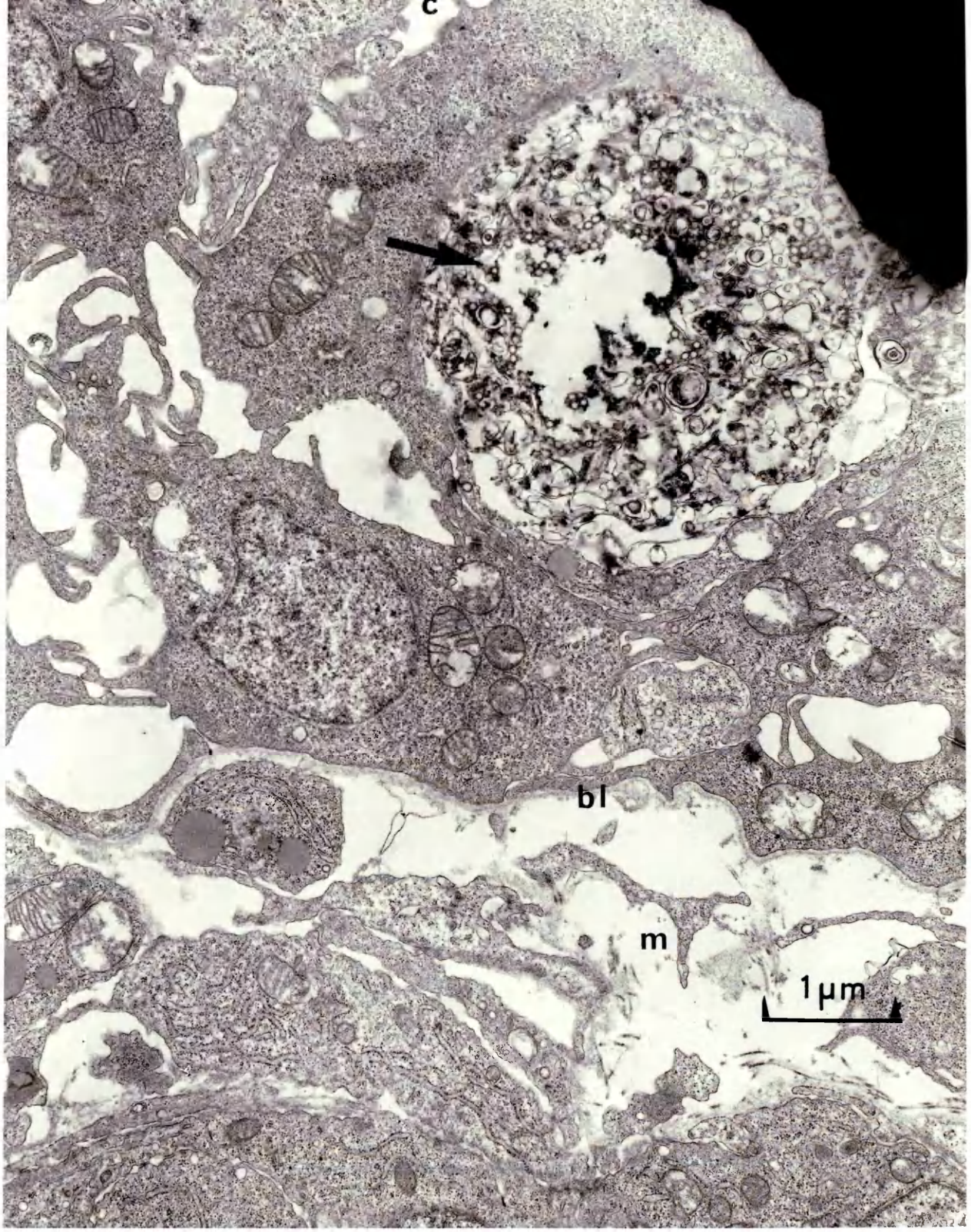
BL = basal lamina D = desmosome

PLATE 56



HSV type 1 day one pock
Extensive cellular breakdown in the chorion (x22,500)

PLATE 57

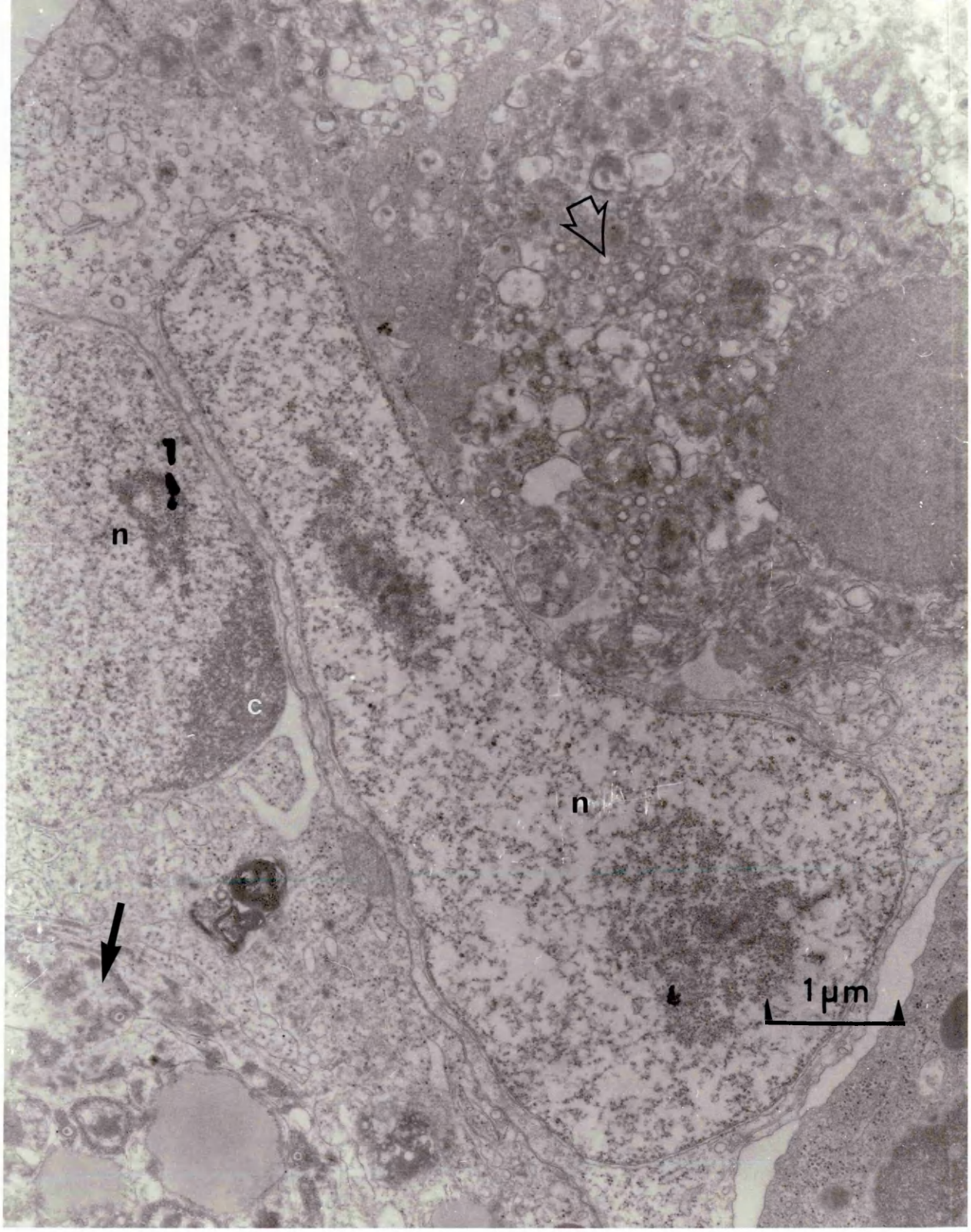


HSV type 1 day two pock

Highly infected cell (arrowed) surrounded by normal uninfected cells (x22,500)

c = chorion m = mesoderm bl = basal lamina

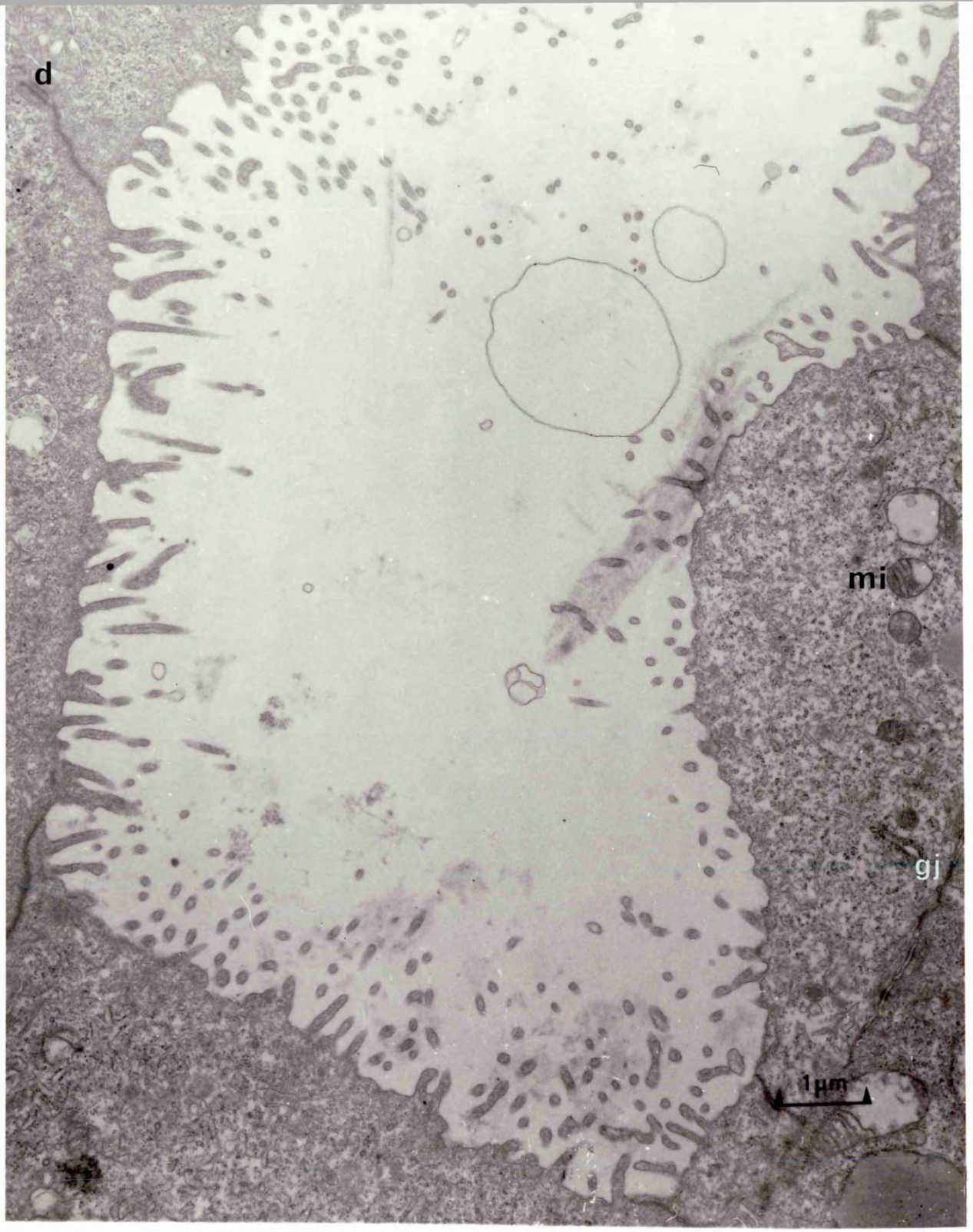
PLATE 58



HSV type 1 day two pock

Infected cells in the chorion (x22,500)
one degenerate cell (arrowed)
one cell containing numerous empty virions (open arrow)
n = nucleus c = marginated chromatin

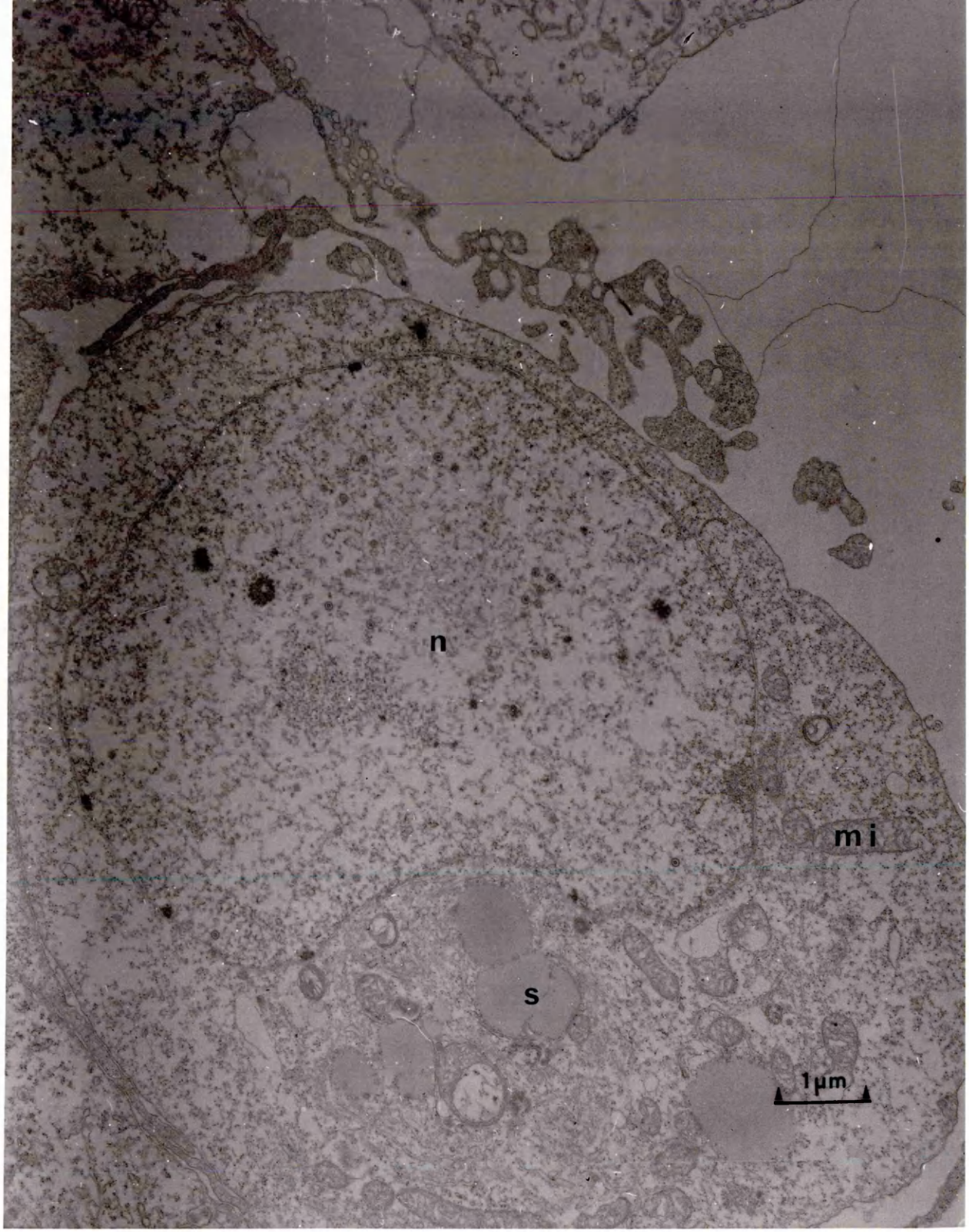
PLATE 59



HSV type 1 day one pock

Infected region of chorion showing an increased number of microvilli at the pock surface (x15,000)
mi = mitochondria d = desmosome gj = gap junctions

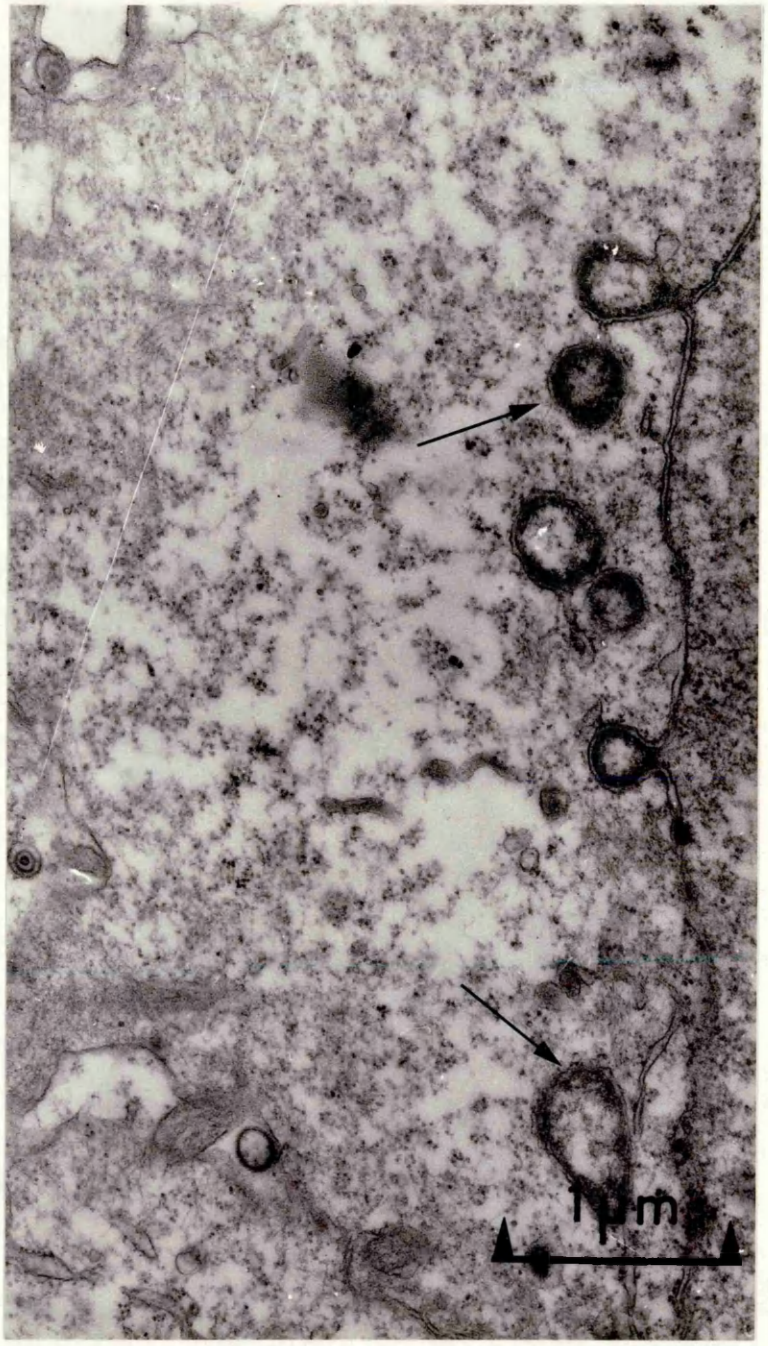
PLATE 60



HSV type 1 day one pock

Infected cell in chorion (x15,000)
Nucleus and cytoplasm are electron translucent and few cell-to-cell bridges are present
n = nucleus mi = mitochondria s = secretory droplets

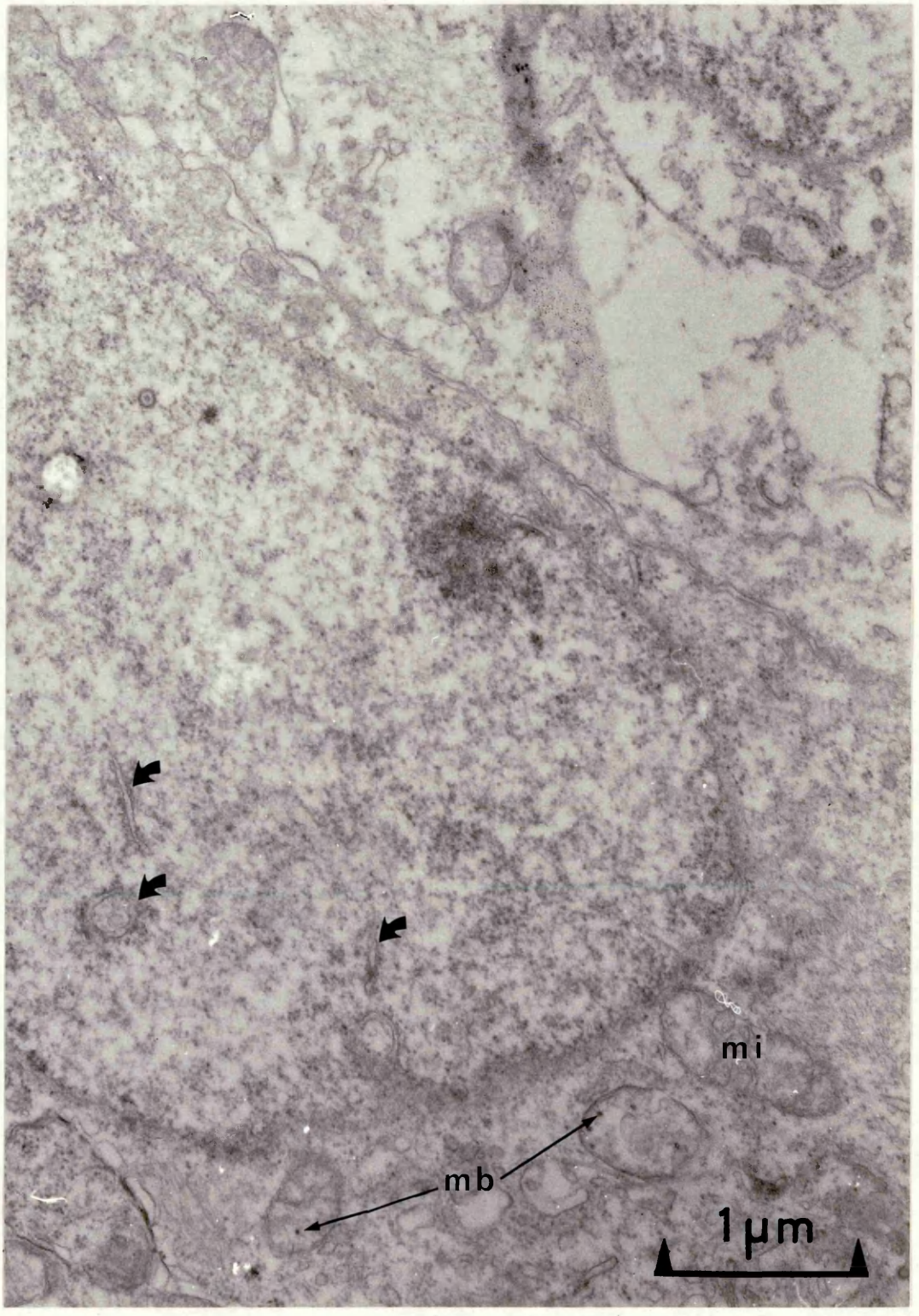
PLATE 61



HSV type 1 day one pock

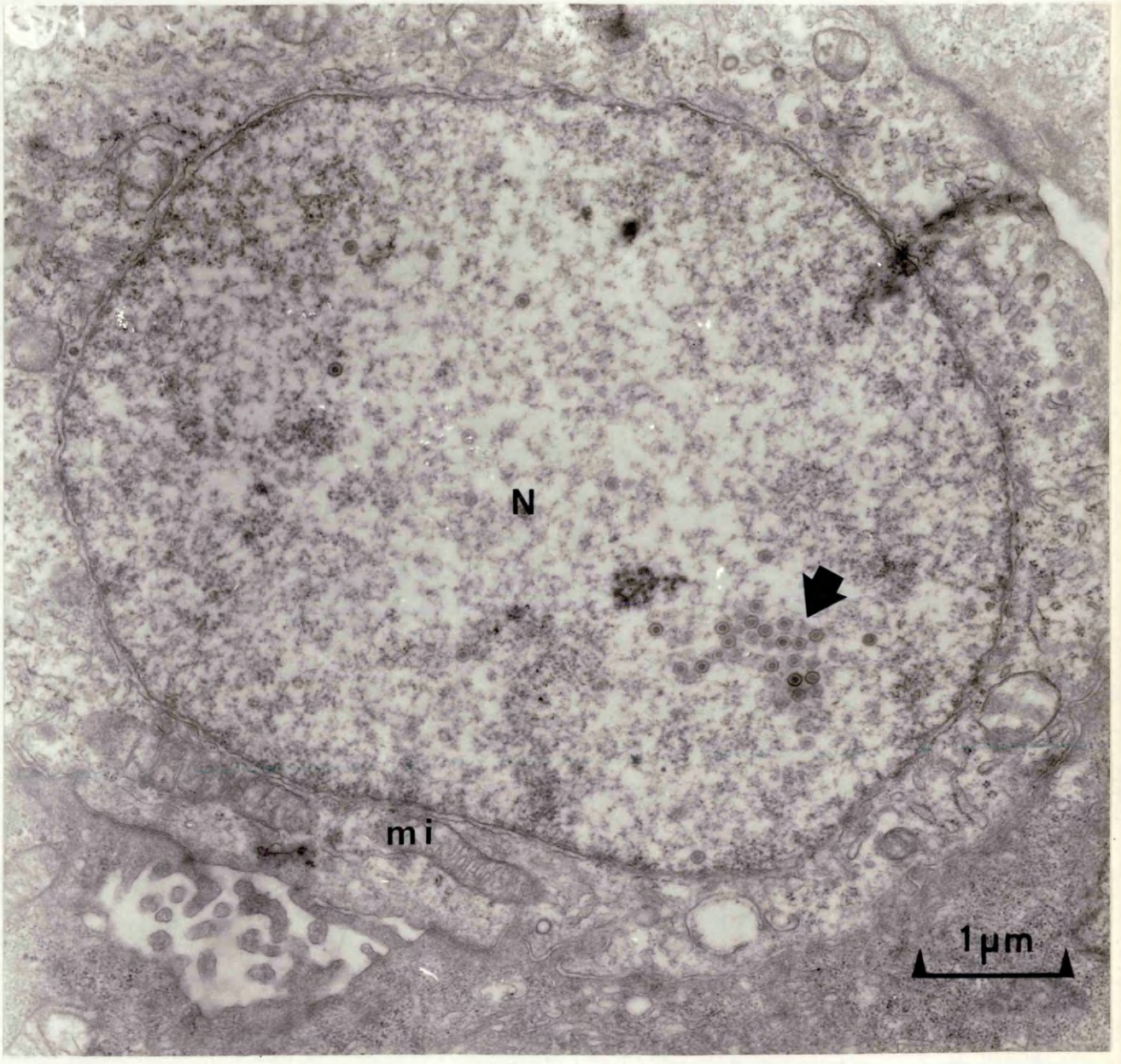
Enveloped virus particles in the cytoplasm with possibly virus-associated changes in the nuclear membrane (arrowed) (x30,000)

PLATE 62



HSV type 1 day one pock
Infected nucleus containing membraneous material (arrowed)
mi = mitochondria mb = microbodies (peroxisomes)

PLATE 63



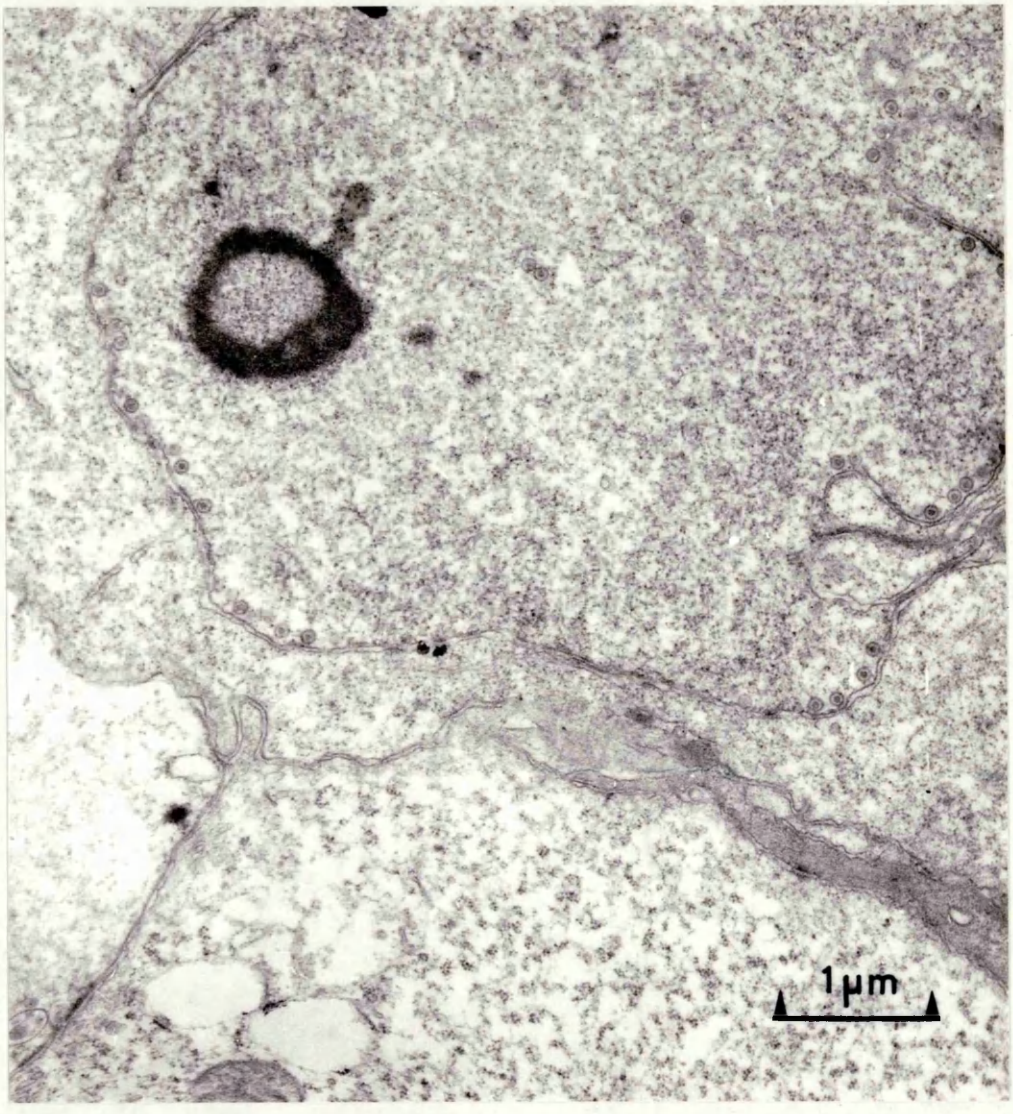
HSV type 1 day one pock

Infected nucleus with virus capsids arranged in small crystalline array (arrowed)

mi = mitochondria N = nucleus

(x22,500)

PLATE 64

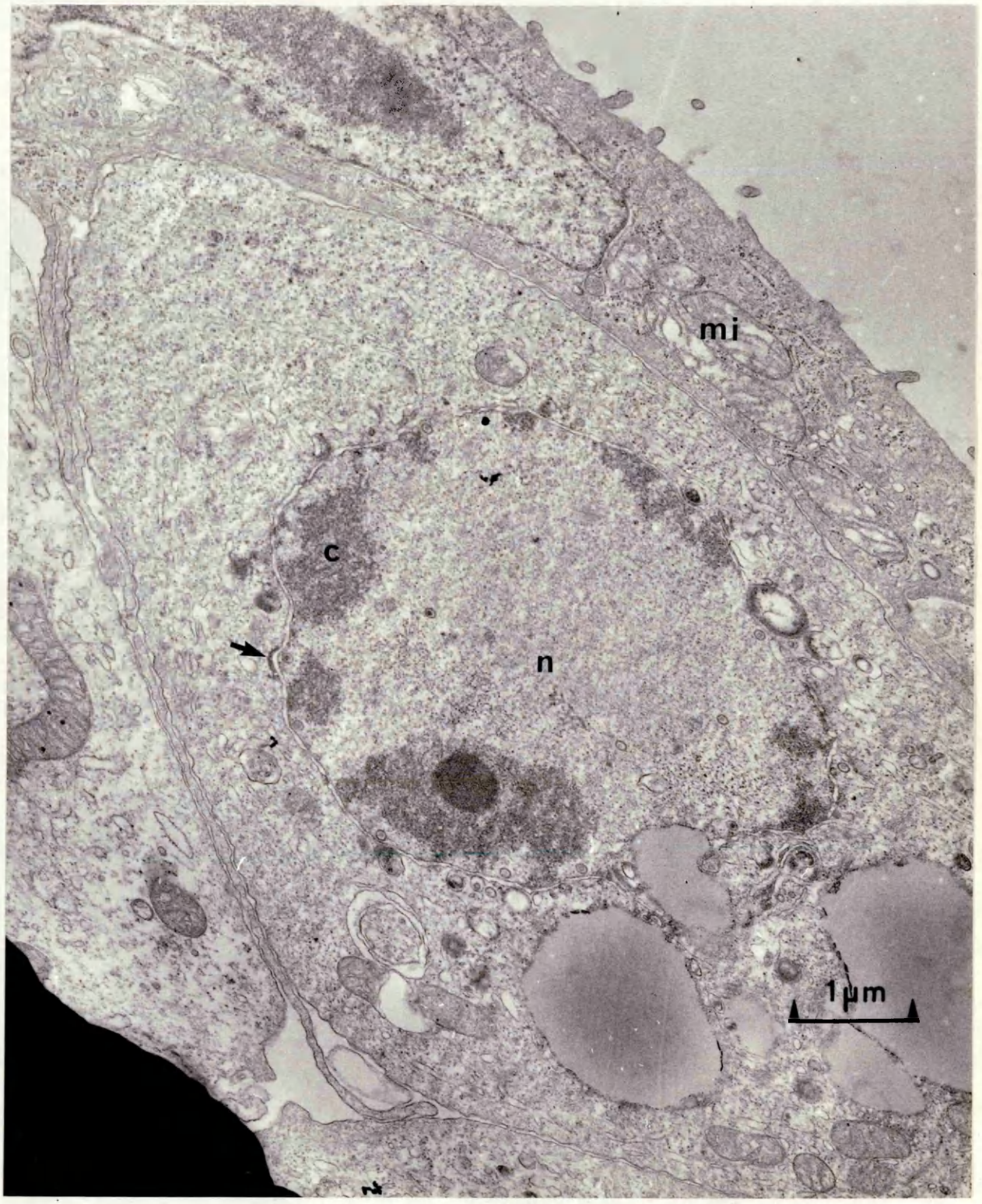


HSV type 1 day one pock

Infected cell (x20,000)

Note virus particles arranged along nuclear membrane
Dense annular structure containing granular material is present in infected nucleus

PLATE 65

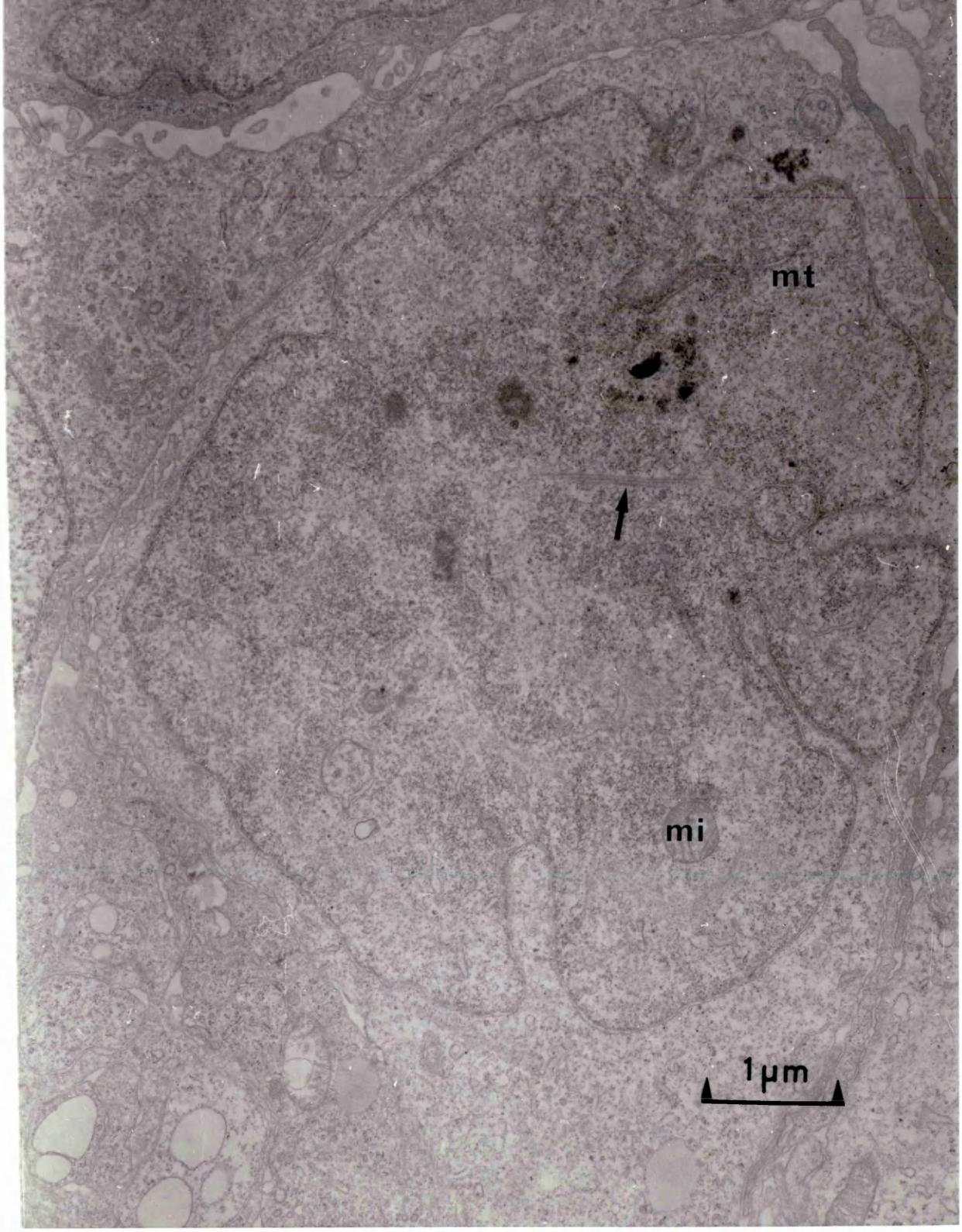


HSV type 1 day one pock

Infected cell at pock surface showing thickening of the nuclear membrane in apposition to virus particles (arrowed) Note dense object associated with marginated chromatin (x20,000)

n = nucleus mi = mitochondria c = chromatin

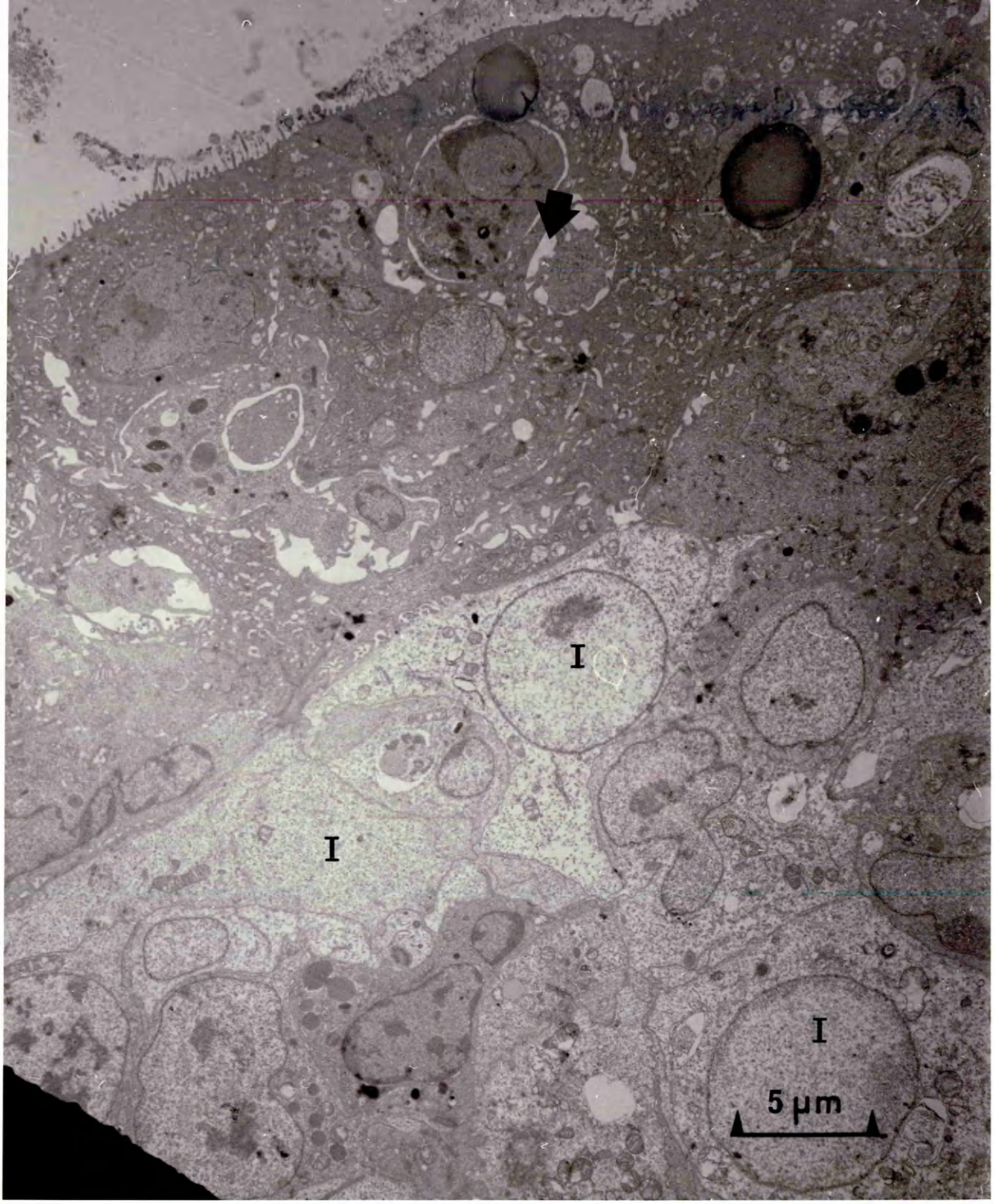
PLATE 66



HSV type 1 day two pock

Highly contorted nucleus containing microtubules (mt), mitochondria (mi) and 22 nm. diameter microtubular structures (arrowed)

PLATE 67

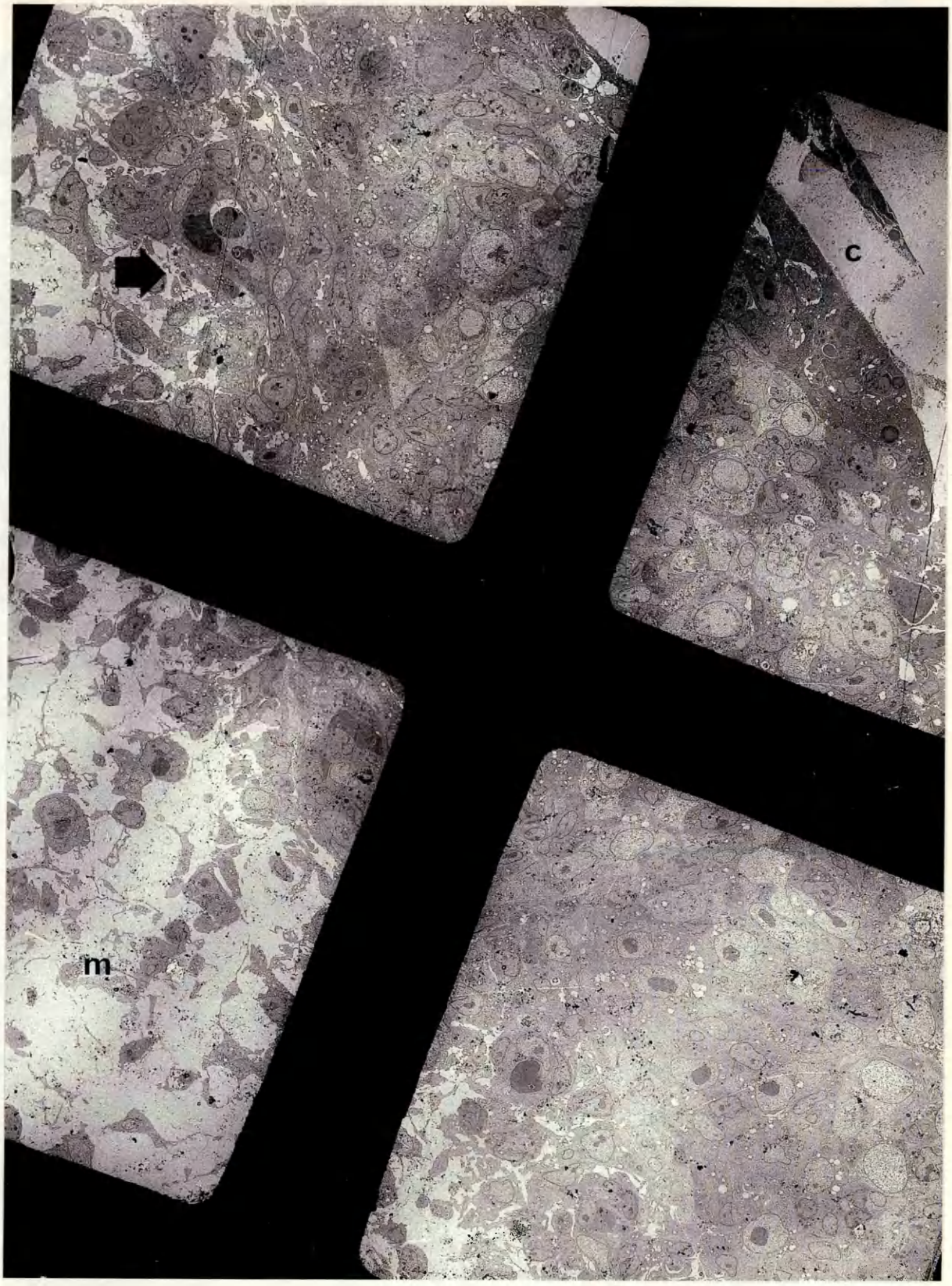


Chorion in HSV type 1 day three pock (x4,500)

The upper cells of the chorion are more electron dense with contorted cytoplasmic bridges (arrowed) whilst those lower down were electron translucent. Note microvilli on pock surface.

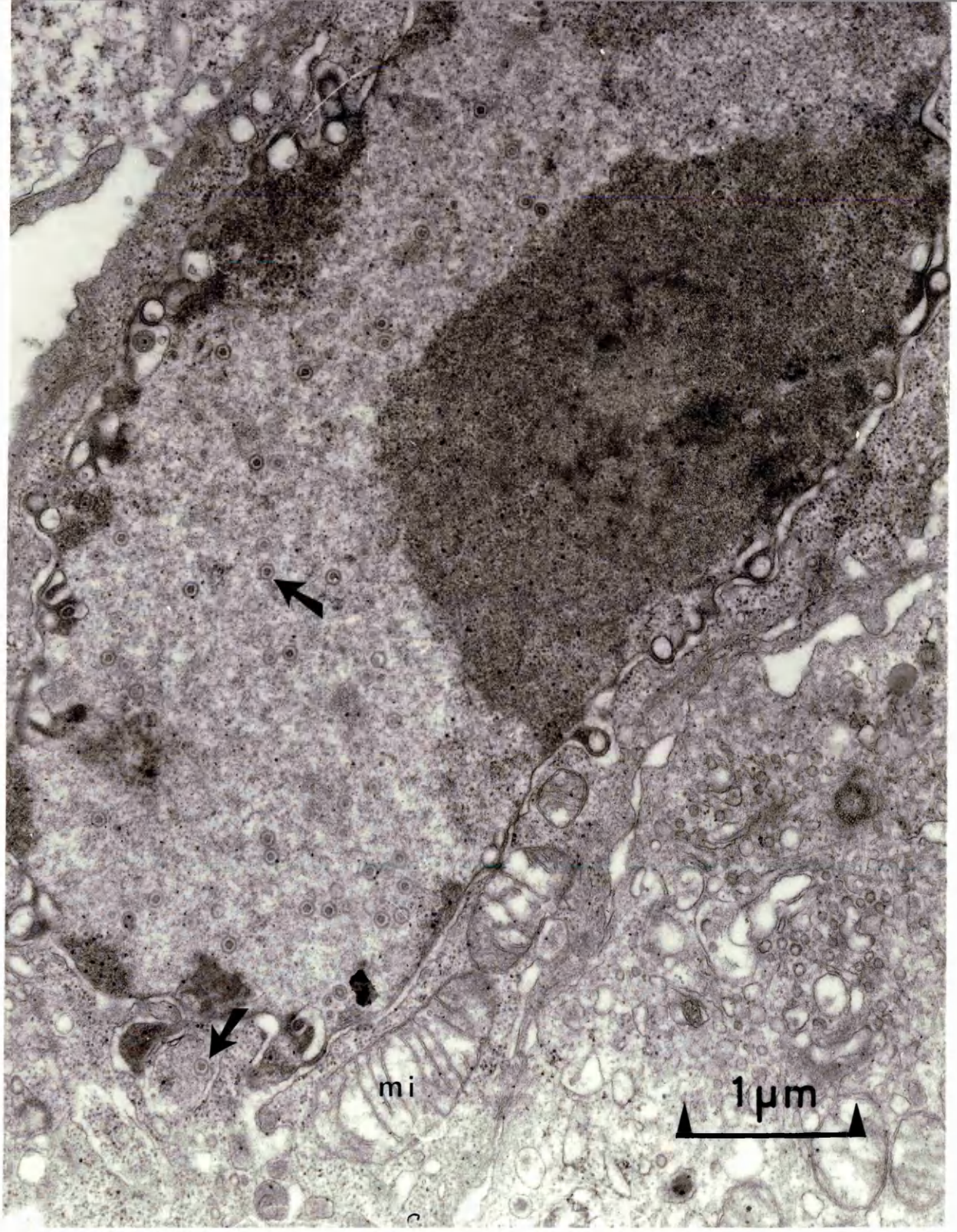
I = cells containing virus particles

PLATE 68



HSV type 1 day three pock at low magnification
Slight infiltration of the mesoderm is evident (arrowed)
c = chorion m = mesoderm

PLATE 69



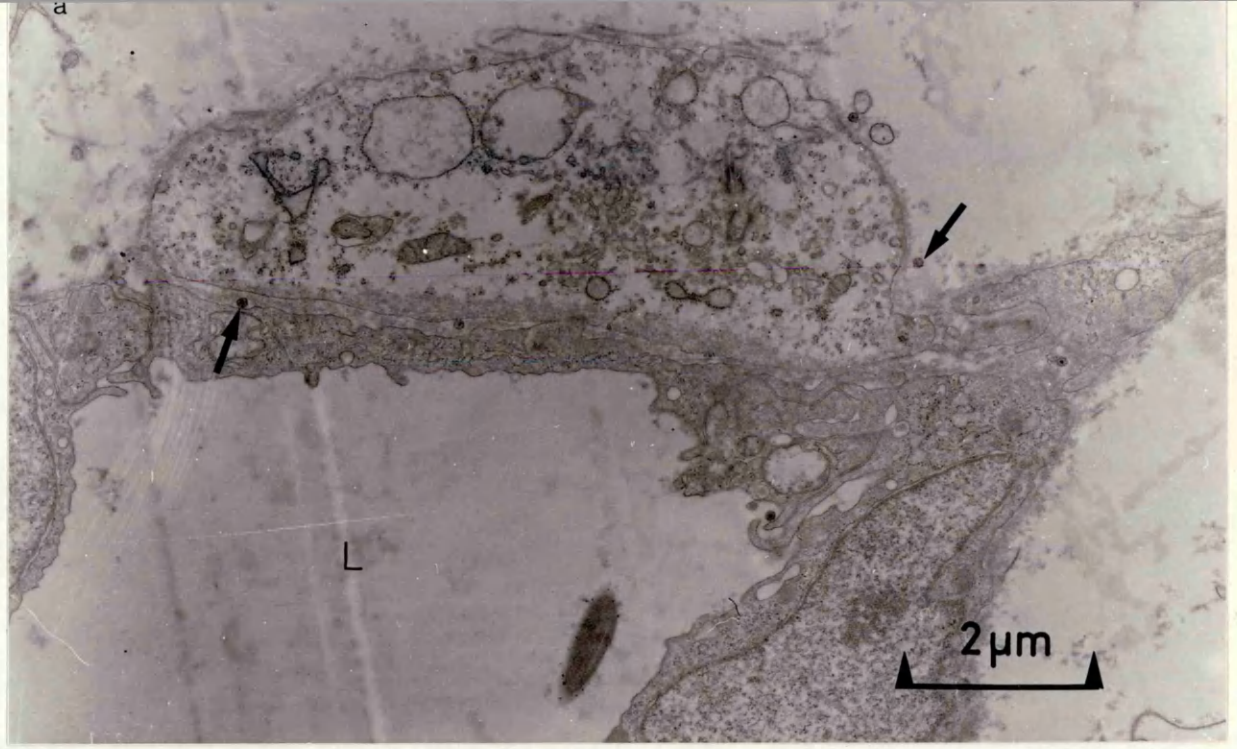
HSV type 1 day three pock

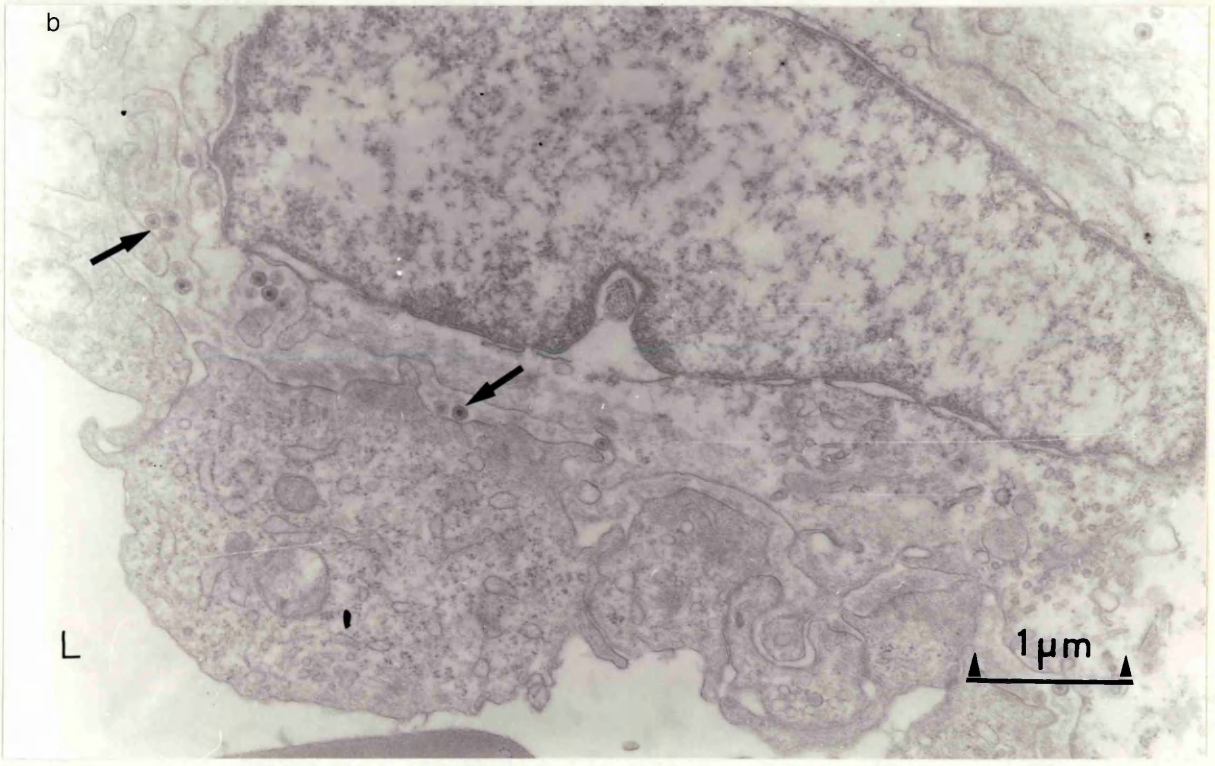
Infected cell with virus capsids in nucleus and cytoplasm (arrowed)

Vacuoles budding from the nuclear membrane are present

c = chromatin (marginated) mi = mitochondria

PLATE 70





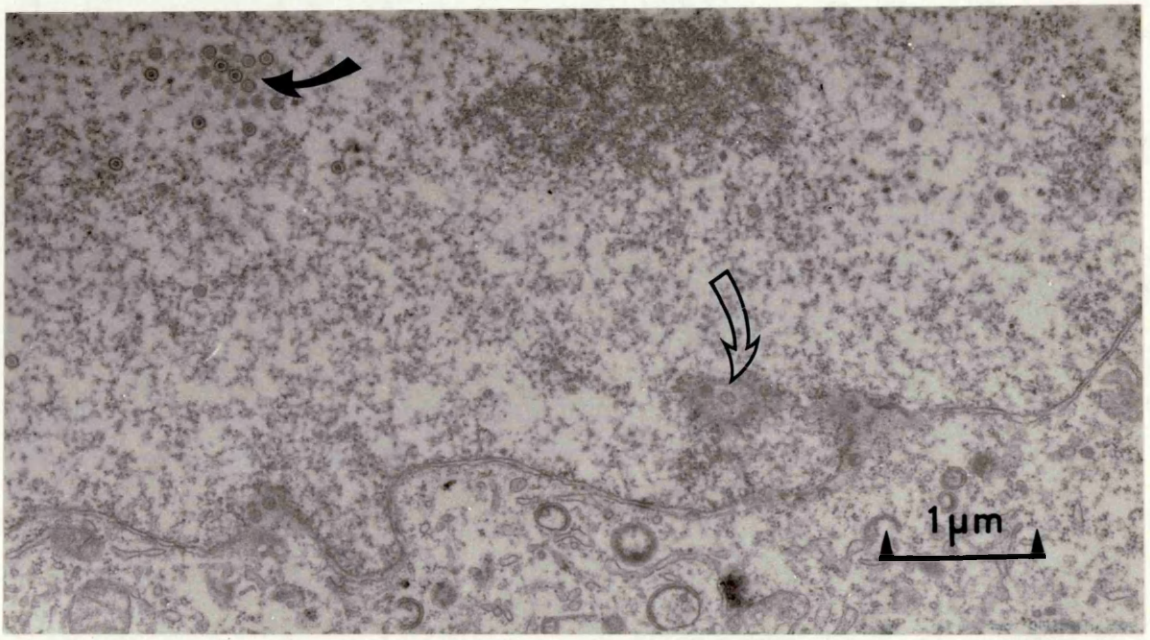
HSV type 1 day seven pock

a) part of a mesodermal blood vessel (x12,600) irregular, fragile structures (arrowed) resembling virus capsids are present in the extra-cellular spaces, but not within cells

b) higher magnification (x20,000) of another area of the same blood vessel showing further examples of these extra-cellular fragile structures

L = lumen of blood vessel

PLATE 71



HSV type 1 day two pock

Infected nucleus showing close resemblance of nuclear pores (open arrow) to virus capsids (closed arrow) (x20,000)

PLATE 72

9.4 Herpes simplex virus type 2 infected CAM

One day after CAM inoculation with HSV type 2, the lesions were similar to those of the HSV type 1 infected CAM with slight piling up of cells in the chorion layer of the pock, which had an open appearance. Cellular reaction in the mesoderm was more pronounced than with day one HSV type 1 and the allantois appeared thickened below the pock even at this early stage. Infected cells occurred throughout the thickness of the chorion and extended to the edges of the pocks. Like type 1 pocks at the same stage there was an increase in the number of microvilli on the surface cells of the chorion compared with uninoculated controls. Within infected cells reduplication of the nuclear membrane occurred more frequently than in type 1 equivalents, as did pseudo-crystalline arrays of virus capsids in infected nuclei. The particles within these small crystals were often markedly hexagonal in appearance (plate 73). In some infected nuclei the capsids were associated with densely staining granules of approximately 65 nm. diameter (plate 74). There was a number of capsids with bizarre cores showing double rings, ribbons or bars and rosettes together with large numbers of empty capsids. At this stage virus was occasionally found in the mesoderm. With the HSV type 2 day one pocks many more virus infected cells were present and pocks appeared to be at a more advanced stage of infection than the type 1 day one pocks.

The pocks produced by the second day after inoculation of the CAM were very large and a single ultra thin section of a pock covered a large area of the 3 mm. diameter specimen grid. The chorion showed increased proliferation both into

and along the surface of the membrane when compared with the HSV type 2 day one pock or the HSV type 1 day two pock, and the mesoderm showed marked cellular infiltration particularly below the pock area of the chorion (plate 75). Inflammatory leucocytes, particularly macrophages and mast cells, were present in both mesoderm and chorion (plate 76). Both these layers were heavily infected with virus (plates 77 and 78). Although the allantois became thickened beneath the pock no virus particles were detected in the allantoic cells. However, there were small numbers of pinocytotic vesicles into these cells from the mesoderm, a feature not observed in controls or HSV type 1 lesions (plate 79). As with the type 1 pocks the virus infected cells were present in greater numbers in the lower, less electron dense, layer of the chorion. The number of surface microvilli increased compared with controls.

HSV type 2 infected cells contained many more virus particles, which were either 'full' or 'empty', compared with those infected with HSV type 1 (plates 80 and 81), and many infected nuclei had contorted nuclear membranes (plates 80 and 82). In contrast to the type 1 pocks, extracellular and cytoplasmic virus particles both enveloped and naked were a common feature of the type 2 pocks (plate 82, 84), as was the relatively large number of empty capsids. There were also enveloped virus particles which appeared to be situated in vesicles in infected nuclei (plate 83), and the perinuclear space of infected nuclei was much disrupted and contained complete virus particles (plate 82). Virus was present at all stages of morphogenesis (plates 83 and 84). Occasionally virus infected cells were in the process

of cell division (plate 85), others in what could be cell fusion (plate 86) and others showed nuclear lysis with nuclear contents and virus particles oozing into the cytoplasm (plate 87). Some rather more electron dense infected cells showed pale patches in the nuclei and collections of 16 to 20 nm. diameter granules associated with viral capsids (plate 88). Other granules associated with capsids which were only seen in HSV type 2 infected CAM cells included some with diameters 42 to 50 nm. (plate 89a,b) and others which were very pleomorphic in structure and 65 to 95 nm. in diameter (plates 81 and 90). Some infected nuclei contained small periodic structures made up of membraneous layers, each of which measured 21 nm. across and was studded with grains 16 nm. in diameter and 8 nm. apart (plate 91). Microfilaments of average width 16 nm. in diameter were also common in infected nuclei (plates 91 and 92), as were small paracrystalline arrays of virus particles suggesting an hexagonal arrangement (plate 86). Moderately electron dense bodies 68 to 80 nm. diameter resembling inner capsids of herpesvirus particles were seen alongside groups of virus particles in infected nuclei (plate 91). These could also be interpreted as tangential sections of the extreme ends of viral capsids. Virus particles were frequently seen in association with chromosome material and these particles often appeared distorted and fragile as if in early stages of formation (plate 93).

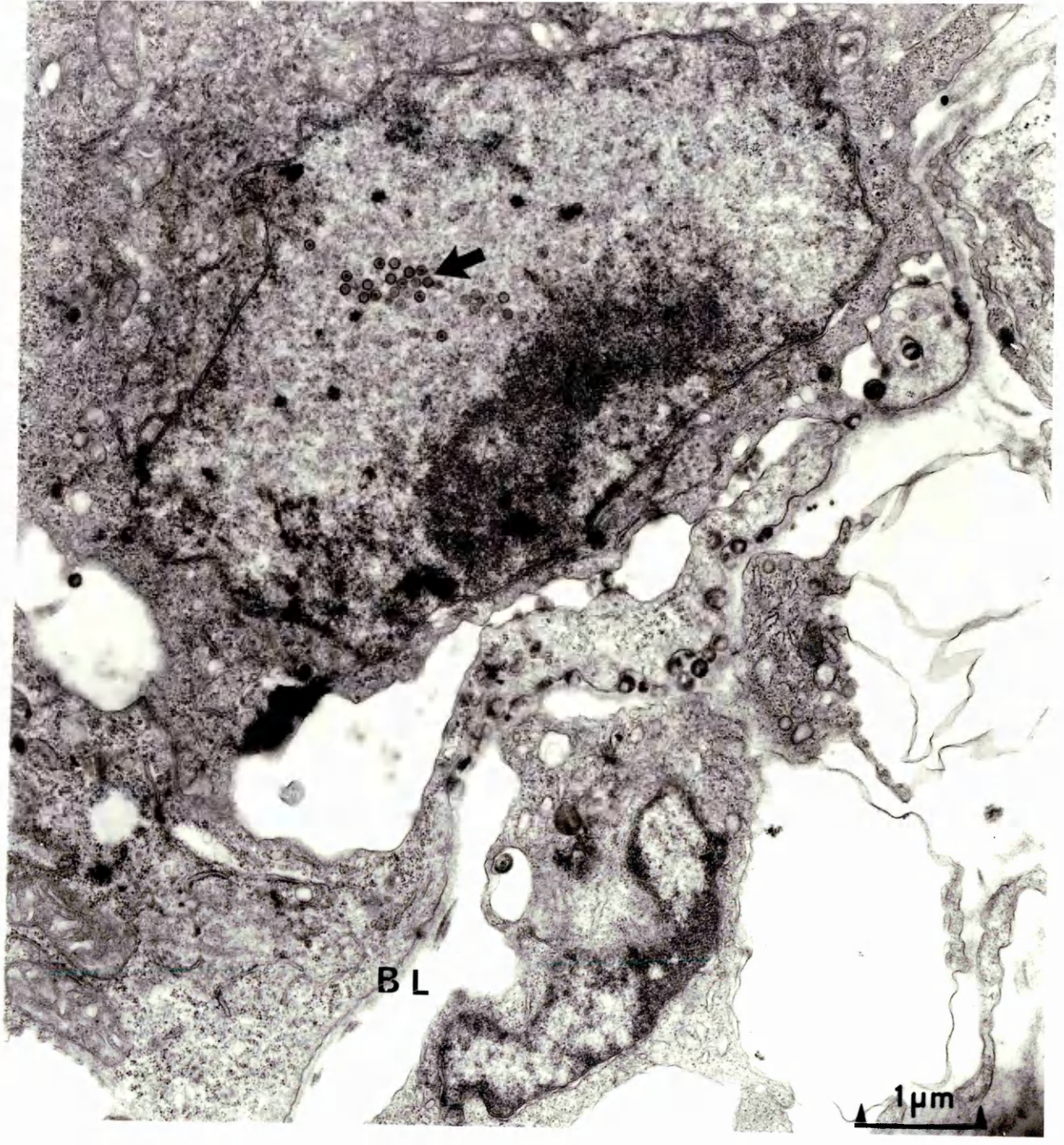
Lattice structures were common in longitudinal, tangential and cross-section in nuclei and cytoplasm of infected cells. In longitudinal section they appeared as filamentous strands 10 nm. wide (plate 94a,b), and in cross-section they were

21 x 21 nm., with a central hole of approximately 10 nm. diameter, and centre-to-centre spacing of 31 nm. (plates 92 and 94b). These structures were seen only in the HSV type 2 infected CAM cells. Other structures frequently seen in the cytoplasm of infected cells had a semi-circular appearance, were variable in length and 53 to 58 nm. across (plate 95).

HSV type 2 pocks continued to increase in size from three to seven days after CAM inoculation. There was also an increase in the number of virus particles and infected cells present. The cells of the chorion and mesoderm were infected (plate 96), but no virus was observed in the cells of the allantois throughout the seven day period of observation. Those non-enveloped particles free in the mesodermal space were surrounded by finely granular material which gave them a fuzzy outline, and they were often close to phagocytic cells (plate 97). The structure of three to seven day pocks was essentially similar to the structure of day two pocks, except that there was a slight increase in the frequency of paracrystalline arrays of virus particles (plate 98) and capsids with bizarre cores. The latter included those with: double cores, annular cores, bar-shaped cores, very dense cores, off-centre cores, off-centre cores with thickened capsid opposite, toroidal cores, flimsy or 'broken up' cores, and cores with an apparent threefold symmetry (plates 84, 99, 100 and 101b).

In haemorrhagic pocks virus was present in the cells lining the lumen of the blood vessels as well as in leucocytes (plate 101a,b). No virus was present in the erythrocytes. Marginated leucocytes were present in the

blood vessels (plate 75), presumably connected with the inflammatory response. The chorion and mesoderm were heavily infected, but no virus was found in the cells of the allantois, although infected cells were in contact with this layer.

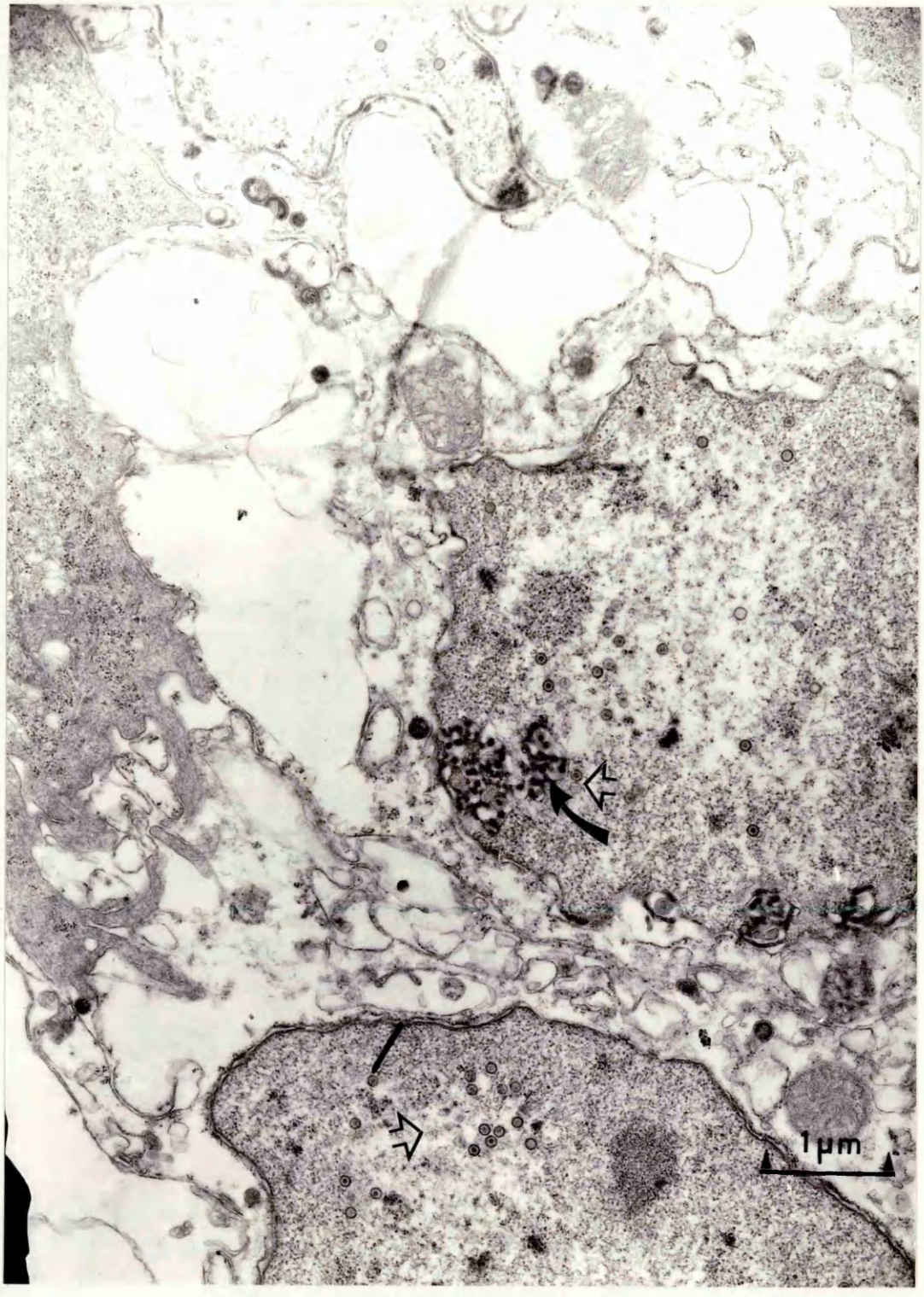


HSV type 2 day one pock

Infected cell in the chorion (x18,900)

Note hexagonal appearance of virus particles in the intranuclear paracrystalline array (arrowed), and the presence of virus particles or envelopes associated with the basal lamina (BL)

PLATE 73

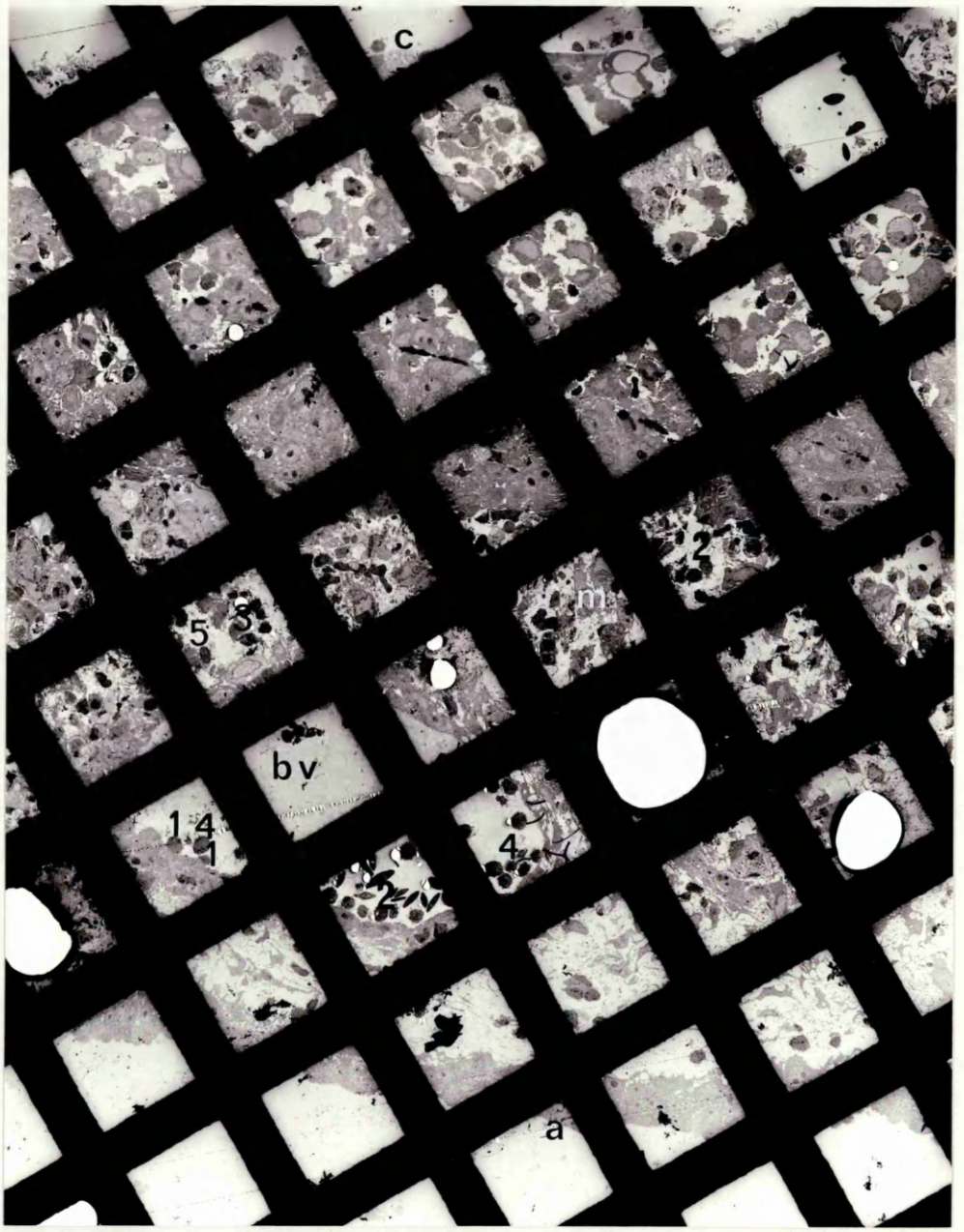


HSV type 2 day one pock

Infected cells in the mesoderm (x18,900)

Dense granules (arrowed) approximately 65 nm. in diameter are present alongside what may be sites of viral synthesis. Note immature particles (open arrow) alongside.

PLATE 74



Part of a day two HSV type 2 pock

c = chorion m = mesoderm a = allantois

bv = blood vessel

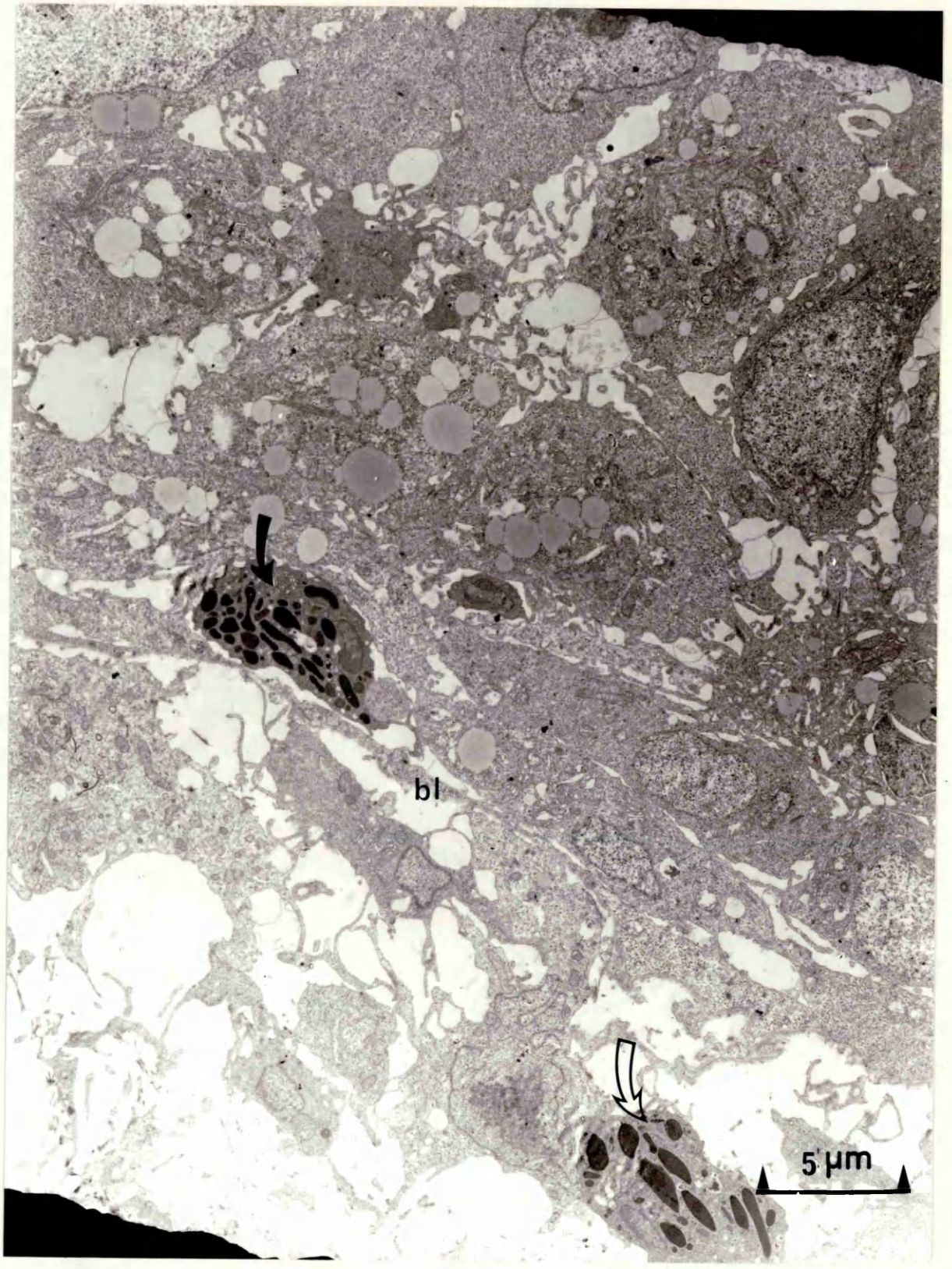
Note inflammatory response

Also present are:

marginated leucocytes	1
non-granular leucocytes	2
mast cells	3
granular leucocytes	4
macrophages	5

(best viewed with a hand lens)

PLATE 75

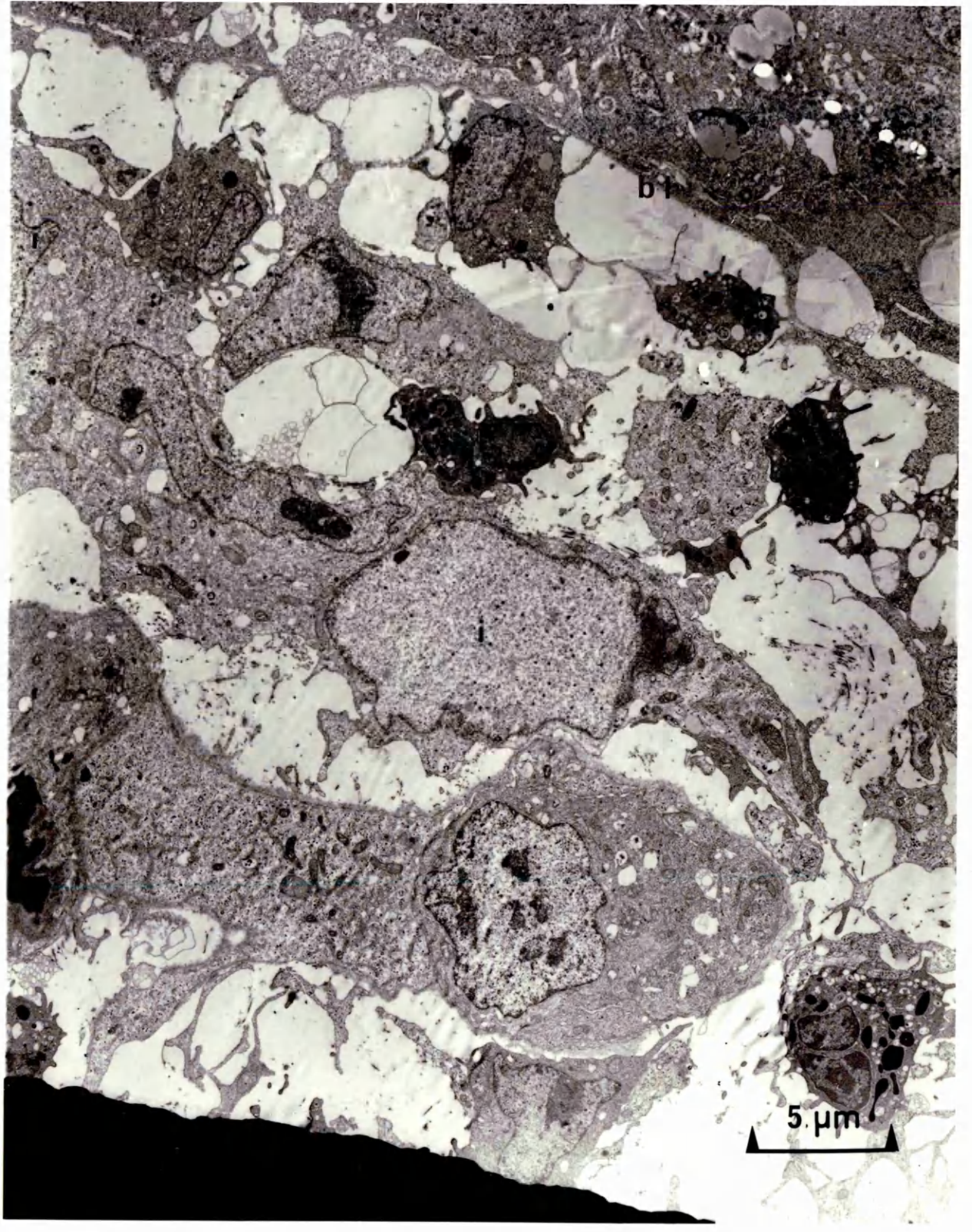


HSV type 2 day two pock

Part of the infected chorion (x4,800)

Note infiltrative cell in the mesoderm (open arrow) and in the chorion (solid arrow)
bl = basal lamina

PLATE 76

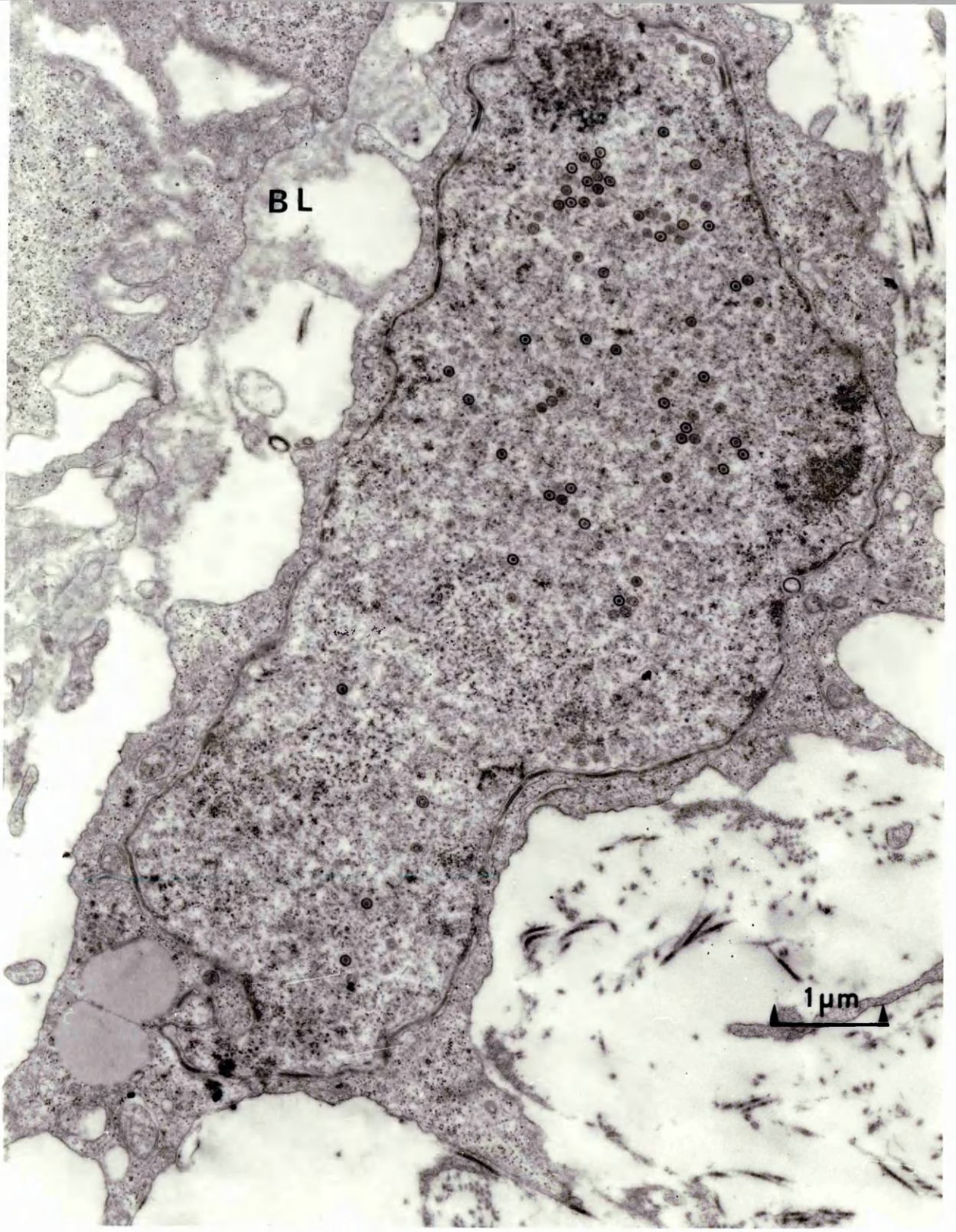


HSV type 2 day two pock

Infected mesoderm (x4,800) Note inflammatory cells

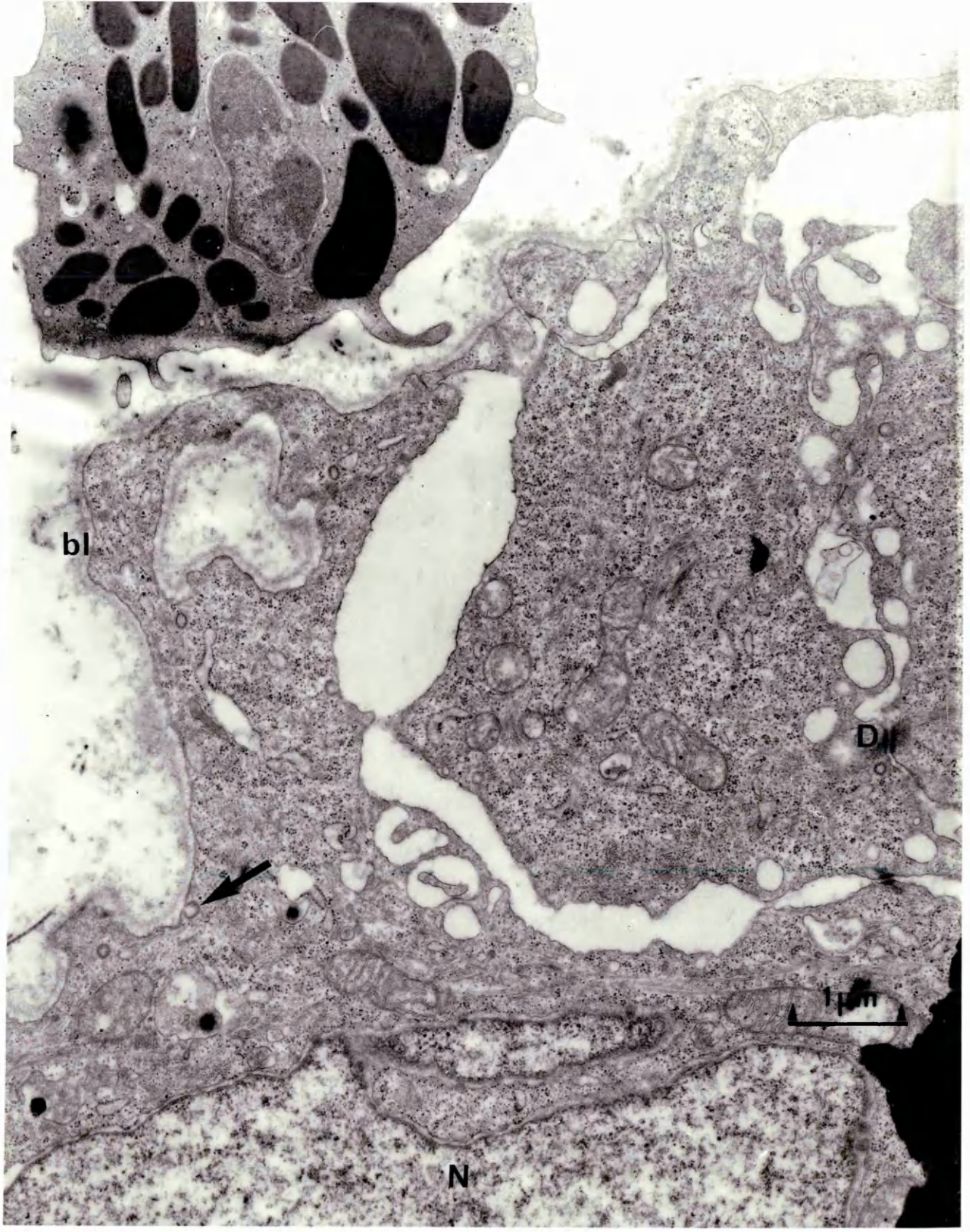
i = infected cells bl = basal lamina

PLATE 77



HSV type 2 day two pock
Heavily infected cell in mesoderm (x18,900)
BL = basal lamina

PLATE 78

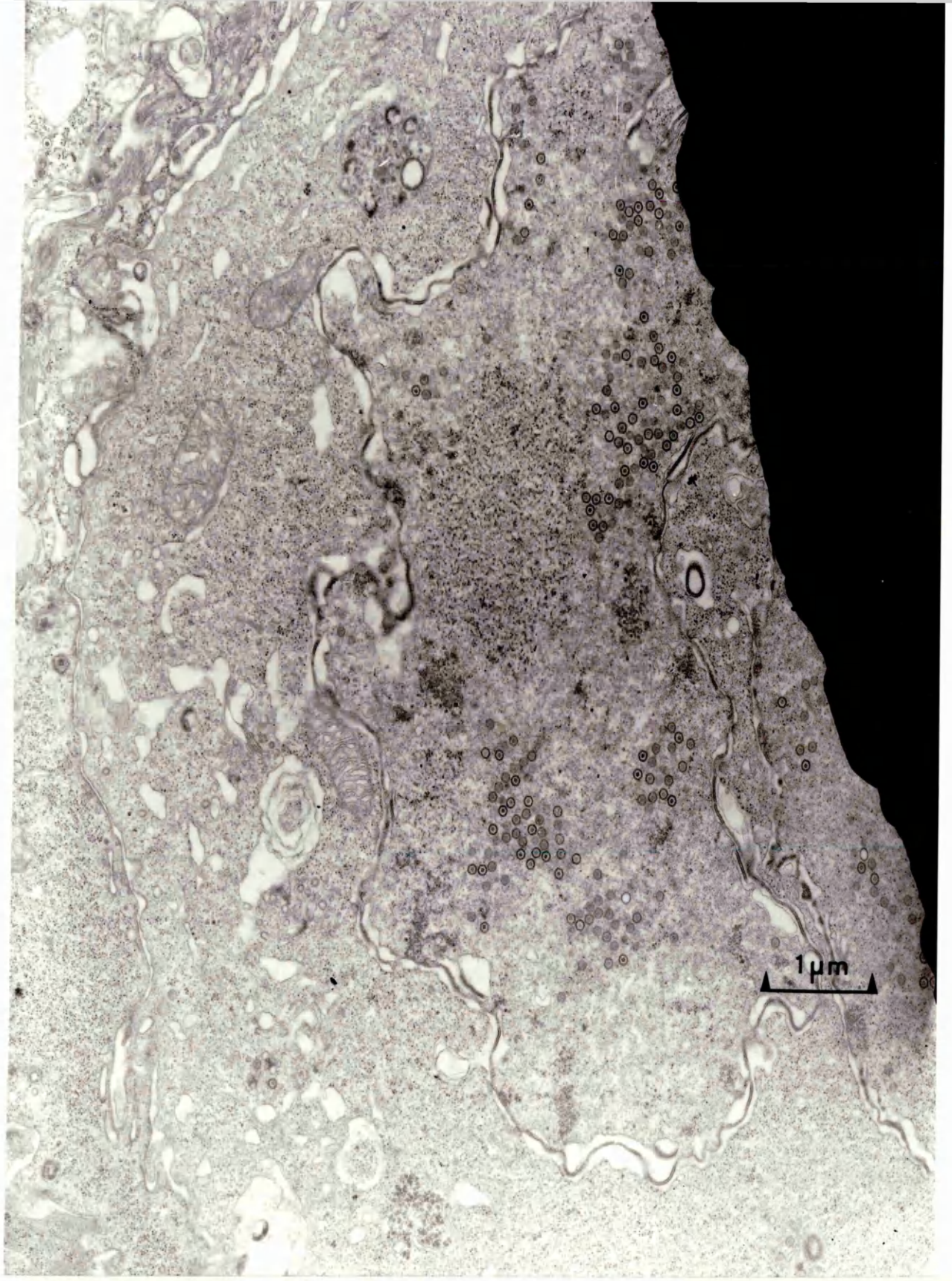


HSV type 2 day two pock

Allantoic layer of CAM (x18,900)

Mast cell in contact with basal lamina (bl)
Note pinocytotic vesicle (arrowed) into allantoic cell
D = desmosome N = nucleus

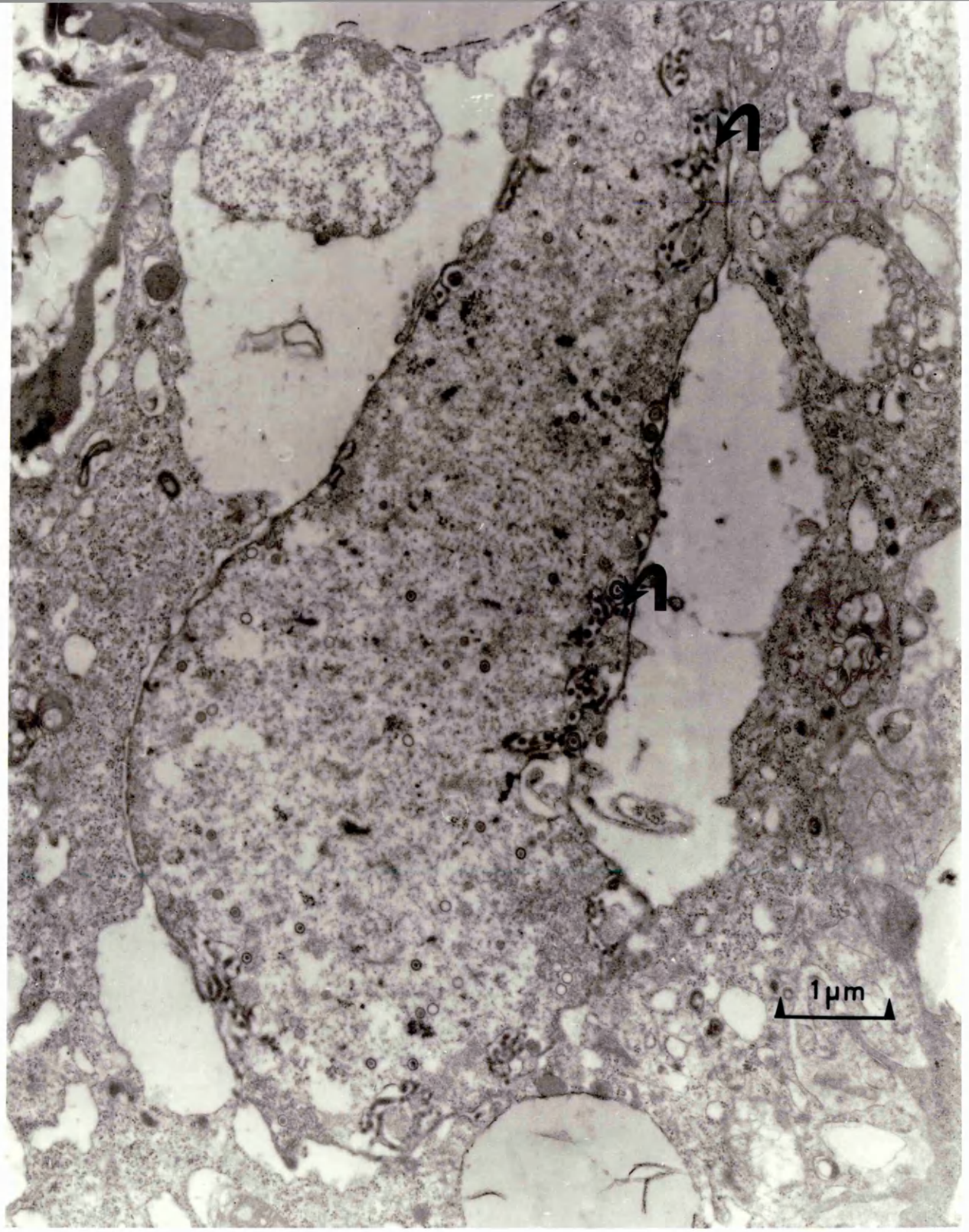
PLATE 79



HSV type 2 day two pock

Infected cell in chorion showing numerous 'full' and some 'empty' particles in the nucleus (x18,900)
Note disruption of the nuclear membrane and shrinkage of the nucleus

PLATE 80

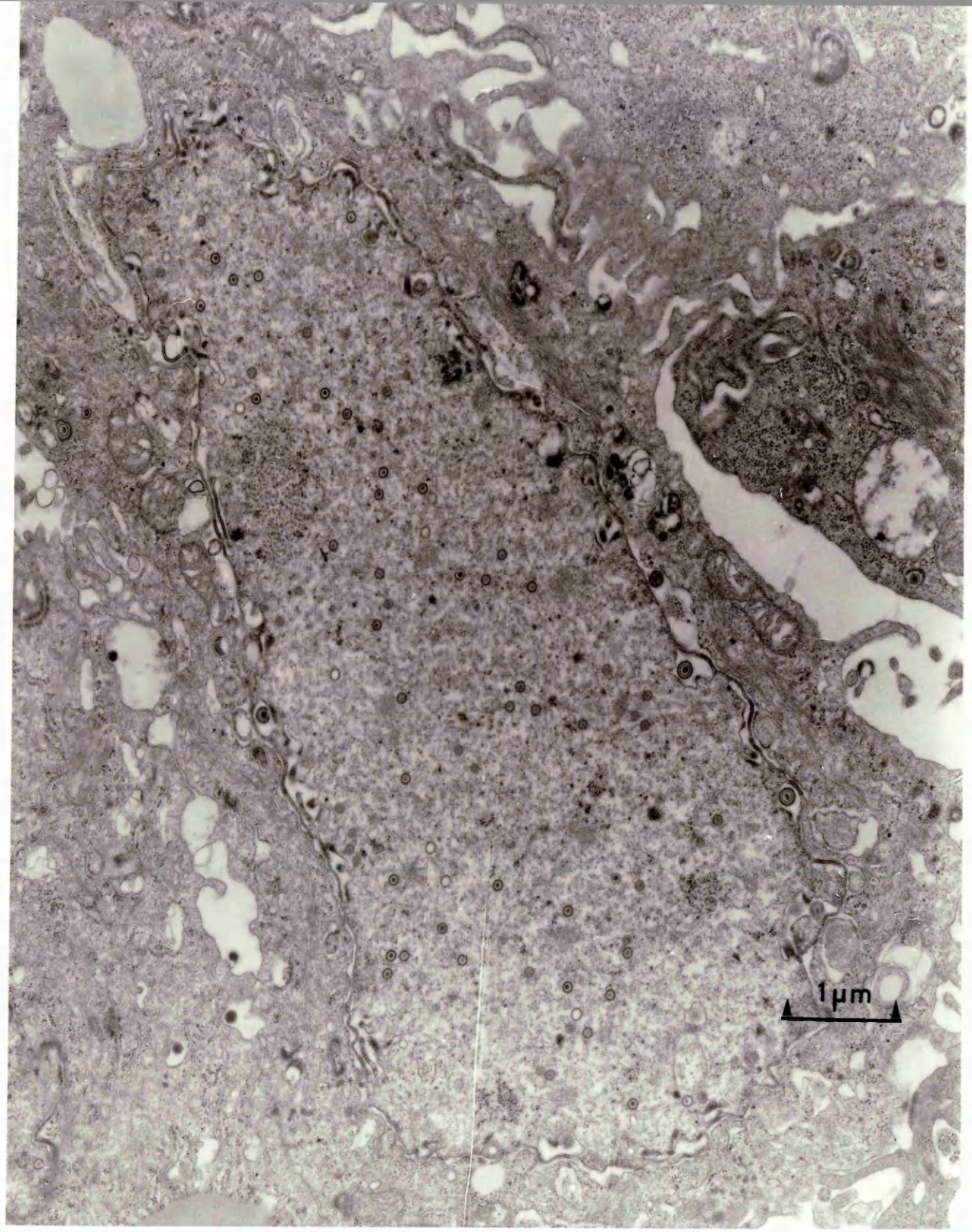


HSV type 2 day two pock

Infected cell in chorion showing numerous 'full' and 'empty' virus particles in the nucleus
Cytoplasm highly vacuolated due to enlargement of the perinuclear space

Note dense granules (arrowed) (x18,900)

PLATE 81

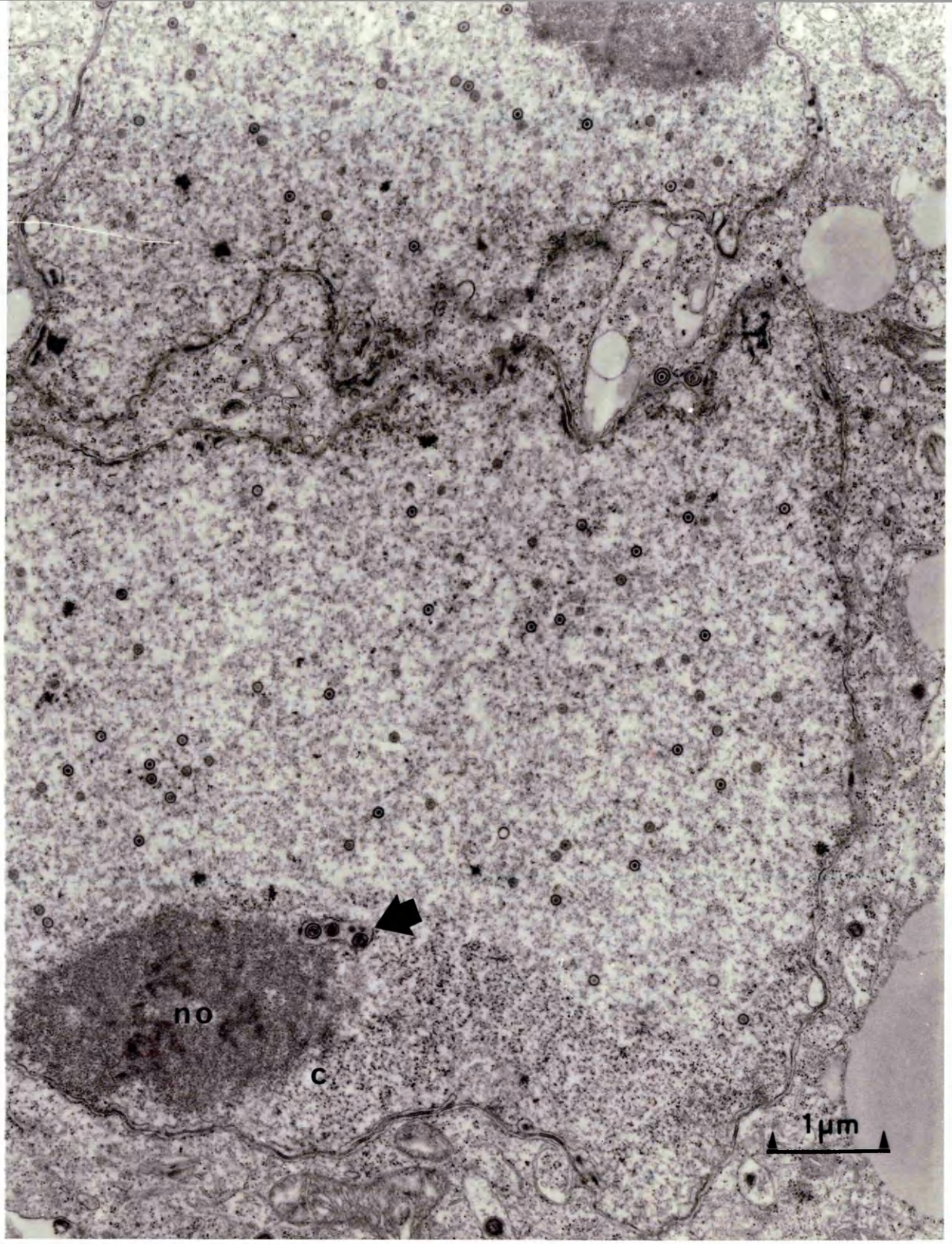


HSV type 2 day two pock

Highly infected cell in chorion with numerous full and empty capsids in the nucleus (x18,900)

Note highly convoluted nuclear membrane

PLATE 82

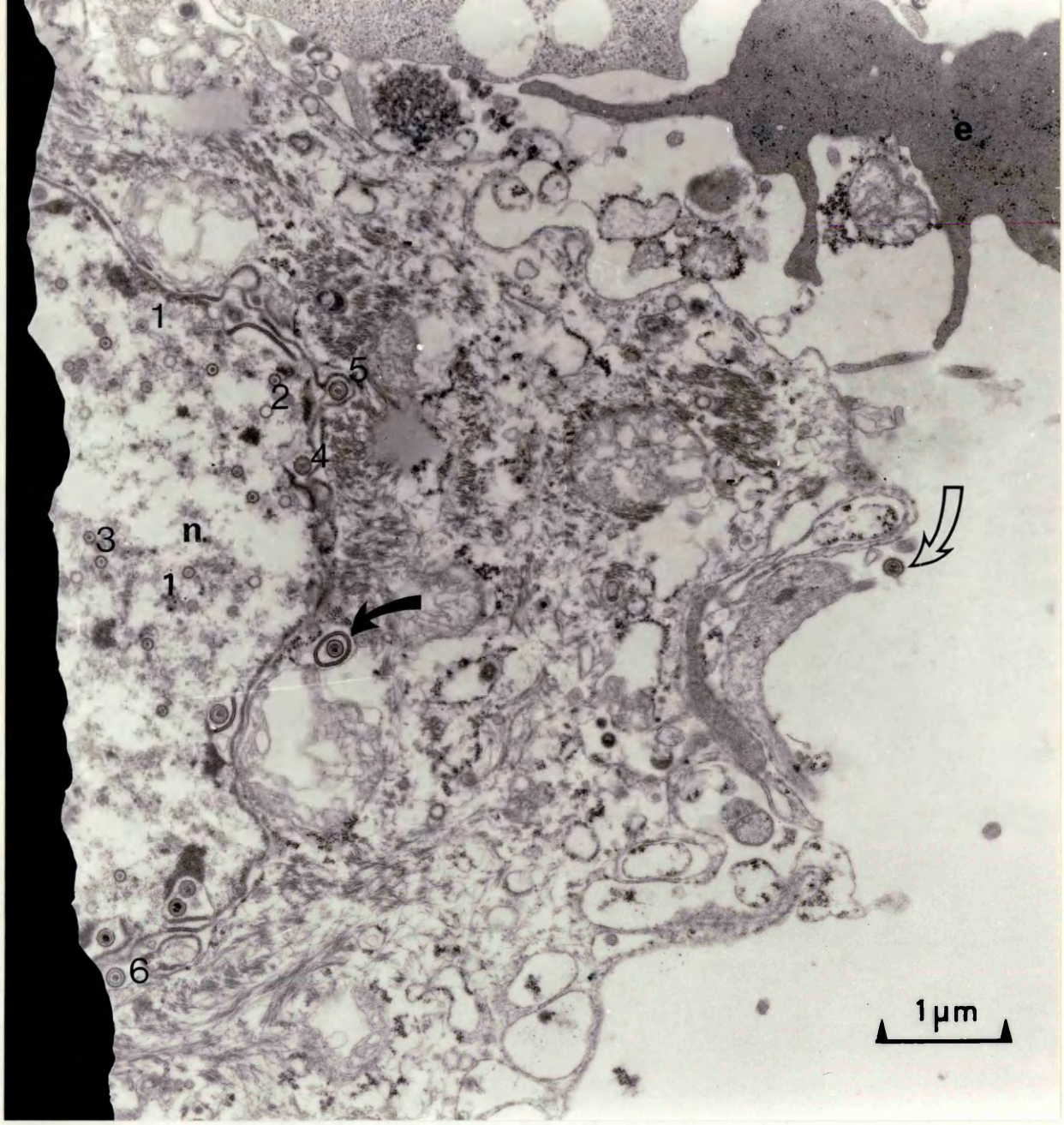


HSV type 2 day two pock

Infected cell at the base of the chorion containing enveloped virus particles within vesicles (arrowed) in the nucleus

c = chromatin no = nucleolus (x18,900)

PLATE 83



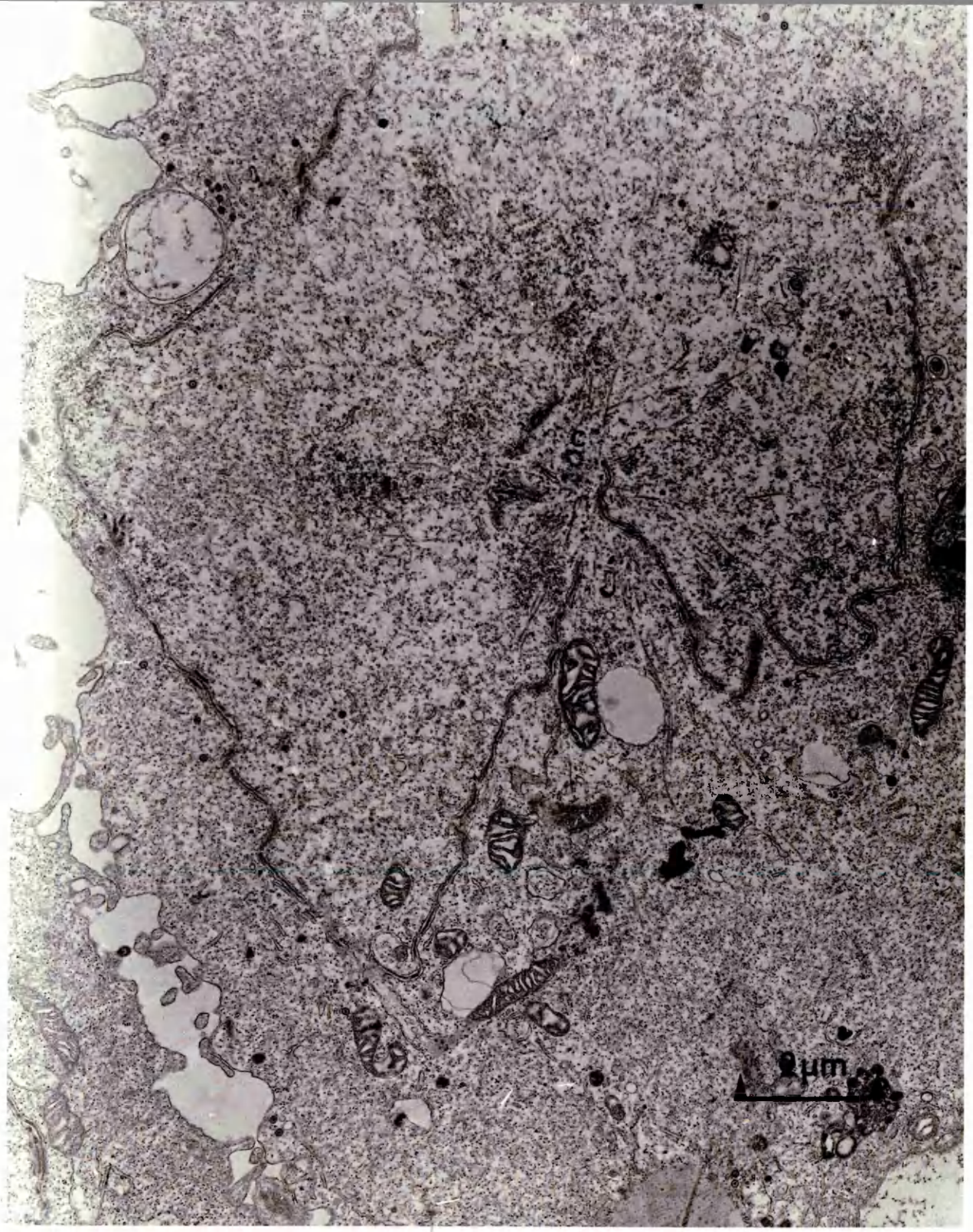
HSV type 2 day two pock

Infected cell in chorion showing particles at all stages of morphogenesis (x18,900)

1 site of capsid formation in nucleus
2 'empty' particles in nucleus
3 'full' particles in nucleus
4 particles budding from the nuclear membrane
5 " " " " " " (further advanced)
6 enveloped particles in the cytoplasm

Note capsid with envelope of reduplicated nuclear membrane (solid arrow), extracellular enveloped virus particle with pronounced fringe (open arrow) and capsids with unusual cores

n = nucleus e = erythrocyte

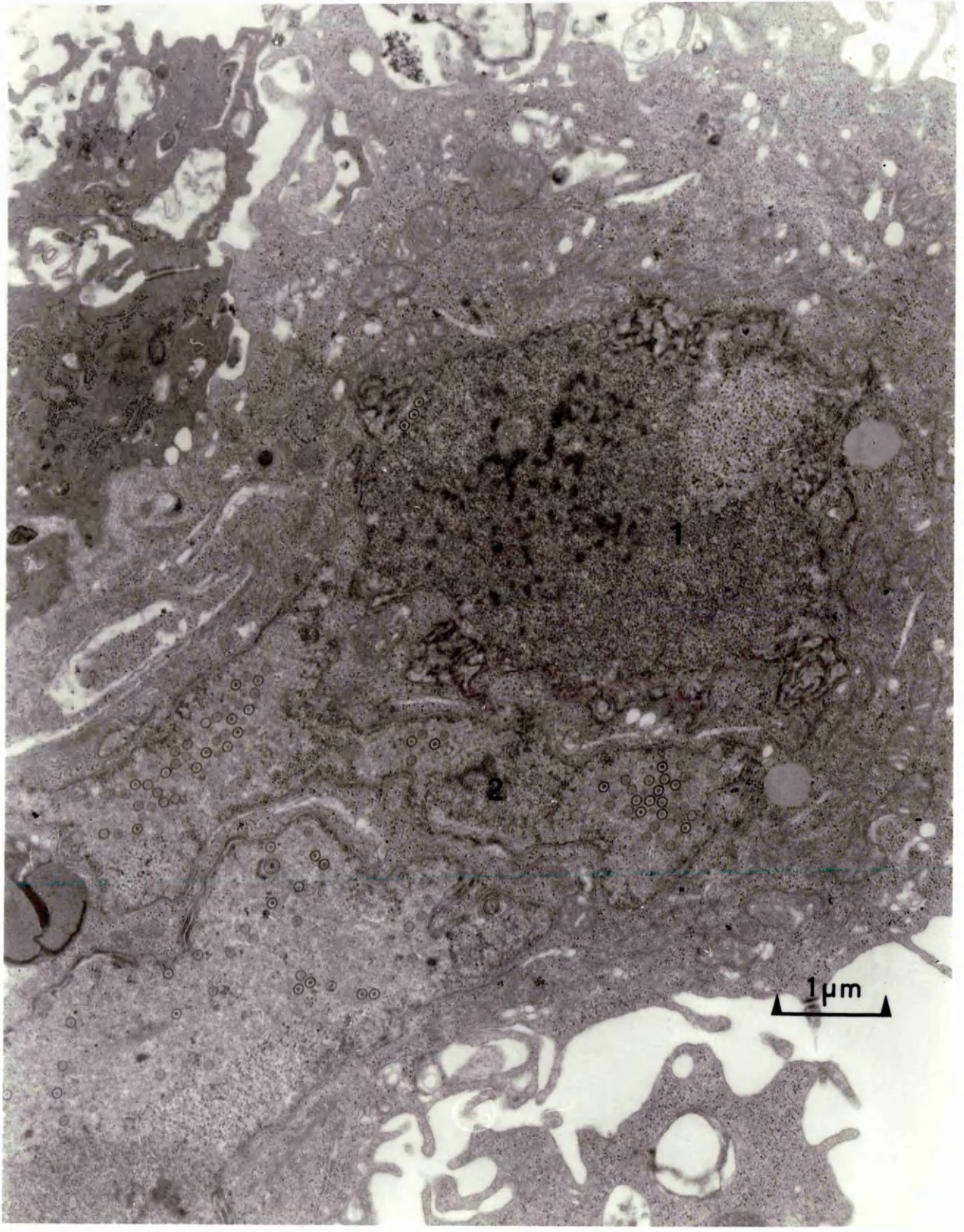


HSV type 2 day two pock

Infected cell in chorion in the process of cell division
c = centriole (x12,000)

Note microtubules, also large number of naked capsids in cytoplasm and disrupted nuclear membrane

PLATE 85

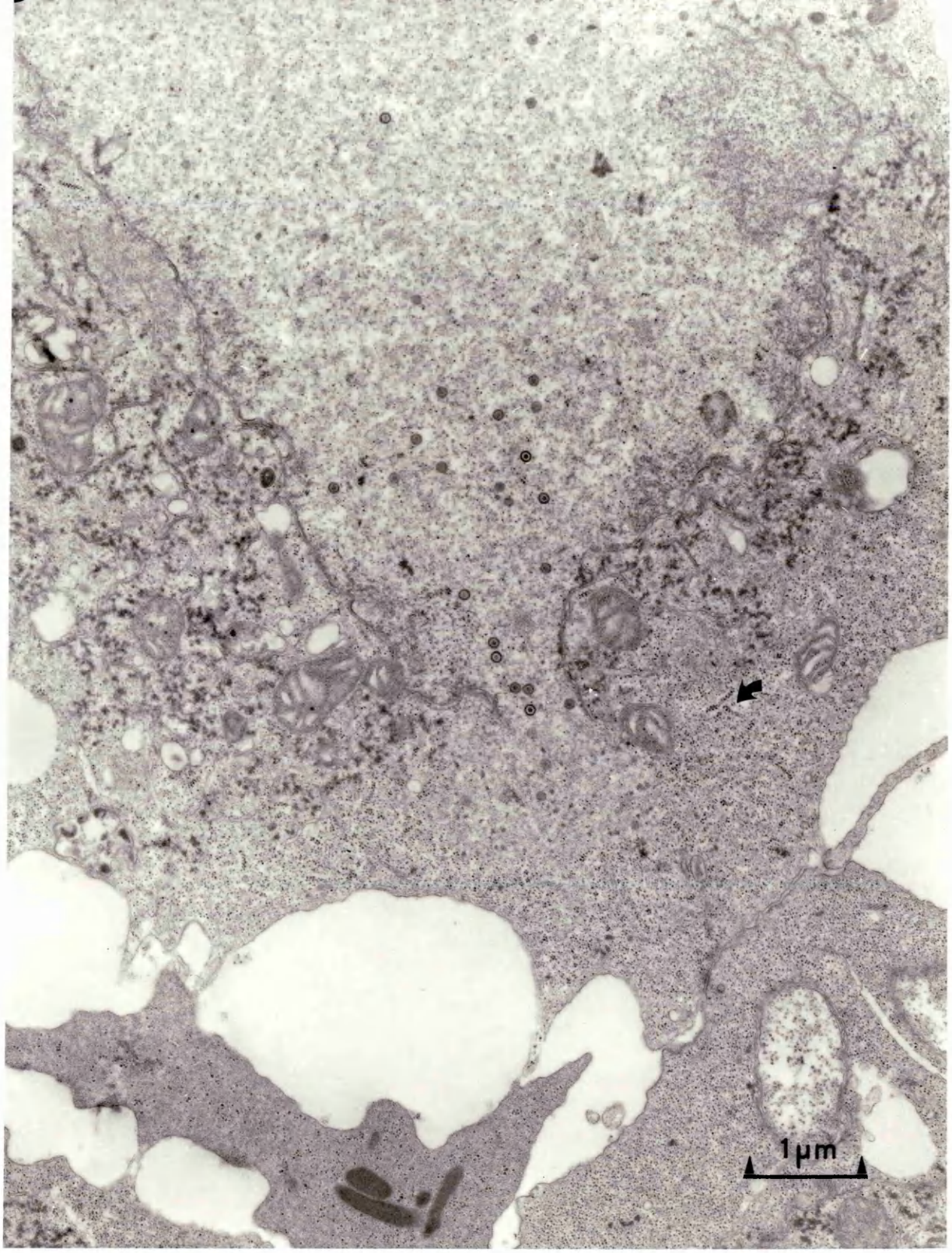


HSV type 2 day two pock

Infected cell in chorion with what appears to be two nuclei (1 and 2)
Note dense granules 42 nm. in diameter in nucleus 1 and small paracrystalline array in nucleus 2

(x18,900)

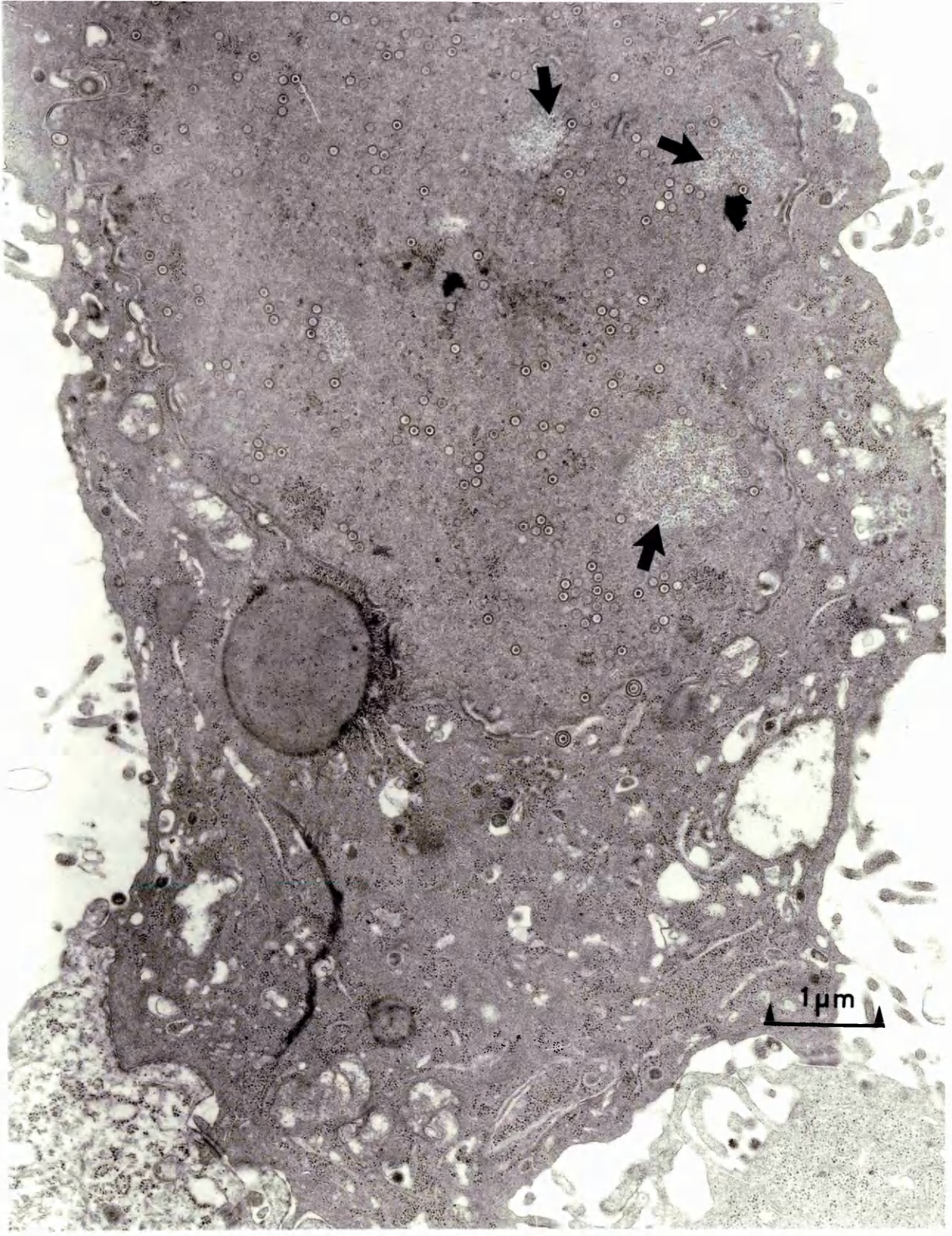
PLATE 86



HSV type 2 day two pock

Infected cell in lower chorion in which the nucleus has lysed and nuclear and cytoplasmic contents are mixed with naked virus capsids (x18,900)
(note polysomes, arrowed)

PLATE 87

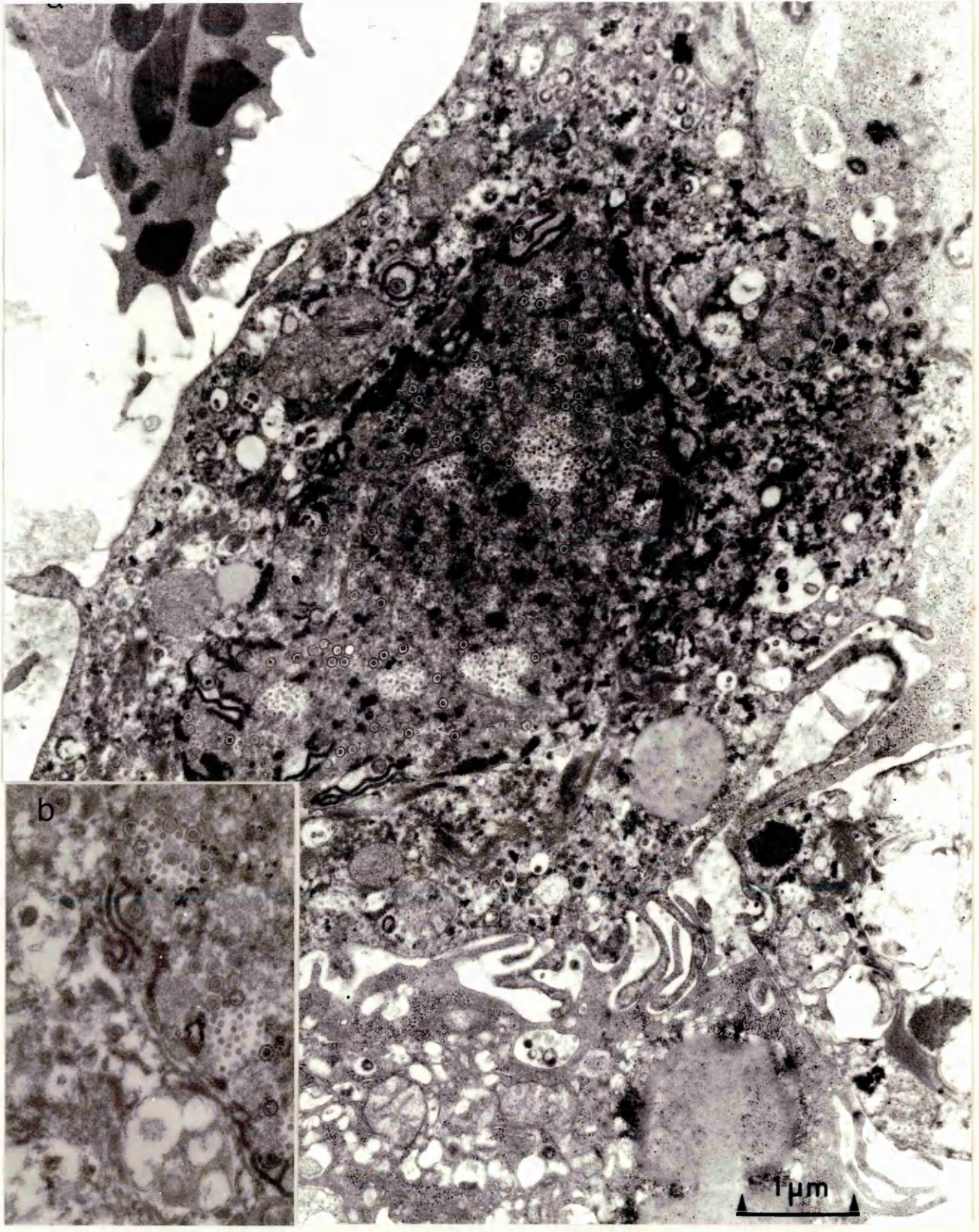


HSV type 2 day two pock

Densely staining highly infected cell in chorion (x18,900)

Pale areas in nucleus containing 16-20 nm. granules (arrowed)

PLATE 88

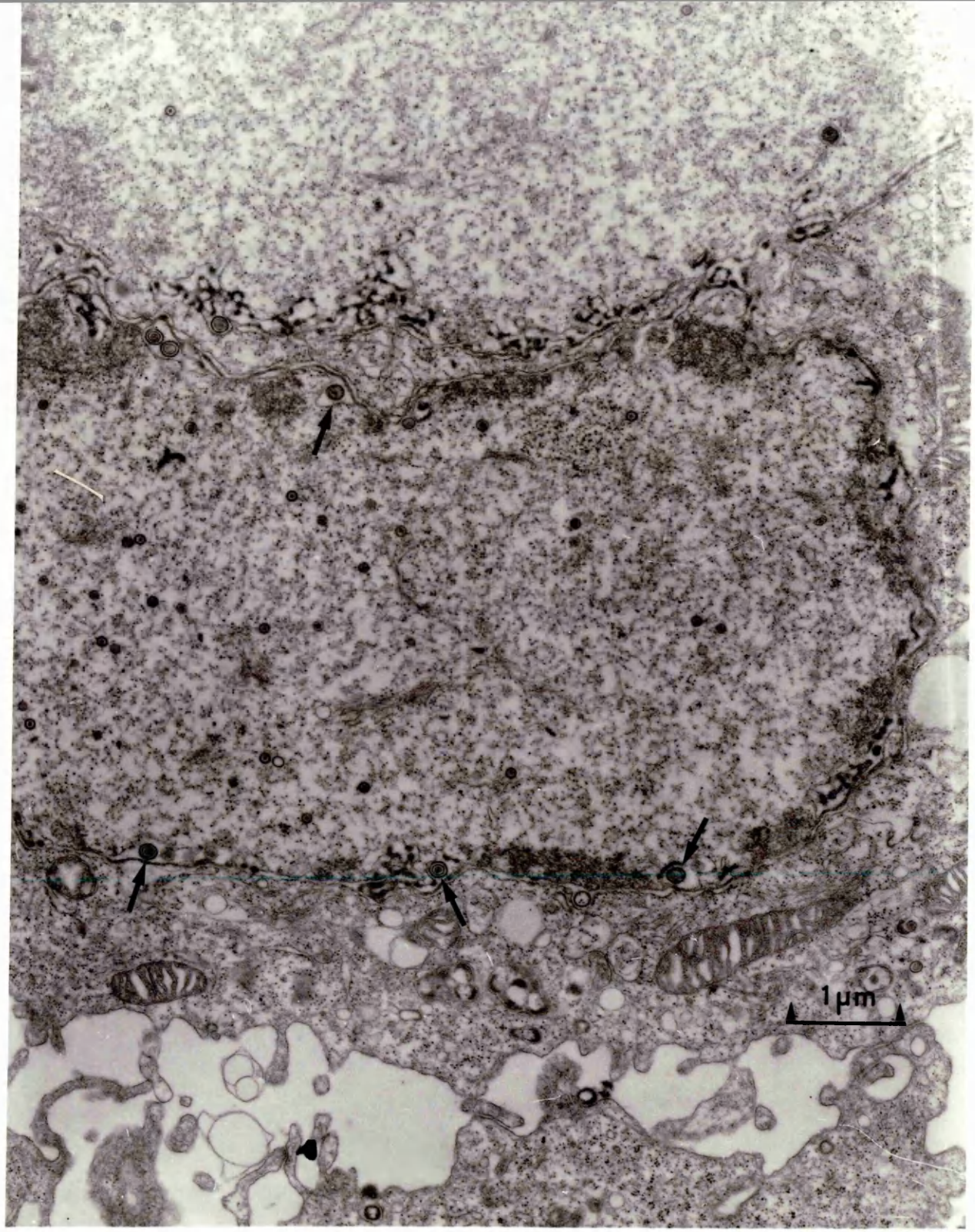


HSV type 2 day two pock

a) highly infected cell in chorion showing dense granules only in the nucleus (x18,900)
Note tubular structures in both nucleus and cytoplasm

b) infected cell in chorion containing numerous dense granules 42-50 nm. in diameter closely associated with virus capsids (x25,200)

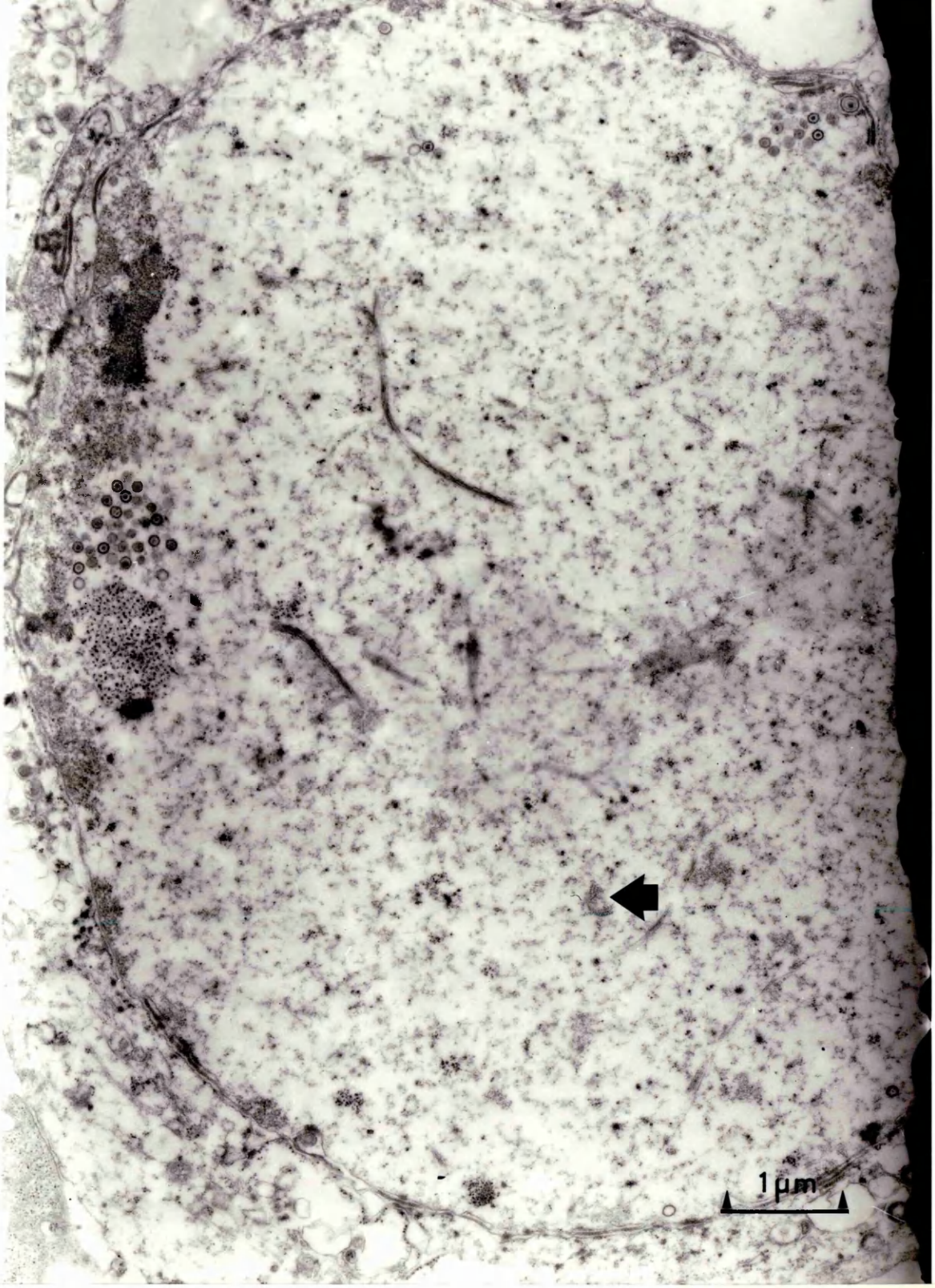
PLATE 89



HSV type 2 day two pock

Two infected nuclei in chorion with pleomorphic 65-95 nm. granules concentrated at the nuclear membrane (x18,900)
Complete virus particles are seen in the perinuclear space (arrowed)

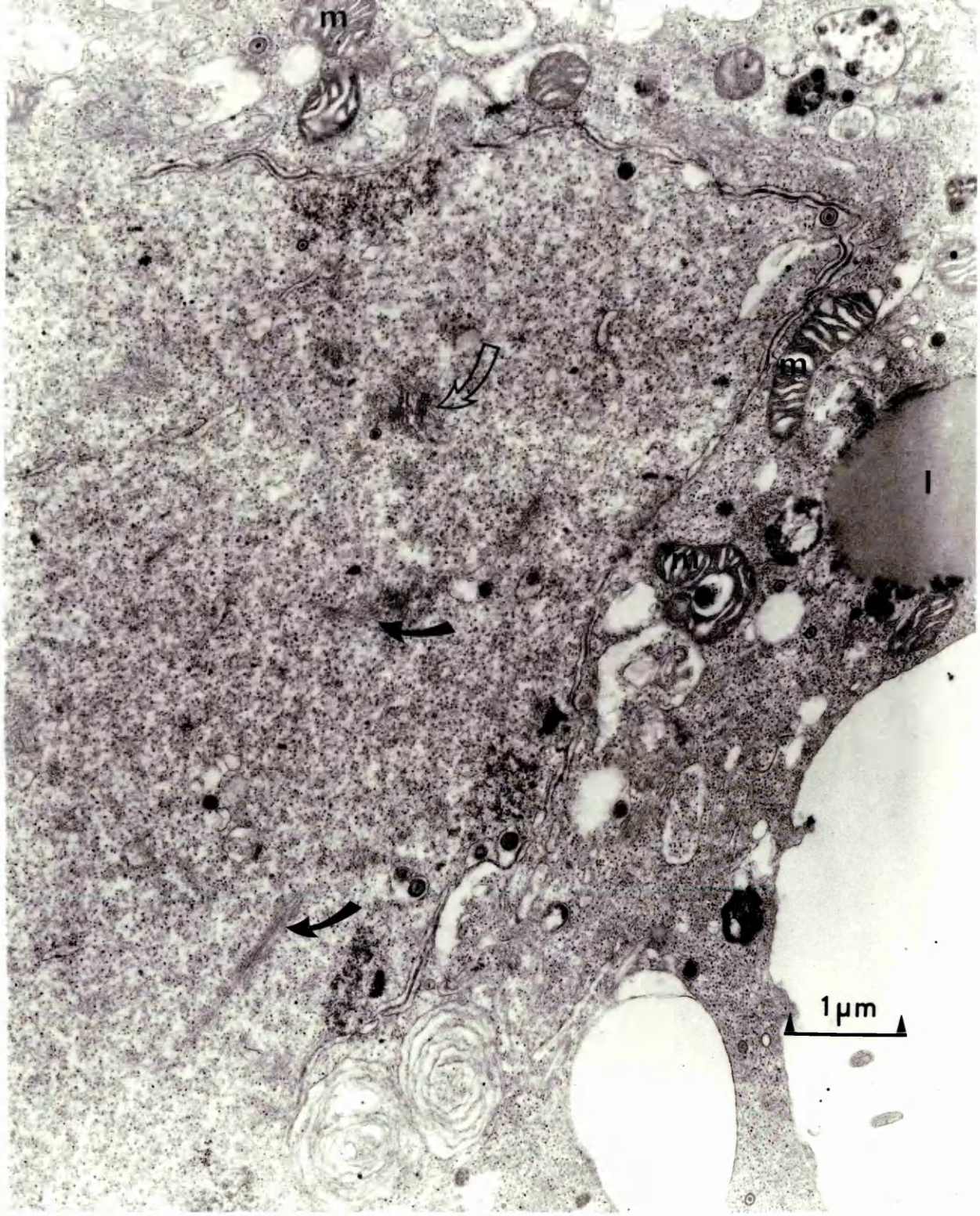
PLATE 90



HSV type 2 day two pock

Infected nucleus in a cell at the base of the chorion containing virus capsids and 68-80 nm. bodies resembling virus cores (x18,900)
Note membraneous structure (arrowed); each membrane is 21 nm. thick, studded with granules 16 nm. in diameter and 8 nm. apart; also present are numerous microfilaments

PLATE 91



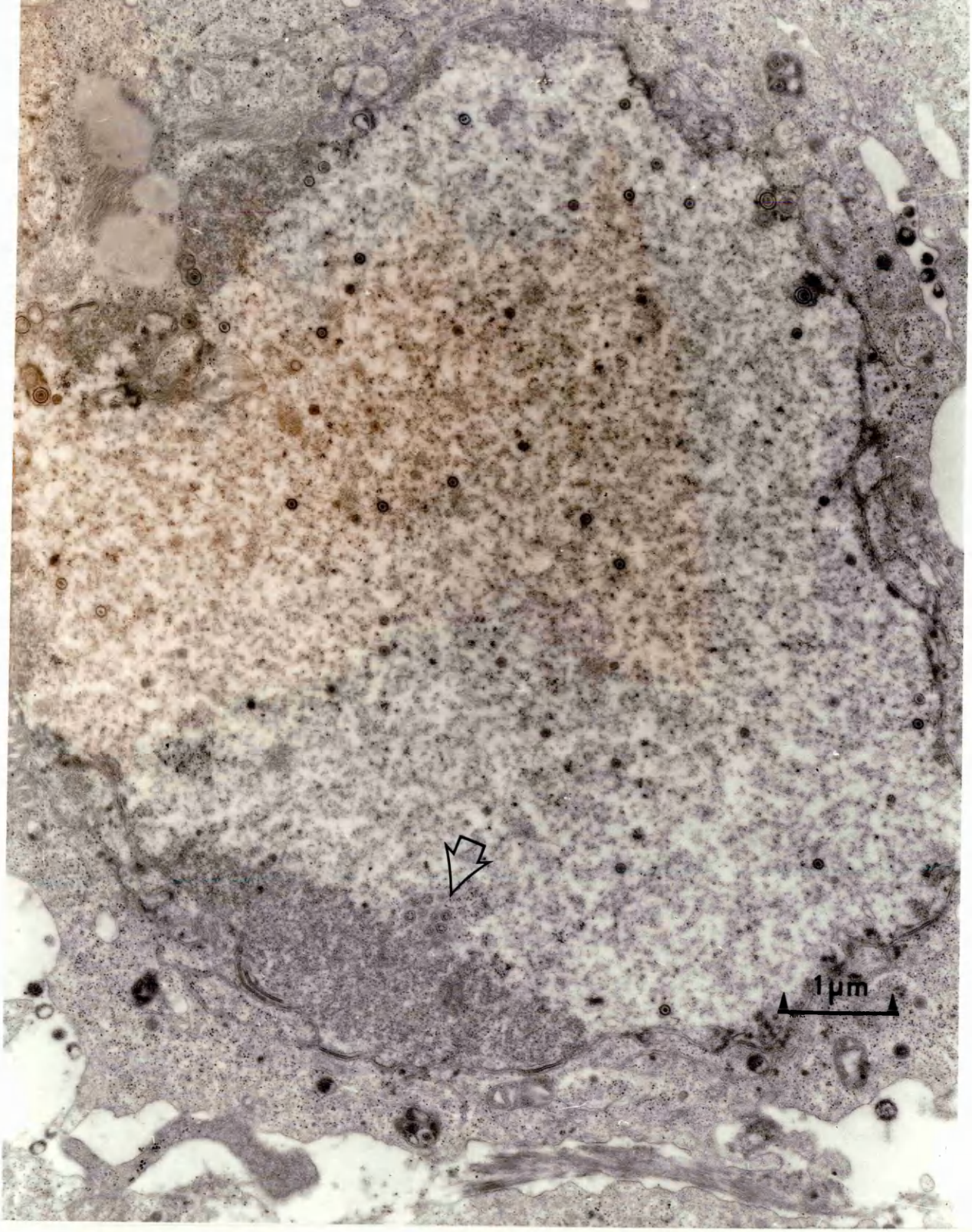
HSV type 2 day two pock

Infected cell in chorion

The nucleus contains numerous 16 nm. microfilaments (arrowed) and lattice structures (open arrow)

Enveloped and naked cytoplasmic virus particles are present; note whorl-like structures in the cytoplasm
m = mitochondria l = lipid droplet (x18,900)

PLATE 92

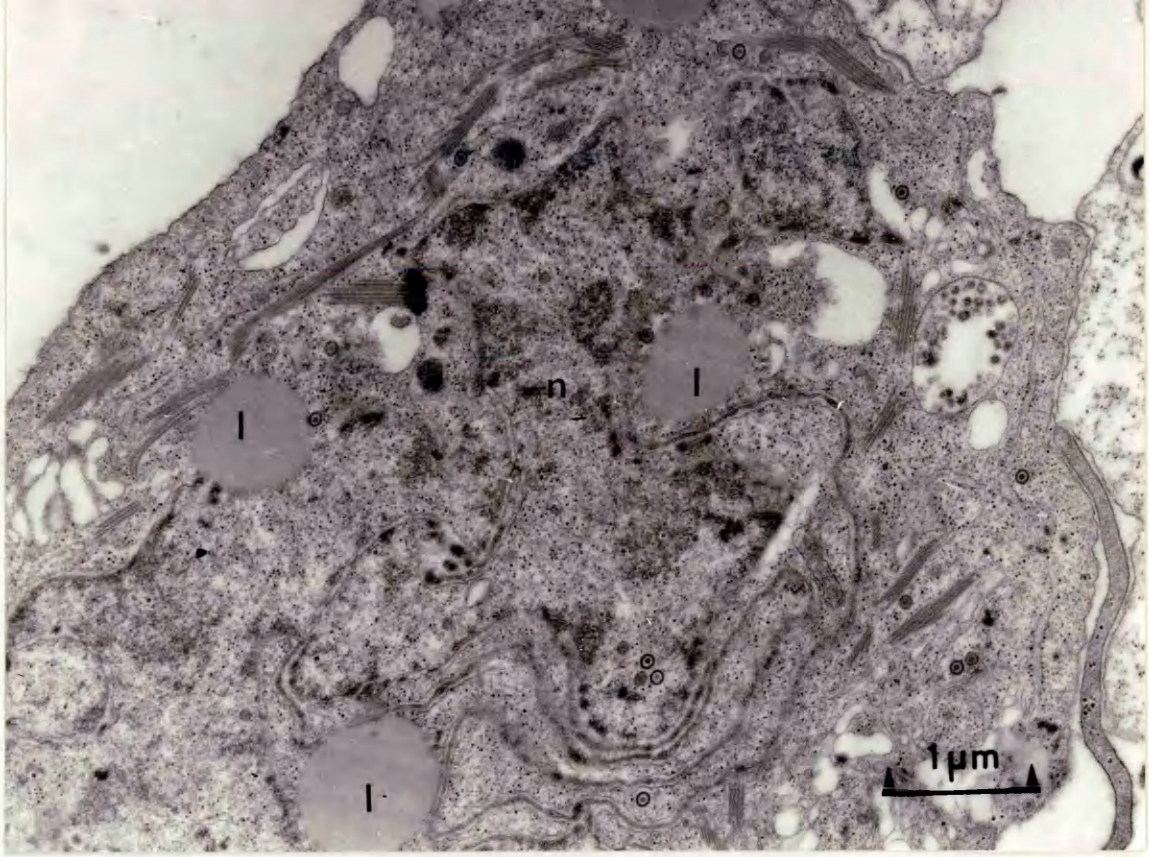


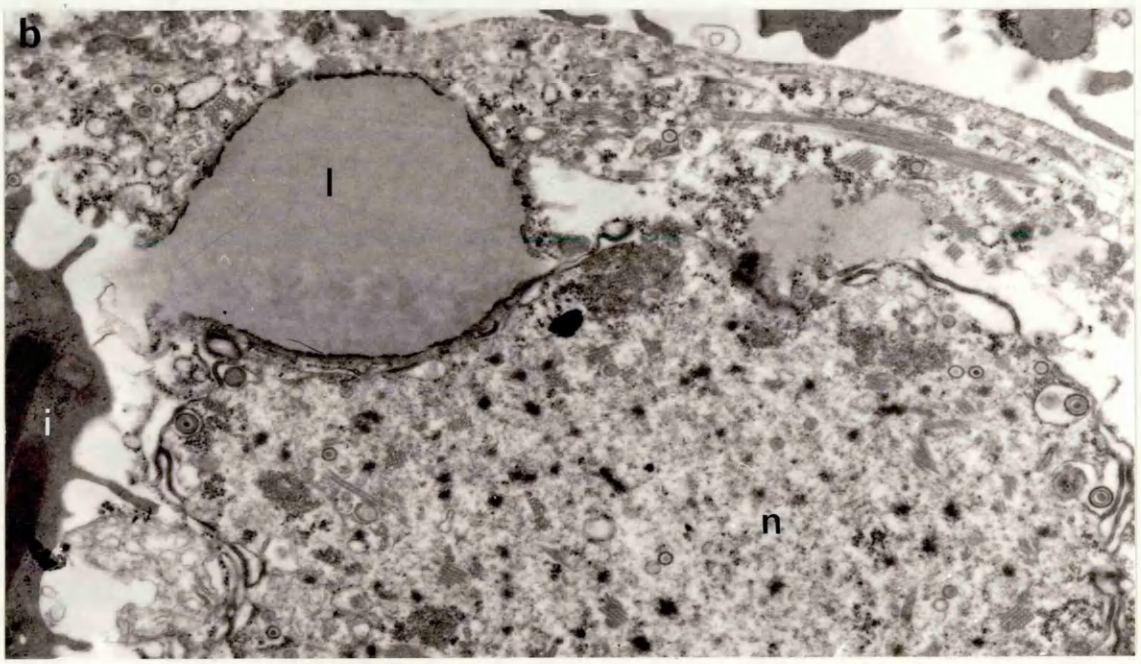
HSV type 2 day two pock

Nucleus of infected cell in mesoderm (x18,900)

Full and empty capsids are present, as well as fragile particles (open arrow) which are associated with chromatin and are suggestive of particles at an early maturation stage

PLATE 93





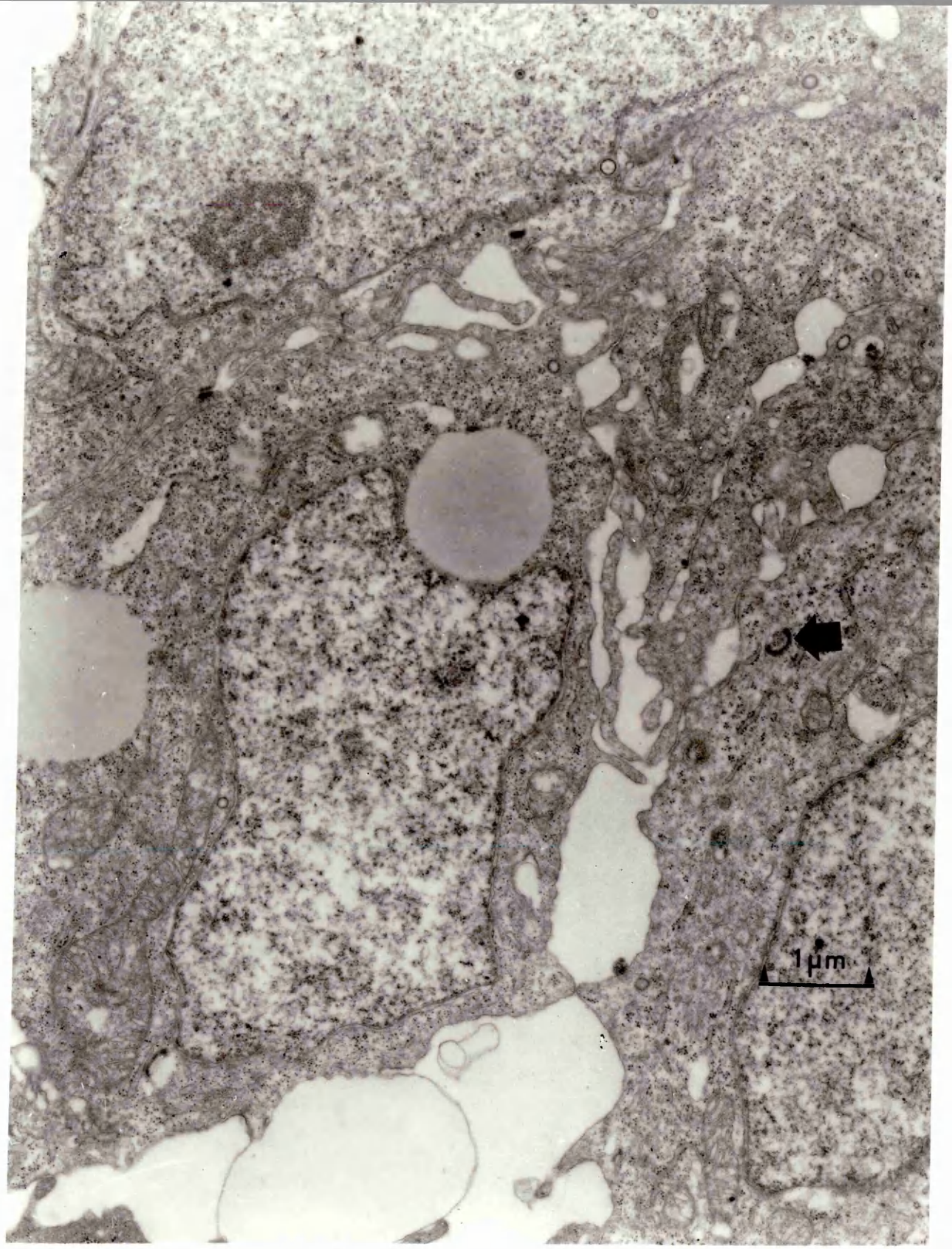
HSV type 2 day two pock

a) highly infected cell in mesoderm showing lattice-like structures in tangential and longitudinal section

b) highly infected cell in chorion with lattice structures in longitudinal, tangential and cross-section in the nucleus and cytoplasm

(x18,900)

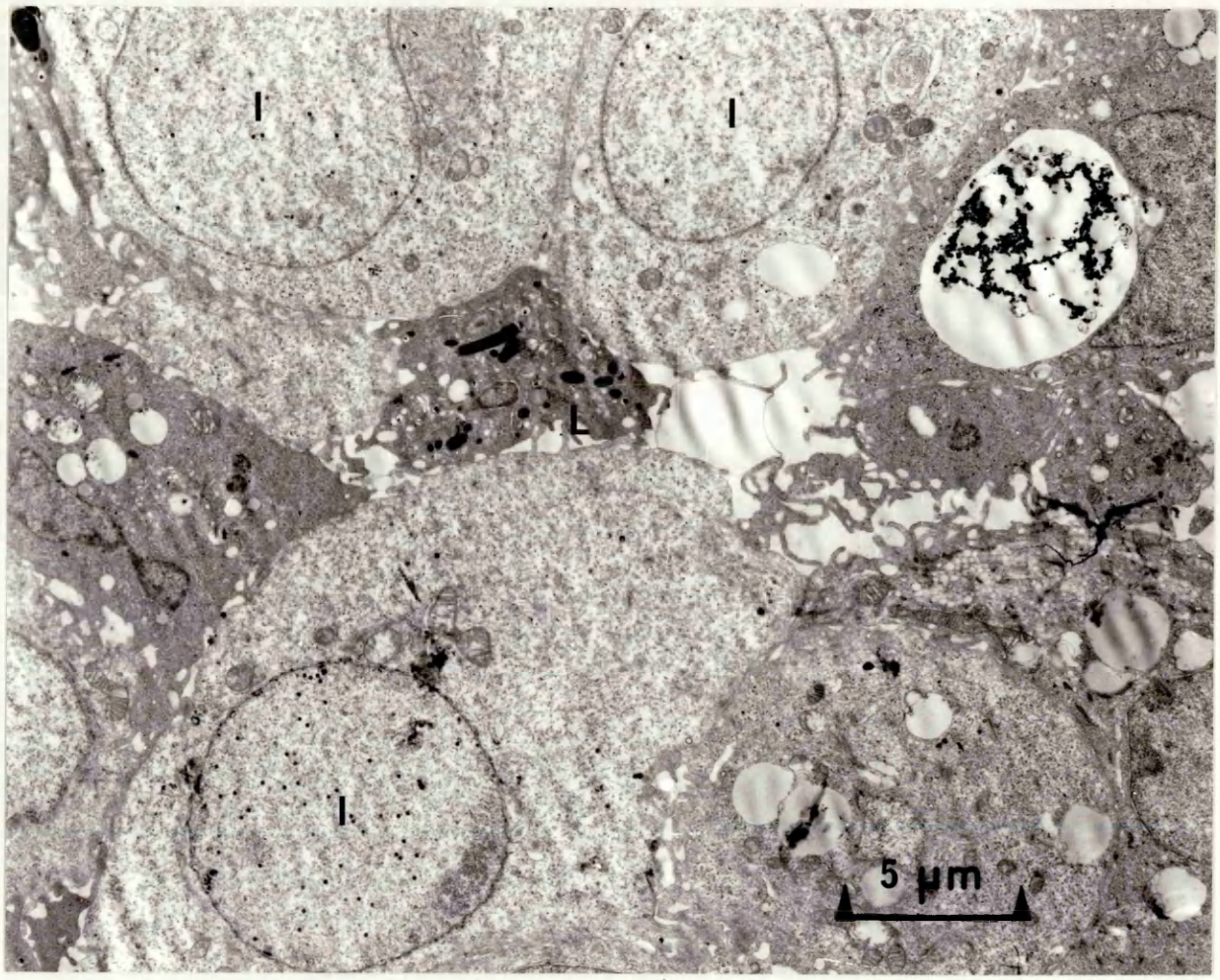
n = nucleus l = lipid droplet i = inflammatory cell



HSV type 2 day two pock

A semi-circular structure 53-58 nm. across is present in the cytoplasm of an infected cell in the mesoderm (no virus particles are seen in the nucleus) (x18,900)

PLATE 95



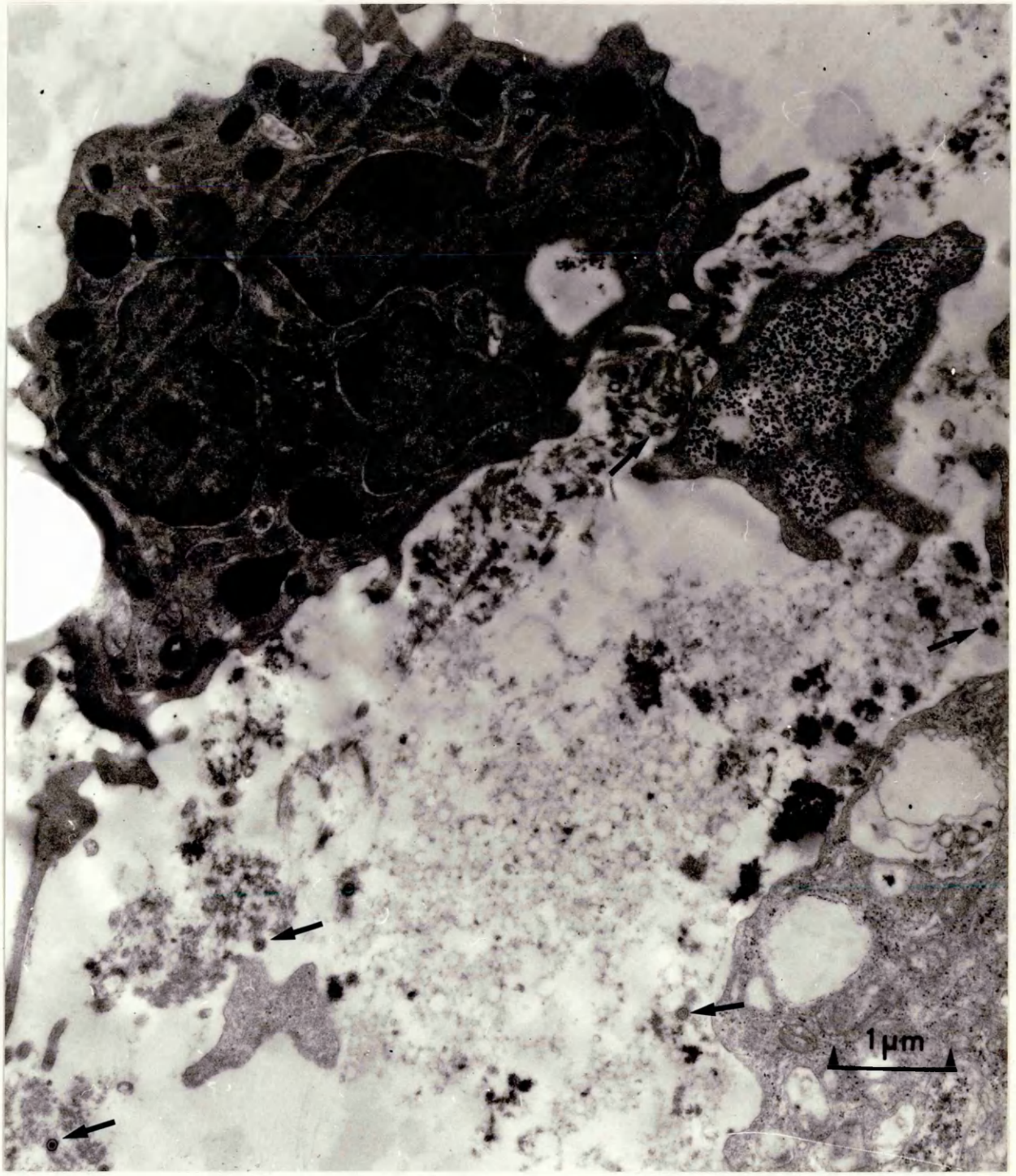
HSV type 2 day five pock

Region of chorion showing numerous virus infected cells (x4,800)

I = infected cells L = leucocyte

(note macrophages)

PLATE 96



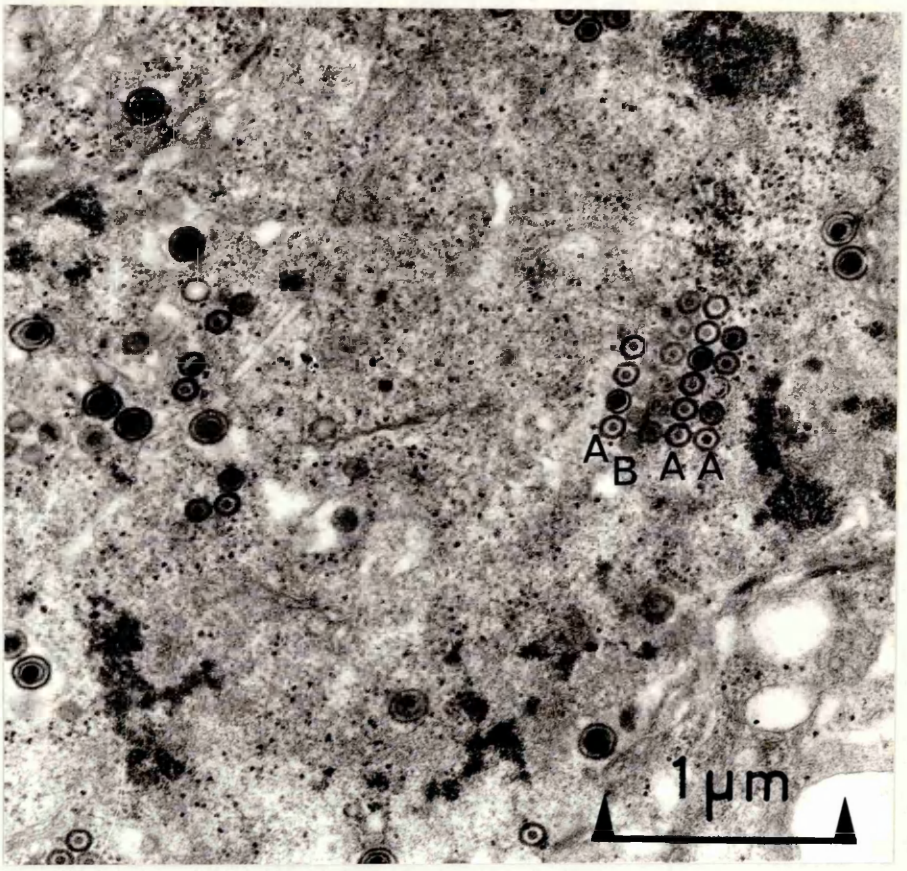
HSV type 2 day three pock

Region of mesoderm showing densely staining macrophage

Note virus capsids free in the mesoderm surrounded with a fine meshwork, giving them a fuzzy outline (arrowed)

(x18,900)

PLATE 97



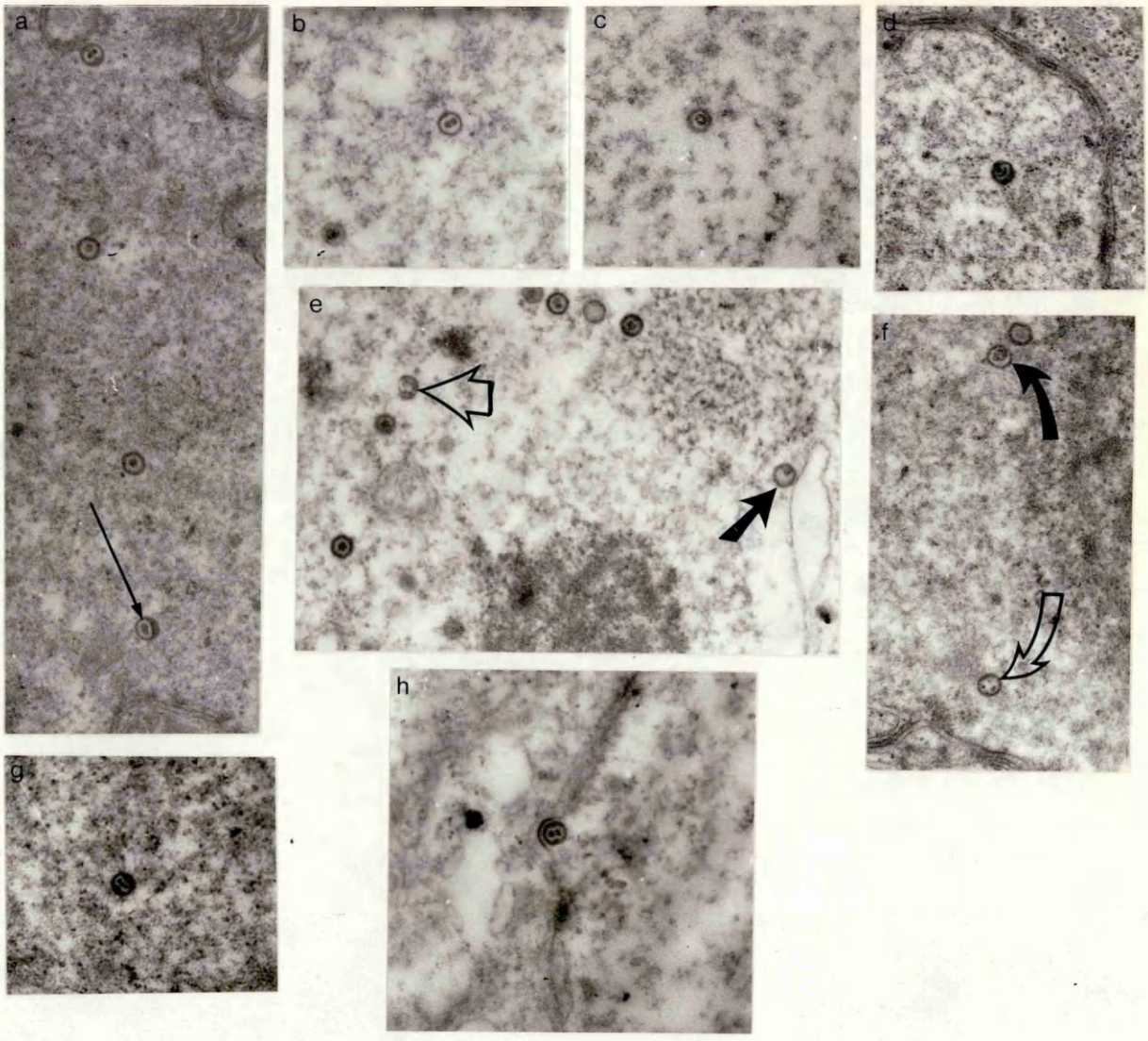
HSV type 2 day three pock

Infected nucleus with virus particles arranged in a para-crystalline formation

The particles immediately in the plane of the section (A) suggest a square symmetry, whilst the particles in row (B) appear to be hexagonally placed in relation to those of row (A)

(x32,000)

PLATE 98



Cores of HSV type 2

a) bar-shaped core with one-sided thickening of the capsid

b) double core

c) annular (hollow) core

d) curved core

e) off-centre core (solid arrow), 'fragile' core (open arrow)

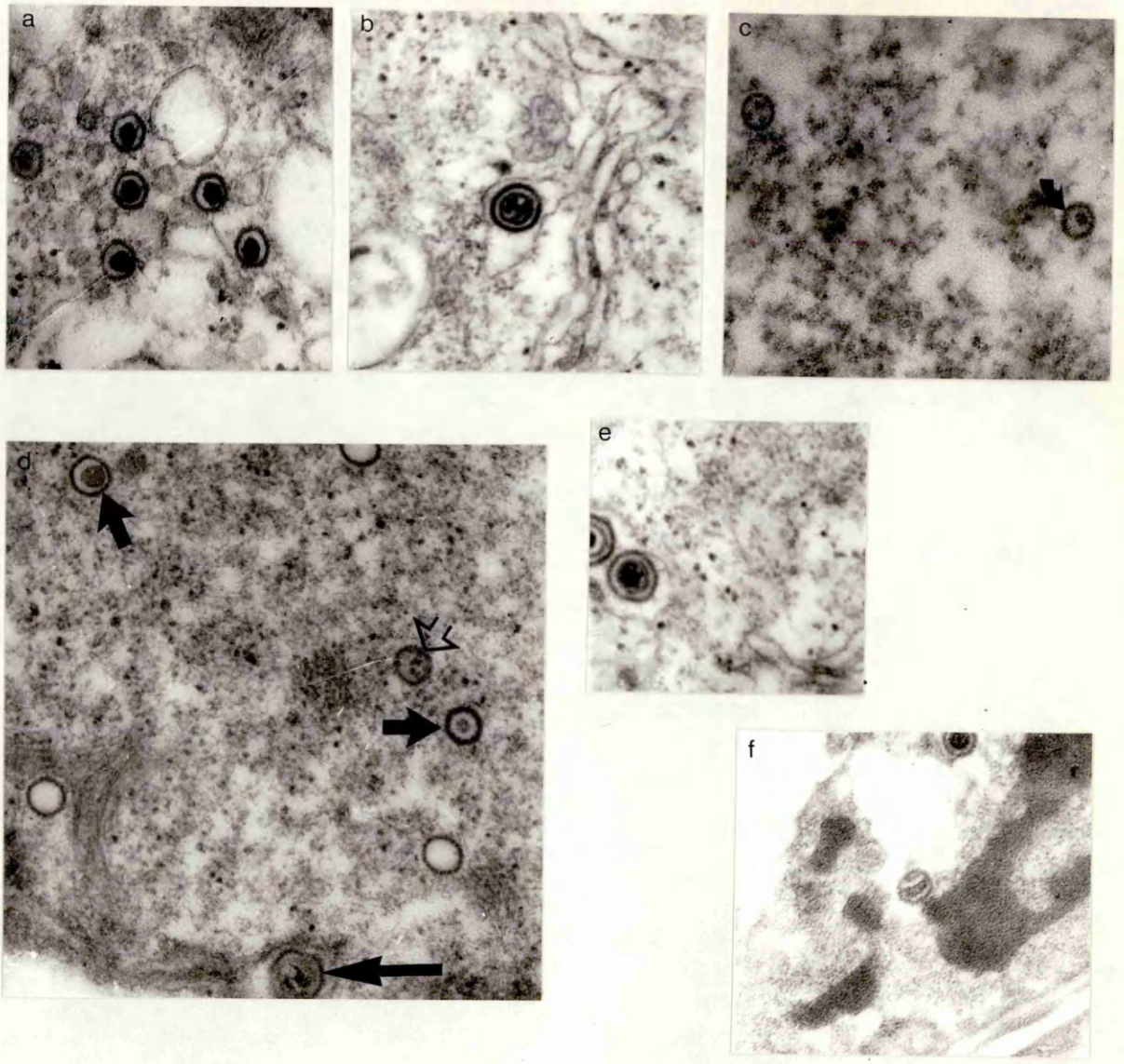
f) apparently broken-up core (open arrow) and toroidal core (solid arrow)

g) bar-shaped core with thickening of the capsid either side of the core

h) double core associated with nuclear membrane

(all magnifications x31,500)

PLATE 99



Cores of HSV type 2

a) densely staining solid cores

b) toroidal shaped core

c) core to one side of capsid (solid arrow) and core showing threefold symmetry

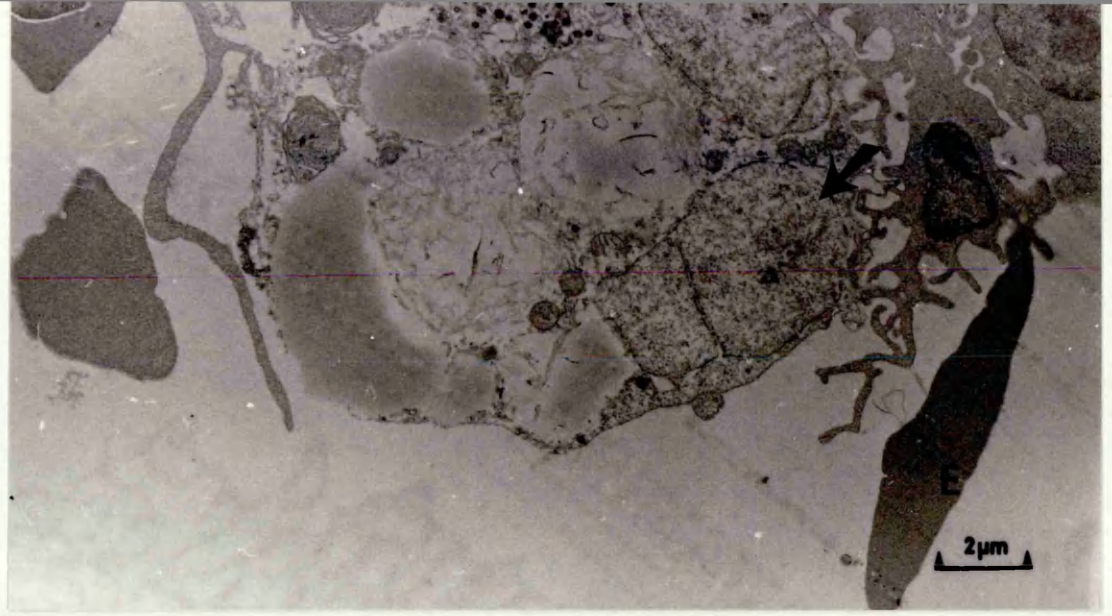
d) pale staining cores (short solid arrows), toroidal core (long solid arrow) and 'broken' core (open arrow)

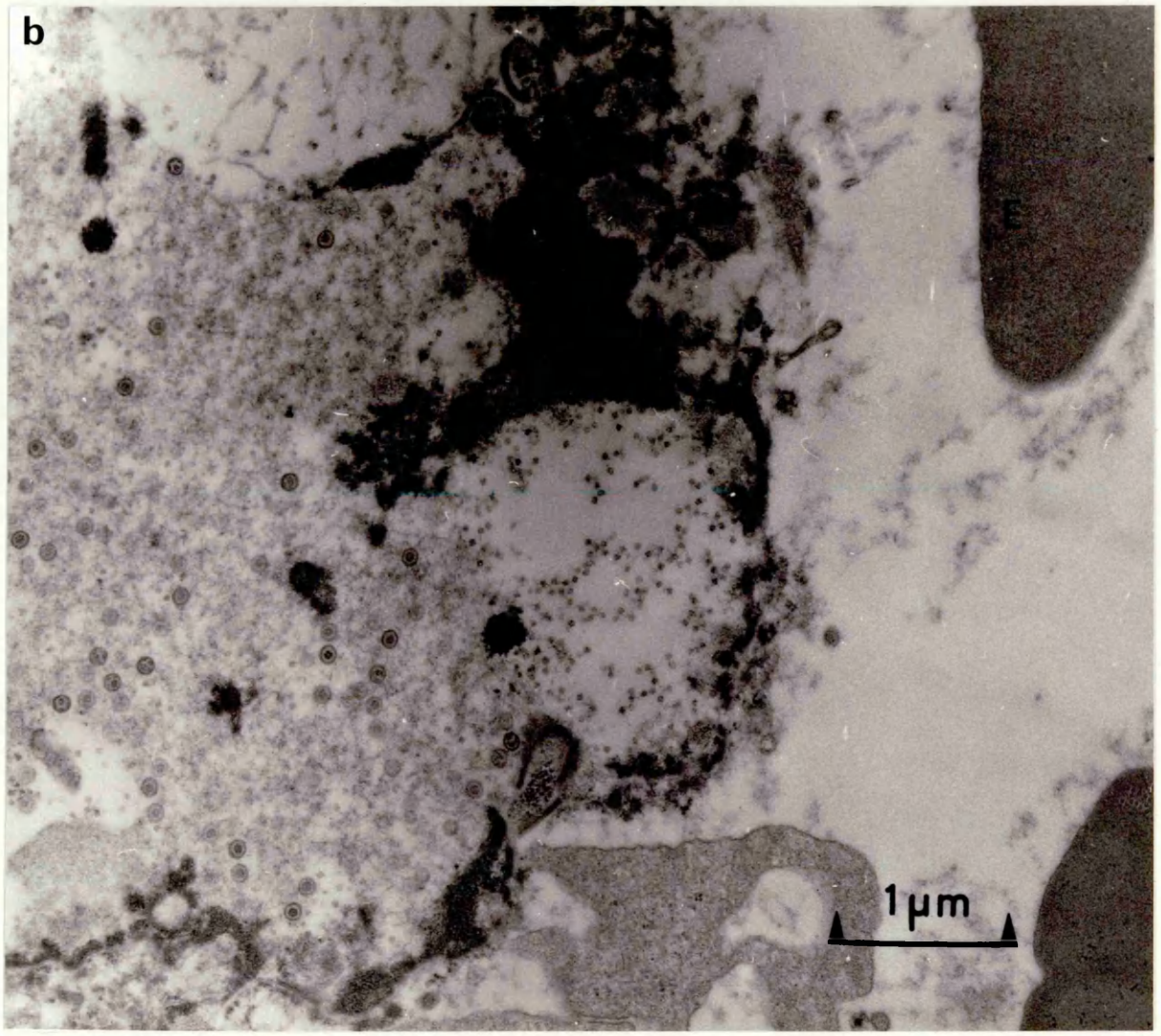
e) densely staining filamentous core

f) particle with no gap between the curve-shaped 'core' and the capsid

(all magnifications x48,000)

PLATE 100





HSV type 2 day three haemorrhagic pock

a) low magnification view of part of blood vessel (x6,000) Virus infected cell (arrowed) is present in the wall of the blood vessel

b) higher magnification of virus infected cell in blood vessel (x24,000) Cell is breaking down and releasing virus particles into the lumen

E = erythrocytes

PLATE 101

D I S C U S S I O N

The use of pock size on the chorioallantoic membrane (CAM) to type isolates of herpes simplex virus (HSV) has been strongly advocated by a number of workers, in particular Hutfield (1967) and Parker and Banatvala (1967). However, others have urged caution in the interpretation of results because of factors other than virus type which may influence lesion size (Nahmias *et al.* 1968). With the strains of virus used in the present study, no difficulty was experienced in differentiating fresh isolates of type 1 virus from those of type 2 using the pock size marker. However, some laboratory strains of type 1 virus, which had received multiple passages on the CAM, produced larger pocks than the fresh isolates, confirming reports made by Coriell and colleagues (1949), Wildy (1955) and Schneweis (1962b). Typing of these laboratory strains was further complicated if large inocula were used, as this resulted in pocks coalescing to form confluent patches which resembled the larger pocks of type 2 strains. Passage of these laboratory strains in human cell cultures before their inoculation onto the CAM resulted in a development of normal small pocks.

In addition to the size of lesions, gross pock structure was an additional useful marker for differentiating highly passaged viruses of either type. The type 1 high passage strains induced necrosis, but not haemorrhages, the latter being a salient feature of type 2 virus induced pocks, particularly when virus was inoculated onto the CAM in high titre. High and low passage virus varied in its capacity

to induce pocks in eggs from different breeds of fowl. The most suitable were White Leghorn eggs, whereas those from Light Sussex - Rhode Island Red crosses gave variable results and occasionally did not support the growth of HSV type 1 strains at all (unpubl. obser.). This suggests an explanation for the report by Gologan and co-workers (1972) who observed that type 1 strains did not induce pocks on the CAM of an unspecified breed of eggs, whilst type 2 strains produced polymorphic pocks which did not increase in size with incubation. They concluded that the type 2 strains were more heterogeneous viruses than type 1. But if such variations in response of breed and age of eggs and passage history of virus can be set aside, then useful deductions on the nature of strains under test can be made from the comparative gross and histological examination of pocks and electron microscopy of infected cells within pocks.

In the present study, in which the development of pathological lesions induced by HSV types 1 and 2 was followed by both optical and electron microscopy, the histological observations of Nahmias and colleagues (1968) were confirmed and extended. They examined the lesions on the CAM of oral and genital strains of HSV by optical microscopy at three or four days post-inoculation. The results presented in this study showed that the early stages of CAM infection were the same with either virus type, and involved proliferative changes of the ectoderm with only slight infiltration of the mesoderm. By two or three days these changes were more pronounced with type 2 strains. The ability to recognise differences between the pocks induced by the two

types of HSV was inversely related to the concentration of virus in the inoculum and consequent density of pocks on the membrane. When inocula of less than 100 pock-forming units were used and lesions were well separated, histological differences between pocks formed by types 1 and 2 viruses were noted by day two post-inoculation. The type-specific differences were most striking from four to seven days post-inoculation when the total CAM involvement of the type 2 strains became obvious. These results are in keeping with the clinical observation that genital strains normally induce more diffuse lesions involving the deeper tissues than do the oral isolates. At this time the inflammatory response involving mast cells, macrophages and leucocytes was maximal. The secretions of the mast cells probably prevented intra-vascular coagulation and induced vaso-dilation, facilitating the migration of leucocytes from the blood vessels to the pock, whilst the increase in the number of macrophages was probably a response to cellular debris arising from the lytic cycle of the virus. The multi-nucleate giant cells seen in the type 2 pocks may have been virus induced or developed from macrophages which had engulfed cellular material (Bloom and Fawcett 1975).

When doing electron microscopic studies it is essential to be aware of the limitations of the technique. Thin sectioning suffers from the problems of selection in that only a comparatively small number of cells can be examined and the plane of section may produce misleading information. Fixed material examined by thin sectioning presents static two dimensional images of dynamic three dimensional structures and the possibility of incorrect interpretation is

considerable, particularly when sequential development is traced. Studies on virus maturation against time are difficult to control, particularly in a tissue like the CAM in which cell numbers are variable so that only comparative studies between viruses at identical times may be worthwhile.

The most striking ultrastructural feature which distinguished HSV type 1 infected CAMs from those of HSV type 2 was the number of virus infected cells present in the pocks. In type 1 infections these reached a maximum two to three days after inoculation. The relative number of infected to uninfected cells declined thereafter, whilst in type 2 infections the number of infected cells continued to increase over the study period of seven days. The number of mature enveloped virus particles in the cytoplasm of type 2 virus infected cells was considerably more than in those of type 1. In the CAM type 2 viruses induced a morphologically more efficient infection with many more virus particles produced. From the results it may be postulated that the presence of virus particles or antigens stimulates the cells of the CAM to proliferate. Virus spread throughout the infected layers of cells in the pocks in a random fashion and virus infected cells occurred alongside apparently normal ones. The increase in the number of microvilli at the surface of pocks induced by both virus types may have been a virus induced phagocytic response. That no virus was found in the allantoic cells of type 2 pocks may reflect the restrictive nature of these cells for HSV. This was supported by the low levels of virus replication following allantoic cavity inoculation or cultivation of the viruses

in allantoic cells *in vitro*. The extra-cellular virus-like structures seen in the mesodermal layer of a type 1 pock at day seven were of interest in that they resembled the extra-cellular structures observed by Cromack (1968) in chick embryo cell monolayers. She suggested that these might be indigenous oncogenic virus particles. In the present study, it is possible that the structures were damaged HSV particles released from macrophages near the blood vessel, or particles which had leaked from the chorion to the mesoderm, but as they were found surrounding one blood vessel and not elsewhere in the mesoderm of this or any other type 1 pock these explanations seem unlikely. They could have been avian herpesviruses rather than HSV, but Brandly (1934) demonstrated that infectious laryngo-tracheitis virus was not transmitted through the egg. This also applied to Marek's disease virus (Solomon *et al*. 1970). Nonetheless, contaminating indigenous viruses cannot be ruled out. Positive identification of these particles from their morphology alone has proved to be impossible.

There was no qualitative difference in the method of entry of HSV types 1 or 2 into CAM cells and both viropexis and fusion were observed. These results confirm those of Hummeler and colleagues (1969). Similarly both virus types showed the standard features of herpesvirus morphogenesis with minor variations. The significance of the 21 nm. tubular and lattice structures seen in HSV type 2 infected CAM cells is obscure, but they may represent excess virus capsid protein material. Kalnins and co-workers (1967) found filamentous structures in adenovirus induced tumours in hamsters, and suggested that filamentous structures

could be considered as 'viral footprints' in neoplastic cells. Aurelian and colleagues (1971) noted similar structures in cultures of cervical tumour cells from which HSV type 2 was isolated. That these structures were seen in CAM cells infected with type 2 virus but not in type 1 infected cells in the present study may be connected with the oncogenic potential of HSV type 2, particularly in view of its reported association with cervical carcinoma. Berthiaume and Joncas (1973) observed 22 nm. lattice-like structures similar in appearance to those present in HSV type 2 infected CAM cells in the nucleus and cytoplasm of HEp2 cells. As this cell line was derived from a human carcinoma of the larynx, a viral-induced origin for these tubules seems a possibility. The morphologically different 22 nm. diameter microtubular structures occasionally found in HSV type 1 infected CAM cells were seen only in longitudinal section and appeared ribbon-like, lacking the lattice structure of those in the type 2 infected cells. In addition, these were present in association with nuclear changes including the presence of cytoplasmic organelles in the nucleus. It is therefore considered that these were cellular microtubules.

Dense granules of various sizes have been found in cells infected with HSV by many workers, but as yet their function is undecided. The 37 nm. diameter granules present in virus infected CAM cells, but not in the uninoculated control cells, were similar in size to the viral cores. These results, whilst not elucidating the nature of dense granules in infected cells, list two further sorts which occur in the cells of the CAM infected with HSV type 2, but not type 1. The 16-20 nm. diameter granules observed in some infected

The type 2 infected cells may exhibit additional granules in the infected nuclei because of a more intensive production of viral antigens, or the granules may be interchromatin structures and condensed nuclear proteins. In either case these would become more easily visible following clearing of the nucleus due to virus-induced condensation and margination of the chromatin. That the HSV type 2 infected cells contain 21 nm. lattice structures and 42-50 nm. and 65-95 nm. dense granules which are not present in HSV type 1 infected cells was shown, but further studies, perhaps involving autoradiographic and immunoferritin techniques as well as biochemical analysis of purified preparations, would be required in order to study the possible role of these structures in virus morphogenesis.

The greater heterogeneity noted in the cores of HSV type 2 compared with those of type 1 may result from one of three alternatives: they are the toroidal cores described by Furlong and colleagues (1972) in various orientations to the plane of section; the cores exist as pieces in various arrangements prior to fusion to form the dense cores described, or they are cores which have been assembled defectively. That the cores are toroidal cannot be excluded, although this fails to explain why structured cores were not found in the type 1 infected cells. Nii and Yasuda (1975) demonstrated that the cores of type 1 and type 2 could exist as toroids. On the other hand Miyamoto (1971) interpreted granular or pale staining cores as precursors of the dense cores, although this may be artefactual due to fixation or staining or the relationship of the particles to the plane of section, especially as Watson and co-workers

(1964) showed that particles with dense cores in one section had hollow cores in a subsequent section. Finally, the structured cores occurred more commonly in the older pocks when numerous virions were present even though the infectious virus titre in the CAM harvests declined at this period. This would favour the view that these were defective virions.

The observation made in this study that the cytoplasm of the type 2 infected cells contained more naked and partially enveloped particles than that of type 1 infected cells was made previously by Schwartz and Roizman (1969a). A number of explanations for this observation can be offered: capsids are partially assembled in the cytoplasm; the nuclear membrane of type 2 infected cells is particularly fragile; they are degenerating enveloped particles; cytoplasmic envelopment is normal for type 2 virus, and virus particles are taken into the cells and de-enveloped just as rapidly as they are released. The assembly of type 2 capsids in the cytoplasm seems unlikely, although there is good evidence that some viral antigens are made there (Miyamoto *et al.* 1971). The present results showed that the 21 nm. lattice structures were present in the cytoplasm as well as the nucleus of type 2 infected cells, but that the granules specific to the type 2 infected cells occurred only in the nuclei. Further evidence tending to eliminate cytoplasmic capsid assembly was that although capsids without cores were present in the cytoplasm, fragile particles thought to be capsids early in their formation were observed in the nucleus but never in the cytoplasm. The nuclear membrane of HSV type 2 infected cells may undergo changes making it more fragile, particularly as ruptured nuclei were observed

in some type 2 infected cells but not in type 1. This occurred despite the larger amount of reduplicated nuclear membrane in the type 2 virus infected cells. Any weakening of the nuclear membrane may be the physical result of envelopment of the larger number of particles produced in the type 2 infected cells. It was proposed by Schwartz and Roizman (1969b) that naked cytoplasmic capsids were enveloped virus particles which had their envelopes removed in the cytoplasm. Why this should occur with HSV type 2 and not type 1 is not clear, although it would explain the similarity in the infectivity yield for the two virus types despite the larger number of particles observed in the type 2 infected cells by thin sectioning. If type 2 virus particles were de-enveloped in the cytoplasm, it might be expected that virus envelope material should be observed in the cytoplasm; autoradiographic or immune electron microscopy may resolve this question. In this study, what appeared to be cytoplasmic envelopment of type 2 virions was occasionally observed, but there was no evidence that capsids already enveloped at the inner lamella of the nucleus were de-enveloped at the outer lamella of the nuclear membrane as postulated by Stackpole (1969) for Lucké tumour herpesvirus. Schwartz and Roizman (1969a) considered that some cells were restrictive for HSV due either to a block on virus envelopment leading to large numbers of virus crystals and naked particles in the cytoplasm, or to the lack of ducts for virus release resulting in de-envelopment of new particles in the cytoplasm. However, in this study the presence of such accumulations of immature virions in HSV type 2 infected cells is certainly

not linked with a restrictive cycle, since HSV type 2 spreads with ease in this tissue.

A further feature which differentiates the two HSV types is the capacity of type 2 virus to spread from the original pock on the CAM to other regions of the egg and embryo (Rodgers 1973 and this study). The rate of spread of the type 2 virus was related to the infectious titre in the inoculum, ranging from no spread or haemorrhages of the CAM with very low inocula to rapid spread and subsequent death of the embryo within six days with larger inocula. Electron microscopy studies showed that the blood vessels of the HSV type 2 infected CAM were affected. It is likely that death of the embryo resulted from a blood-borne disseminated infection. It is also possible that the greater involvement of the CAM by the type 2 strains compared with those of type 1 resulted in physical impairment of the membrane leading to stress of the embryo making it more susceptible to virus infection. Inoculation of HSV type 1 in amounts equal to or exceeding those of type 2 did not induce spread beyond the CAM, indicating that restriction of the type 1 virus to the inoculation site was truly type-specific and not concentration dependent, although all inocula were less than 10^7 TCD_{50} per 0.1 ml.

Shaffer and Enders (1939) noted that inoculation of a probable type 1 strain onto the CAM did not result in spread of the virus from the CAM. These workers, quoting reports from Goodpasture and Anderson's laboratory thought that the non-virulence of their strain of HSV for the embryo after CAM inoculation was due to the lack of innervation of the membrane. The situation in the egg may well parallel

human infections in which spread of HSV to the brain is said to be along nerve fibres for type 1 and by the blood stream for type 2 (Craig and Nahmias 1973).

Anderson (1940), working with the HF strain, observed spread of infection from the inoculated CAM to the embryo after six or seven days' incubation. As this virus strain was originally isolated from a lip lesion in 1925 (Flexner and Amos 1925) there had been ample opportunity for mutation and selection or mixture to occur, because of numerous passages, and later observations throw doubt on the type identity of HF strains. From the results of Wheeler (1964) it appears that his HF strain behaved as a type 2 virus and was more virulent for chick embryos than the HPF strain, a derivative of the HF strain. Subsequently Dowdle and colleagues (1967) examined a number of so-called HF strains from different sources by kinetic neutralisation tests and found some to be type 1 and others type 2 strains. The possibility that the HF strain was originally composed of a mixed population of virions from which subsequent laboratory passages selected particles with increased virulence cannot be excluded. This makes it difficult to assess Anderson's results.

In the present study there was evidence that serial egg passage in excess of 24 times increased the spreading capability of the type 1 strains and increased the pathogenicity for the CAM and embryo of the type 2 strains. Further passage up to 36 times in eggs produced no fundamental progressive change in these properties, but it is possible that continued egg passage in excess of 100 times could dramatically alter the virus characteristics in eggs.

Indeed Taniguchi (1966) showed that a fresh isolate from eczema herpeticum (presumably type 1) which had received six CAM passages did not spread beyond the ectoderm of the inoculated CAM and no virus was obtained from the allantoic fluid or embryo. In contrast, after 87 to 101 serial CAM passages this strain spread rapidly throughout the egg and killed the embryo in four days.

That the type 2 virus appears to be mesodermotropic was shown in sections, and as the primitive mesoderm gives rise to the blood vessels, this might explain the apparent greater ease of infection of the blood vessels of the CAM by the type 2 virus. The tendency for the highly egg-passaged type 1 strains to spread to other regions of the egg may reflect an increase in their tissue tropism for the mesoderm, and it would be interesting to examine lesions of these pocks for evidence of virus penetration beyond the chorion.

Although type 2 virus spread to the allantoic fluid rapidly and could be recovered from this cavity in large quantities for at least six days, yet when either virus type was inoculated directly into the allantoic sac, the concentration of virus declined, and in only one case (type 2 strain PAR) was any virus present after six days' incubation. This suggests that the cells lining the allantoic cavity are less susceptible to infection with HSV. In support of this are the low yields of infectious virus of either type obtained from the allantoic cells *in vitro* and the failure to find infected allantoic cells by electron microscopy after HSV type 2 inoculation of the CAM. The pinocytotic vesicles observed in the allantoic cells of

HSV type 2 inoculated CAMs may have been virus-induced particularly as these structures were not observed in the allantois of control or type 1 infected CAMs. After CAM inoculation two routes of spread to the allantoic fluid may be postulated: virus is shed continuously over the six day period into the allantoic cavity from the infected CAM cells either by cell-to-cell spread including infection of the allantoic cells, or by mechanical leakage of virus through the allantoic layer. Alternatively, following the initial infection of the CAM virus spreads to the allantoic fluid and also to the embryo. It is then excreted into the allantoic cavity from the embryo, so maintaining high concentrations of virus in the fluid. If the latter hypothesis were correct, the reverse does not occur, that is, spread to the embryo does not follow inoculation of the allantoic fluid. After allantoic inoculation with the egg adapted strains, the decline in the titre of infectious virus was slower, and in one case an increase in titre occurred without spread to the embryo. This indicates an increased capacity for replication of the egg adapted strains in the cells lining the cavity. With these strains, the possibility of their spread to and replication in embryo organs other than those examined, although unlikely, cannot be discounted.

It seems likely that after CAM inoculation the type 2 strains spread to the embryo by infection of the cells of the blood vessels lying in the mesoderm, spread to the embryo being always associated with haemorrhage of the CAM. Although no herpesvirus intra-nuclear inclusions could be identified in the endothelial cells of the blood vessels by optical microscopy, there were virus particles seen

within these cells by electron microscopy. It was found that the virus had spread to the embryo brain, heart and gut to a similar extent, supporting a haematogenous spread throughout the embryo, rather than spread to the viscera via the blood stream followed by neural spread as suggested by Anderson (1940).

Apart from differences in the capacity of the two types of virus to spread within the tissues of the egg, there were some differences and similarities in the yield of infectious virus from the infected CAM *in ovo* and from the various egg tissues *in vitro*. However, accurate quantitative comparisons between the two virus types in CAM cells *in ovo* was difficult because of proliferation of the chorion cells following virus infection and the spread of the type 2 virus within the egg from the inoculated CAM. Although the results in this study were insufficient to draw too many conclusions, nonetheless it is significant that the titres of infectious virus recovered from homogenised CAMs at three and six days post-inoculation showed a much slower rate of decline for type 2 than for type 1 virus. It would appear that with HSV type 1 strains, replication occurred at an early stage and was then interrupted so that after six days the quantity of virus in the CAM harvests dropped dramatically. This was more pronounced following administration of low concentration inocula. With the higher titred type 2 inocula, spread of virus to the allantoic fluid had occurred by the third day and to the embryo by the sixth day, whilst with low titred inocula no spread had occurred. This could explain the more rapid decline in infectious type 2 virus between day three and day six with the higher

concentration inocula compared with the low concentration inocula, following loss of infectious virus from the CAM into the allantoic fluid and blood stream. In general, the rate of virus increase was similar for both virus types, except that with the high concentration type 1 inoculum the rate was somewhat slower. This may reflect a truly slower replication rate following administration of high doses of type 1 virus, or that viral multiplication in CAM cells may have been interrupted at an earlier stage than with the lower inocula, so giving the impression of a slower replication rate.

Previous quantitative studies on CAM yields are not in complete agreement. Shaffer and Enders (1939) showed that after initial replication, a decrease in the virus population occurred five days after CAM inoculation, whilst Scott and co-workers (1953) observed an equilibrium of titre from the CAM at three days post-inoculation. Taniguchi (1966) showed that with a fresh isolate (probably type 1), virus replication progressed for the first two days post-CAM-inoculation, and was followed by a sharp decrease in virus yield, due, it was suggested, to the action of interferon. The weight of the CAM continued to increase to the sixth day post-inoculation and decreased on the seventh. From this it was clear that although virus replication was interrupted on the second day after CAM inoculation, cellular proliferation in the form of pocks continued up to the sixth day, and thereafter resolution of the pocks caused a decline in CAM weight. Taniguchi also showed by fluorescent antibody techniques that only a small number of cells in the pocks contained virus, a finding confirmed in this

study by electron microscopy. The present study showed that type 2 virus replication and pock formation continued uninhibited, findings also observed for egg-adapted strains by Yoshino and colleagues (1961).

When egg-borne factors were removed by cultivation of the viruses in cell cultures of egg tissues, the growth patterns differed somewhat from those *in ovo*. No significant type differences were observed in the growth curves in chick embryo fibroblasts or brain cell cultures, which contrasts with the type-specific spread differences *in ovo*. This indicates that factors other than embryo tissue tropism result in spread differences. That the type 2 strain produced more infectious virus in the chick embryo CAM cell cultures than the type 1, correlates with the higher titres from the CAM *in ovo*, and with the larger number of virus particles seen in sections with the type 2 strains. Indeed the findings in the CAM tissues *in vitro* paralleled those *in ovo*. The poor growth of both virus types in allantoic cell cultures correlates with their lack of growth in the allantoic cavity *in ovo*, but there are differences between HSV types 1 and 2. It is not clear why the CAM cell cultures should be less sensitive to infection with the type 1 virus, and the allantoic cell cultures to infection with either virus type, except that the fibroblasts and brain cell cultures were embryo-derived, whilst the CAM and allantoic cell cultures came from the CAM.

It was possible to distinguish fresh isolates of HSV type 1 from those of type 2 by the temperature of incubation of CAM-inoculated eggs. The type 2 virus strains and laboratory strains of type 1 grew more readily on the CAM at high

temperatures than did the freshly isolated type 1 strains. These results correlated well with the lower virulence for the CAM of the freshly isolated type 1 strains compared with the type 2. The ability of viruses to replicate at high temperatures, correlated with an increase in virulence, has been shown for the *Poxviridae* (Bedson and Dumbell 1961) and polioviruses (Lwoff 1962). Zygraich and Huygelen (1973) have also shown that mutants of HSV type 2 incapable of replication at elevated temperatures were less neurovirulent for rabbits. The results presented here for HSV strains in eggs differ from those of Longson (1971) and Ratcliffe (1971) who found that type 2 strains did not replicate in cell cultures at high temperatures, but agree with Wheeler (1964) who found that a type 2 strain replicated better in cell cultures at 40°C than did a type 1.

The inability of the type 2 strains to replicate in cell cultures at supra-optimal temperatures has been attributed to the thermolabile nature of both the type 2 virus-induced enzyme thymidine kinase (Thouless and Skinner 1971) and the instability of the type 2 virus DNA (Crouch and Rapp 1972). Thymidine kinase, involved in DNA synthesis, is required for HSV replication, but Kitt and Dubbs (1963a,b) have shown that virus-induced enzyme is not essential provided cellular enzyme is available. Marcon and Kucera (1975) pointed out that in human cells inoculated with HSV type 2 and incubated at 42°C, only cellular DNA was synthesised, whilst at 41°C both cellular and viral DNA was produced, even though the type 2 virus-induced enzyme is readily inactivated at 39°C. From these results it would appear that the type 2 virus

genome can utilise the cellular enzyme for DNA synthesis at elevated temperatures. Jamieson, Gentry and Subak-Sharpe (1974) demonstrated that in actively dividing cells virus-induced deoxyprymidine kinase is not essential for HSV replication and Thouless and Wildy (1975) have shown that the deoxyprymidine kinase molecule is responsible for the thymidine kinase activity of HSV. From the results of these workers and those of the present study, it follows that in actively dividing cells such as those present in the proliferating pocks on the CAM, the type 2 virus would not be restricted by the heat lability of its own enzyme, particularly as Buddingh (1970) found that egg tissue metabolism is highly active between 12 to 15 days of incubation. The converse of this would be the resting confluent cell monolayers in which the virus-induced enzyme would be essential for virus replication, and cultures infected with HSV type 2 strains at high temperatures would not produce virus, due to the thermolabile nature of the virus-induced thymidine kinase. This may explain the inconsistency in the results of Ratcliffe (1971) and Crouch and Rapp (1972) who could differentiate strains by growth at differing incubation temperatures in some cells, but not in others, perhaps reflecting the state of the cells and the availability of their thymidine kinase at the time of inoculation, rather than an effect of cell type. If it were true that in actively dividing cells virus-induced thymidine kinase was not essential, then both virus types should replicate equally well on the CAM at high temperatures which was in fact the case with laboratory strains, and fresh isolates of HSV type 2. Despite the comparatively thermostable

nature of the type 1 enzyme as was shown by Thouless and Skinner (1971), the freshly isolated type 1 strains did not replicate so readily on the CAM at high temperatures. This correlated with the lower infection rate for the cells of the CAM, as was shown by virus yield studies and electron microscopy. Under the conditions of high incubation temperature and lower virulence, the effects of interferon and thermal inactivation of virus would be accentuated, particularly as the thermal stability of virions and their capacity to grow at elevated temperatures are distinct characteristics (Bedson and Dumbell 1961, Yoshino *et al.* 1968). In the present study similar inoculum concentrations were used, yet there is the possibility that the reduction in pock numbers on the CAM at temperatures up to 39.5°C compared with 36°C, with a given strain, is due to thermal inactivation of virus particles within the inoculum before the infection is established. Also, the results from egg inoculation would appear to correlate with the human infection in which HSV type 2 strains show a predilection to infect the genitalia, a warm region of the body, compared with the lip and other body surfaces commonly colonised by type 1.

Under controlled conditions the size and morphology of pocks on the CAM provide a useful means for distinguishing fresh isolates of HSV type 1 from those of type 2 and further indicators are the detection of type 2 virus in the allantoic fluid of CAM inoculated eggs and their ability to replicate on the CAM at elevated temperatures. However, laboratory adapted strains were found to give unpredictable results in the tests studied.

If extensive studies with HSV are planned, it is advisable that stock pools of virus should be prepared with the minimum of laboratory manipulation in order to reduce the risk of mutation and selection of populations with properties different from the original virus. Laboratory manipulation may be responsible for the variation in results from one laboratory to another, leading to the suggestion that HSV exists as a spectrum (Roizman *et al.* 1970). From the results presented here and those of other work which has been quoted, it is established that laboratory strains of herpes simplex virus occupy the intermediate positions in a spectrum of which the fresh isolates of each type are the extremes.

A C K N O W L E D G E M E N T S

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A P P E N D I X 1

MEDIA USED IN BIOLOGICAL STUDIES

Unless otherwise stated all media were sterilised by autoclaving at 10 lb. pressure per square inch for 15 minutes (115°C) after an initial free steaming period of 15 minutes. All media were pre-warmed to 37°C prior to use.

1 Media used in egg work

1.1 Penicillin streptomycin broth saline (PSBS)

Isotonic saline	100 ml.
Hartley digest broth . . .	10 ml.
"Crystamycin" (penicillin 200,000 units per ml. + streptomycin 0.2 gm. per ml.) .	0.1 ml.

The saline and broth were sterilised by autoclaving. The components were mixed under aseptic conditions and the solution stored at 4°C.

1.2 Vaseline preparation

For dispensing, the vaseline preparation was loaded into a 10 ml. syringe (without needle) using a wooden spatula. The mixture was stored at room temperature.

2 Media used in cell cultures

2.1 De-ionised water

All water used to make up media was distilled once in a metal still, then passed through an Elgastat de-ioniser, distributed in 25 ml. and 100 ml. bottles, and sterilised by autoclaving. Sterile water was stored at 4°C.

2.2 Phosphate buffered saline (PBS) solution A

Phosphate buffered saline (Dulbecco and Vogt 1954) was obtained from Oxoid Ltd. in tablet form. Each tablet was dissolved in 100 ml. de-ionised water and the solution sterilised by autoclaving. Solutions were stored at 4°C.

The constituents of the commercial PBS solution A in a final litre of solution were as follows:

NaCl	0.8 gm.
KCl	0.02 gm.
Na_2HPO_4	0.115 gm.
KH_2PO_4	0.02 gm.

2.3 Antibiotics

2.3.1 "Crystamycin" This was obtained in ampoules from Glaxo Laboratories Ltd., and stored at room temperature. The contents of two ampoules were taken up in a total of five ml. sterile de-ionised water, so that the final solution contained 200,000 units of penicillin and 0.2 gm. of streptomycin per ml. The solution was stored at 4°C in glass bijou bottles. When added to 100 ml. of medium, 0.1 ml. of the final medium contained 200 units per ml. penicillin and 200 µg. per ml. streptomycin.

2.3.2 "Fungizone" This was obtained in ampoules from Squibb and Sons, Inc. and stored at 4°C. The contents of one ampoule were taken up in 10 ml. of sterile de-ionised water and the final solution contained 5 mg. amphotericin B and 4.1 mg. sodium deoxycholate per ml. The solution was stored at 4°C in glass bijou bottles and was added to reagents used to prepare primary cell cultures from egg products. When added to 100 ml. of medium, 0.1 ml. of the final medium contained 5 µg. per

ml. amphotericin B and 4.1 µg. per ml. sodium deoxycholate.

2.4 Washing fluid

This was prepared by mixing, using aseptic precautions, 100 ml. PBS solution A and 0.1 ml. "Crystamycin". Solutions were prepared and used as required.

2.5 Hanks' Balanced Salt Solution (BSS) (Hanks & Wallace 1949)

This was obtained in powder form from Oxoid Ltd., and was prepared by dissolving the contents of one sachet of solution A in one litre of sterile de-ionised water and the contents of one sachet of solution B in another litre of sterile de-ionised water to give 10 times concentrated stock solutions. Each solution was sterilised by Seitz filtration using an HP/EKS, 14 cm. diameter pad which had been pre-washed with 300 ml. sterile PBS solution A. These solutions were distributed aseptically into sterile 500 ml. bottles and stored at 4°C. For use 100 ml. of the stock solution A was mixed with 100 ml. of the stock solution B and made up to one litre with sterile de-ionised water. This working solution was distributed in bottles in 100 ml. volumes and sterilised by autoclaving at 8.5 lb. pressure per square inch for 10 minutes (112-114°C). Bottles were stored at 4°C ready for use. The constituents of the commercial Hanks' BSS in the solution when ready for use were as follows:

Sachet A		Sachet B	
NaCl	4.358 gm./l.	NaCl	3.642 gm./l.
KCl	0.4 gm./l.	$CaCl_2$	0.14 gm./l.
Na_2HPO_4	0.06 gm./l.	$MgCl_2$	0.1 gm./l.
KH_2PO_4	0.06 gm./l.	$MgSO_4$	0.1 gm./l.
phenol red	0.02 gm./l.	dextrose	1.0 gm./l.

2.6 Trypsin

A stock solution of 2% trypsin (Difco 250) in Hanks' BSS was made by shaking the suspension well and then warming at 37°C in a water bath for 30 minutes or more until the trypsin was almost completely dissolved. The solution was filtered through Whatman filter paper No. 1 and N/1 sodium hydroxide added to adjust the pH to 6.6 - 6.8. The solution was filtered by Seitz filtration and stored in 25 ml. quantities at 4°C.

Working solutions were prepared freshly as required, under aseptic conditions, by mixing 100 ml. PBS solution A and 10 ml. 2% stock solution of trypsin.

The trypsin working solutions for primary cells were made up freshly as required from the following sterile constituents:

Hanks' BSS	100 ml.
2% Trypsin stock solution	11 ml.
4.4% $NaHCO_3$	0.7 ml.
"Crystamycin"	0.1 ml.
"Fungizone"	0.1 ml.

2.7 Versene

A 1% solution of sodium versenate (the di-sodium salt of sequestric acid) was made in PBS solution A by warming the suspension at 37°C for 30 minutes. The solution was dispensed into four ml. aliquots, sterilised by autoclaving and stored at 4°C.

2.8 Trypsin-versene mixture

This solution was made up freshly as required under aseptic conditions by mixing 100 ml. PBS solution A with four ml. versene and 2.5 ml. trypsin stock solution.

2.9 Phenol red

A 0.2% solution of phenol red was made by dissolving one gm. of Gurr's phenol red powder in 2.5 ml. N/1 sodium hydroxide and making up the final volume to 500 ml. with sterile de-ionised water. The solution was filtered through Green's "Hyduro" 904½, 38.5 cm. diameter filter paper, and stored unsterile at 4°C.

2.10 Sodium bicarbonate solution ($NaHCO_3$)

A stock 4.4% solution of sodium bicarbonate was made by dissolving 44 gm. sodium bicarbonate in 950 ml. sterile de-ionised water and adding 50 ml. of 0.2% phenol red solution. Carbon dioxide was allowed to bubble through the solution for one hour and the solution became a cherry red colour. The solution was immediately distributed into bijou bottles which were filled to the brim and tightly capped. The solutions were sterilised by autoclaving and stored at 4°C.

2.11 Foetal calf serum

Sterile foetal calf serum was obtained in 400 ml. quantities from Flow Laboratories and was stored at -30°C. When required it was distributed in 10 ml. volumes in sterile bottles and stored at 4°C.

2.12 Eagle's Minimum Essential Medium (Eagle's MEM)

This medium (Eagle 1959) was obtained as a sterile times 10 concentrated stock solution without sodium bicarbonate or antibiotics from Burroughs Welcome and Co. For use the stock solution was diluted tenfold under aseptic conditions using sterile de-ionised water. Both the stock solution and the diluted medium were stored at 4°C.

2.12.1 Growth medium Eagle's MEM growth medium was prepared from the following sterile constituents:

Eagle's MEM	100 ml.
Foetal calf serum	10 ml.
Sodium bicarbonate (4.4%) . .	1.5 ml.
"Crystamycin"	0.1 ml.
"Fungizone" (when used) . . .	0.1 ml.

2.12.2 Maintenance medium Eagle's MEM maintenance medium was prepared from the following sterile constituents:

Eagle's MEM	100 ml.
Foetal calf serum	2 ml.
Sodium bicarbonate (4.4%) . .	2.5 ml.
"Crystamycin"	0.1 ml.
"Fungizone" (when used) . . .	0.1 ml.

2.13 Synthetic medium 199 (SM 199)

This medium (Morgan, Morton and Parker 1950) was obtained as a sterile tenfold concentrated stock solution without sodium bicarbonate or antibiotics from Burroughs Welcome and Co., and was treated as previously described for Eagle's MEM.

2.13.1 Growth medium SM 199 growth medium was prepared from the following sterile constituents:

SM 199	100 ml.
Foetal calf serum	2 ml.
Sodium bicarbonate (4.4%) . .	2.5 ml.
"Crystamycin"	0.1 ml.

2.13.2 Maintenance medium SM 199 maintenance medium was prepared from the following sterile constituents:

SM 199	100 ml.
Foetal calf serum	1 ml.
Sodium bicarbonate (4.4%) . .	4 ml.
"Crystamycin"	0.1 ml.

All growth and maintenance media were prepared using aseptic techniques.

A P P E N D I X 2

REAGENTS AND MATERIALS USED IN ELECTRON MICROSCOPY STUDIES

1 Negative staining

1.1 Grids

Athene 400 mesh grids of the old format were used and these were supplied by Smethurst Ltd.

1.2 Formvar

This was supplied as a dry powder from Shawinigans and was stored in a desiccator to prevent absorption of moisture.

A stock 2% solution was made by dissolving one gm. of formvar in 50 ml. electron microscopy grade ethylene dichloride (Taab Laboratories). The solid was always added to the solvent and the mixture shaken vigorously to encourage solution. Any remaining solid dissolved on standing overnight. The stock solution was stored in glass-stoppered bottles in a desiccator until required.

The working solution was made up freshly as a 0.2% solution by mixing 45 ml. ethylene dichloride and 5 ml. stock 2% formvar solution.

1.3 Formvar-carbon-coating of grids

A clean dry glass slide was dipped into 50 ml. 0.2% working strength formvar solution in a Coplin jar, removed and drained. When dry the film was cut *in situ* into suitable sized squares using a sharp scalpel, and these squares were floated off onto the surface of glass-distilled water by carefully submerging the slide with formvar film. In

reflected light the formvar films could be seen as pale opalescent squares floating on the surface of the distilled water. Athene 400 grids were placed (matt side down) on top of the films and the films and attached grids were collected from the water by carefully placing pieces of Whatman No. 1 filter paper over the films. Filter papers with adherent formvar films and grids were collected, dried and carbon-coated. Carbon-coating of grids was performed in an Edwards High Vacuum Coating Unit model E12E4 using carbon rods and a current of 90 amps at 10 volts to produce the arc.

1.4 Phosphotungstate negative stain (PTA)

A 3% solution was made by dissolving potassium phosphotungstate in 100 ml. glass-distilled water and the pH was adjusted to 6.3 using fresh 10% aqueous potassium hydroxide. Any turbidity remaining after standing at room temperature for 24 hours was removed by centrifugation. The stain was stored at room temperature and used as required.

1.5 Bovine plasma albumin

This was used at a 0.05% solution in distilled water to aid even distribution of specimens on grids for negative staining. Solutions were stored at 4°C.

2 Thin sectioning

2.1 Grids

Three types of grids were used: the Smethurst Athene 400 and 200 mesh, and Taab 50 μ grids. Prior to use these grids were dipped into a 0.05% solution of polybutene (BP

Chemicals) in xylene and dried on Whatman No. 1 filter paper. This procedure enhanced adhesion of sections to grids and had no adverse effect on sections or staining.

2.2 Cacodylate buffer

This was made by dissolving 21.4 gm. sodium cacodylate (Taab Laboratories) in one litre glass-distilled water (0.1 M solution) and adjusting the pH to 7.2 - 7.4 using concentrated hydrochloric acid. Solutions were stored at 4°C until required.

2.3 Glutaraldehyde

Glutaraldehyde was obtained from Taab Laboratories as a specially purified (Electron Microscopy Grade) 25% aqueous solution. The stock solution was stored in the dark at room temperature and working 3% solutions were made freshly as required by dispersing 3 ml. of 25% glutaraldehyde in 22 ml. cacodylate buffer.

2.4 Osmium tetroxide (OsO_4)

A stock 2% solution of OsO_4 (Johnson Matthey) was made by dissolving the contents of a 0.5 gm. ampoule in 25 ml. cacodylate buffer. The ampoule was prepared as follows: the label was removed from the ampoule, the exterior was washed clean in cold water, and after drying the ampoule was scored completely round with a glass file and heat fractured. The two halves of the ampoule and contents were dropped into a glass universal screw-cap bottle containing the buffer. As OsO_4 has a dangerous pungent vapour, it was always handled in a fume cupboard. Solution was completed by standing the crystals plus buffer overnight at 4°C. The stock solution was stored at 4°C

in the tightly capped universal bottle suitably padded with corrugated paper in a screw-capped tin (those supplied in smallpox kits were found to be ideal). Stored in this way the solution remained stable for many months without darkening. The working 1% solution was made by mixing equal volumes of the stock 2% solution and cacodylate buffer.

2.5 Ethanol series

A graded ethanol series was made by mixing appropriate amounts of dry absolute ethanol and cacodylate buffer. The 50% and 70% solutions were stored at 4°C and the 90%, 95% and absolute ethanol solutions were stored at room temperature. (Percentages refer to the concentration of ethanol).

2.6 Epoxy propane (propylene oxide)

This was obtained from BDH and was supplied ready for use. Care was taken to keep atmospheric water vapour from the epoxy propane by keeping it securely stoppered at all times.

2.7 Resin mixtures

A total of six resin mixtures was examined in preliminary tests for this study and one was found to give consistently satisfactory results judged by trimming, sectioning and staining of specimens. This resin was described by Mollenhauer (1964) and the formula is given in 2.7.1 below. All volumes were measured using disposable 25 ml. Sterilin graduated medicine measures, with the exception of the dibutyl phthalate and DMP-30 which were measured using one ml. disposable syringes without needles.

2.7.1 Resin 1 Epon-araldite (Mollenhauer 1964) This was made from the following constituents:

Component	Proportion	Volume for 1 CAM specimen
Epon 812 (resin)	5 parts	15 ml.
DDSA[1] (HY964) (hardener)	11 "	33 "
Araldite (CY212) (resin)	3 "	9 "
Dibutyl phthalate (plasticizer)	0.8 "	2.4 "
DMP-30[2] (accelerator)	0.4 "	1.2 "

This resin was polymerised at 60°C for 24 hours and produced excellent blocks.

Other resin mixtures which were tested but not found so suitable are detailed below.

2.7.2 Other resin mixtures

A.	Araldite (CY212)	10 ml.
	DDSA	10 ml.
	Dibutyl phthalate (plasticizer)	1 ml.
	DMP-30 (accelerator)	0.5 ml.

This resin (Glauert and Glauert 1958) was polymerised at 60°C for 24 hours and produced blocks of variable quality.

B.	Taab embedding resin	50 ml.
	DDSA (hardener)	30 ml.
	MNA[3] (hardener)	20 ml.
	DMP-30 (accelerator)	2 ml.

This resin was polymerised at 60°C for both 24 and 48 hours and the resultant blocks were of variable quality.

C.	(i) Epon 812 (resin)	12.4 ml.
	DDSA (hardener)	20 ml.

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[1] Dodecenyl succinic anhydride (HY964).

[2] Tri-di-methylamine methyl phenol (DY064).

[3] Methyl nadic anhydride (HY906).

(ii) Epon 812 (resin) . . . . 50 ml.
MNA (hardener) . . . . 44.5 ml.

(i) and (ii) were mixed in the proportions 10 ml. (i) to 40 ml. (ii) and 1 ml. DMP-30 added. Blocks were polymerised at 60°C for 24 hours. This formula produced hard blocks difficult to trim.

(i) and (ii) were mixed in the proportion 15 ml. (i) to 35 ml. (ii) and 1 ml. DMP-30 added. Blocks were polymerised at 60°C for 24 hours. This produced blocks difficult to section.

D. Epon 812 (resin) . . . . 50 ml.
DDSA (hardener) . . . . . 30 ml.
MNA (hardener) . . . . . 20 ml.
DMP-30 (accelerator) . . . . 2 ml.

This resin was polymerised at 60°C for 24 hours, and produced blocks with numerous bubbles, which were difficult to section.

E. Epon 812 (resin) . . . . 12.5 ml.
MNA (hardener) . . . . . 11.1 ml.
DMP-30 (accelerator) . . . . 2 ml.

This resin was polymerised at 60°C for 24 hours and produced soft blocks difficult to trim, section and stain.

2.8 Optical stains for 1 μ resin embedded sections

2.8.1 Toluidine blue (1%) A solution of 1% borax was prepared by dissolving one gm. sodium borate in 100 ml. distilled water, and to 50 ml. of this was added 0.5 gm. toluidine blue (Trump *et al.* 1961) which was dissolved by shaking and allowing to stand for 24 hours. The resultant 1% toluidine blue in 1% borax solution was filtered through a Whatman No. 1 filter paper and stored in a dark glass-stoppered bottle at room temperature.

2.8.2 Azure II (0.1%) This stain was prepared in a similar way to that described for toluidine blue, excepting that 0.05 gm. Azure II was dissolved in 50 ml. 1% borax solution (Jeon 1965). Suitable staining time for each stain was found to be one minute at 60°C.

2.9 Stains for ultra thin sections

2.9.1 Uranyl acetate A 5% solution of uranyl acetate (BDH) was made by dissolving one gm. in 20 ml. glass-distilled water. The solid was found to dissolve completely upon standing overnight at room temperature. The solution was filtered through a Whatman No. 50 filter paper and stored in a dark glass-stoppered bottle at room temperature. This stain could be stored satisfactorily for a few weeks.

2.9.2 Lead citrate (modified from Venebale and Coggeshall 1965). One pellet AR grade sodium hydroxide was dissolved in 15 ml. cold boiled glass-distilled water. This solution was added to 0.1 gm. lead citrate (K&K Laboratories) in a glass screw-capped universal bottle and dissolved by shaking. A further 10 ml. cold boiled glass-distilled water was added, and the stain was stored tightly capped at room temperature. This stain could be kept satisfactorily for a few weeks.

APPENDIX 3

1 Egg passage details of strains used in high passage studies

CAMs were removed from eggs and homogenised in 10 ml. sterile distilled water as previously described. Dilutions in tenfold steps were prepared in sterile distilled water and these were inoculated on to the CAMs of further eggs and CAMs with 100+ pocks were used for further passages. Strains were passaged at different times to prevent cross-contamination.

1.1 Egg passage of HSV type 1 strain HFEM (on two separate occasions)

CAM pass level	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum
1	4	10^{-2}	3	10^{-2}
2	3	10^{-2}	3	10^{-2}
3	4	10^{-3}	3	10^{-1}
4	3	10^{-3}	2	10^{-2}
5	4	10^{-1}	3	10^{-2}
6*	3	10^{-3}	2	10^{-3}
7	4	10^{-3}	5	10^{-2}
8	3	10^{-2}	2	10^{-2}
9	4	10^{-2}	5	10^{-2}
10	4	10^{-2}	3	10^{-2}
11	3	10^{-3}	4	10^{-2}
12*	4	10^{-2}	2	10^{-2}
13	4	10^{-3}	2	10^{-2}
14	7	10^{-3}	3	10^{-1}
15	3	10^{-2}	2	10^{-2}

contd ...

(continuation)

CAM pass level	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum
16	3	10^{-3}	5	10^{-2}
17	2	10^{-1}	2	10^{-2}
18*	5	10^{-3}	5	10^{-3}
19	3	10^{-3}	2	10^{-2}
20	2	10^{-3}	5	10^{-1}
21	2	10^{-3}	3	10^{-1}
22	3	10^{-3}	4	10^{-2}
23	6	10^{-2}	3	10^{-3}
24*	2	10^{-3}	4	10^{-2}
25	4	10^{-3}	-	-
26	5	10^{-3}	-	-
27	2	10^{-1}	-	-
28	4	10^{-1}	-	-
29	3	10^{-3}	-	-
30*	4	10^{-1}	-	-
31	2	10^{-3}	-	-
32	2	10^{-3}	-	-
33	2	10^{-3}	-	-
34	2	10^{-3}	-	-
35	2	10^{-3}	-	-
36*	4	10^{-3}	-	-

* samples stored at -70°C

- = not done

1.2 Egg passage of HSV type 1 strain 22101/71 and HSV type 2 strain LOV

	HSV type 1 strain 22101/71		HSV type 2 strain LOV	
CAM pass level	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum
1	3	10^{-2}	4	10^{-3}
2	3	10^{-1}	3	10^{-4}
3	3	10^{-1}	4	10^{-3}
4	2	10^{-3}	3	10^{-3}
5	3	10^{-2}	4	10^{-3}
6*	2	10^{-3}	3	10^{-4}
7	5	10^{-3}	4	10^{-4}
8	2	10^{-3}	3	10^{-4}
9	5	10^{-2}	4	10^{-3}
10	3	10^{-2}	4	10^{-3}
11	4	10^{-2}	3	10^{-3}
12*	2	10^{-2}	4	10^{-3}
13	2	10^{-2}	4	10^{-3}
14	3	10^{-2}	7	10^{-3}
15	2	10^{-3}	3	10^{-3}
16	5	10^{-2}	3	10^{-3}
17	2	10^{-1}	2	10^{-3}
18*	5	10^{-3}	5	10^{-3}
19	2	10^{-2}	3	10^{-3}
20	5	10^{-2}	2	10^{-3}
21	3	10^{-2}	2	10^{-3}
22	4	10^{-1}	3	10^{-3}
23	3	10^{-2}	6	10^{-3}
24*	4	10^{-2}	2	10^{-2}

contd ...

(continuation)

HSV type 1 strain 22101/71			HSV type 2 strain LOV	
CAM pass level	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum	Days of incubation prior to harvest	Dilution of CAM homogenate as inoculum
25	-	-	4	10^{-3}
26	-	-	5	10^{-3}
27	-	-	2	10^{-3}
28	-	-	4	10^{-2}
29	-	-	3	10^{-2}
30*	-	-	4	10^{-3}
31	-	-	2	10^{-1}
32	-	-	2	10^{-3}
33	-	-	2	10^{-3}
34	-	-	2	10^{-3}
35	-	-	2	10^{-4}
36*	-	-	4	10^{-3}

* samples stored at -70°C

- = not done

APPENDIX 4

The following tables are graphically represented in the 'Results' section 7.

1 Yield of infectious virus from chick embryo fibroblast cell cultures (CEFC)

Hours after initiation of infection at which cultures were harvested	HSV type 1 strain 22101/71	HSV type 2 strain 9889/70
0	1.0×10^5*	3.0×10^4
1	1.0×10^4	1.0×10^4
3	5.0×10^3	1.0×10^3
4	3.0×10^3	3.0×10^2
7	3.0×10^4	3.0×10^3
19	1.0×10^5	1.0×10^4
21	1.0×10^5	2.0×10^4
24	1.0×10^5	8.0×10^4
27	2.0×10^5	1.0×10^5
30	3.0×10^5	1.0×10^5
43	3.0×10^5	3.0×10^4
45	3.0×10^5	1.0×10^4
48	3.0×10^5	3.0×10^3
54	3.0×10^5	3.0×10^3
67	1.0×10^5	2.0×10^3
72	1.0×10^5	3.0×10^2
91	3.0×10^4	1.0×10^2
96	1.0×10^4	3.0×10^1
200	0	0

* titres of infectious virus expressed as TCD_{50} per 0.1 ml. of the harvests

2 Yield of infectious virus from chick embryo allantoic cell cultures (CEAC)

Hours after initiation of infection at which cultures were harvested	HSV type 1 strain 22101/71	HSV type 2 strain 9889/70
0	8.0×10^1*	1.0×10^2
1	1.0×10^1	4.0×10^1
2	8	2.5×10^1
4	3.0×10^3	2.5×10^2
15	3.0×10^3	4.0×10^2
18	5.0×10^1	4.0×10^2
21	4.0×10^1	3.0×10^2
24	4.0×10^1	3.0×10^2
27	2.5×10^1	3.0×10^2
39	1.0×10^1	3.0×10^2
42	1.0×10^1	3.0×10^2
45	1.0×10^1	3.0×10^2
48	1.0×10^1	3.0×10^2
51	1.0×10^1	3.0×10^2
63	1.0×10^1	2.5×10^2
72	1.0×10^1	2.0×10^2
91	5	2.0×10^2
96	5	8.0×10^1
195	0	0

* titres of infectious virus expressed as TCD_{50} per 0.1 ml. of the harvests

3 Yield of infectious virus from chick embryo brain cell cultures (CEBC)

Hours after initiation of infection at which cultures were harvested	HSV type 1 strain 22101/71	HSV type 2 strain 9889/70
0	3.0×10^3 *	3.0×10^3
1	3.0×10^2	3.0×10^2
2	3.0×10^3	3.0×10^1
4	3.0×10^4	3.0×10^2
15	3.0×10^4	7.0×10^2
18	3.0×10^4	1.0×10^3
21	5.0×10^5	4.0×10^4
24	1.0×10^5	2.0×10^4
27	1.0×10^5	7.0×10^3
39	1.0×10^5	5.0×10^3
42	3.0×10^4	2.0×10^3
45	3.0×10^4	2.0×10^3
48	3.0×10^4	2.0×10^3
51	3.0×10^4	2.0×10^3
63	3.0×10^4	2.0×10^3
72	3.0×10^4	2.0×10^3
91	1.0×10^4	2.0×10^3
96	5.0×10^3	2.0×10^3
195	0	0

* titres of infectious virus expressed as TCD_{50} per 0.1 ml. of the harvests

4 Yield of infectious virus from chick embryo CAM cell cultures (CECC)

Hours after initiation of infection at which cultures were harvested	HSV type 1 strain 22101/71	HSV type 2 strain 9889/70
0	1.0×10^3*	2.0×10^2
1	3.0×10^2	3.0×10^1
2	1.0×10^3	3.0×10^2
4	2.0×10^3	4.0×10^2
15	4.0×10^2	4.0×10^3
18	3.0×10^2	5.0×10^3
21	3.0×10^2	3.0×10^4
24	3.0×10^2	1.0×10^4
27	3.0×10^2	6.0×10^2
39	3.0×10^2	5.0×10^2
42	3.0×10^2	5.0×10^2
45	3.0×10^2	4.0×10^2
48	3.0×10^2	3.0×10^2
51	3.0×10^2	3.0×10^2
63	1.0×10^2	2.0×10^2
72	3.0×10^1	2.0×10^2
91	5	1.0×10^2
96	5	1.0×10^2
195	0	0

* titres of infectious virus expressed as TCD_{50} per 0.1 ml. of the harvests

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Infection, Haemorrhage and Death of Chick Embryos after inoculation of Herpes Simplex Virus Type 2 on to the Chorioallantoic Membrane

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SUMMARY

When the chorioallantoic membrane of the embryonated hen's egg was inoculated with herpes simplex virus type 2 strains, an infection resulted which spread rapidly throughout the egg. Haemorrhage and death occurred in the embryo, and haemorrhage was also observed in the chorioallantoic membrane. Virus was recovered from the infected chorioallantoic membrane, allantoic fluid, amniotic fluid and selected organs of the embryo. In contrast, similar inoculation of herpes simplex virus type 1 strains gave no haemorrhage in chorioallantoic membrane or embryos, embryos did not die and virus was recovered only from the inoculated chorioallantoic membrane. Inoculation of either type into the allantoic cavity did not result in spread of virus to the embryo. Prolonged adaptation of herpes simplex viruses to growth in eggs eventually resulted in spread of type 1 virus from the chorioallantoic membrane to other regions of the egg and there were also marginal increases in virulence of type 2 virus.

It is generally recognized that there are two types of herpes simplex virus (HSV), which can be differentiated by various tests, one of which is the appearance of the pocks they produce on inoculation on to the chorioallantoic membrane (CAM) of fertile hen's eggs (Hutfield, 1967; Parker & Banatvala, 1967; Nahmias *et al.* 1968). Preliminary observations in this laboratory indicated another difference between type 1 and type 2 strains in pathogenicity for the whole egg. This was explored in detail with four strains of each type, of which three were established laboratory strains and one was a fresh isolate. Type 1 laboratory strains were HFEM, HIL, WAL and the fresh isolate was 22101; type 2 laboratory strains were LOV, PAR, 17152, and the fresh isolate was 9889. An outline study was also made with a further 21 HSV strains; nine strains were type 1 and twelve were type 2. Of the nine type 1 strains, five had had several passages in tissue culture and four were fresh isolates; of the twelve type 2 strains five had had several passages in tissue culture and seven were fresh isolates. All strains were typed by pock characteristics on the CAM and appearance of c.p.e. in tissue culture; laboratory strains were also studied by the temperature marker test (Longson, 1971; Ratcliffe, 1971). Five of the eight strains used in the detailed study were supplied by Professor P. Wildy (Birmingham University) and had been further typed by the kinetic neutralization test (Rawls *et al.* 1968). Infectivities of the eight strains studied in detail were assayed in human embryo lung cell cultures using the 50% end-point dilution method (Reed & Muench, 1938), titres varied from $2{\cdot}1 \times 10^6$ to $4{\cdot}7 \times 10^7$ TCD_{50} per ml for type 1 strains and from $2{\cdot}1 \times 10^5$ to $3{\cdot}2 \times 10^7$ TCD_{50} per ml for type 2 strains.

Fertile Leghorn hens' eggs were incubated at 36 °C and after 11 days 0·1 ml undiluted stock virus pool was inoculated on to the CAM which was exposed using the false air sac technique. Great care was taken to ensure that during the drilling of the eggs the membranes remained intact and any eggs showing signs of bleeding were discarded. Harvests of allantoic

Table 1. *Spread of types 1 and 2 herpes simplex viruses in the egg*

	HSV type 1 strains				HSV type 2 strains															
	HFEM 22101				HIL WAL				LOV				PAR				17152				9889			
Days of harvest...	1	2	3	6	1	2	3	6	1	2	3	6	1	2	3	6	1	2	3	6	1	2	3	6
CAM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluids																								
Allantoic	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Amniotic	−	−	−	−	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+
Embryo																								
Heart	−	−	−	−	−	−	+	+	−	−	+	+	−	−	+	+	−	+	+	+	−	+	+	+
Gut	−	−	−	−	−	+	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+
Brain	−	−	−	−	−	+	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+

+ = herpes virus recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests.

− = herpes virus not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests.

fluid, CAM, amniotic fluid, heart, gut and brain were made 1, 2, 3 and 6 days post-inoculation, taking the following precautions to prevent cross-contamination. A syringe with a fine gauge needle (26 G) was inserted 4 to 5 mm into the blunt end of the egg, and allantoic fluid was withdrawn slowly. After carefully cutting away the dropped region of the CAM with the shell still attached, the amniotic sac was lifted with blunt forceps and the amniotic fluid was removed with a syringe. The embryo was removed from the amniotic sac by carefully cutting the amniotic membranes so as to prevent contact between the embryo and allantoic fluid. The embryo was washed free of amniotic fluid in three changes of 150 ml normal saline, and cut longitudinally to expose the heart which was removed together with the heart blood. Samples of liver and intestine were removed at this stage. The brain was aspirated using a syringe with a coarse gauge needle (18 G), which was inserted into the back of the head between the eyes. The shell and CAM removed earlier were separated to harvest the CAM.

Allantoic cavity inoculation was performed by inserting a fine gauge needle under the shell and directly inoculating the allantoic fluid with 0·1 ml undiluted stock virus pool. Allantoic fluid and brain were harvested as described above.

Harvests were stored at −70 °C. Virus in the harvests was demonstrated by inoculation of harvests on to the CAMs of further eggs, and observation of the CAM for development of typical herpes pocks within 3 days. Allantoic and amniotic fluids were inoculated undiluted, heart and gut material were inoculated as 40% (v/v) suspensions in penicillin/streptomycin broth saline (PSBS), brain was inoculated as a 20% (v/v) suspension in PSBS and CAM was homogenized and inoculated at a 10^{-3} dilution in distilled water.

Each strain produced pocks on the CAM typical of the type to which the strain belonged, i.e. type 1 pocks were less than 0·5 mm in diam. and type 2 pocks were greater than 1 mm after 3 days. Type 2 always caused a haemorrhagic condition of the CAM. No haemorrhage was seen after inoculation of the CAM with type 1 strains. Three days after CAM inoculation with type 2 strains, the embryos were also haemorrhagic and often dead. Neither death nor haemorrhage of embryos occurred when CAMs were inoculated with type 1 strains even after prolonged incubation.

Type 1 viruses were recovered only from harvests of CAM. All other regions of the egg harvested after inoculation of type 1 viruses on to the CAM failed to yield pocks on further passage in eggs, indicating that HSV type 1 had not spread beyond the CAM. Results were identical for all type 1 strains on all days tested. (Table 1).

In contrast, all type 2 strains tested spread rapidly from the CAM to other regions of the egg, appearing in the allantoic fluid 1 or 2 days after CAM inoculation, in the amniotic fluid 2 days after inoculation and in the embryo harvests 2 or 3 days after inoculation. All selected parts of the embryo yielded virus. The results were identical for all type 2 strains tested (Table 1). Similar results were obtained when HSV type 2 virus strain 9889 (fresh isolate) was diluted 10^{-1} to 10^{-4} although progressive dilution resulted in delayed spread to the embryo. When even higher dilutions of the type 2 strain were inoculated on the CAM, so that inocula contained only 5 to 20 pock forming units, no haemorrhage of the CAMs was observed, and no virus was recovered from the embryo. Under these conditions, embryos hatched normally. In contrast spread of type 1 virus beyond the CAM was never demonstrated even using the stock virus pools undiluted. The titres of type 1 virus in these pools were comparable with the titres of the type 2 virus pools.

Herpes simplex virus type 1 strain 22101 and HSV type 2 strain LOV were examined after 12 and 24 serial egg passages. The nature and rate of spread were unaffected with either strain after 12 egg passages, but after 24 egg passages the type 2 strain spread more rapidly to the organs of the embryo, appearing in the allantoic fluid and brain only 1 day after CAM inoculation. The type 1 strain spread into the allantoic fluid after 4 days but did not spread to the brain of the embryo.

Following inoculation of either type 1 or type 2 virus into the allantoic fluid, no virus was recovered from the embryo brain. The amount of virus recovered from the allantoic fluid declined with time.

Further studies with 21 other strains showed that all 12 type 2 strains spread from the CAM to the allantoic fluid within 3 days, whilst none of the 9 type 1 strains had spread beyond the CAM 3 days after inoculation.

These results show that type 2 herpes simplex viruses spread rapidly from the inoculated CAM to other regions of the egg including the embryo, whereas type 1 viruses were restricted to the inoculation site. The rate of spread of type 2 virus was related to the concentration of infectious virus in the inoculum. Following inoculation with small concentrations of virus spread was delayed and with even smaller amounts the CAM was not haemorrhagic and no virus was recovered from the embryo which, apparently unaffected, hatched normally. Inoculation of HSV type 1 in concentrations equal to or exceeding those of type 2 did not induce spread beyond the CAM, indicating that restriction of type 1 virus to the inoculation site was truly type specific and not concentration dependent.

There was evidence that frequent serial passages in eggs somewhat increased the spreading potential of the type 1 strain. After 24 egg passages the type 1 strain had spread into the allantoic fluid 4 days after CAM inoculation, although embryos were not infected even 6 days post-inoculation. Type 2 virus at the 24th egg passage had become even more pathogenic for the CAM, causing greater haemorrhage and necrosis. It was also more rapidly pathogenic for the embryo, and the rate of spread to the allantoic cavity was also increased.

As observed by Nahmias *et al.* (1968), HSV type 2 infection of the CAM produced lesions involving all three layers of the membrane, that is ectoderm, mesoderm and endoderm, whilst HSV type 1 lesions were restricted to the outer ectoderm layer. It was interesting that in this study type 2 HSV apparently passed through the CAM, perhaps infecting all three germ layers on route, to reach the allantoic fluid. It could be recovered from the fluid in

large quantities for at least 6 days, but virus inoculated directly into the allantoic fluid declined in concentration. This suggests that the cells lining the allantoic cavity are either insusceptible, or are infected only with difficulty from the allantoic fluid side of the membrane. Since virus inoculated into the allantoic fluid did not spread to the embryo it seems likely that after inoculation of HSV type 2 on to the CAM, virus infection of the embryo is not via the allantoic fluid, but is due to virus penetration of the blood vessels lying in the mesoderm layer of the CAM. Spread of virus to the embryo was always associated with haemorrhage of the CAM. When a small dose of HSV type 2 was inoculated onto the CAM few pocks were produced, the CAM was not haemorrhagic, and there was little if any spread of virus to the embryo. It is possible that spread was prevented because the few pocks formed were remote from blood vessels. Anderson (1940) using the HF strain and Wildy & Holden (1954) using the multiple egg passaged derivative strain HFE observed death of embryos. These observations were made prior to dividing HSV into two types and a comparative study of HF strains obtained from several sources revealed that some cultures were type 1 while others were type 2 (Dowdle *et al.* 1967).

Anderson (1940) showed that vascular spread of herpes virus within the egg was possible by demonstrating herpes virus intra-nuclear inclusions in the endodermal cells lining the embryonic blood vessels and by isolating virus from the allantoic artery 2 days after inoculation of the CAM.

The reason for the restriction of type 1 strains to the inoculation site on the CAM compared with the rapid spread of type 2 strains to other regions of the egg can at present only be assumed to be due to the relatively superficial nature of the primary CAM lesion. It appears that this property of invasiveness is reproducible and type specific, although caution is necessary in interpreting results observed with strains which have had multiple egg passages. Recovery of infectious virus from the allantoic fluid three days after inoculation of a herpes strain onto the carefully preserved intact CAM, taken together with the appearance and size of the CAM lesions produced, could prove a useful additional test in the differentiation of type 1 from type 2 herpes simplex viruses.

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